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Development of the acoustically evoked behavioral response in zebrafish to pure tones

David G. Zeddies[1,*] and Richard R. Fay[1,2,†]

[1]*Parmly Hearing Institute, Loyola University – Chicago, Chicago, Illinois, USA* and [2]*Department of Psychology, Loyola University – Chicago, Chicago, Illinois*

*Present address: Center for Comparative and Evolutionary Biology of Hearing, University of Maryland, College Park, MD 20742, USA
†Author for correspondence (e-mail: rfay@luc.edu)

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Summary

Zebrafish (*Danio rerio*) were placed in small wells that could be driven vertically with a series of calibrated sinusoids. Video images of the fish were obtained and analyzed to determine the levels and frequencies at which the fish responded to the stimulus tones. It was found that fish 4 days post fertilization (dpf) did not respond to the stimulus tones, whereas fish 5 dpf to adult did respond. It was further found that the stimulus thresholds and frequency bandwidth to which the fish responded did not change from 5 dpf to adult; indicating that the otolithic organ adaptations for high-frequency hearing are already present in larval fish. Deflating the swimbladders in adult fish eliminated their response, which is consistent with sensing sound pressure. Deflating the swimbladder in larval fish did not affect their thresholds, which is consistent with sensing the particle motion of the fluid directly. Because adult fish with Weberian ossicles have a greater input to the inner ear for a given sound pressure level (SPL), the finding that the adult and larval fish respond at the same SPL with intact swimbladders suggests that the acoustic startle response threshold is adjusted as the fish develop in order to maintain appropriate reactions to relevant stimuli.

Key words: otolith organs, hearing, swimbladder, Weberian ossicles, escape response, *Danio rerio*, zebrafish.

Introduction

Psychophysical studies suggest that goldfish have a rich and complex sense of hearing that shares many features with other vertebrates, including humans (for reviews see Fay, 1988; Fay and Simmons, 1998). Although goldfish have proven to be a productive model for studying hearing, it is zebrafish that are the more widely used model organism in general. Zebrafish are especially popular for developmental studies, due, in large part, to the fact that the embryos and early larval stages are transparent (Nüsslein-Volhard et al., 2002). Much has been learned about vertebrate development using zebrafish, and their popularity continues to grow now that many tools of molecular biology are being applied.

With regard to hearing ability, fish are often categorized either as 'hearing generalists' or 'hearing specialists' with the distinction being that hearing specialists have morphological adaptations that aid in the detection of sound pressure (Popper and Fay, 1998). The ear of the hearing generalist is thought to function as an accelerometer responding directly to the particle motion of the sound field (de Vries, 1950; Fay and Olsho, 1979), whereas the specialist ear receives acoustic energy that has been re-radiated from the swimbladder or a nearby bubble (von Frisch, 1938). Because the gas in the swimbladder (or other bubble) is compressible the volume changes with sound pressure and the energy radiated to the ear is proportional to pressure.

Zebrafish, like goldfish, are otophysan hearing specialists that have specialized bones known as Weberian ossicles that mechanically connect the swimbladder to the sacculi (the end organs involved in hearing). The inner ear anatomy of zebrafish is also similar to goldfish (Platt, 1993), and leads to the expectation that zebrafish will have similar hearing capabilities as goldfish. Recent evidence is consistent with this view. Higgs et al. (2002) measured the auditory brainstem response (ABR) of juvenile (the smallest fish were 25 mm) and adult zebrafish, and adult goldfish. They found that zebrafish had thresholds similar to goldfish, the same bandwidth (hearing range; 100–4000 Hz), and the same best frequency (800 Hz). Higgs et al. (2002) also showed that despite the fact that hair cells are continuously being added to the sensory epithelium during growth, there was no change in threshold, bandwidth, or best frequency during this developmental period for zebrafish. In another study on smaller zebrafish, however, Higgs et al. (2003) found that the highest frequency at which an ABR measurement could be obtained increased linearly as the fish developed from 10–13 mm (200 Hz) to >25 mm (4000 Hz), a development pattern they correlated with the development of the Weberian ossicles. Other studies on other species of fish have also found changes in auditory sensitivity in developing fish (Atlantic herring: *Clupea harengus*, Blaxter

and Hoss, 1981; Damselfishes: *Pomacentridae*, Kenyon, 1996; Croaking gonrami: *Trichopsis vittata*, Wysocki and Ladich, 2001).

It is known that larval zebrafish perform a startle response in reaction to sudden acoustic stimuli at an early stage when the fish begin free swimming (typically 5 days post fertilization, dpf) and they are approximately 3.5 mm in length (e.g. Kimmel et al., 1974). A swimming response, indistinguishable from the startle response, can be evoked by touch at an earlier age (2 dpf) indicating that the appearance of the acoustic startle response is probably not limited by motor development (Kimmel et al., 1974). The appearance of the acoustic startle response does coincide with the development of morphological specializations for hearing, including calcification of the otoliths and inflation of the swimbladder (Eaton and DiDomenico, 1986). The characteristics of the acoustic stimuli to which larval zebrafish are responsive, however, have not been studied quantitatively. Here we use acoustically evoked behavioral responses (AEBRs) to follow the development of sound sensitivity by determining the levels and frequencies to which larval and adult zebrafish respond.

Materials and methods

Animal supply

Wild-type AB zebrafish (*Danio rerio* Hamilton 1822) were maintained at 28°C in a colony at Northwestern University in the laboratory of Joseph Takahashi. For breeding, one female and one male (typically) adult fish were placed in breeding tanks at night and the eggs collected the following morning. Eggs were washed in 'egg water' (Westerfield, 1994) and kept in small beakers. Hatching invariably occurred at 2 days post fertilization (2 dpf). Larvae were then raised in 2 l plastic tanks. (All procedures at 28°C.) For experiments, batches of fish were transported to Loyola University Chicago where they were kept in similar 2 l plastic tanks at 28°C. Testing usually took place the day after transporting, but some were tested up to 3 days after transporting and none were tested on the same day as transporting. The youngest animals tested were 4 dpf and the oldest were 8 months. For experiments, larval fish were transferred to the testing chamber using a pipette; adult fish were transferred by net. Except in the case of swimbladder deflation, the fish were not anaesthetized before or during the experiment. Animal rearing and experimental procedures were approved by the Animal Care and Use Committees at both Northwestern University and Loyola University Chicago.

