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Ultrasound detection in the Gulf menhaden requires gas-filled bullae and an intact lateral line

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SUMMARY

Clupeiform fish species, including the Gulf menhaden (*Brevoortia patronus*) that belong to the subfamily Alosinae, can detect ultrasound. Clupeiform fishes are unique in that they have specialized gas-filled bullae in the head associated with the ear *via* the bulla membrane and with the lateral line *via* the lateral recess membrane. It has been hypothesized that the utricle of the inner ear is responsible for ultrasound detection through a specialized connection to the gas-filled bullae complex. Here, we show that the lateral line and its connection to the gas-filled bullae complex *via* the lateral recess are involved in ultrasound detection in Gulf menhaden. Removal of a small portion of the lateral line overlying the lateral recess membrane eliminates the ability of Gulf menhaden to detect ultrasound. We further show that the gas-filled bullae vibrates in response to ultrasound, that the gas-filled bullae are necessary for detecting ultrasound, and that the bullae connections to the lateral line *via* the lateral recess membrane play an important role in ultrasound detection. These results add a new dimension to the role of the lateral line and bullae as part of the ultrasonic detection system in Gulf menhaden.

Key words: ultrasound detection, lateral line, bullae, Gulf menhaden.

INTRODUCTION

Hearing abilities in fish species are generally limited to frequencies below 3 kHz and many fish only respond to the particle motion component of the sound field (Hawkins, 1981). However, in the early 1990s it was reported that ultrasonic sonar pulses were an efficient way of repelling certain clupeiform fish species from the water intakes of power plants (Dunning et al., 1992; Nestler et al., 1992). It was not clear whether the fish responded to the ultrasonic component of the sonar or whether they responded to low frequency by-products. Later, behavioural and physiological studies showed that shad and menhaden species (members of the subfamily Alosinae) exhibited a clear and intensity graded response when exposed to pure ultrasound (Mann et al., 2001; Mann et al., 1998; Mann et al., 1997; Plachta and Popper, 2003; Wilson et al., 2008). The ability to detect ultrasound appears to be limited to the Alosinae, as it has not been found in other clupeiform fish species, including Pacific herring, bay anchovy and scaled sardines (Mann et al., 2001; Mann et al., 2005). Ultrasound detection of the Alosinae is hypothesized to be an anti-predator response to echolocating toothed whales (Mann et al., 1997; Plachta and Popper, 2003), which use intense ultrasonic signals to detect and catch prey (Au, 1993).

Over the past 15 years, the mechanism of ultrasound detection in Alosinae has been an enigma. The inner ear has been hypothesized as the ultrasound detector in Alosinae because of its unique anatomy (Higgs et al., 2004; Mann et al., 1997; Plachta and Popper, 2003). In all clupeids, gas-filled tubes on each side of the head extend from the swimbladder and expand to gas-filled bullae encapsulated in bone. These bullae are mechanically connected to both the inner ear (macula utriculus) and the lateral line *via* thin membranes – the bulla membrane and the lateral recess membrane, respectively (Blaxter et al., 1981; Denton and Blaxter, 1976; Denton et al., 1979; Hoss and Blaxter, 1982). The gas-filled bullae complex and its connection to the lateral line through the lateral recess membrane is believed to have a function in schooling behaviour and is found in most clupeiform species (Blaxter et al., 1981). Furthermore, clupeiform species have a very specialized utricular macula, divided into three distinct regions with large numbers of hair cells with long ciliary bundles (Denton and Gray, 1979; Popper and Platt, 1979). In the Alosinae, the support for the middle section of the utricular macula was found to be particularly thin compared with that of other clupeiform species, leading to the suggestion that this part of the inner ear is key to ultrasound detection in the Alosinae (Higgs et al., 2004). Furthermore, single unit recordings of ultrasound sensitive neurons were made in regions of the brain typically associated with the auditory system; the secondary octaval population and the descending octaval nucleus (Plachta et al., 2004). However, the hypothesis that the utricle mediates ultrasound detection has not been verified experimentally and there is at present no direct evidence that the inner ear of fish acts as an ultrasound detector.

