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# Development of ultrasound detection in American shad (*Alosa sapidissima*)

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**Summary**

**It has recently been shown that a few fish species, including American shad (*Alosa sapidissima*; Clupeiformes), are able to detect sound up to 180 kHz, an ability not found in most other fishes. Initially, it was proposed that ultrasound detection in shad involves the auditory bullae, swim bladder extensions found in all members of the Clupeiformes. However, while all clupeiformes have bullae, not all can detect ultrasound. Thus, the bullae alone are not sufficient to explain ultrasound detection. In this study, we used a developmental approach to determine when ultrasound detection begins and how the ability to detect ultrasound changes with ontogeny in American shad. We then compared changes in auditory function with morphological development to identify structures that are potentially responsible for ultrasound detection. We found that the auditory bullae and all three auditory end organs are present well before fish show ultrasound detection behaviourally and we suggest that an additional specialization in the utricle (one of the auditory end organs) forms coincident with the onset of ultrasound detection. We further show that this utricular specialization is found in two clupeiform species that can detect ultrasound but not in two clupeiform species not capable of ultrasound detection. Thus, it appears that ultrasound-detecting clupeiformes have undergone structural modification of the utricle that allows detection of ultrasonic stimulation.**

Key words: ultrasound detection, bullae, utricle, American shad, *Alosa sapidissima*.

## Introduction

It had long been thought that the ability to detect ultrasonic frequencies was limited within the vertebrates to echolocating bats and cetaceans (e.g. Sales and Pye, 1974; Grinnell, 1995; Nachtigall et al., 2000). It has recently been discovered, however, that some fish species also can detect frequencies in the ultrasonic range. Blueback herring (*Alosa aestivalis*) and alewives (*Alosa pseudoharengus*) avoid ultrasonic pulses in field settings (Nestler et al., 1992; Ross et al., 1993, 1996), and American shad (*Alosa sapidissima*) show behavioural (Plachta and Popper, 2003) and physiological (Mann et al., 1997, 1998, 2001) responses to sounds up to 180 kHz. With the exception of a few reports in cod (*Gadus morhua*; Astrup and Møhl, 1993, 1998), all other reports of ultrasound detection have been in fishes in the order Clupeiformes, with the ability perhaps restricted to one subfamily within the Clupeiformes, the Alosinae (Mann et al., 2001).

While the mechanism used for high-frequency detection is understood in most species that can detect frequencies higher than 1.5 kHz [e.g. the Weberian ossicles of the Cyprinidae (von Frisch, 1938; Fay and Popper, 1974) or the accessory air sac of the Mormyridae (von Frisch, 1938; Yan and Curtsinger, 2000; Fletcher and Crawford, 2001)], the mechanistic basis for ultrasonic detection in clupeiform fishes remains unclear. As in other teleosts, there are three auditory end organs in the inner ear of clupeiform fish: the utricle, saccule and lagena. Fishes in the order Clupeiformes, however, have a modified inner ear in which the utricular epithelium is divided into anterior, middle and posterior maculae (Tracy, 1920; O'Connell, 1955; Best and Gray, 1980), as opposed to the single utricular epithelium found in all other vertebrates (e.g. Platt, 1984). The clupeiform ear is surrounded by one (in sprat, *Sprattus sprattus*) or two (in most other clupeiformes) gas-filled bubbles, called the prootic and pterotic bullae, which are themselves connected to the swim bladder *via* a thin tube (O'Connell, 1955; Allen et al., 1976; Blaxter and Hunter, 1982). The prootic bulla is connected directly to the middle utricular epithelium by a thin 'elastic thread' (Best and Gray, 1980). Fluid motion through the fenestra (a narrow opening at the bulla–utricle interface) also physically couples the bulla and the middle utricular epithelium. Pressure waves impinging upon a clupeiform fish cause the swim bladder and auditory bullae to vibrate (Denton and Blaxter, 1979). Vibrations of the

bullae may induce movements of the middle utricular epithelium (Denton and Gray, 1979; Best and Gray, 1980), thus forming an indirect pathway transferring pressure information detected by the swim bladder or bullae to displacement information detected by the inner ear. The indirect pathway allows the ear to respond to higher frequencies than would be possible without this pathway (Blaxter and Hoss, 1981).

It was first hypothesized (Mann et al., 1998) that the prootic bulla was responsible for ultrasound detection in Clupeiformes *via* the indirect pathway described above. While the bulla may be involved in ultrasound detection, it is now clear that it is not solely responsible because, while all Clupeiformes have prootic bullae connected to a tripartite utricle (Blaxter and Hunter, 1982), not all of these species detect ultrasound (Mann et al., 2001). Thus, there clearly must be an additional specialization in those clupeoids that have been shown to detect ultrasonic frequencies.

One powerful technique for assessing the functional basis of auditory abilities has been to examine the development of structural and functional attributes together. In mammals and birds, development of the middle ear bones coincides with an expansion of detectable frequencies (Ehret and Romand, 1981; Saunders et al., 1986; Geal-Dor et al., 1993). Similarly, zebrafish (*Danio rerio*; Cypriniformes) show an increase in maximum detectable frequency coincident with development of the Weberian ossicles (Higgs et al., 2003). Atlantic herring (*Clupea harengus*; Clupeiformes) larvae show a steep increase in behavioural responsiveness to an acoustic stimulus coincident with bulla inflation (Blaxter and Batty, 1985) and also an increase in responsiveness to predatory attack as the bulla fills (Fuiman, 1989; Blaxter and Fuiman, 1990). Interestingly, there is no change in behavioural responsiveness coincident with bulla inflation in Atlantic menhaden (*Brevoortia tyrannus*; Clupeiformes) and bay anchovy (*Anchoa mitchilli*; Clupeiformes) when using a mechanical stimulus (Higgs and Fuiman, 1996, 1998), suggesting that changes seen in clupeoid predator studies were due to detection of auditory (pressure), rather than mechanosensory (displacement), information.

The purpose of the current study was to investigate the development of hearing and auditory morphology in the American shad, particularly with regard to the onset of ultrasound detection. We used a combination of behavioural, physiological and morphological techniques to show how the onset of ultrasound detection coincides with the development of apparent specializations in the utricle of American shad. We also compare the utricle of adult shad with the utricle in three other clupeoid species, one that can detect ultrasound and two that cannot, to show that the observed utricular specialization may be restricted to ultrasound-detecting species.

## Materials and methods

### *Animal care and rearing*

Day 2 posthatch American shad (*Alosa sapidissima* Wilson) larvae were obtained from the State of Maryland Cedarville Fish Hatchery and transported directly to the University of Maryland. Larvae were placed into a 208 litre elliptical tank where they were raised for the duration of this study. Larger shad used for morphological examination were obtained from our lab stock. All animals were kept at 0‰ salinity. Larvae were initially reared at 19–20°C, with temperature raised to 23–24°C at 30 mm total length (*TL*) to increase growth rate. Larvae were reared on *Artemia salina* nauplii until 30 mm, at which size they were gradually switched to dry food. All procedures used in this study were approved by the University of Maryland Institutional Animal Care and Use Committee.