Experimental apparatus

Controlled vibratory stimuli were delivered to a platform made of qtr inch thick translucent plastic by a vertically oriented Bruel & Kjaer Type 4810 shaker (Fig. 1). Fish were placed in 12 mm diameter wells (standard 24-well polystyrene tissue-culture dishes; Costar/Corning, Corning, NY, USA), that were secured to the plastic platform with clips and screws. Eight larval fish were used in an experiment, with one fish placed in each of the central eight wells. A Tucker-Davis Technologies (TDT Inc, Gainesville, FL, USA) System 3 was used to control the shaker and also to monitor platform movement through an accelerometer. The TDT System 3 was used to generate single-tone stimuli that were then amplified by a Crown D-75A power amplifier. To attenuate amplifier noise and match impedance with the Bruel & Kjaer shaker, the output of the amplifier was connected to a circuit in which a 25 Ω power resistor was placed in parallel with the series combination of a 4 Ω power resistor and the shaker.

A custom-designed graphical user interface (GUI) was created in Matlab v6 (Mathworks) to control the TDT System 3 *via* Active-X. Video sequences during the experiments were digitally captured in the Matlab GUI from the output of a Panasonic wv-BP330 black and white video camera connected to a Videum VO video-capture board (Winnov LP, Sunnyvale, CA). Active-X software was used to control the video-capture board from Matlab (ActiVideo, Inc, Laguna Hills, CA, USA).

Stimuli

Stimuli consisted of sinusoids of 120 ms duration with 20 ms cosine squared rise-fall times. The stimulus frequencies were 100, 150, 200, 300, 400, 600, 800 and 1200 Hz. Because greater current is required at higher frequencies to produce a given displacement, 1200 Hz was the highest frequency that we could test using the shaker.

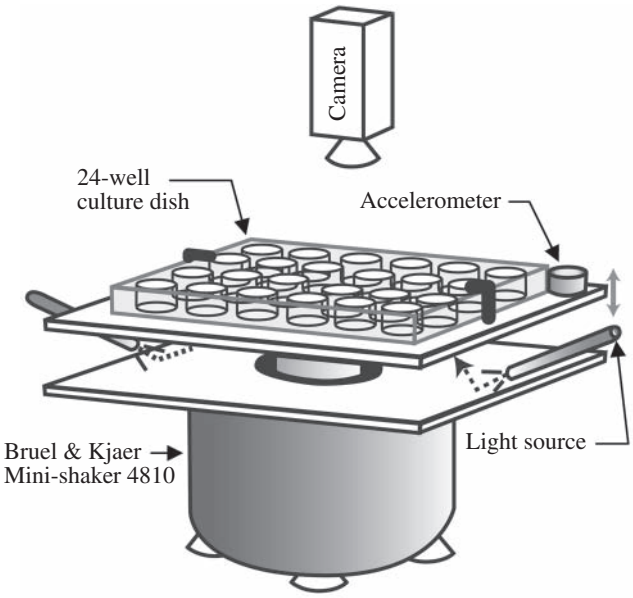

Fig. 1. Equipment setup used to evoke responses in larval zebrafish. A standard, 24-well culture dish was secured to a ¼ inch thick, translucent, plastic platform using screw-down fasteners. The platform was securely mounted onto a Bruel & Kjaer Mini-shaker Type 4810 so that the platform could be vertically displaced. Another plastic platform was mounted on the non-moving body of the shaker and illuminated obliquely to provide diffuse, uniform illumination to the underside of the moving platform. An accelerometer was mounted on the moving platform and a video camera was placed above. The same set up was used for adult fish, except that a single plastic well was mounted in place of the 24-well culture dish.

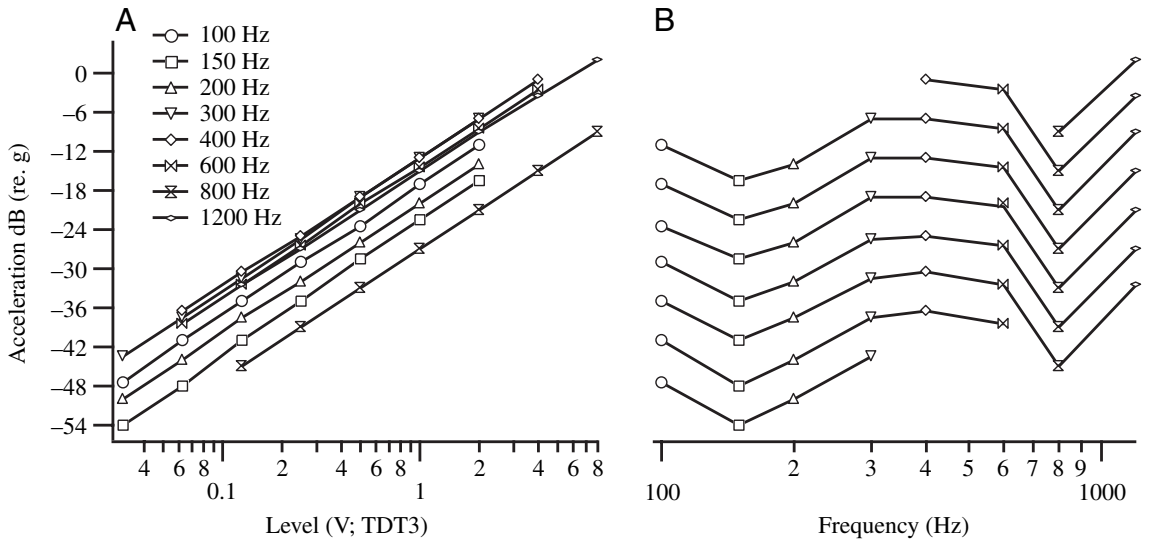

Fig. 2. The RMS acceleration of the platform for the 24-well culture dish with the central eight wells filled to the standard depth. (A) The RMS acceleration of the platform for the test frequencies as a function of the output voltage from the Tucker-Davis System 3. (B) The same acceleration data plotted as a function of frequency.