In the present study, we used volume reconstructions derived from micro-computed tomography (micro-CT) images to compare the head anatomy of a clupeid that can detect ultrasound, the Gulf menhaden (*Brevoortia patronus*, Goode 1878), and a species that cannot, the scaled sardine (*Harengula jaguana*, Poey 1865). The imaging data indicated a more elaborate channeling of the lateral line system overlying the bullae in the Gulf menhaden. Therefore, we tested the hypothesis that this portion of the lateral line was involved in ultrasound detection in Gulf menhaden by mechanically removing the lateral line system overlying the bullae and subsequently measuring the neural response using auditory evoked potentials (AEPs). Further, we tested the role of the gas-filled bullae connected to the lateral line. We found that the lateral line system overlying the bullae (i.e. the lateral recess) is a necessary part of the ultrasonic detection system in Gulf menhaden and that the bullae are the transducing element making it possible for the Gulf menhaden to detect ultrasound.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida.

Micro-CT imaging

One Gulf menhaden and one scaled sardine were scanned in air using a micro-CT scanner (Scanco Medical AG, Brüttisellen, Switzerland) with scan x-ray source settings of 55 kVp/143 μA. Isotropic voxel reconstructions were obtained for the scaled sardine (20 μm) and menhaden (16 μm). Segmentation and three-dimensional (3D) reconstructions of micro-CT images were performed using AMIRA 4.1.2 (Mercury Computer Systems, San Diego, CA, USA). Labelling the structures of the acousticolateralis system followed previously published studies (Blaxter et al., 1981; Dario and Pinna, 2006; Denton and Blaxter, 1976; Denton et al., 1979).

AEPs

Twenty Gulf menhaden and seven scaled sardines were used in the experiments. The fish were anaesthetized with MS-222 and placed in a fish holder 5 cm below the water surface in a 17 l test tank. Tones of 20 ms in duration, gated with a Hanning window, were transmitted using an ITC-1042 transducer (International Transducer Cooperation, Santa Barbara, CA, USA) for emission of 40 kHz tones or a UW30 transducer (Lubell Labs Inc., Columbus, OH, USA) for emission of 600 Hz tones. The transducers were connected to a Tucker-Davis-Technologies AEP workstation (Tucker-Davis-Technologies, Inc., Alachua, FL, USA). The UW30 transducer was placed in air and the ITC-1042 transducer was placed in the tank. The tone was presented 10 times per second. Measurements of the sound field were made with a TC4013Reson hydrophone (Reson A/S, Slangerup, Denmark) and showed no frequencies outside the test frequency. To record the AEPs, a recording needle-electrode was placed intracranially, a reference needle-electrode was placed in the dorsal musculature, and a ground needle-electrode was placed in the tail musculature. The AEP signals were amplified with a TDT HS4 amplifier 100,000 times and bandpass filtered from 5 to 3000 Hz with a notch filter at 60 Hz. Evoked potential sweeps were 40 ms in duration and were measured by averaging the amplified evoked potentials 200 times.

Laser vibrometer measurements

Eight Gulf menhaden and seven scaled sardines were used in the laser vibrometer measurements. The skin, muscle tissue and bone encapsulating the upper part of the right bulla dorsal to the lateral recess were removed, leaving the gas-filled bulla exposed. The fish was placed on its left side and all measurements were taken directly from the exposed right gas-filled bulla. After the first set of measurements, the air in the right bulla was replaced with Ringer solution and measurements were repeated. Vibrational measurements were acquired with a Polytec CLV laser vibrometer