### *Behaviour*

Fish were filmed approximately once per week from 2 July to 3 September 2002 (Table 1). All fish in the tank were exposed to the sound, but only those fish that were in the field of view at stimulus onset were recorded and used for data analysis. Thus, there is no way of knowing if fish were tested more than once, but, due to the apparently random distribution of fish in the tank, we do not feel this biased our results. Fish were recorded for approximately 3 s before sound presentation, 3 s during sound presentation and 3 s after sound was stopped. Images were recorded through a digital video camera (512 pixels×492 pixels, 30 frames $s^{-1}$; Sanyo Corp., San Diego, CA, USA) connected to a frame grabber (Pinnacle DC 10+) using MGI Videoware software on a PC. Video files were compressed with VirtualDub software (1:50, Intel Indeo 5.1® Codec) to reduce file size. Response was scored as positive if fish made an obvious C-start (Eaton et al., 1977) at the onset of sound presentation. A response was labelled a C-start if the flexion was faster than one video frame (33.3 ms). As the C-start is a fixed action pattern initiated by Mauthner cells (Eaton et al., 1977), the degree of body flexion does not change developmentally (Kimmel et al., 1974; Taylor and McPhail, 1985; Eaton and DiDomenico, 1986), providing an objective measure for the onset of startle responses. The proportion of

Table 1. *Date of experiment, age, total length and responsiveness of American shad larvae to 90 kHz sound presentation*

| Date | Age (dph) | Mean *TL* (mm) | *N* |
|---|---|---|---|
| 2 July 2002 | 31 | 18.0 | 31 |
| 9 July 2002 | 38 | 22.9 | 22 |
| 16 July 2002 | 45 | 25.0 | 16 |
| 23 July 2002 | 52 | 25.0 | 10 |
| 6 August 2002 | 66 | 28.0 | 3 |
| 13 August 2002 | 73 | 43.0 | 9 |
| 28 August 2002 | 88 | 46.7 | 14 |
| 3 September 2002 | 94 | 67.5 | 9 |

*N* represents the total number of fish examined to determine percent responding shown in Fig. 3.

dph, days posthatching; *TL*, total length.

fish responding in each experiment was recorded. To obtain the daily mean size of fish in the tank for each set of experiments, all fish in the field of view were measured before the first experiment of the day commenced.

Tone bursts were presented in the following order: 10 kHz, 50 kHz, 90 kHz, 120 kHz, 120 kHz, 90 kHz, 50kHz and 10 kHz. Tone bursts were narrow band around the frequency of interest with no significant energy in low-frequency ranges (see Fig. 1 for example at 90 kHz). Each frequency was played twice to ascertain possible effects of presentation order on responsiveness. Presentation order had no significant effect on the proportion of fish responding within a frequency (after arcsine square-root transformation; Zar, 1984; $P>0.05$) so responses were lumped together by frequency irrespective of presentation order. Tone bursts were generated with a function generator (182A; Wavetek, Everett, WA, USA) gated through a Hewlett-Packard pulse generator with a 2 ms rise/fall time and amplified (Techron 5507). Tone bursts were played from an ITC-1042 (International Transducer Corp., Santa Barbara, CA, USA) underwater transducer. Sound pressure levels (rms) were measured with a precalibrated hydrophone and were as follows: 10 kHz=145–150 dB re 1 μPa; 50 kHz=155–160 dB re 1 μPa; 90 kHz=145–150 dB re 1 μPa; 120 kHz= 135–140 dB re 1 μPa. Because of amplifier limitations, it was not possible to play the 120 kHz tone as loud as the other frequencies but all intensity levels were well above the threshold for hearing at each frequency (see below).

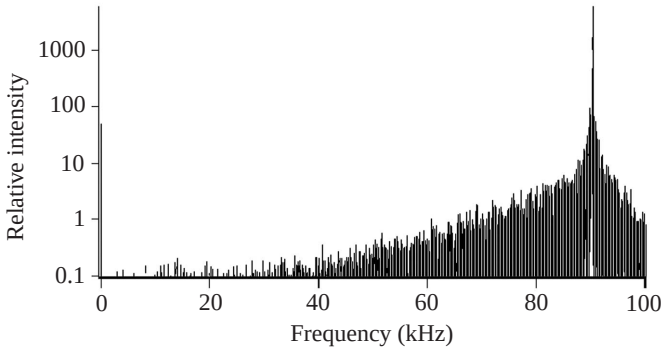


Fig. 1. Sound spectrum analysis (fast Fourier transform) of 90 kHz tone burst used in behavioural stimulation. Dominant energy is at 90 kHz, with rapidly diminishing power at lower and higher frequencies.

### *Auditory brainstem response (ABR) recording*

Due to the extreme fragility of American shad larvae, we were not able to obtain auditory brainstem recordings (ABRs) from any animals smaller than 30 mm *TL*. Thus, we cannot ascertain when ABR first occurs in response to ultrasound. We were able to determine, however, how the response changes with later development. Following the protocols of Higgs et al. (2002a, 2003), fish were held in place with a small piece of netting wrapped around the trunk. A tube with flowing water was placed into the fish's mouth to hold the head stable and to irrigate the gills. This arrangement held the fish securely in place so no muscle relaxants or anaesthetics were necessary. Fish were lowered underwater so that the top of the head was at least 5 cm under the water surface. Tone bursts of frequency 0.1 kHz, 0.2 kHz, 0.4 kHz, 0.6 kHz, 0.8 kHz, 1 kHz, 2 kHz and 4 kHz were presented *via* a UW-30 (University Sound Inc., Buchanan, MI, USA) underwater speaker placed 25 cm below the fish. Tone bursts of 40 kHz, 60 kHz, 80 kHz and 90 kHz were presented *via* an ITC-1042 underwater transducer. All tone bursts had a 10 ms duration and were gated through a Hanning window with a 2 ms rise/fall time. Tone bursts were presented at alternating starting phases of 90° and 270° to cancel stimulus artifacts in the response recordings. The speakers were connected to a McIntosh amplifier, which was in turn connected to a Tucker-Davis Technologies (TDT, Alachula, FL, USA) physiology apparatus. Tone bursts were generated using TDT SigGen software and played from the computer through the TDT system. Output levels were calibrated with a precalibrated hydrophone (InterOcean Systems, San Diego, CA, USA) for sonic frequencies and an LC-10 hydrophone for ultrasonic frequencies.

