

High-throughput behavioral screening method for detecting auditory response defects in zebrafish

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Abstract

We have developed an automated, high-throughput behavioral screening method for detecting hearing defects in zebrafish. Our assay monitors a rapid escape reflex in response to a loud sound. With this approach, 36 adult zebrafish, restrained in visually isolated compartments, can be simultaneously assessed for responsiveness to near-field 400 Hz sinusoidal tone bursts. Automated, objective determinations of responses are achieved with a computer program that obtains images at precise times relative to the acoustic stimulus. Images taken with a CCD video camera before and after stimulus presentation are subtracted to reveal a response to the sound. Up to 108 fish can be screened per hour. Over 6500 fish were tested to validate the reliability of the assay. We found that 1% of these animals displayed hearing deficits. The phenotypes of non-responders were further assessed with radiological analysis for defects in the gross morphology of the auditory system. Nearly all of those showed abnormalities in conductive elements of the auditory system: the swim bladder or Weberian ossicles. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

A forward genetics approach using zebrafish is an attractive strategy for elucidating the genetics of hearing and balance. This approach relies on the generation of random mutations that can affect the function of genes crucial to the inner ear and requires high-throughput screening of the progeny of mutagenized fish to detect mutant phenotypes. So far, screening for inner ear mutants has involved either observation of gross morphological features in transparent embryos or simple behavioral tests such as the observation of swimming patterns. With such approaches, 78 inner ear and lateral line mutants were recovered from two large-scale chemical mutagenesis screens (Malicki et al., 1996;

Whitfield et al., 1996). A behavioral screen for motility problems including balance-defective swimming behavior (Granato et al., 1996) has led to the isolation of an additional 15 mutations in eight loci affecting the function of the sensory cells of the inner ear (Nicolson et al., 1998). Additional inner ear mutants have also been detected in an ongoing large-scale retroviral mutagenesis screen (Amsterdam et al., 1999).

Although the success of these screens on zebrafish larvae is unquestionable, more sophisticated methods may be required to detect late-onset phenotypes affecting the adult inner ear and the lateral line sensory maculae. Here we present an automated, high-throughput behavioral screening method for assessing hearing in adult zebrafish that relies on the observation of the acoustically-triggered startle response, which in the zebrafish is likely to be initiated by the Mauthner system and other components of the brainstem escape network (Kimmel et al., 1974; Eaton et al., 1977a,b; Hatta and Korn, 1998). This startle response, an unconditioned

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behavior, is a rapid and massive acceleration resulting from a brisk contraction of the body axial muscles. It can be triggered by acoustic and visual stimuli and can be modulated by input from the lateral line organs (Faber and Korn, 1975; Korn and Faber, 1975). With this behavioral assay, 36 adult zebrafish, restrained in visually isolated compartments, can be simultaneously assessed for responsiveness to near-field 400 Hz sinusoidal tone bursts. Automated, objective determinations of responses are achieved with an image analysis program that subtracts images taken with a CCD video camera before and after stimulus presentation. Up to 108 fish can be screened per hour. To validate the reliability of our assay, we tested over 6500 wild-type and mutagenized fish. The phenotypes of non-responders were further assessed with radiological analysis for defects in the gross morphology of the auditory system. These studies demonstrated that the behavioral assay described is capable of detecting defects in the auditory system.

2. Methods and results

2.1. Animals

All experiments were performed on AB/AB, Tu/Tu, and AB/Tu strains of adult zebrafish. Fish were maintained in standard laboratory conditions as described previously (Bang et al., 2001). The majority represented F1 generation of ethyl-nitroso-urea (ENU) mutagenized animals. ENU mutagenesis was performed using standard protocols (Mullins et al., 1994; Solnica-Krezel et al., 1994).

2.2. Acoustic generation and delivery

The general organization of the equipment required for testing auditory responses in fish is described schematically in Fig. 1. Fish were tested in individual cubicles of two 18-compartment styrene boxes (Flambeau, Weldon, NC) filled with water to a height of 25 mm. Each compartment measured 35 × 35 mm and the boxes were covered with plexiglass. The walls of each compartment were painted white so that fish were visually isolated from each other. The cubicles were placed on the center of a 1.26-cm thick plate of plexiglass (53 cm²) to which a 38 cm (15 in.) loudspeaker (Radio-Shack, Tandy Corporation, Fort Worth, TX) was bolted to form a closed acoustic system. The vertical motion of the plate, driven by the loudspeaker, was transmitted to fluid motion of the water in the container.