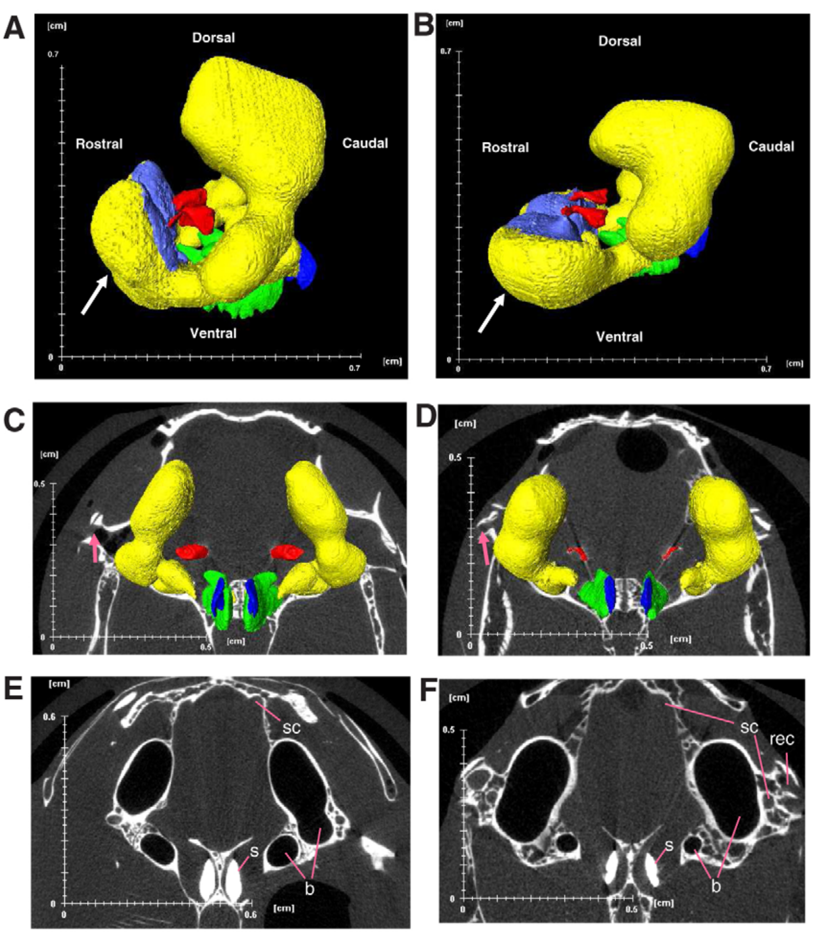

Fig. 1. Two dimensional (2D) images and three dimensional (3D) reconstructions of bullae, perilymph of bullae, and otoliths of a scaled sardine and a Gulf menhaden. (A,B) Sagittal views of the 3D reconstructions in the (A) scaled sardine and (B) Gulf menhaden. (C,D) Caudal views of the 3D reconstructions in the (C) scaled sardine and (D) Gulf menhaden with 2D images illustrating the positioning of the bulla and lateral recess relative to the body surface. Bulla, yellow; perilymph of bulla, light blue; utricle, red; saccule, green; lagena, dark blue; rostral body of bulla, white arrow; approximate location of lateral recess membrane, pink arrow. (E,F) 2D images of the (E) scaled sardine and (F) Gulf menhaden illustrating the channelling of the lateral recess. sc, semicircular canals; b, bulla; s, saccule; rec, lateral recess.

(Polytec Inc., Irvine, CA, USA; sensitivity: 5 mm s^{-1} V^{-1}). The unfiltered signals were acquired for 40 ms and measured by averaging the laser vibrometer signal 100 times. To minimize fluctuation of the acoustic stimuli, the laser was aimed at the same location in the tank throughout the experiment for the same fish. The fish was moved to point the laser on the bulla or the muscle tissue.

RESULTS

Micro-CT imaging

Comparisons of 3D reconstructions of Gulf menhaden and scaled sardine show clear anatomical differences. These distinctions include the shape of the two bullae, the orientation of the bulla and utricle, and the positioning of the bulla and lateral recess relative to the body surface (Fig. 1). In Gulf menhaden, the rostral body of each bulla is positioned ventral to the utricle, rather than the anterior position observed in the scaled sardine (Fig. 1A,B). Furthermore, in Gulf menhaden, the distances of the bulla and lateral recess to the body surface are much shorter than those in the scaled sardine (Fig. 1C,D). In addition, the channelling of the lateral recess in Gulf menhaden is more elaborate than the channelling in the scaled sardine (Fig. 1E,F).