Recording of auditory responses was also accomplished through the TDT system, running TDT BioSig software and a 50 ms recording duration. A recording electrode was placed just under the skin of the fish, along the midline just dorsal to the opercular edge. A reference electrode was placed under the skin along the midline, posterior to the eyes. A ground electrode was placed in the water near the body of the fish. All electrodes (Rochester Electromedical Inc., Tampa, FL, USA) had stainless steel tips coated with fingernail polish as insulation, with the tip of the electrode remaining uninsulated. The remainder of the electrode was covered in waterproof insulation so recording underwater was not a problem. Electrode leads were connected to a TDT HS4 head stage, which was integrated into the rest of the physiological apparatus. Responses to stimulation were sent through a 60 Hz notch filter to reduce electrical artifact. For each frequency and intensity, 400 responses were averaged together. Threshold responses were defined for each frequency as the sound level at which stereotypical ABR responses were first observed (see Fig. 2A). This visual inspection method is common in ABR studies across vertebrates (Walsh et al., 1986; Hall, 1992) and gives comparable results to more statistical approaches (Mann et al., 2001; Brittan-Powell et al., 2002). For statistical comparison of threshold over development, fish were lumped into one of four size classes; 30–39 mm *TL* ($N$=6), 40–55 mm *TL* ($N$=3), 75–90 mm *TL* ($N$=10) and >100 mm *TL* ($N$=2). No fish were tested more than once. Thresholds were compared across size classes with two-way analysis of variance (ANOVA; Zar, 1984). Because of a change in calibration procedures in our laboratory, care must be taken when comparing current thresholds with those in our previous work (Mann et al., 1997, 1998, 2001). The current technique results in thresholds approximately 20–30 dB re 1 μPa lower than our previous reports. While we feel our new calibration technique may be more accurate (Higgs et al., 2002b), it does not change

the conclusions of our previous work, just the threshold numbers.

*Morphology*

For the first 100 days posthatch (100 dph; mean *TL*=32 mm), 4–5 larvae were removed from the tank every 4–7 days. Animals were anaesthetized with MS-222 (tricaine methanesulfonate; Sigma) and immediately examined under a dissecting microscope. *TL* was measured and the inflation of the auditory bulla (visible as a silver bubble when inflated) and the number and position of otoliths present were recorded. Both bulla inflation and otolith number were easily visible through the skin of larvae until at least 20 mm *TL*. After recording external development, larvae were killed with an overdose of MS-222 and immediately placed into 4% paraformaldehyde until sectioned.

After at least 24 h of fixation, animals were embedded in Immunobed (Polysciences Corp., Warrington, PA, USA) plastic resin and sectioned at 10 μm. For all size classes, sections were made in both sagittal and frontal planes. Sections were mounted on slides, dried for at least 24 h on a 40°C slide warmer, cleared, stained with cresyl violet and coverslipped with Permount (Sigma). Sections were examined and the state of utricular development was recorded. Images were captured with a digital camera (Magnafire, Optronics Inc., Goleta, CA, USA) directly connected to the microscope.

For comparative purposes, the utricles of at least three adults of American shad, gulf menhaden (*Brevoortia patronus*; Clupeiformes), bay anchovy and scaled sardine (*Harengula jaguana*; Clupeiformes) were fixed in 4% paraformaldehyde, embedded and sectioned in Immunobed and mounted onto slides. Menhaden and scaled sardine were collected from the field at Mote Marine Laboratory (Sarasota, FL, USA), and bay anchovies were collected from the field in Chesapeake Bay, MD, USA. Field-collected animals were immediately killed in MS-222 and fixed in 4% paraformaldehyde. American shad adults were obtained from our lab stock.

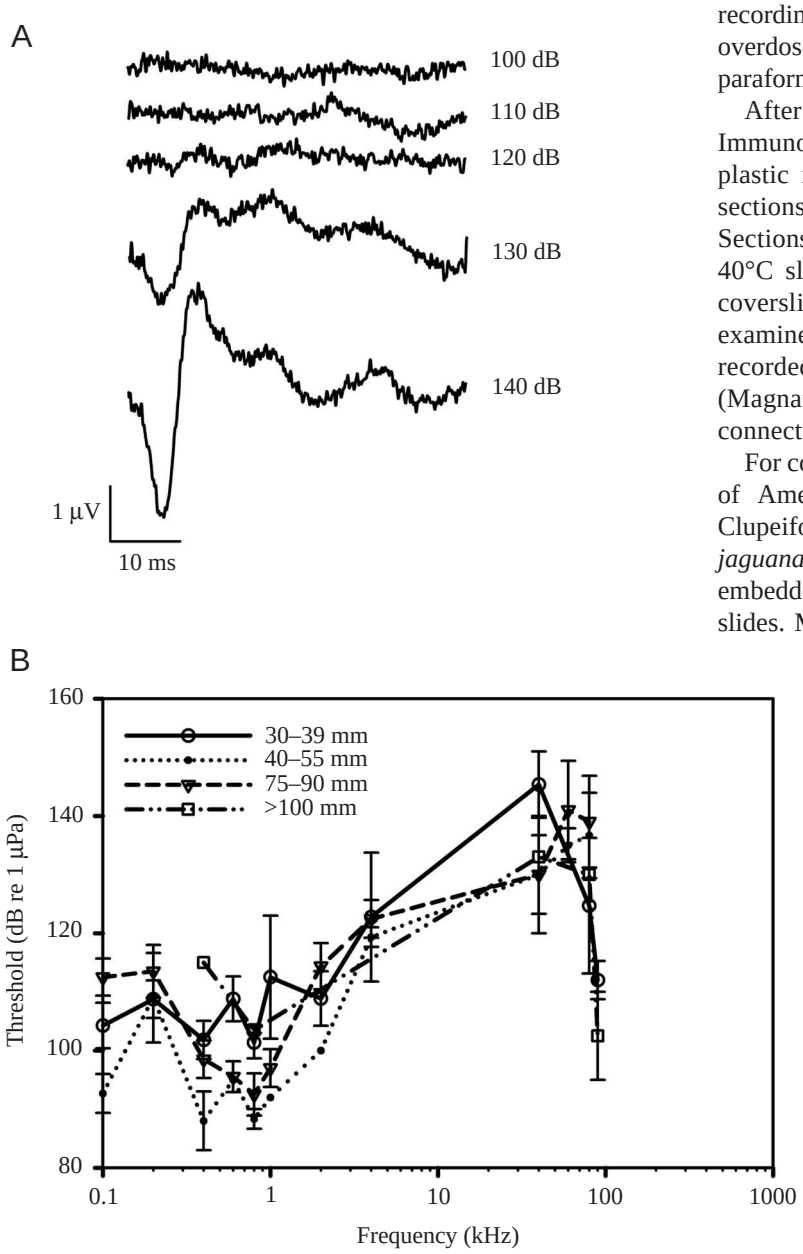


Fig. 2. (A) Example response waveform (lowpass filtered at 3000 Hz) for a 32 mm total length (*TL*) American shad juvenile to a 90 kHz tone burst. In the example trace, the response is first seen at 120 dB re 1 μPa as a characteristic dip and rise approximately 6 ms after the beginning of sound presentation. The response increases in amplitude and decreases in latency as sound intensity increases. Sound levels are expressed in dB re 1 μPa. Response waveforms were similar at all stimulus frequencies except 100 Hz and 200 Hz, which had more peaks. (B) Auditory brainstem response thresholds in American shad larvae (30–39 mm and 40–55 mm *TL*), juveniles (75–90 mm *TL*) and adults (>100 mm *TL*) in response to tone bursts from 0.1 kHz to 90 kHz.