This system is conceptually similar to that used by Fay to measure directional sensitivity in goldfish (Fay, 1984) and by Eaton et al., to examine acoustically-

evoked startle responses in goldfish (Eaton et al., 1988). Furthermore, similar techniques are used to calibrate hydrophones. The combination of the small dimensions of our test chambers (including the shallow, 2.5 cm, depth) and moderate sound frequency allows us to assume that the entire water column is moving as a unit and that the sound pressure at any depth in the column is the product of the acceleration of the column, the density of the fluid and the depth (Schloss and Strasberg, 1962). For a column of our dimensions this assumption is valid for frequencies of 50–1000 Hz.

The acoustic stimulus was a 400 Hz sinusoidal tone burst of 40 ms duration produced with a tone-burst generator (Tektronix FG 501A 2 MHz) and phase-locked with voltage initially rising from the zero crossing. The output of the tone-burst generator was attenuated (Model 350 600 Ω attenuator set, Hewlett-Packard, Loveland, CO) and then amplified with a Crown D7 (Crown Audio, Inc., Elkhart, IN) amplifier to 6 V peak-to-peak. The individual components of the stimulus generation system are diagrammed in Fig. 1B. A 400 Hz tone-burst was chosen based on data from behavioral audiograms of goldfish (Fay, 1969). Morphological (Platt, 1993; Bang et al., 2001) and electrophysiological (Hatta and Korn, 1998) studies suggest that the zebrafish auditory system is very similar to that of the goldfish, which is sensitive to a frequency range of 200–1000 Hz with best sensitivity at 400 Hz (Fay, 1969; Fay and Ream, 1986).

To calibrate the acoustic delivery system, we measured the vertical motion of the plexiglass plate by spectral analysis of acceleration using a B&K accelerometer (Bruel and Kjaer model 4290, Naerum, Denmark) coupled to a charge amplifier and interfaced with a DSP board (Ariel TMS32020/C25, Highland Park, NJ) in a PC computer. This system performed analog to digital conversion and fast Fourier transformation of the accelerometer output using MATLAB software (The Mathworks, Natick, MA). Input stimuli were 400 Hz tone-bursts produced with the acoustic delivery system described above. During development and characterization of the acoustic delivery system, we also used digitally synthesized chirps (see Teoh et al., 1997) converted with a data acquisition board with its on-board processor (DSP-16+ with TMS32020/C25, Ariel Highland Park, NJ) using conversion rates specified by Ariel sysID software (SYSID Labs, Berkeley, CA) and passed through an anti-aliasing filter and adjustable attenuator.

Spectral analysis of acceleration in response to the chirp provided assessment of the transfer function of the loudspeaker system. While many variants of this acoustic delivery system were assessed, the particular choice and arrangement of loudspeaker and plexiglass plate described above was chosen because it produced a resonance close to 400 Hz. Spectral analyses of stimu-