Acceleration

In order to determine the acceleration of the water in the wells, long-duration stimuli (>20 s) of the test frequencies were delivered to the 24-well culture dish mounted on the platform. The central 8 wells of the culture dish were completely filled with water and then 2 ml were removed from each one to attain a standard water volume. The RMS output of the accelerometer was measured using an HP 3581A wave analyzer. Fig. 2A shows that, as expected, doubling the stimulus level doubles the acceleration (i.e. the slopes of the curves are 6 dB per stimulus level doubling). Although the acceleration amplitude is a linear function of the voltage to the shaker, the acceleration amplitude is not constant with respect to frequency. This is illustrated in Fig. 2B where there is 6 dB between the lines but the lines are not flat as a function of frequency.

Sound pressure level

The wells in which the larval fish were placed are too small for commercially available hydrophones, so to measure the sound pressure level inside the wells, a small probe tube was constructed by carefully gluing cellophane to the end of a 1 mm diameter, 30 mm-long stainless-steel tube to form a drum. The stainless-steel probe tube was coupled to a Bruel & Kjaer (B&K) Precision Sound Level Meter (type 2235) using a 10 mm flexible tube (the gap between the probe tube and the microphone tube was ~1 mm). The probe was then placed near a calibrated Bruel & Kjaer 8103 hydrophone in various places within a larger water tank (2 l). Fig. 3 shows the output of the 8103 hydrophone and our probe at different frequencies. The difference between the hydrophone and the probe is about 40 dB across the frequencies tested (Fig. 3, Probe attenuation), and was used to calibrate the probe tube system for use underwater.

The probe was rigidly affixed near the center of one of the wells in the culture dish such that the probe moved with the dish when the stimuli were presented. Having the probe move with the wells measures the sound pressure that the fish would experience and prevents the artifactual measurement of sound pressure due to the probe changing depth with vertical vibration. The tip of the probe was 3 mm from the bottom of the well.

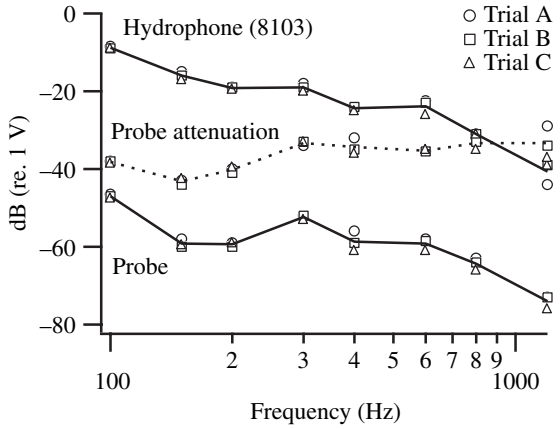

Fig. 3. Calibration of the probe tube microphone. A custom-made probe tube was attached to the microphone of a Bruel & Kjaer Type 2235 Precision Sound Level Meter. The tip of the probe tube was placed near a calibrated Bruel & Kjaer 8103 hydrophone and the sound pressure level (SPL) of both the hydrophone and sound level meter with the probe were measured for different frequencies at a stimulus level of 1 volt RMS applied to a UW 30 speaker in a cylindrical tank. Measurements were made at three different locations in the tank (Trials A, B and C). Probe attenuation, the SPL difference between the hydrophone and the sound level meter with probe, was used to calibrate the probe for use under water.

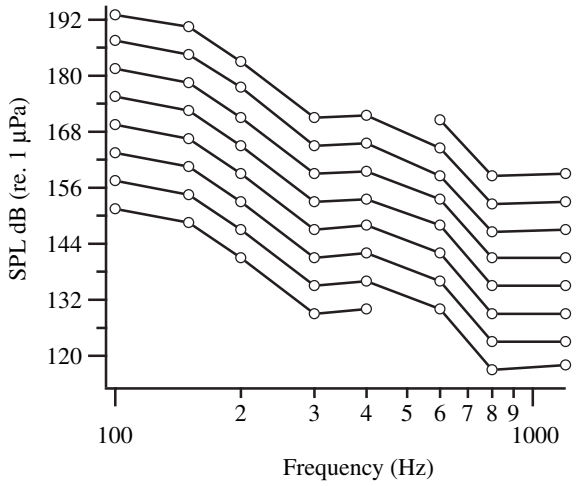

Fig. 4. Sound pressure level (SPL) in the wells of the 24-well culture dish. The probe tube was used to measure the SPL in the wells for the frequencies and levels used in experiments (see text). Note that approximately 6 dB separates the lines because each line represents a doubling of voltage to the shaker.

Sound pressure was measured at each frequency and level (Fig. 4). To test repeatability, the sound pressure levels at all levels and frequencies were measured in three separate trials. In between each of the trials, the culture dish was removed from the platform, emptied, the 8 center wells were completely filled with water, and then 2 ml were removed to obtain the standard amount of water in each well. The growth of sound pressure was 6 dB per doubling of the voltage to the shaker at all frequencies and little difference was evident among the three trials. We then tested whether the depth of water in the well affected the measured sound pressure levels. The well with the probe was first completely filled, then 1, 1.5 and 2 ml were removed, and finally the well was emptied. (The other 7 wells were kept with the standard amount of water, i.e. filled less 2 ml, so that the overall mass would not change much.) It was found that changing the depth of the water did not change the measured sound pressure level, even when the well contained no water. It is inferred that due to flexing of the well walls and water surface, the speed of sound in these small wells is close to that in air and as a consequence the apparent acoustic impedance of water in the wells is close to that of air.

Samples of the stimuli at the highest levels used in the experiments were recorded. Fig. 5 shows the time waveform of the stimuli (right insets), an example of two periods from the stimuli (from ~60 ms, left inserts), and the Fast-Fourier transform (FFT) of the stimuli for 100, 600, and 1200 Hz. The stimuli contain little harmonic distortion. The most distorted waveform occurs at 600 Hz where the first harmonic (1200 Hz) is 36 dB attenuated relative to the fundamental (600 Hz). In all other stimuli the first harmonic is attenuated at least 40 dB relative to the fundamental. Subharmonics are attenuated by about 80 dB for all the frequencies tested.