## Results

*Behaviour*

Shad larvae never showed evasive responses to 10 kHz, 50 kHz or 120 kHz stimulation, even up to 70 mm *TL*, only responding to 90 kHz stimulation. From 18 mm to 25 mm mean *TL*, only 10–40% of the fish responded to the 90 kHz sound presentation but by ≥28 mm *TL* 70–100% of the fish responded (Fig. 3). Thus, the period between 25 mm and 28 mm *TL* appears to be a critical window in the development of ultrasound detection.

*Physiology*

By 30 mm *TL*, the smallest size that we were able to test with ABR, all fish responded behaviourally to 90 kHz. We also found that all fish ≥30 mm *TL* responded to ultrasound at the level of ABR (Fig. 2B). There was no significant difference in auditory threshold ($P$=0.5, $N$=21 for size class main effect) for fish from 30 mm to

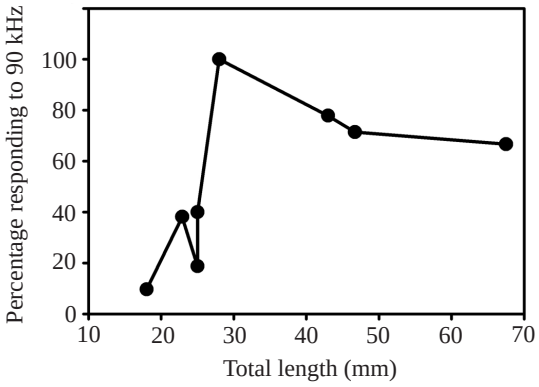


Fig. 3. The percentage of fish visible on video recordings that responded to 90 kHz sound presentation over development.

>100 mm *TL*. In the 30–39 mm size class, all fish responded to tone bursts from 0.1 kHz to 90 kHz. From 0.1 kHz to 4 kHz, the threshold was in the range of 101–122 dB re 1 μPa, while in the ultrasonic frequencies, (40–90 kHz) threshold for 30–39 mm fish was in the range of 112–145 dB re 1 μPa. This was similar to the values for the larger fish (>75 mm *TL*), who had a range of sonic (0.1–4 kHz) thresholds of 92–115 dB re 1 μPa and ultrasonic thresholds of 102–135 dB re 1 μPa (Fig. 2B).

### *Auditory structure*

In adult American shad, the prootic bulla sits directly in front of the utricle (Fig. 4A). The bulla is subdivided into a gas-filled and a fluid-filled portion by a flexible bulla membrane (Fig. 4B,C). The bulla membrane is directly connected to the anterior portion of the middle macula of the utricle by an 'elastic thread' (*sensu* Denton and Gray, 1979; Fig. 4B,C). The utricular middle macula in adults is suspended from the rest of the utricle by a thin connection approximately one cell layer thick (Fig. 4B,C,G).

The utricular epithelium of newly hatched shad larvae is continuous, with no division into anterior, posterior and middle maculae, a situation that continued until at least 12.5 mm *TL* (Fig. 4D). By 16 mm *TL*, the utricle first shows division into a tripartite structure (Fig. 4E). At 16 mm *TL*, the three portions of the utricular maculae could be differentiated from one another but the three components were still well connected (Fig. 4E). The connection between the middle macula and the rest of the ear continued to thin so that by 26 mm *TL* the middle macula was connected to the anterior and posterior portions of the utricle by a sheet of cells approximately one cell layer thick (Fig. 4F) and looked similar to the utricular suspension seen in adults (Fig. 4G).

Shad larvae hatch with no evidence of an auditory bulla and never show bulla inflation up to 11 mm *TL* (Fig. 5). By 12 mm *TL*, the prootic bulla begins to inflate and at ≥13 mm, all larvae examined had inflated prootic bullae (Fig. 5).

At hatching, shad larvae have two otoliths in each ear, the lapillus and sagitta, which overlay the utricular and saccular epithelia, respectively (Fig. 5). There is no sign of an asteriscus (lagenar otolith) until 15 mm, at which point ~30% of the larvae examined had asteriscus (Fig. 5). By 18 mm, all larvae had asteriscus (Fig. 5) overlying a small lagenar epithelium.

On a comparative level, the utricle of adult American shad is similar to that of gulf menhaden but different from those of bay anchovy or scaled sardine (Fig. 6). The middle macula of both shad and menhaden is very loosely attached to the rest of the utricle, connected only by tissue approximately one cell layer thick (Fig. 6A,B). By contrast, both anchovy and sardine middle maculae have a firmer base of attachment to the rest of the utricle (Fig. 6C,D) and resemble the situation seen in shad larvae (Fig. 4E) more than in adult shad (Fig. 6A).

## Discussion

The enhanced responsiveness to 90 kHz stimulation in both behavioural and physiological experiments suggests an enhanced relevance of this frequency to shad. The behavioural responses to 90 kHz stimulation were clearly startle responses, with a characteristic C-start (Eaton et al., 1977) and apparent increases in swimming speed. That shad never responded behaviourally to frequencies other than 90 kHz, even though ABR showed the ear could clearly detect 10 kHz and 50 kHz tone bursts, suggests that these other frequencies did not startle the juvenile shad. Based on the ABR results, shad also seemed especially sensitive to the 90 kHz relative to lower frequency ultrasonic tones. The enhanced sensitivity, both behavioural and physiological, fits in well with previous results. Plachta and Popper (2003) found enhanced behavioural responsiveness in adult shad to frequencies of 70–110 kHz, with 90 kHz eliciting the most responses and <70 kHz or >110 kHz showing little response. Plachta and Popper (D.T.T.P. and A.N.P., unpublished data) found many single units in the shad that were especially responsive to 90 kHz as well.

It has been previously hypothesized (Mann et al., 1998, 2001; Astrup, 1999) that the response of American shad to ultrasonic frequencies is an adaptation for avoiding predation by echolocating odontocetes. The current results add some support to this hypothesis, although the role of odontocete clicks in shad escape behaviour has yet to be explicitly tested. Pacific herring (*Clupea pallasi*) show evasive responses to a simulated echolocation click (Wilson and Dill, 2002), although due to the broadband nature of the stimulus used it is not clear whether or not they respond to ultrasonic frequencies. While there are species- and habitat-specific differences in frequencies used in echolocation signals, many odontocetes emit echolocation clicks with a peak frequency of 90–100 kHz (Au, 2000). It may be this component of echolocation signals that has driven the evolution of shad ultrasound detection, although more experiments must be conducted before this can be determined.