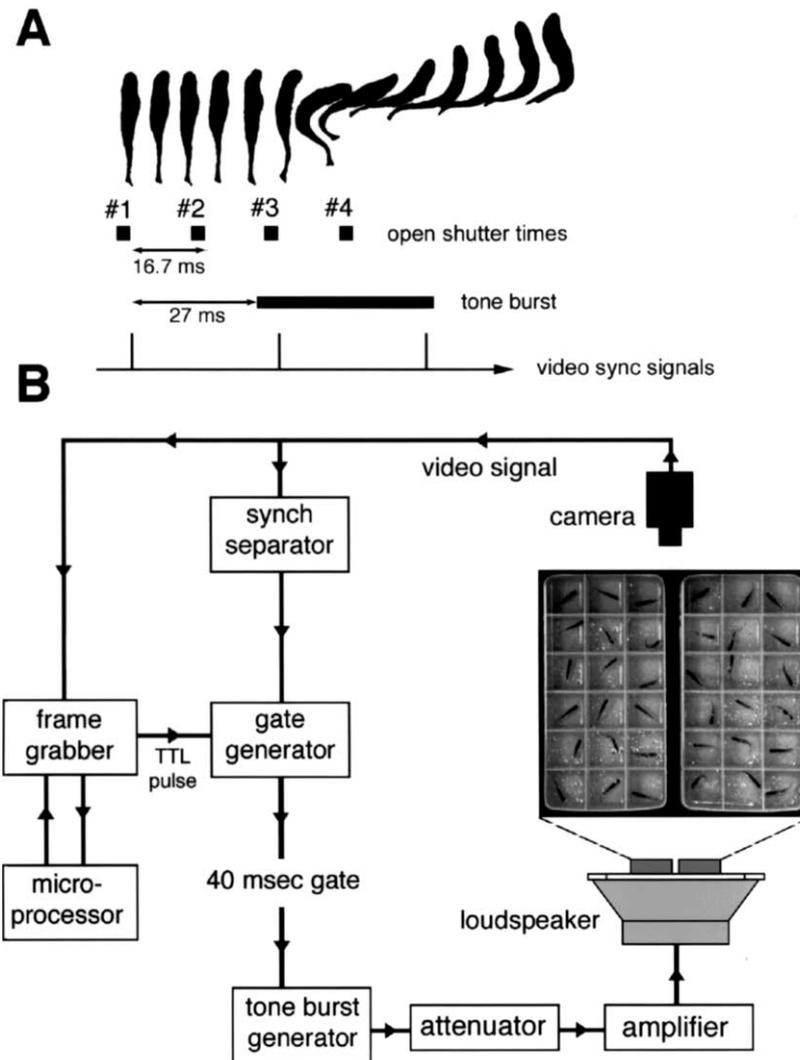


Fig. 1. (A) Video frames were acquired and de-interlaced into fields representing images collected at 16.7 ms intervals. The camera shutter was electronically opened to image acquisition for 4 ms at the end of the 16.7 ms interval. The 40-ms tone-burst was generated at precise times relative to the acquisition of the video frames. The startle response (after Kimmel, 1972) is depicted relative to the frame acquisition times. (B) The core of the automated screening device is a gate generator, which synchronized a computer-controlled TTL pulse (produced by the frame grabber) to the timing pulse extracted from the video signal. The tone-burst was attenuated and amplified before being delivered to the loudspeaker. Two sets of cubicles, filled with water, were placed at the center of a thick plate of plexiglass bolted to the loudspeaker. A fish was placed in each of the 36 cubicles.

lus-induced acceleration at the onset (first 20 ms) of a 400 Hz tone indicated that the primary energy of the signal was at 400 Hz with relatively little energy at other frequencies (Fig. 2). Stimulus magnitude varied from the center to periphery of the plate by only a few dB (data not shown). Lack of variation was later corroborated by the observation that the probability of response of the fish to acoustic stimulation did not change with cubicle location. An unattenuated 6 V sinusoidal signal of 400 Hz into the loudspeaker produced motion of the plate with an acceleration of around 0.2 g, or 200 cm/s^2 (Fig. 2). For our near-field stimulus, pressure will vary linearly with depth—being maximal at the base of the water column near the sound source and approaching a value dominated by atmospheric pressure at the surface (Schloss and Strasberg, 1962). Assuming the animals

tested were on average in the middle of the 2.5 cm column of water, the sound pressure level delivered to them with an unattenuated signal would have been 325 dynes ($200 \text{ cm/s}^2 \times 1.25 \text{ g}$ of water for every square cm of surface area of the plate). This corresponds to an unattenuated sound pressure level of 32.5 Pa for near-field (displacement mode) acoustic stimulation of the fish. Most fish responded consistently with a startle response when the stimulus level was attenuated by 5–15 dB, indicating response thresholds lower than 6–16 Pa. Because our measurements did not take into account a likely additional attenuation due to coupling between the loudspeaker plate and the fluid-filled compartment boxes, the actual sound pressure levels required to generate a startle response are probably somewhat less than this.