To accommodate adult fish, some experiments used a larger, single well (120×80×40 mm) in which three adult fish were

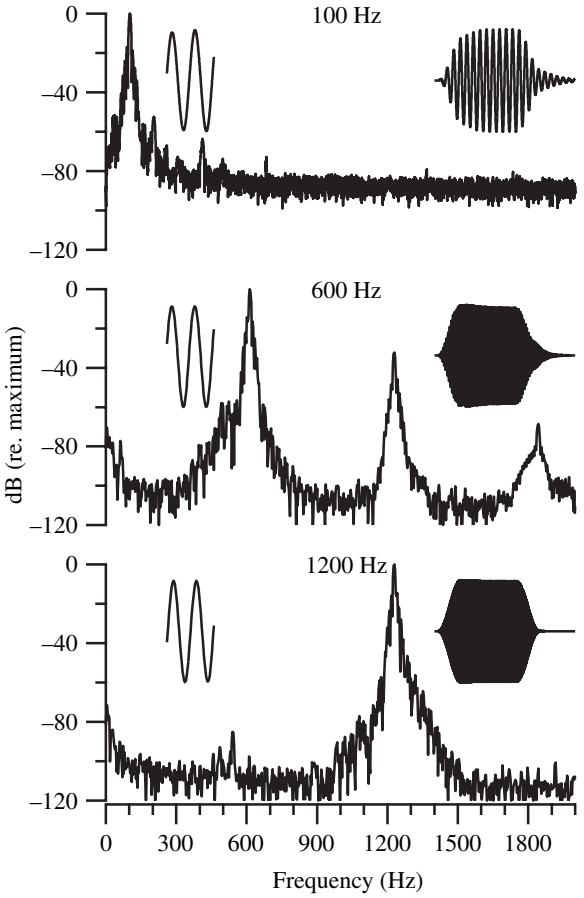

Fig. 5. Sound pressure time waveforms (right insets), examples of two cycles (left insets), and the spectra for three frequencies (100, 600 and 1200 Hz) at the highest stimulus levels used in experiments.

placed during an experiment. The mass of the filled large well was greater than the 24 small wells (eight filled), which exaggerates mechanical resonance, and the acoustics in the large well were also less well behaved than in the small wells. Sound pressure varied to some extent at different locations and with water column depth. The sound pressure used to plot results from the large well were measured in the center of the well at a depth of 15 mm from the bottom.

Data collection and analysis

Each data set consisted of 56 trials (eight frequencies and seven levels – see above). The 56 trials were randomized and each one presented once at intervals of 105 ± up to 30 s. Two data sets were completed during an experiment (except for a few cases with deflated swimbladders). Five seconds of video were recorded during each trial at 25 frames per second (40 ms per frame, 480×360 pixels). The tone began 2.4 s after the video recording was started; i.e. starting at the 60th frame and lasting until the end of the 62nd frame.

Larval zebrafish are able to initiate a startle response (also called ‘escape response’) in about 10 ms following the onset of intense acoustic stimuli (Kimmel et al., 1974; Kimmel et al., 1980; Eaton et al., 1981). Rapid responses to intense stimuli

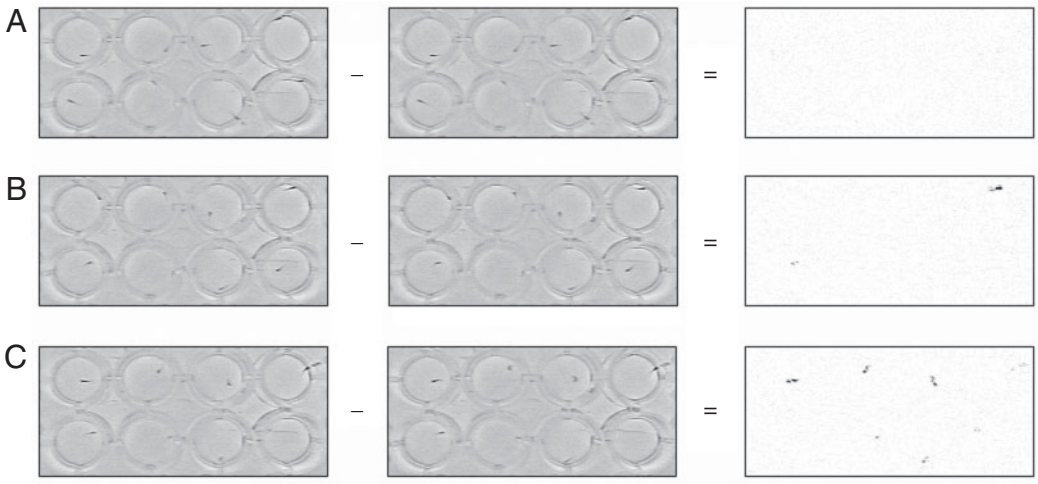

Fig. 6. Examples of video frame subtraction. (A,B) Show the subtraction of two different consecutive frames for the fish in quiet. In A, no movement occurs between frames and the resulting difference image (column 3) contains only noise. In B, two fish move resulting in two darker patches in the difference image. C shows several fish moving in the presence of the stimulus. Note that in areas where no movement takes place, the noise is small and consistent in the difference images. Fish are 4.5 mm (16 dpf).

have been shown to involve Mauthner cell activation (for a review of the Mauthner cell's involvement in escape behavior see Eaton et al., 2001). However, lower intensity acoustic stimuli evoke less vigorous escape behaviors with increased latency (Kimmel et al., 1974, Eaton and Kimmel, 1980), and larval zebrafish perform similar escape behaviors with increased latencies when the Mauthner cells are absent (Kimmel et al., 1974; Kimmel et al., 1980). Because we used long, gated stimuli in order to limit spectral content and because the video frame rate was too low, it was not possible for us to differentiate between Mauthner-mediated responses and other types of movement by the fish. Our primary interest was in determining if fish would respond to the sounds, regardless of whether or not the responses were Mauthner-mediated. Therefore, we defined any distinguishable movement associated with the stimulus as a response.