AEP and the lateral line system

We tested the possibility that the lateral line system overlying the gas-filled bullae (i.e. the lateral recess and the lateral recess membrane) could be important in ultrasound detection in Gulf menhaden. A fraction of the lateral line system (where it is connected to the lateral recess and the lateral recess membrane) on both sides of the head was mechanically removed by scraping off the gelatinous layer (Fig. 2). The menhaden were exposed to ultrasonic pulses at 40 kHz with a sound pressure level of 178(±4) dB re. 1 μPa (r.m.s.) and a duration of 20 ms, and a control 600 Hz tone pulse with a sound pressure level of 120(±3) dB re. 1 μPa (r.m.s.) with a duration of 20 ms before and after lateral line removal. AEP (Corwin et al., 1982) recordings were used to measure sound detection abilities. There was a strong neural response at 40 kHz (Fig. 3A,C) before mechanical removal of a fraction of the lateral line, but after removal the response at 40 kHz disappeared. The mean response before and after removal was significantly different

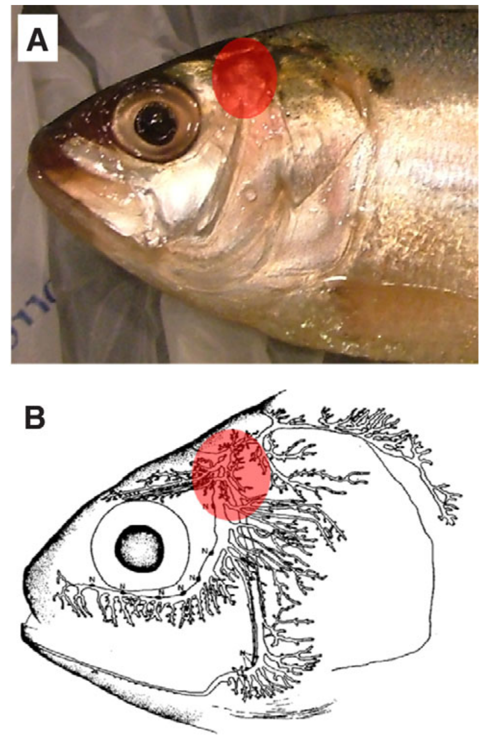

Fig. 2. (A) Picture of a Gulf menhaden. Marked area shows the removed part of the lateral line. Only the outermost skin was scraped off and no muscle or bone tissue was removed. (B) Schematic drawing of the lateral line in Atlantic menhaden. Marked area shows the removed part of the lateral line. N=8 [Modified from Hoss and Blaxter (Hoss and Blaxter, 1982).]

(Student's paired t-test, P<0.001). However, the response at 600 Hz (Fig. 3B,C) was strong both before and after lateral line removal (P=0.97).

The gas-filled bullae

To test the role of the gas-filled bullae complex in ultrasound detection, a laser vibrometer was used to measure the vibrational