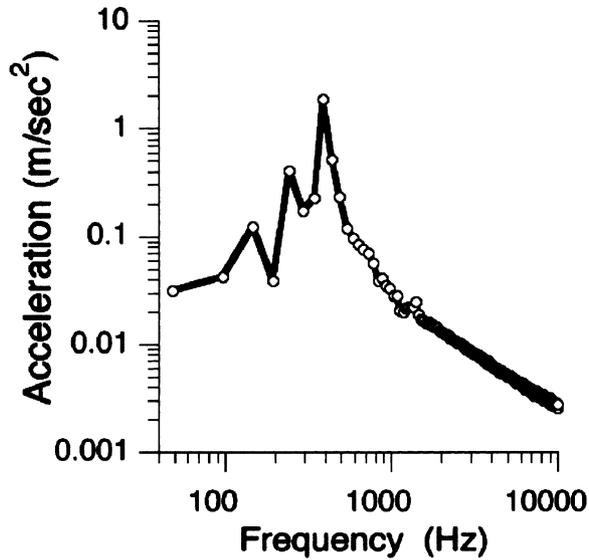


Fig. 2. Spectral analysis of motion of the plate supporting the fish containers shows most of the energy is concentrated at 400 Hz. Motion is expressed as acceleration in m/s^2 . Measurements were taken at the onset (first 20 ms) of the 400 Hz tone burst delivered unattenuated to the loudspeaker. Most tests were performed with 5–15 dB attenuation of this signal.

2.3. Synchronization of the acoustic stimulus with the frame grabber

Images were captured with a CCD-camera (Hitachi KP-M1, Denshi America, Woodbury, NY) placed 1 m above the fish (Fig. 1B). A Computer TV zoom (1:1.2/12.5/75) lens (Cybersense Inc., Nickolasville, KY), was positioned so that images of the 36 fish filled the video frame. In each test for the startle response, two successive frames were acquired at normal video rates (30 per s) with an LG-3 Frame Grabber (Scion Corporation, Frederick, MD) attached to a PowerPC 750 (G3) computer (Apple Computer Inc., Cupertino, CA) and later de-interlaced to produce four successive fields so that images were acquired every 16.7 ms. The camera was electronically shuttered to acquire images in the final 4 ms of each 16.7 ms interval (Fig. 1A). Images were acquired and manipulated using SCION IMAGE 1.62 software (Scion Corporation).

To synchronize the stimulus delivery to frame acquisition, we extracted timing pulses (video sync pulses) from the composite video signal using a video sync separator (Colorado Video model 315, Boulder, CO). Video sync pulses occur every 33.4 ms at the onset of each frame. A gate generator was built which accepted both the video sync pulses and a computer generated TTL pulse. This device produced a 40-ms gate synchronized to the first incoming video sync pulse that occurred following the computer generated TTL pulse. The gate was produced with a 27-ms delay after the video sync pulse was received. Timing of the gate was calibrated with an oscilloscope and has an error of

around 0.1 ms. The gate was used to generate phase-locked tone bursts from the Tektronics tone-burst generator.

The computer-generated TTL control pulse triggered both the frame acquisition and the gated tone burst at time = 0 ms (T_0). This set the times of image acquisition for fields #1, 2, 3 and 4 at $t_1 = -4.0$ to 0.0, $t_2 = 12.7$ –16.7, $t_3 = 29.3$ –33.3, and $t_4 = 46.0$ –50.0 ms (see Fig. 1A). The tone-burst began at $t = 27$ ms. Thus, the position of the fish is monitored in a time window from 19.1 to 23.1 ms after presentation of the tone. If the fish has moved from its position monitored 16.7 ms earlier (in a window of from 2.4 to 6.4 ms after the tone) it was scored as responding to the tone. The time of presentation of the tone-burst was empirically determined to produce a response of the fish in field #4, but not in field #3. Thus, comparison of fields #2 and 4 by the image analysis algorithm (below) indicated movement of the fish in response to sound while comparison of fields #1 and 3, served as a control, indicating any movement in the absence of sound (i.e. any motion due to normal swimming).