To determine whether the fish responded to a stimulus, a statistical description of their movement was used. A frame-by-frame subtraction in which the first frame was subtracted from the second, then the second from the third, and so on, was performed on the 5 s of video for all 56 trials in a data set. With no movement (or without fish in the wells) there was little difference in individual pixel intensity on successive frames (Fig. 6A). The noise that was present, the differences in pixel intensity when no movement occurred, was consistent and small compared with pixel intensity changes caused by movement of the fish. For example, areas with no movement in Fig. 6A–C are light colored and consistent, whereas in areas where there is fish movement in Fig. 6B,C there are dark areas of large changes in pixel intensity. A threshold value above the noise was set (the same for all experiments) such that any change in pixel intensity above this threshold could be used to define movement by the fish.

The acoustic stimulus was presented 2.4 s into the 5 s video recording of each trial. The first 2.4 s of each trial therefore represent the free-swimming behavior of the fish in quiet. The number of pixels with intensity changes above threshold in a moving window of three consecutive frames were collected for the first 2.32 s of all 56 trials in a data set. A histogram was then constructed of the frequency of occurrence for the number of pixels above threshold (amount of fish movement) for the fish in quiet. The histogram was fit with a single exponential equation (decreasing from a maximum at zero). An exponential distribution is expected if the eight fish act as infrequently-moving, individual particles. With the probability density function in place for the fish in quiet, the number of pixels above threshold during the stimulus can be compared. A positive response to the stimulus was recorded when the number of pixels that changed during the stimulus would occur by chance less than 1 in 10,000 times (P<0.0001) for the distribution of the fish in quiet for that data set. Fig. 7 shows an example of a histogram and the fitted exponential equation for a data set of the fish in quiet. For this data set, a positive response to a stimulus would be registered if more than 91 pixels were above threshold (Fig. 7, arrowhead). This is a very conservative criterion, but gives results that seem to match well with visual impressions. Visual inspection of the video recordings produced almost identical results as the statistical analysis in a blind scoring of trials in initial experiments (C. Buck, P. Sigafus and D. G. Zeddies, personal observations).

Adult fish were more active than larvae, and the adults' movement in quiet was not well fit by an exponential equation. The responses were distinct so, as in other studies (e.g. Kimmel et al., 1974), the video records were scored by visual inspection for fish in the large well.

Choosing the cosine-squared rise and fall time for stimuli

Zebrafish will respond rapidly to short acoustic bursts, but such short signals are intrinsically broadband with energy at frequencies other than the nominal sinusoidal frequency. Therefore, to limit the spectral content, we chose relatively long (120 ms), cosine-squared gated stimuli. However, because these fish perform escape response with latencies of ~10 ms (Kimmel et al., 1974), it is not clear when we would expect the fish to respond to 120 ms stimuli that are gradually gated. To assess whether gating time affects response thresholds, we determined the thresholds, using the analysis described above, for rise and fall times of 2.5, 5, 10, 20, 40 ms

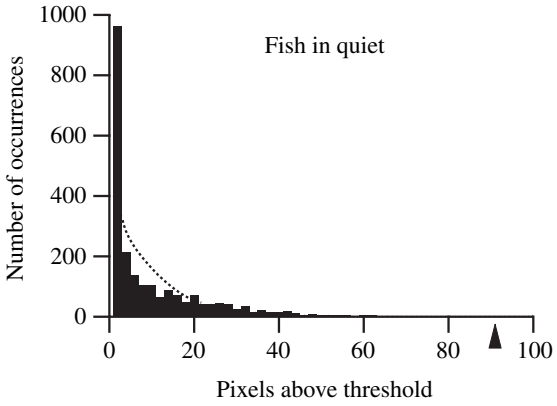

Fig. 7. Example of a typical histogram showing the movement of larval fish in quiet. For a data set consisting of 56 trials, the first 2.32 s of each trial were frame subtracted and the number of pixels above the noise threshold was determined for a moving window of three frames. The frequency of occurrence of the number of pixels above the noise threshold is plotted as a histogram, and a single exponential equation was fit to the histogram (dotted line; goodness of fit λ=0.1012). In this case, a positive response would be registered if the cumulative number of pixels above the noise threshold when the tone was on (frames 60 to 62) was >91 (P<0.0001; arrowhead).

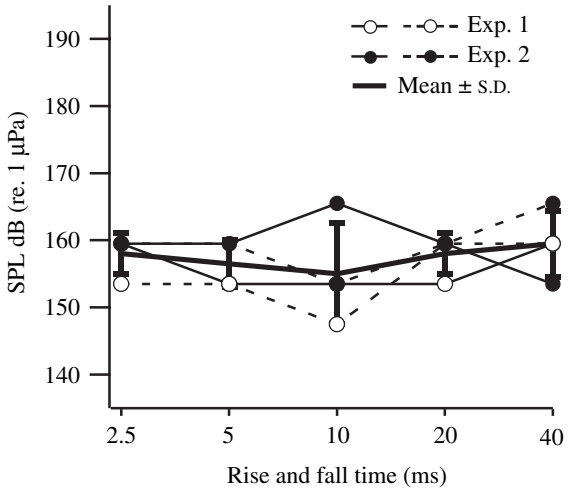

Fig. 8. AEBR thresholds in larval fish for cosine-squared gated, 120 ms, 400 Hz tones as a function of the rise–fall time.

(randomized presentation of rise-fall times and levels). No clear trend was found (Fig. 8), so a rise-fall time of 20 ms was arbitrarily chosen as the standard because we knew that the 20 ms rise-fall produced clean stimuli in our mechanical system (see above).

Choosing intervals for stimulus presentation

To maximize the number of presentations that can be presented during an experiment the interval between presentations must be minimized. Too frequent presentation could cause the fish to habituate to the stimuli, so we tested whether the response threshold changed when stimuli were presented at different intervals. Seventy five consecutive presentations of a 400 Hz tone were presented at 3 min intervals at a level that was previously determined to be just above threshold. A positive response was obtained for each of these presentations. Knowing that an AEBR could be induced every 3 min, trials were created to test whether a recently performed response would affect the probability of the subsequent responses. A trial consisted of an initial AEBR induced by the suprathreshold 400 Hz tone followed by a test tone of a randomly selected level and an interval of 60, 90 or 150 s. Each new trial was presented 3 min after the conclusion of the previous trial. A total of 30 trials were presented during an experiment. The threshold for response was determined to be the same for the intervals tested (data not shown). A standard interval of 105 s with a random variation of ± up to 30 s was selected.