Once developing shad could detect sounds, their sensitivity to these sounds as measured by ABR did not change developmentally. There have been only a few studies on the development of auditory sensitivity in fish, with the results varying between species. In rays (*Raja clavata*; Corwin, 1983)

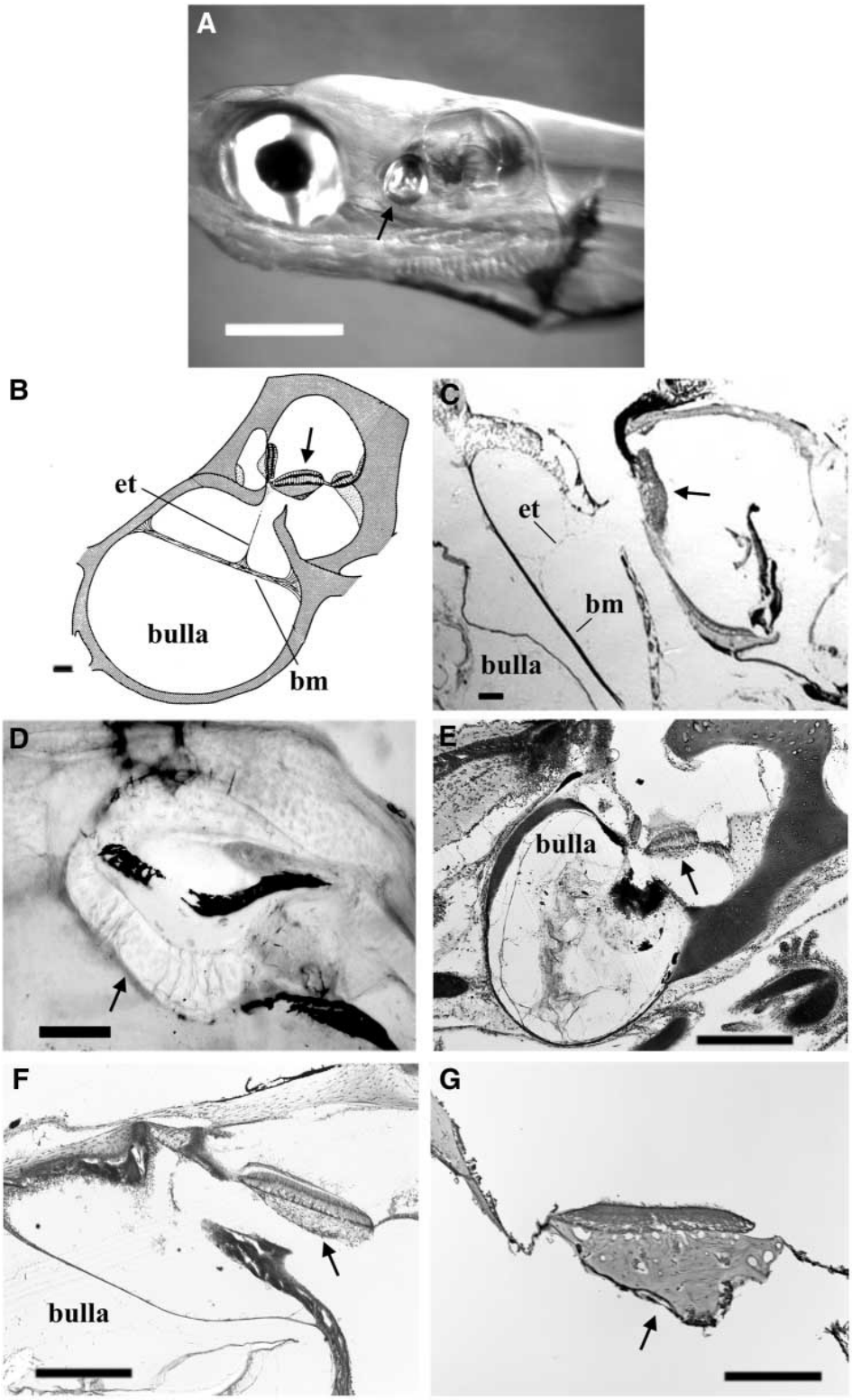


Fig. 4. (A) Relationship of the prootic bulla to the inner ear of American shad, as demonstrated in a 12.5 mm *TL* larva. The prootic bulla (arrow) sits just anterior to the utricle. (B) Diagram (modified from Denton and Gray, 1979) and (C) transverse section showing the relationship of the prootic bulla to the utricle in adult American shad. The bulla is connected to the middle macula (arrow) of the utricle by an 'elastic thread' (as defined in Denton and Gray, 1979) connected to the bullar membrane. et, elastic thread; bm, bullar membrane. Sections through the utricle of (D) 12 mm *TL*, (E) 16.5 mm *TL*, (F) 26 mm *TL* and (G) adult American shad. Arrows in B, C, E, F, G and H represent the middle utricular epithelium. Scale bar in A=1 mm, in B=100 μm and in C,E–H=10 μm. Orientation of plates B–G is as shown in A (anterior is to the left and dorsal up in all cases).

and damselfish (*Pomacentrus partitus*; Kenyon, 1996), auditory sensitivity (as measured by auditory nerve recordings) improves (threshold decreases) with development, perhaps due to increases in the number of sensory hair cells in the ear. In gouramis (*Trichopsis vittata*; Wysocki and Ladich, 2001) and red sea bream (*Pagrus major*; Iwashita et al., 1999), small changes in sensitivity are seen for some frequencies, although the changes are not consistent developmentally. In goldfish (*Carassius auratus*; Popper, 1971) and zebrafish (Higgs et al., 2002a, 2003), there is no change in auditory sensitivity with development, even though there are significant increases in hair cell number (Higgs et al., 2002a, 2003).

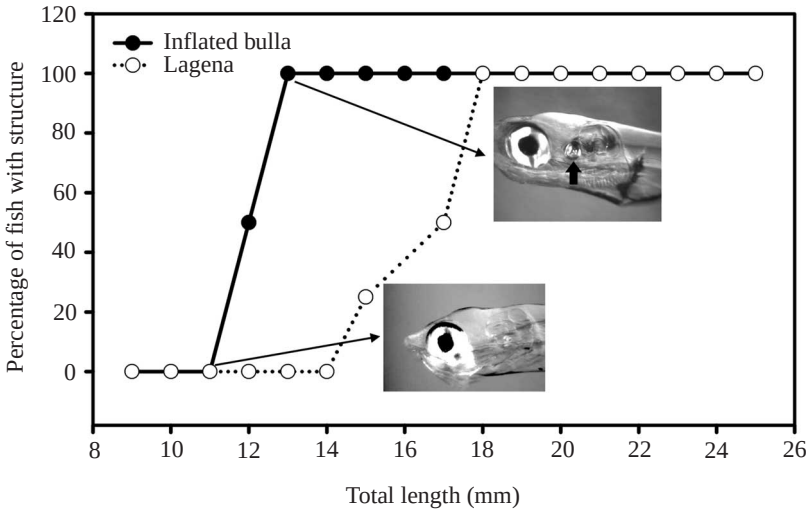


Fig. 5. The percentage of American shad larvae with a fully inflated bulla (*N*=5 for each data point, solid line) or a lagenar otolith (*N*=7–10 for each data point, broken line) over development (all larvae had utricular and saccular otoliths at hatching). Inset photographs show a 9 mm larva with no auditory bulla and only two otoliths per ear and a 14 mm larva with a filled auditory bulla (thick arrow).