We have not attempted to measure the onset, or the latency, of the startle response, but have focused exclusively on the presence of a response. If the fish responded earlier than 19 ms after the tone-burst, its position would still be different in the 19–23 ms time window and it would be scored as responding. If the fish began its response after 23 ms, it would be scored as not responding. If the fish began its response during the 19–23 ms window, the amount of movement would determine whether or not it was scored as responding based upon the results of the automated analysis (described below). We know from previous studies that the adult zebrafish produces a robust startle response movement within 13 ms (Kimmel, 1972). Because the response of the fish to the acoustic stimulus was probabilistic, each session consisted of ten tests at 2-min intervals. This 2-min time interval was empirically determined to minimize habituation to the stimulus.

2.4. Computer analysis of response

The image analysis algorithm (Fig. 3) is based upon mathematical manipulation of images acquired before and after the presentation of a tone-burst. By subtracting the image after the tone burst (field #4, shown in panel B of Fig. 3) from that taken before the tone burst (field #2, Fig. 3, panel A), we produced a double image of any fish that moved (Fig. 3, panel C). In panel C, the image of the fish before the sound can be seen in white and that after the sound can be seen in black. With no movement of the fish, the difference image is gray. In the example in Fig. 3, all fish moved in response to sound except the fish in the third cubicle from the top on the rightmost column (indicated with a black square).