Deflating the swimbladder

Adult and larval fish were anesthetized using a 5000:1 dilution of MS-222 (ethyl *m*-aminobenzoate). When the fish became unresponsive they were placed on a translucent panel and viewed through a dissecting scope that was illuminated from below to make the swimbladder visible. We deflated the anterior, posterior, or both chambers of the swimbladder in adult zebrafish using an M1 needle syringe to draw out the gas. In larvae, a combination of a sharp glass pipette and the M1 needle was used to deflate the swimbladder. After experiments, adult fish were dissected to evaluate the status of the swimbladder. When both chambers were deflated then no gas-filled bladder was seen upon dissection; indicating that the chambers did not refill during the experiment. When only the anterior or posterior chamber was deflated then both chambers of the swimbladder were easily identified during dissection; suggesting that the remaining chamber could, at least in part, refill the deflated chamber. Images of the larval fish were taken before and after experiments. After the experiment, the swimbladder could not be seen indicating that, as in adults, the swimbladder does not reinflate during the course of an experiment.

Results

AEBR thresholds

The 4 dpf fish did not respond to the stimuli at the levels and frequencies presented, but 5 dpf and older fish did respond. Fig. 9 shows the AEBR thresholds in SPL (top panel) and acceleration (bottom panel) as a function of frequency for larval fish ranging from 5 to 26 dpf. For each experiment shown in Fig. 9, the stimulus set (consisting of 8 frequencies and 7 levels presented in randomized order; see Materials and methods) was presented twice (different randomization for each set). The thresholds determined for each frequency were consistent between the stimulus sets. In only 4% of the cases (3 of 72) did the threshold determined at a particular frequency differ by more than 6 dB between the presentations of the stimulus set. ‘Gaps’ were seen in 6% of the cases (9 of 144) where a positive response was attained

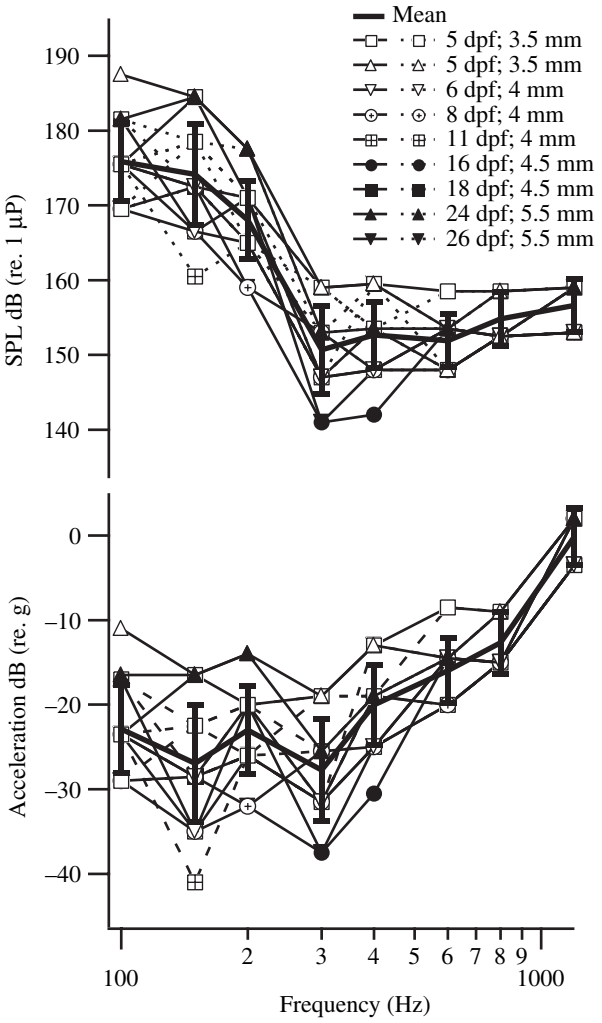

Fig. 9. AEBR thresholds in larval fish of different ages shown as SPL (top) and acceleration (bottom). The mean ± S.D. for the 5–26 dpf fish is plotted as thick black lines. An experiment for a group of animals consisted of two sets of trials (see Materials and methods). Therefore, each group has two plotted curves using the same symbols but connected with different lines (solid for trial set 1, and dashed for trial set 2). Two different groups of 5 dpf fish were tested, so 5 dpf appears twice (with different symbols).

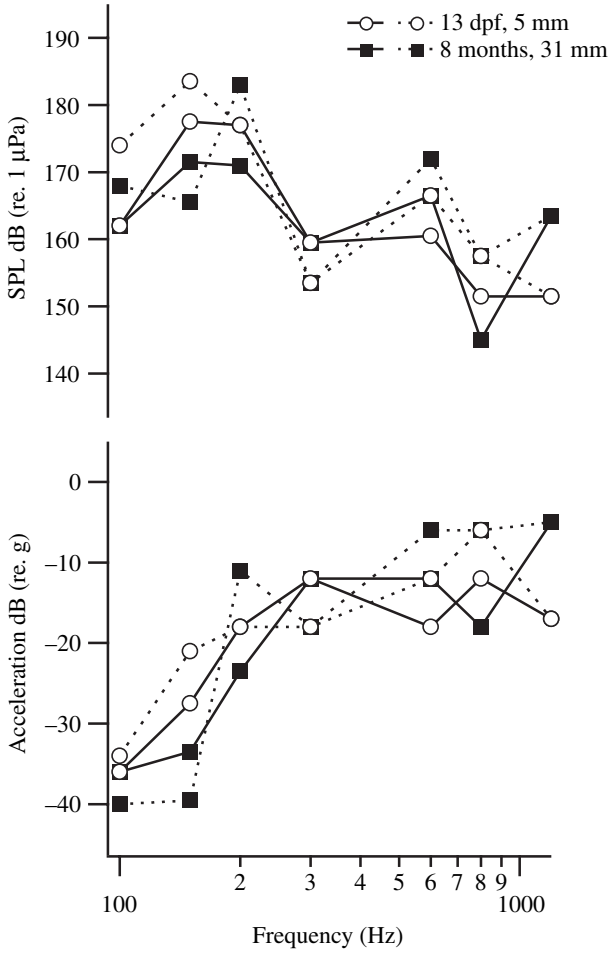

Fig. 10. AEBR thresholds for adult and larval fish in a single large well. The top panel is SPL, the bottom panel is the same data plotted as a function of acceleration.

at a low level and then the fish failed to respond at the next higher level but again responded at the subsequent higher level. When a gap occurred the lowest level at which a positive response was obtained was considered threshold. There were no obvious developmental shifts in thresholds or frequency bandwidth as a function of age for animals between 5 and 26 dpf.