We have previously hypothesized (Higgs et al., 2003) that in those species possessing an indirect pathway for pressure information to reach the ear, functional auditory development may be more constrained by the conductive pathway of sound to the ear than by the sensorineural pathway of hair cell addition. The results of the current study lend support to this hypothesis. Once the bullae are filled in American shad, there is no change in auditory sensitivity. In Atlantic herring, the pressure sensitivity of bullar movements does not change once the bulla is filled (Blaxter et al., 1981), thus delivering the same amount of stimulation to the utricular maculae for a given pressure regardless of fish size. The same mechanism should also be working in American shad. A given sound pressure should move the bulla the same amount, regardless of fish size. If this is the mechanism of higher frequency sound detection in shad, as has been shown for other Clupeiformes (Blaxter and Hoss, 1981), then one should expect no change in auditory sensitivity with shad development once the bullae are filled, as was shown by our ABR results.

Bulla inflation was complete by 14 mm, much before the size at which animals responded to ultrasound (26 mm). This strongly suggests that an inflated bulla is not sufficient itself for ultrasound detection. Also, other clupeoid fishes with prootic bullae [bay anchovy, scaled sardine and Spanish sardine (*Sardinella aurita*); Clupeiformes] do not detect ultrasound (Mann et al., 2001) so the presence of an inflated bulla is not sufficient for ultrasound detection. This does not mean that the auditory bullae play no role in ultrasound detection. Indeed, we suspect the bullae are an important part of the ultrasonic pathway but the results do lead us to suggest that more than just an auditory bulla is necessary to allow detection of ultrasonic frequencies.

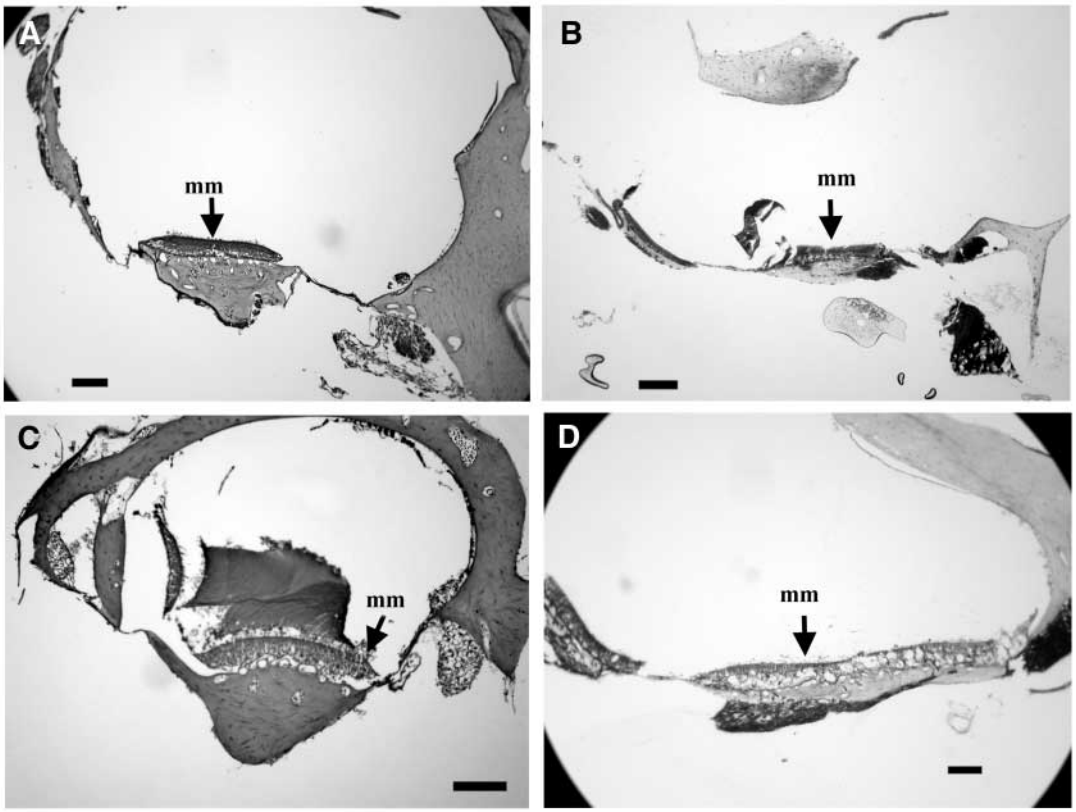


Fig. 6. Structure of the middle utricular macula (mm) in adult (A) American shad, (B) Gulf menhaden, (C) bay anchovy and (D) scaled sardine. Shad and menhaden (A,B) can detect ultrasound while anchovy and sardine (C,D) cannot.

While we did not expect the lagena to play a role in ultrasound detection, the frequency responses of the individual auditory end organs of fish are still too poorly understood to rule this out *a priori*. The shad lagenae first develop at 18 mm, but ultrasound detection did not begin until 26 mm. Thus, the lagena is not a limiting factor in ultrasound detection and it is doubtful that lagenar specializations play a role in this ability.

While still not definitive proof, development of utricular specializations was coincident with the onset of behavioural responsiveness to 90 kHz stimulation. The connection between the middle macula and the rest of the utricle gradually thinned so that by 26–35 mm the connection became as thin as that seen in the adult. It was during this size range that responsiveness to 90 kHz first began. Examination of this utricular specialization in adults of other species in Clupeiformes lends further support to this hypothesis. The two species (shad and menhaden) that have been shown in previous studies to respond to ultrasound (Mann et al., 2001) have a very thin connection between the middle macula and the rest of the epithelium, while those two species that are not responsive to ultrasound (bay anchovy and scaled sardine) have a much more robust utricular suspension. Before the role of the utricular suspension in shad ultrasound detection can be definitively proven, single- or at least multi-unit recordings must be made from the different utricular epithelia of clupeoid fishes. Despite this caveat, we would argue that the correlation between utricular structure and response to ultrasound is highly supportive of this hypothesis.

We posit the following hypothesis for ultrasound detection in American shad. As sound waves impinge upon the air-filled bulla, the sound causes the air-filled chambers to move. This results in vibration of the middle epithelium due to the motion of the fluids in the chamber and the direct connection to the bullar membrane *via* the elastic thread (Denton and Gray 1979; Denton et al., 1979). While it was previously thought that movement of the middle macula decreased rapidly for frequencies greater than 1 kHz (Denton et al., 1979), recent data obtained using a noninvasive vibration measurement technique originally developed by Rogers and Hastings (1989) show that movement of the bulla, and therefore presumably of the middle macula, resumes at frequencies above ~40 kHz (M. C. Hastings, unpublished data). The looser connection of the middle epithelium found in American shad and menhaden, as compared with species that do not detect ultrasound, may allow higher sensitivity to bullar vibrations due to its greater freedom of movement.