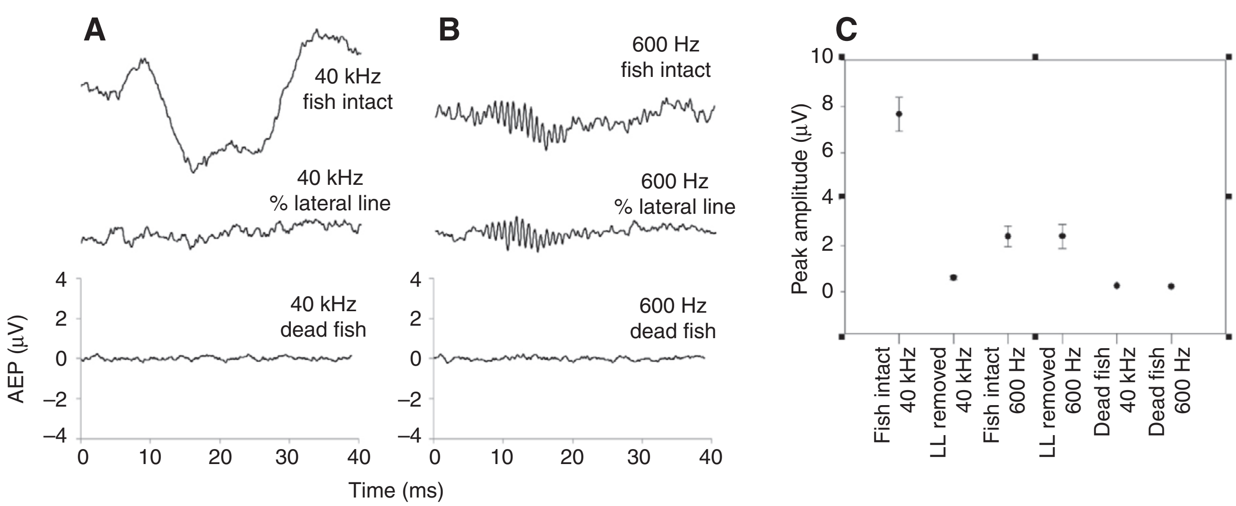

Fig. 3. Auditory evoked potential (AEP) waveforms and peak amplitude measurements showing the effect of removing a fraction of the lateral line (LL) system in Gulf menhaden. (A,B) AEP waveforms from intact fish, fish with a fraction of the lateral line removed (% lateral line) and dead fish controls in response to an acoustic stimulus at 40 kHz (A) and at 600 Hz (B). (C) Mean (±s.e.) peak amplitude for the different treatments. N=8.

amplitude in response to an acoustic stimulus of 40 kHz. There was a strong vibrational response from the gas-filled bulla from both the Gulf menhaden and the scaled sardine when exposed to 40 kHz (Fig. 4). The mean vibrational response from the gas-filled bulla was significantly stronger than the mean vibrational response from the Ringer-filled bulla (Student's paired *t*-test, Gulf menhaden P=0.007 and scaled sardine P=0.006) and adjacent muscle tissue (Gulf menhaden P=0.009 and scaled sardine P=0.006). A significant difference was not found between the mean vibrational response of the Ringer-filled bulla and the adjacent muscle tissue (Gulf menhaden P=0.09, scaled sardine P=0.36).

AEP measurements were also conducted on eight Gulf menhaden before and after both bullae were filled with Ringer solution (Fig. 5) to test for the importance of the gas-filled bullae in ultrasound detection. The bullae were filled from the dorsal side of the head with a syringe to avoid damage to the lateral line. Filling of the bullae reduced the neural response at 40 kHz considerably, and there was a significant difference in the mean AEP before and after filling of the bullae (Student's paired *t*-test, P=0.004), However, filling of the bullae produced only minor effects on the neural response at 600 Hz, which were not significant (P=0.97).

DISCUSSION

For more than 15 years, the sensory organ and the mechanism responsible for ultrasound detection in Alosinae has been a mystery. Here, we present data that support the hypothesis that the lateral line and its connection to the gas-filled bullae *via* the lateral recess and lateral recess membrane are involved in ultrasound detection in Gulf menhaden. This finding is somewhat surprising because until now the lateral line was believed to be a close-range system, which can detect low frequency hydrodynamic stimuli (<100 Hz) caused by moving objects a few body lengths away from the fish or other current generating sources such as streams or ocean currents (Coombs and Braun, 2003; Denton and Gray, 1983). The present study adds a new dimension to the role of the lateral line system in detecting ultrasound, either through the response of the sensory cells to ultrasound or through its role as a mechanical connection to the inner ear. Separate neural recordings from the inner ear and lateral line primary afferents need to be conducted to elucidate which organs are involved in ultrasound detection.