To test the responses of adult fish, a larger container was used (it should be noted that the acoustics inside the large well were not as well behaved as in the smaller wells, see Materials and methods). Fig. 10 shows the response thresholds of adult fish, 8 months of age, and the response thresholds of 13 dpf larval fish also tested in the large single well. (Three adult fish were tested together, and eight larval fish in their respective experiments.) The thresholds and bandwidth for the adult and larval fish were approximately the same.

The swimbladder

At 4 dpf the fish were not responsive to our acoustic stimuli. However, 5 dpf and older fish did respond and the thresholds and bandwidth for the responses did not appear to change with age. It is widely recognized that the general behavior of 5 dpf zebrafish is different from 4 dpf zebrafish. At 4 dpf the fish are mostly sessile (inactive and lying on the bottom or sides of the container) whereas 5 dpf fish are free swimming and active. The swimbladder also inflates at 5 dpf (Fig. 11). In adult goldfish, postsynaptic potentials in the Mauthner cell can all but be eliminated by deflating the swimbladder (Canfield and Eaton, 1990). And, there is evidence that the swimbladder may be required for the startle response in adult zebrafish. In a screen of mutagenized zebrafish, virtually all fish that failed to perform a startle response to a loud 400 Hz tone had evident morphological defects in the conductive pathway including the swimbladder and Weberian ossicles (Bang et al., 2002).

Deflating both chambers of the swimbladder in adult fish

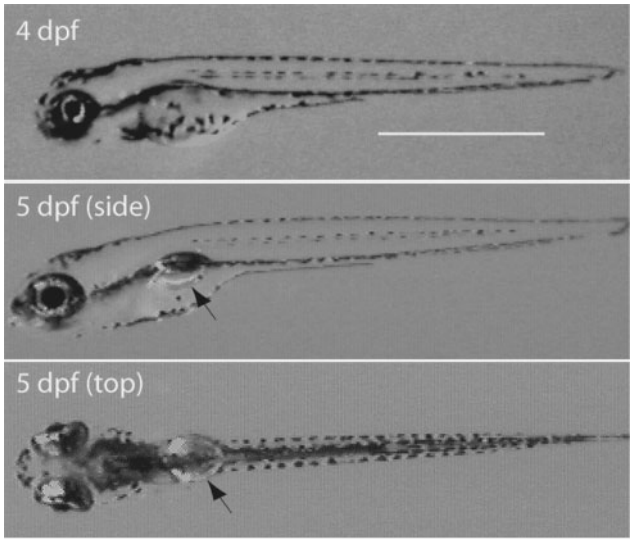

Fig. 11. Images of 4 and 5 dpf zebrafish larvae. The swimbladder has inflated and is clearly visible in 5 dpf animals (arrows), but not in the 4 dpf animals. Scale bar, 1 mm.

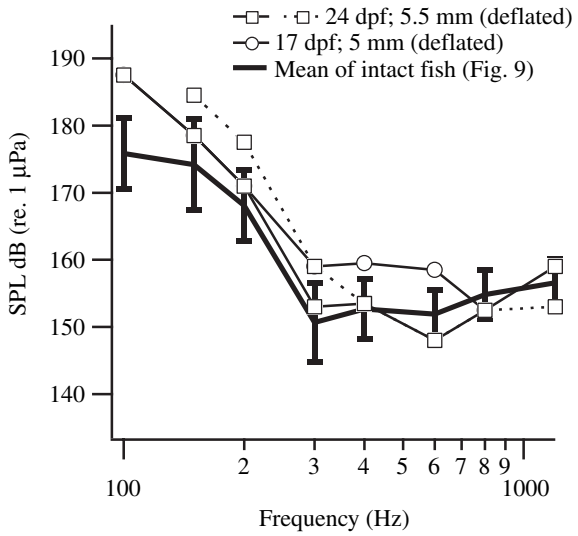

Fig. 12. AEBR thresholds for larval fish in which the swimbladder was deflated. For comparison, the mean ± S.D. of the 5 to 26 dpf intact fish (from Fig. 9) is also shown.

essentially eliminated the AEBR. Only two positive responses were registered in four separate experiments (2 positive responses in 280 trials; data not shown). Fig. 12, however, shows that larval fish with deflated swimbladders still responded near the mean thresholds determined for larval fish with intact swimbladders (all of these experiments on larval fish were conducted in the small wells and analyzed as described in Materials and methods). Adult fish continued to respond to the stimuli when only the anterior or posterior swimbladder was deflated (data not shown). We had expected that deflating the anterior chamber would be sufficient to elevate the thresholds because the anterior chamber is directly connected to the sacculi *via* the Weberian ossicles. Our presumption is that the intact posterior chamber was able to re-inflate the anterior chamber.

Discussion

Frequency was meaningful in these experiments

Larval and adult zebrafish respond to the long, slowly rising and falling, pure-tone stimuli by performing AEBRs. Because the stimuli were smoothly ramped on (and off) with 20 ms cosine-squared gating, there is not a distinct (broadband) onset to which the fish could be responding. Also, in these relatively long stimuli there was little energy outside of the fundamental frequency, which together with the relatively long duration rise-fall times suggests that the fish responded specifically to the stimulus frequencies.