In effect, one can regard the connection between the middle epithelium and the rest of the ear as a spring-like mechanism. Oscillations of the bullar membrane would alternately pull the middle epithelium towards the auditory bulla and then push it back toward the otolith *via* the elastic thread connection and impinging oscillatory movement of the perilymph through the fenestra at the bulla–utricle interface. The structure overlying the epithelium should stay stable during these bullar vibrations, and the relative movement between the otolith or cupula and the hair cell epithelium would result in depolarizations of the hair cells. The extremely thin connection between the middle epithelium and the rest of the utricle is essentially a spring with miniscule mass that would require little energy to stretch, thus making this epithelium more sensitive to vibrations at ultrasonic frequencies. In essence, the system may resemble a very crude place-type mechanism, whereby part or all of the middle epithelium in ultrasound-detecting Clupeiformes responds to ultrasound due to possible changes in stiffness along its length associated with the variations in thickness, just as part of the basilar membrane responds to ultrasound in bats and dolphins (Echteler et al., 1994). The response of such a spring-controlled system would be ‘flat’ over its frequency bandwidth so that the sensitivity to ultrasound should be about the same at all frequencies to which the bulla responds. Indeed, Mann et al. (1997, 2001) demonstrated that hearing sensitivity in ultrasound-detecting species is about the same from 40 kHz to >100 kHz. Our hypothesis leaves the enhanced sensitivity we found at 90 kHz unexplained (Mann et al., 1997, 2001 did not test 90 kHz tone bursts), so finer scale analyses of bullar vibration need to be conducted to determine if there is a bullar resonance around 90 kHz.

If our hypothesis for the mechanism of ultrasound detection is correct, one can then suggest a relatively direct path to the evolution of ultrasound detection. Many freshwater fishes show hearing specializations that are thought to have arisen to enhance detection of higher frequencies in shallow waters, where low frequencies propagate only very short distances (Rogers and Cox, 1988). While it is not known where clupeoid fishes arose, it is likely that they did evolve in freshwater, and thus development of higher frequency hearing up to 4 kHz, as found in all species in this group (Mann et al., 2001), may be associated with hearing in shallow waters. Once these species invaded the oceans and were subject to selective pressures imposed by echolocating dolphins, some clupeoids may have evolved a simple change in the thickness of the middle sensory epithelium in the utricle, thereby facilitating detection of sounds at higher frequencies than in other related species. In effect, the middle epithelium was ‘preadapted’ for hearing higher and higher frequencies needed for ultrasound detection.

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## References

**Allen, J. M., Blaxter, J. H. S. and Denton, E. J.** (1976). The functional anatomy and development of the swimbladder–inner ear–lateral line system in herring and sprat. *J. Mar. Biol. Assoc. UK* **56**, 471-486.

**Astrup, J.** (1999). Ultrasound detection in fish – a parallel to the sonar-mediated detection of bats by ultrasound-sensitive insects? *Comp. Biochem. Physiol. A* **124**, 19-27.

**Astrup, J. and Møhl, B.** (1993). Detection of intense ultrasound by the cod *Gadus morhua*. *J. Exp. Biol.* **182**, 71-80.