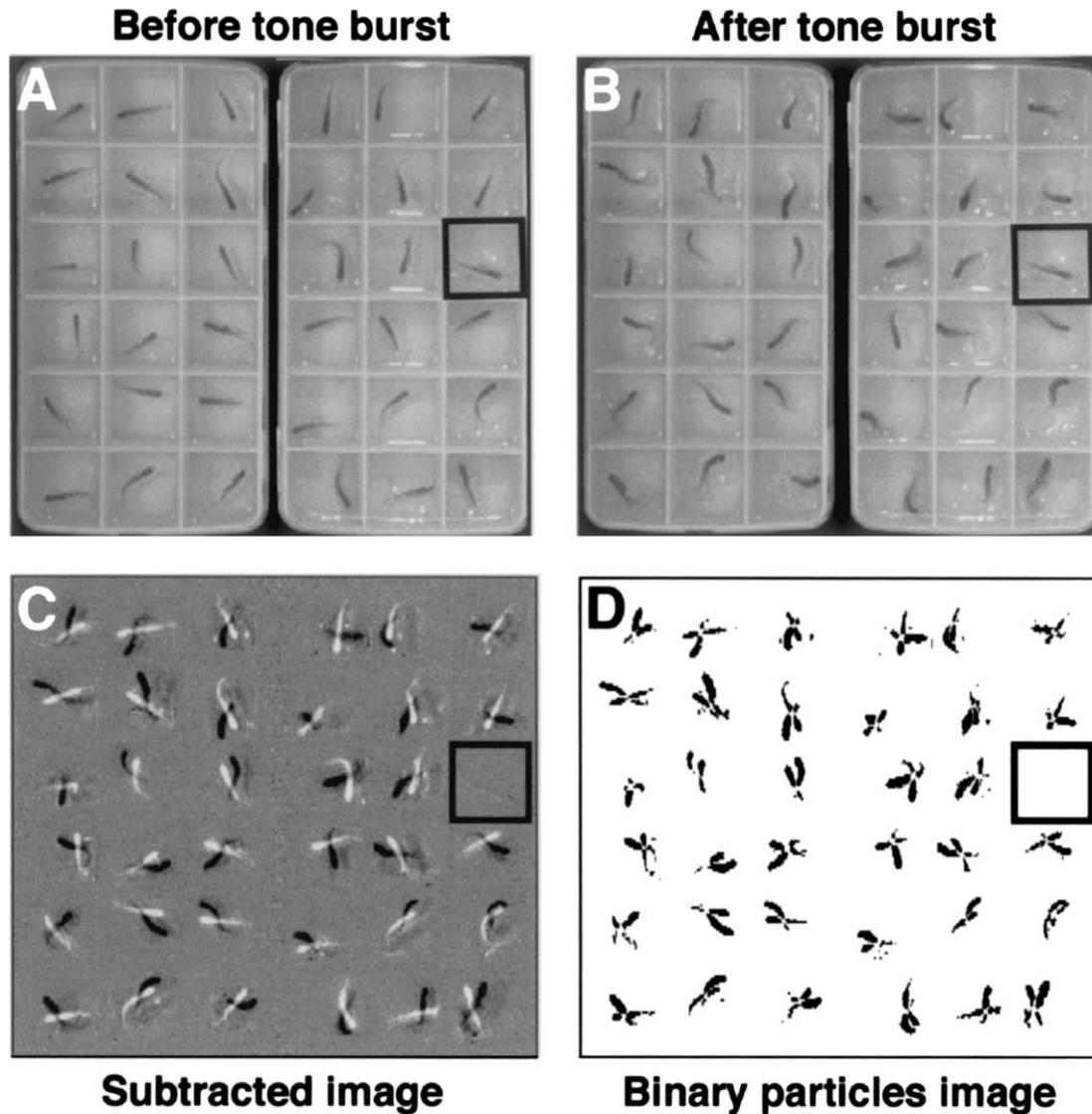


Fig. 3. Images of 36 fish, taken before (A) and after (B) presentation of the tone-burst, were subtracted to give a difference image (C). Two images of the fish appeared in the difference image (C) if the fish moved. No image appeared if the fish did not move. The image in (C) was produced following 8-bit pixel subtraction of (B) from (A), dividing the resulting value by 2, and then adding 128 to the difference such that a pixel difference of zero shows as gray, positive numbers are darker and negative numbers are lighter. In this image all but one fish (enclosed in a block box, third down on the right) responded to the tone-burst. The binary image (D) was produced mathematically to allow automated determination of the location of each fish that moved.

To automate assessment of the startle response, we developed a series of mathematical manipulations of the images that, in the end, indicated either the presence or absence of a startle response to the tone-burst. Subtracting the 8-bit images as described above yielded pixel values with a range of -255 to 255 with values near 0 where there was no difference between the images. The absolute values of these pixels were made binary after choosing a threshold to create an image such as that in Fig. 3D. All contiguous high pixel values were defined as a particle. We wrote a simple set of SCION IMAGE macros (available upon request) to produce a list of particle location and areas. In order to assign each of these particle locations to a corresponding test cubicle,

four reference points were taken from an acquired image of the compartment boxes. A group of MICROSOFT EXCEL macros (available upon request) were written to map the cubicles onto the binary image to locate the position of each particle within the cubicles. The total surface area of all the particles assigned to each cubicle was determined. Responses were assessed as positive if the total particle surface area for each cubicle was greater than an empirically determined value.

To compare subjective with objective determinations, ten trials for each of 205 fish were assessed by an observer (by comparing display of the two video images such as those shown in panels 3A and B) and with the computer algorithm described above. Each trial com-

prised an assessment of the startle response in the presence and absence of sound stimulus for a total of 4100 comparisons of subjective with objective analyses. Of these 4100 cases, the observer reported a response 1880 times and no response 2220 times. There were 55 cases where the computer indicated a response, but the observer did not, and 12 cases, where the observer indicated a response, but the computer did not. Thus, the objective and subjective assessments showed 99.5% agreement in detecting positive responses and 97.6% agreement in detecting no response.

2.5. Behavioral testing procedures

Fish were brought from the animal facility and allowed to acclimate for at least 1 h to the testing room environment. Subsequently, they were placed in the water-filled cubicles and left to acclimate to the confinement of the cubicles for at least an additional 10 min before testing. Animals were tested in three stages to ensure that those identified as non-responders were consistently non-responsive. In the first stage, each fish was tested ten times at 2-min intervals. Results of stage I testing for 504 fish are plotted in Fig. 4. Fish were generally not scored as responding in the absence of the stimulus (Fig. 4, lower panel). No fish produced a