The hair cell is the sensory unit in both the lateral line and the inner ear. The adequate stimulus is an aspect of the particle motion (particle displacement, velocity or acceleration), depending on which level of a sensory organ is regarded (Kalmijn, 1989; Sand and Karlsen, 2000). However, the frequency range of detection for the inner ear and lateral line is limited to less than 1000 Hz, if there is no specialized transducer mechanism. To detect higher frequencies, it is necessary to transduce the energy retained in the acoustic pressure wave of a sound field into detectable particle motion (Fay and Popper, 1974; Kalmijn, 1989; Sand and Enger, 1973). This can be solved by having a gas-filled structure in connection with the hair cells (Karlsen, 1992a). This type of adaptation is found in the Otophysans (goldfish, catfish), where bony structures, the Webarian ossicles, mechanically connect the swimbladder to the inner ear (Fay and Popper, 1999). The connection expands the frequency range detectable by the inner ear. In clupeiform fish species, the proposed transducer (i.e. the gas-filled bulla) is connected to both the inner ear and the lateral line (Blaxter, 1981), which may allow both structures to detect the pressure wave of the sound field (Braun and Grande, 2008). We have shown the importance of the gas-filled bulla as a transducing element by filling this structure with Ringer solution, which caused elimination of the neural response to ultrasonic stimuli. Another important finding is that the inner ear is not affected by any of these procedures, because the response at 600 Hz is retained. It is possible that the response at 600 Hz may not have been affected by filling of the bullae with Ringer solution because of the possibility of a gas bubble retained in the ventral part of the bullae near the utricle. Alternatively, the response could come from the saccule of the inner ear.

Most species of clupeids have gas-filled bullae connected to the inner ear and lateral line (Blaxter et al., 1981). The data acquired using the laser vibrometer show that the bulla vibrates in response to ultrasound, even in a species that does not detect ultrasound. How is it then that some species of clupeids can detect ultrasound and others cannot? Based on the volume reconstructions, there are clear anatomical differences between Gulf menhaden and scaled sardine. In Gulf menhaden, the bullae are shaped differently and positioned closer to the body surface compared with the bullae in the scaled sardine. In addition, the channelling of the lateral recess in Gulf menhaden is more

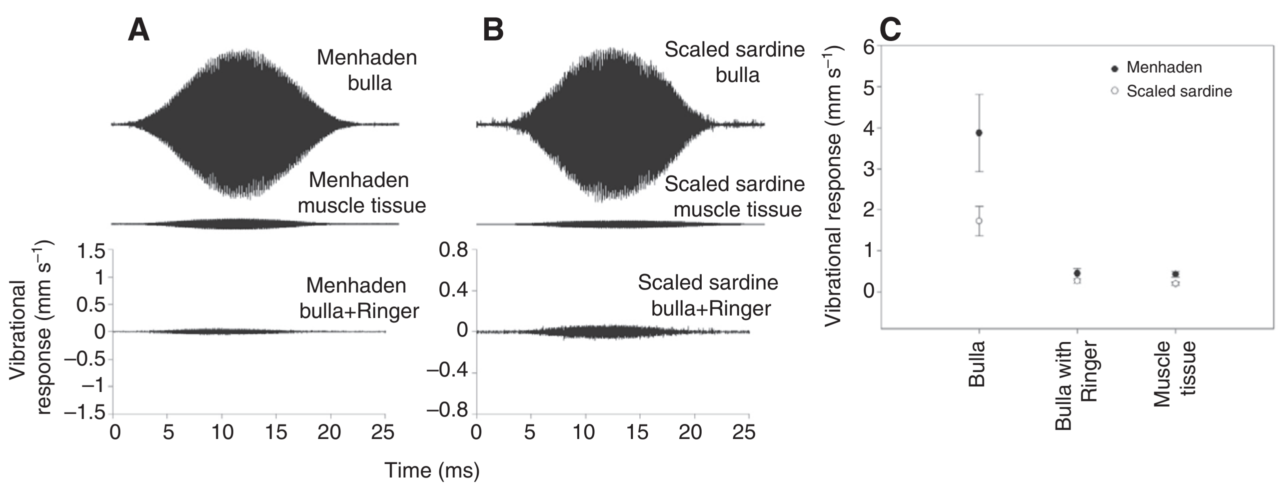

Fig. 4. Vibrational responses of the bulla measured with a laser vibrometer in response to an acoustic stimulus at 40 kHz. Measurements were made directly from the bulla, from adjacent muscle tissue control site, and from the bulla after it was filled with Ringer solution. (A) Example response from one Gulf menhaden. (B) Example response from one scaled sardine. (C) Mean (± s.e.) vibrational response. Gulf menhaden N=8, scaled sardine N=7.