**Astrup, J. and Møhl, B.** (1998). Discrimination between high and low repetition rates of ultrasonic pulses by the cod. *J. Fish Biol.* **52**, 205-208.
**Au, W. W. L.** (2000). Echolocation in dolphins. In *Hearing by Whales and Dolphins* (ed. W. W. L. Au, A. N. Popper and R. R. Fay), pp. 364-408. New York: Springer-Verlag.
**Best, A. C. G. and Gray, J. A. B.** (1980). Morphology of the utricular recess in the sprat. *J. Mar. Biol. Assoc. UK* **60**, 703-715.
**Blaxter, J. H. S. and Hoss, D. E.** (1981). Startle response in herring: the effect of sound stimulus frequency, size of fish and selective interference with the acoustico-lateralis system. *J. Mar. Biol. Assoc. UK* **61**, 871-879.
**Blaxter, J. H. S. and Hunter, J. R.** (1982). The biology of the clupeoid fishes. *Adv. Mar. Biol.* **20**, 1-223.
**Blaxter, J. H. S. and Batty, R. S.** (1985). The development of startle responses in herring larvae. *J. Mar. Biol. Assoc. UK* **65**, 737-750.
**Blaxter, J. H. S. and Fuiman, L. A.** (1990). The role of the sensory systems of herring larvae in evading predatory fishes. *J. Mar. Biol. Assoc. UK* **70**, 413-427.
**Blaxter, J. H. S., Denton, E. J. and Gray, J. A. B.** (1981). The auditory bullae-swimbladder system in late stage herring larvae. *J. Mar. Biol. Assoc. UK* **61**, 315-326.
**Brittan-Powell, E. F., Dooling, R. J. and Gleich, O.** (2002). Auditory brainstem responses in adult budgerigars (*Melopsittacus undulatus*). *J. Acoust. Soc. Am.* **112**, 999-1008.
**Corwin, J. T.** (1983). Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: continual increases in hair cell number, neural convergence, and physiological sensitivity. *J. Comp. Neurol.* **217**, 345-356.
**Denton, E. J. and Blaxter, J. H. S.** (1979). The mechanical relationships between the clupeoid swimbladder, inner ear and lateral line. *J. Mar. Biol. Assoc. UK* **56**, 787-807.
**Denton, E. J. and Gray, J. A. B.** (1979). The analysis of sound by the sprat ear. *Nature* **282**, 406-407.
**Denton, E. J., Gray, J. A. B. and Blaxter, J. H. S.** (1979). The mechanics of the clupeid acoustico-lateralis system: frequency responses. *J. Mar. Biol. Assoc. UK* **59**, 27-47.
**Eaton, R. C., Bombardieri, R. A. and Meyer, D. L.** (1977). The mauthner-initiated startle response in teleost fish. *J. Exp. Biol.* **66**, 65-81.
**Eaton, R. C. and DiDomenico, R.** (1986). Role of the teleost escape response during development. *Trans. Am. Fish. Soc.* **115**, 128-142.
**Echteler, S. M., Fay, R. R. and Popper, A. N.** (1994). Structure of the mammalian cochlea. In *Comparative Hearing: Mammals* (ed. R. R. Fay and A. N. Popper), pp. 134-171. New York: Springer-Verlag.
**Ehret, G. and Romand, R.** (1981). Postnatal development of absolute auditory thresholds in kittens. *J. Comp. Physiol. Psychol.* **95**, 304-311.
**Fay, R. R. and Popper, A. N.** (1974). Acoustic stimulation of the ear of the goldfish (*Carassius auratus*). *J. Exp. Biol.* **61**, 243-260.
**Fletcher, L. B. and Crawford, J. D.** (2001). Acoustic detection by sound-producing fishes (Mormyridae): the role of gas-filled tympanic bladders. *J. Exp. Biol.* **204**, 175-183.
**Fuiman, L. A.** (1989). Vulnerability of Atlantic herring larvae to predation by yearling herring. *Mar. Ecol. Prog. Ser.* **51**, 291-299.
**Geal-Dor, M., Freeman, S., Li, G. and Sohmer, H.** (1993). Development of hearing in neonatal rats: air and bone conducted ABR thresholds. *Hear. Res.* **69**, 236-242.
**Grinnell, A. D.** (1995). Hearing in bats: an overview. In *Hearing by Bats* (ed. A. N. Popper and R. R. Fay), pp. 1-36. New York: Springer-Verlag.
**Hall, J. W.** (1992). *Handbook of Auditory Evoked Responses.* Boston: Allyn and Bacon.
**Higgs, D. M. and Fuiman, L. A.** (1996). Ontogeny of visual and mechanosensory structure and function in Atlantic menhaden *Brevoortia tyrannus. J. Exp. Biol.* **199**, 2619-2629.
**Higgs, D. M. and Fuiman, L. A.** (1998). Associations between behavioral ontogeny and habitat shifts of clupeoid larvae. *J. Mar. Biol. Assoc. UK* **78**, 1281-1294.
**Higgs, D. M., Souza, M. J., Wilkins, H. R., Presson, J. C. and Popper, A. N.** (2002a). Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). *JARO* **3**, 174-184.
**Higgs, D. M., Souza, M. J., Wilkins, H. R., Presson, J. C. and Popper, A. N.** (2002b). Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). Erratum. *JARO* **3**, 222.
**Higgs, D. M., Rollo, A. K., Souza, M. J. and Popper, A. N.** (2003). Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *J. Acoust. Soc. Am.* **113**, 1145-1154.
**Iwashita, A., Sakamoto, M., Kojima, T., Watanabe, Y. and Soeda, H.** (1999). Growth effects on the auditory threshold of red sea bream. *Nippon Suisan Gakkaishi* **65**, 833-838.
**Kenyon, T. N.** (1996). Ontogenetic changes in the auditory sensitivity of damselfishes (Pomacentridae). *J. Comp. Physiol. A* **179**, 553-561.
**Kimmel, C. B., Patterson, J. and Kimmel, R. O.** (1974). The development and behavioral characteristics of the startle response in the zebra fish. *Dev. Psychobiol.* **7**, 47-60.
**Mann, D. A., Lu, Z. and Popper, A. N.** (1997). A clupeid fish can detect ultrasound. *Nature* **389**, 341.
**Mann, D. A., Lu, Z., Hastings, M. C. and Popper, A. N.** (1998). Detection of ultrasonic tones and simulated dolphin echolocation clicks by a teleost fish, the American shad (*Alosa sapidissima*). *J. Acoust. Soc. Am.* **104**, 562-568.
**Mann, D., Higgs, D., Tavolga, W., Souza, M. and Popper, A. N.** (2001). Ultrasound detection by clupeiform fishes. *J. Acoust. Soc. Am.* **109**, 3048-3054.
**Nachtagall, P. E., Lemonds, D. W. and Roitblat, H. L.** (2000). Psychoacoustic studies of dolphin and whale hearing. In *Hearing by Whales and Dolphins* (ed. W. W. L. Au, A. N. Popper and R. R. Fay), pp. 330-363. New York: Springer-Verlag.
**Nestler, J. M., Ploskey, G. R., Pickens, J., Menezes, J. and Schilt, C.** (1992). Responses of blueback herring to high-frequency sound and implications for reducing entrainment at hydropower dams. *N. Am. J. Fish. Manag.* **12**, 667-683.
**O'Connell, C.** (1955). The gas bladder and its relation to the inner ear in *Sardinops caerulea* and *Engraulis mordax. Fish. Bull. US* **56**, 501-533.
**Plachta, D. T. T. and Popper, A. N.** (2003). Evasive responses of American shad (*Alosa sapidissima*) to ultrasonic stimuli. *Acoust. Res. Lett. Online* **4**, 25-30.
**Platt, C.** (1984). Cellular bases for gravistatic reception by invertebrates and vertebrates. In *Comparative Physiology of Sensory Systems* (ed. L. Bolis, R. D. Keynes and S. H. P. Maddrell), pp. 563-586. Cambridge: Cambridge University Press.
**Popper, A. N.** (1971). The effects of fish size on auditory capacities of the goldfish. *J. Aud. Res.* **XI**, 239-247.
**Rogers, P. H. and Cox, M.** (1988). Underwater sound as a biological stimulus. In *Sensory Biology of Aquatic Animals* (ed. J. Atema, R. R. Fay, A. N. Popper and W. N. Tavolga), pp. 131-149. New York: Springer-Verlag.
**Rogers, P. H. and Hastings, M. C.** (1989). Non-invasive vibration measurement system and method for measuring amplitude of vibration of tissue in an object being investigated. *US Patent No. 4 819 643*. 11 April 1989.
**Ross, Q. E., Dunning, D. J., Thorne, R., Menezes, J. K., Tiller, G. W. and Watson, J. K.** (1993). Response of alewives to high-frequency sound at a power plant intake on lake Ontario. *N. Am. J. Fish. Manag.* **13**, 291-303.
**Ross, Q. E., Dunning, D. J., Menezes, J. K., Kenna, M. J., Jr and Tiller, G.** (1996). Reducing impingement of alewives with high frequency sound at a power plant intake on lake Ontario. *N. Am. J. Fish. Manag.* **16**, 548-559.
**Sales G. and Pye, D.** (1974).*Ultrasonic Communication by Animals.* London: Chapman and Hall.
**Saunders, J. C., Relkin, E. M., Rosowski, J. J. and Bahl, C.** (1986). Changes in middle-ear admittance during postnatal auditory development in chicks. *Hear. Res.* **24**, 227-235.
**Taylor, E. B. and McPhail, J. D.** (1985). Ontogeny of the startle response in young coho salmon *Oncorhynchus kisutch. Trans. Am. Fish. Soc.* **114**, 552-557.
**Tracy, H. C.** (1920). The membranous labyrinth and its relation to the precoelomic diverticulum of the swimbladder in clupeoids. *J. Comp. Neurol.* **31**, 219-257.
**von Frisch, K.** (1938). The sense of hearing in fish. *Nature* **141**, 8-11.
**Walsh, E. J., McGee, J. and Javel, E.** (1986). Development of auditory-evoked potentials in the cat. I. Onset of response and development of sensitivity. *J. Acoust. Soc. Am.* **79**, 712-724.
**Wilson, B. and Dill, L. M.** (2002). Pacific herring respond to simulated odontocete echolocation sounds. *Can. J. Fish. Aquat. Sci.* **59**, 542-553.
**Wysocki, L. E. and Ladich, F.** (2001). The ontogenetic development of auditory sensitivity, vocalization and acoustic communication in the labyrinth fish *Trichopsis vittata. J. Comp. Physiol. A* **187**, 177-187.
**Yan, H. Y. and Curtsinger, W. S.** (2000). The otic gasbladder as an ancillary auditory structure in a mormyrid fish. *J. Comp. Physiol. A* **186**, 595-602.
**Zar, J. H.** (1984). *Biostatistical Analysis.* Second edition. Englewood Cliffs, NJ: Prentice-Hall.