The response was probably mediated by the sacculus

We found that these fish responded to frequencies up to 1200 Hz (the highest that we could test). Although there is some energy at harmonic frequencies (~40 dB down from the fundamental) there is very little energy at sub-harmonic frequencies (~80 dB down from the fundamental). Therefore, it is unlikely that these fish could have been responding to a low-frequency component of the high-frequency stimulus. This suggests that the response is mediated through the saccular pathway because it is the only otolithic end organ shown to respond at these relatively high frequencies in otophysan fishes (Fay, 1981). It is also consistent with the finding of Bang et al. (2002) that most mutations affecting the startle response (to a 400 Hz tone) had morphological defects in the saccular transmission pathway, and with studies showing that deflation of the swimbladder (the input to the sacculi) in goldfish makes the startle response less likely (e.g., Canfield and Eaton, 1990).

Although it is possible that the AEBR is mediated through the lateral line system at the low frequencies, it is unlikely in these experiments because the stimuli would poorly activate the lateral line. In these experiments the fish were accelerated vertically with the water, thus little or no movement of the water relative to the fish is expected. Pressure differences across the fish are also not expected. The vertical displacement creates sound pressure in the wells, but because the wavelength is much greater than the dimensions of the wells the fish could not experience a pressure gradient across different regions of their bodies.

Adult zebrafish responded to sound pressure, but larval zebrafish responded to acceleration

We found that deflating both swimbladder chambers in the adult fish effectively eliminated the AEBR, but deflating the swimbladder in larval fish did not affect their thresholds. The elimination of the AEBR with swimbladder deflation in adults is consistent with previous studies, most notably Canfield and Eaton (1990). That the response of the adult zebrafish was affected by swimbladder deflation argues that they were responding to sound pressure in our experiments. Adult

zebrafish are well equipped to detect sound pressure under water. Otophysan fishes, such as goldfish and zebrafish, are hearing specialists in which the swimbladder is coupled to the sacculi *via* Weberian ossicles. The swimbladder acts as a pressure transducer because its volume varies in proportion to pressure – i.e. the swimbladder expands and contracts in the presence of sound pressure because swimbladder gas is compressible (Rogers and Cox, 1988). In adult fish the Weberian ossicles are an efficient mechanical linkage that connects the swimbladder to the sacculi, giving up to about 40 dB gain in pressure sensitivity (e.g. Poggendorf, 1952).

Although the Weberian ossicles had not yet formed in our larval zebrafish (the first morphological evidence for Weberian oscicle formation occurs at 7 mm; Higgs et al., 2003), it could still be possible for the swimbladder to provide pressure information to the sacculi due to the close proximity of the swimbladder to the inner ears of such small animals (e.g. van Bergeijk, 1967). Our finding that deflating the swimbladder in larval zebrafish does not change their thresholds indicates that, unlike the adults, the larval fish were not responding to sound pressure. Therefore, the larval fish are probably responding to direct acceleration of the otolith organs in a manner similar to a hearing generalist fish such as a toadfish (Fay and Edds-Walton, 1997). It is worth noting here that because the scattered acoustic energy of a spherical bubble is proportional to the sixth power of the bubble's radius (see e.g. Pierce, 1994; pp. 428–430), small swimbladders in larval fish may not radiate much energy.

In these experiments the bandwidth and thresholds for larvae and adult were the same

To our surprise, we found that the bandwidth and thresholds of the startle response in adult and larval (>5 dpf) zebrafish were the same. That the bandwidths were the same indicates that the necessary apparatus for processing high frequency information is in place at 5 dpf. In general, otophysan fishes hear to higher frequencies than non-specialist fishes (Fay, 1988), and otophysans are characterized by the presence of Weberian ossicles that mechanically couple the swimbladder to the sacculi. Disrupting the Weberian ossicle chain, however, does not necessarily make fish deaf to the high frequencies, it just raises the sound intensities required to stimulate the ear (Poggendorf, 1952; Ladich and Wysocki, 2003). The fish still have inner ear hair cell/auditory nerve 'channels' that respond best at relatively high frequencies (Fay, 1997). In the larval fish that we tested (<6 mm) the Weberian ossicles had not yet formed, but it appears that the sacculus already has the ability to process high-frequency information.

The development of AEBR thresholds are difficult to interpret because adult and larval fish respond to different components of the acoustic stimulus. When the Weberian ossicles become functional the input to the sacculus increases for a given sound level. Thus, it might be expected that the AEBR thresholds in adult fish would be lower than the thresholds in larvae. In these experiments, the AEBR thresholds are the same for adult and larval fish, arguing that the threshold for activation (downstream from the sacculus, perhaps at the Mauthner cell) is being adjusted; possibly to ensure proper reactions to biologically relevant stimuli. The adjustment in activation threshold is consistent with feed-forward inhibition of the Mauthner-cell circuitry underlying escape responses in goldfish (for review see Faber et al., 1989) that can be potentiated by sound stimuli (Oda et al., 1998).

Canfield and Rose (1996) reported that largemouth bass (*Micropterus salmoides*) feeding on guppies produce ~170 dB (re 1 μPa, ~200 Hz), and in the same paper used ~150 dB (re 1 μPa, ~500 Hz) to elicit escape responses from goldfish. From our measurements, both adult and larval zebrafish would respond to the bass strike and the ~150 dB (re 1 μPa, ~500 Hz) used to elicit responses from goldfish. One has to imagine though, that what is dangerous to a larval fish and warrants reaction may not be dangerous to an adult fish. In these experiments it may be coincidental that the response thresholds are the same in adult and larvae (although it does mean that the sacculus is receiving greater input for a given acoustic stimulus at threshold in adult fish). With larval fish responsive to particle motion (the so-called 'near field component' that decays rapidly with distance) and adult fish responsive to pressure (that can propagate over long distances in the far field) the thresholds measured in SPL could be different if the animals were tested further from the sound source.

While it is possible to conclude from the frequency bandwidth that the otolithic organ adaptations for high-frequency hearing are already present in larval fish, we do not know if the absolute hearing sensitivity was still developing. We do not know, for example, if the sensitivity of the hair cells and tuned primary afferents of the sacculus are different in larval and adult fish. In adult fish the AEBR threshold is ~60 dB greater than the ABR threshold (Higgs et al., 2002), indicating that there is at least a 60 dB range within which the fish can hear but do not startle. Since we do not have comparable ABR data for larval fish (<10 mm), we cannot exclude the possibility that they only detect (hear) intense sounds and that any detectable sound induces an AEBR.

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