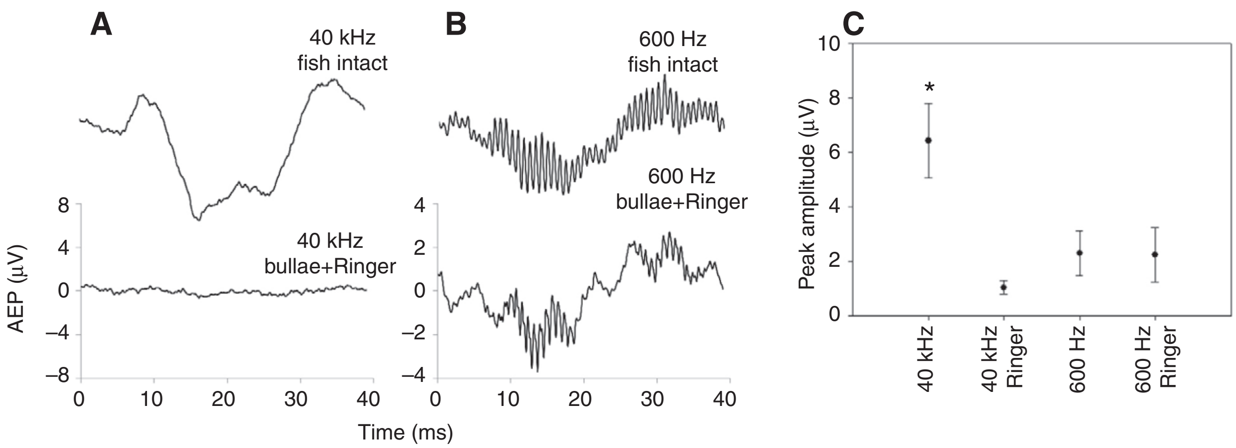

Fig. 5. AEP waveforms and peak amplitude measurements showing the effect of filling the bullae complex with Ringer solution in Gulf menhaden. (A) Response to a 40 kHz acoustic stimulus of intact fish and of fish with bullae filled with Ringer solution. (B) Response to a 600 Hz acoustic stimulus of intact fish and of fish with bullae filled with Ringer solution. (C) Mean peak amplitude response to a 40 kHz and 600 Hz acoustic stimulus before and after filling the bullae with Ringer solution (means ± s.e.). *N*=4.

elaborate than the channelling in the scaled sardine. These anatomical differences may explain why Gulf menhaden are capable of detecting ultrasound and scaled sardines are not. Alternatively, the structure of hair cells, and their orientation and position may be different in Gulf menhaden and scaled sardine; differences in the utricular macula may be another possible cause (Higgs et al., 2004). These anatomical differences warrant further experimental scrutiny.

The lateral line, in general, is important for detecting the hydrodynamic movements caused by nearby prey or predators when they are within a few body lengths of the fish (Sand and Bleckmann, 2008). This adaptation is a key feature for detection when the incoming predator is another fish. However, other important predators in the marine environment include toothed whales, which are fast swimmers. Thus, the detection of hydrodynamic waves caused by a moving toothed whale when it is at close range may not offer enough time for menhaden to successfully avoid predation. However, toothed whales not only alarm their prey with hydrodynamic stimuli at close range but also echolocate, providing ultrasonic cues for fish that can detect these signals. The newly discovered function of the acousticolateralis system allows members of the Alosinae not only to detect a moving toothed whale at very close range but also to detect the ultrasonic signals produced by an approaching echolocating toothed whale at greater distances. This adaptation will probably expand the range over which the lateral line and inner ear can detect the toothed whale and thus possibly increase the chance of survival. This evolutionary arms race where echolocating toothed whales emit high frequency sounds to find food and fish detect these signals to avoid predation is very similar to the terrestrial arms race between many bats and moths (Miller and Surlykke, 2001).

The responses of the lateral line and the inner ear can be difficult to distinguish because of common receptor elements and overlapping frequency ranges (Karlsen, 1992a; Karlsen, 1992b). In the Clupeidae, it is even more complicated because the two systems are mechanically interconnected *via* the gas-filled bullae (Blaxter, 1981). The findings in the present study add to this intriguing complexity by demonstrating that the lateral line not only detects low frequency hydrodynamic stimuli but also is somehow involved in ultrasound detection.

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