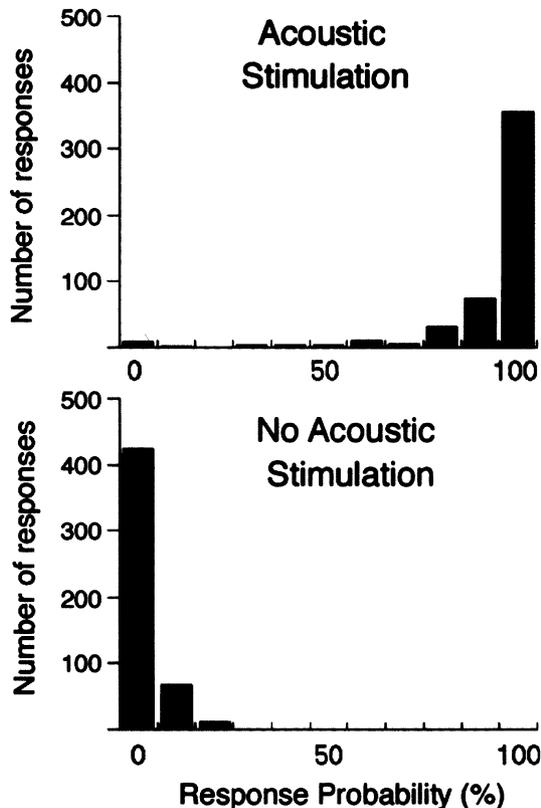


Fig. 4. The percentage probability of a response in the presence (upper panel) and absence (lower panel) of the tone burst is plotted for 504 fish, each tested ten times.

positive response more than 20% of the time in the absence of the stimulus. Most of the fish showed responses to acoustic stimulation most of the time (Fig. 4, upper panel), with 95.5% responding more than 50% of the time. Those responding to less than 50% of the trials were considered to be non-responders and subjected to a stage II testing the following day. The same criterion of a 50% response rate was applied to stage II, and those fish that failed stage II were subjected to a stage III testing at least 1 week later. We subjected 6596 fish to this behavioral screen at age 3–4 months. As the three different genetic backgrounds we used demonstrated differences in sensitivity to the acoustic stimulus, the intensity of the test stimulus was adjusted for each of the strains as follows: 5 dB attenuation for WT:Tu/Tu, 10 dB attenuation for WT:AB/Tu and 15 dB attenuation for WT:AB/AB.

After the three stages of testing, 73 fish were consistently found unresponsive to the auditory stimulus, although they did respond to other stimuli (generally a tap to the side of the tank). This was approximately 1% of the population tested. Because this is a relatively large percentage for subsequent breeding, we developed a radiological screen to examine accessory ear structures: the swim bladder and the Weberian ossicles, both of which enhance sensitivity to acoustic stimulation in these fish. Those fish consistently non-responsive after three stages were subjected to subsequent radiological analysis.

2.6. Radiological analysis

Initial X-ray imaging was performed as follows. Zebrafish were anesthetized in Tricaine (Sigma), at a concentration of 0.04–0.1 mg/ml fish water. They were subsequently oriented in wells molded by embedding glass pipettes in 1%-liquid-agarose-filled petri dishes. Dishes containing zebrafish oriented for lateral or ventral views were placed on Kodak SO-253 High-Speed Holographic Film (10.2 × 12.7 cm) in a Faxitron cabinet radiography system (Faxitron Series, Hewlett-Packard, Model # 43855A, manufactured in November 1984) in complete darkness. The zebrafish were X-rayed for 3 min, at 60–80 kV depending on the age and size of the animals, at a distance of 60 cm from the source (ca. 70R per min dosage). After imaging, the zebrafish were immediately revived by placement in fresh fish water. When necessary, revival was facilitated by forcing fish water through the gills with a pipette. The films were developed following the manufacturer's suggested protocol. X-ray images were photographed with a Leica MZ12 dissecting microscope, at 25–50 × magnification, and imported into ADOBE PHOTOSHOP for further processing.

Later radiography used a Specimen Radiography System (Model MX-20, Faxitron X-ray Corporation,

Wheeling, IL) and 18 × 24 cm mammography film plates and screens (Mamoray HDR-C, Mamoray Detail Intensifying Screen, Agfa-Gevaert NV, Mortsel, Belgium). Fish were exposed at 50 cm from the beam source for 5 s at 24 keV and promptly recovered from anesthesia after transfer back into system water (Bang et al., 2001). The films were developed with an automated developer (Mamoray Compact MR COM).

Zebrafish, like other otophysans, have an accessory hearing apparatus that increases hearing sensitivity. It comprises four tiny bones called Weberian ossicles which mechanically couple movement of the air-filled swim bladder to the ear fluid (Weber, 1820; Chranilov, 1926; Popper, 1973; Finneran and Hastings, 2000). These ossicles have been recently described in the zebrafish (Bang et al., 2001) and, as in other species, are located adjacent to the cervical vertebrae as shown in Fig. 5B.

X-ray analysis was performed on 64 of the 73 non-responders. Of these, 26 had abnormal swim bladders. Specifically, the swim bladder lacked the characteristic constriction in the middle of the structure and was not in close apposition to the tripus (the caudal bone of the Weberian ossicular chain) (compare Fig. 5E to D). Another 26 had abnormalities that included the cervical vertebral column. Specifically, the axis of the cervical vertebrae was severely tilted at the level of the articulation between the first and second vertebrae, along which are located the two rostral-most Weberian ossicles (compare Fig. 5G to F). Eight of the fish had no apparent abnormalities in the Weberian ossicles or in the swim bladder. For four fish, we could not unambiguously assess the presence or absence of anatomical defects. None of the defects described above have been inherited by the F2 generation. Fish with normal acoustically mediated startle responses generally showed no morphological abnormalities on X-ray analysis. Radiological analysis of 102 fish with normal responses showed only one with morphological abnormalities of the auditory conductive system. We conclude that our behavioral hearing assay efficiently detects defects of the auditory conductive system.

3. Discussion

3.1. Validation of the behavioral assay

We describe a simple high-throughput assay for auditory function in adult zebrafish, with which it is possible to screen over 500 fish per (8-h) day. Evidence that this screen can detect auditory defects comes from our finding that 1% of the fish tested, displayed hearing loss. Nearly all of those showed defects in the conductive elements of the auditory system, the swim bladder or Weberian ossicles, which are important for hearing

(Popper and Fay, 1973). This finding also provides evidence that the startle response in our paradigm is due to auditory stimuli and not mediated through other sensory systems. Defects in the swim bladder or Weberian ossicles should not alter responses mediated by the lateral line organ or proprioception receptors.

The startle response is an unconditioned and relatively simple brainstem reflex that can be produced by a variety of stimuli. In the case of acoustically evoked startle response, discharge in fibers of the eighth nerve is transmitted monosynaptically to the reticulospinal neurons of the hindbrain (Furshpan, 1964; Hatta and Korn, 1998). In the zebrafish larva, reticulospinal system neurons, the Mauthner cell as well as its homologs, MiD2cm and MiD3cm, are necessary for a rapid side-wise bend of the body axis, which is the first component of the startle reflex (Kimmel et al., 1980; Liu and Fetcho, 1999). These cells synapse on motoneurons and interneurons in the spinal cord where they activate muscle contraction. In the absence of these reticulospinal cells, the startle response is an order of magnitude slower (Liu and Fetcho, 1999). It is plausible that the screening method presented here would detect mutations in genes controlling general components of the startle response. None of the animals we have examined so far, however, have had obvious defects in non-auditory components of this behavior, such as touch-evoked startle (Furshpan, 1964; Hatta and Korn, 1998).

Nearly 1% of the animals examined showed no response to the acoustic test. In this regard, the use of radiological assessment proved beneficial for quick identification of fish with auditory conduction problems. Obviously, the screen trades the ability to test very large numbers of animals for sophistication of analysis of auditory deficits. Once identified, animals not responding to the acoustic stimulus with a startle response can be outcrossed and the progeny subjected to more sophisticated electrophysiological and morphological analysis of phenotype.

Our calibration of the acoustic system indicated that almost all of the energy in the acoustic stimulus is concentrated in a 400 Hz peak with minimal lower and higher frequency distortion. Several observations suggest the saccule as the organ mediating the response in our assay. Goldfish, with an inner ear similar to that of the zebrafish (Platt, 1993), hear best at frequencies of around 400 Hz (Fay, 1969). Recordings of electrophysiological activity in the inner ear indicate that responses in the saccule range from around 150 to 800 Hz (Furukawa and Ishii, 1967; Fay and Ream, 1986). Analysis of responses in Mauthner cells to acoustic input show a similar range of responses (Casagrand et al., 1999). In addition, fibers innervating the saccule are known to synapse upon Mauthner cells (Zottoli, 1977; Lin and Faber, 1988), which initiate the startle response. These and other experiments suggest that a decision to

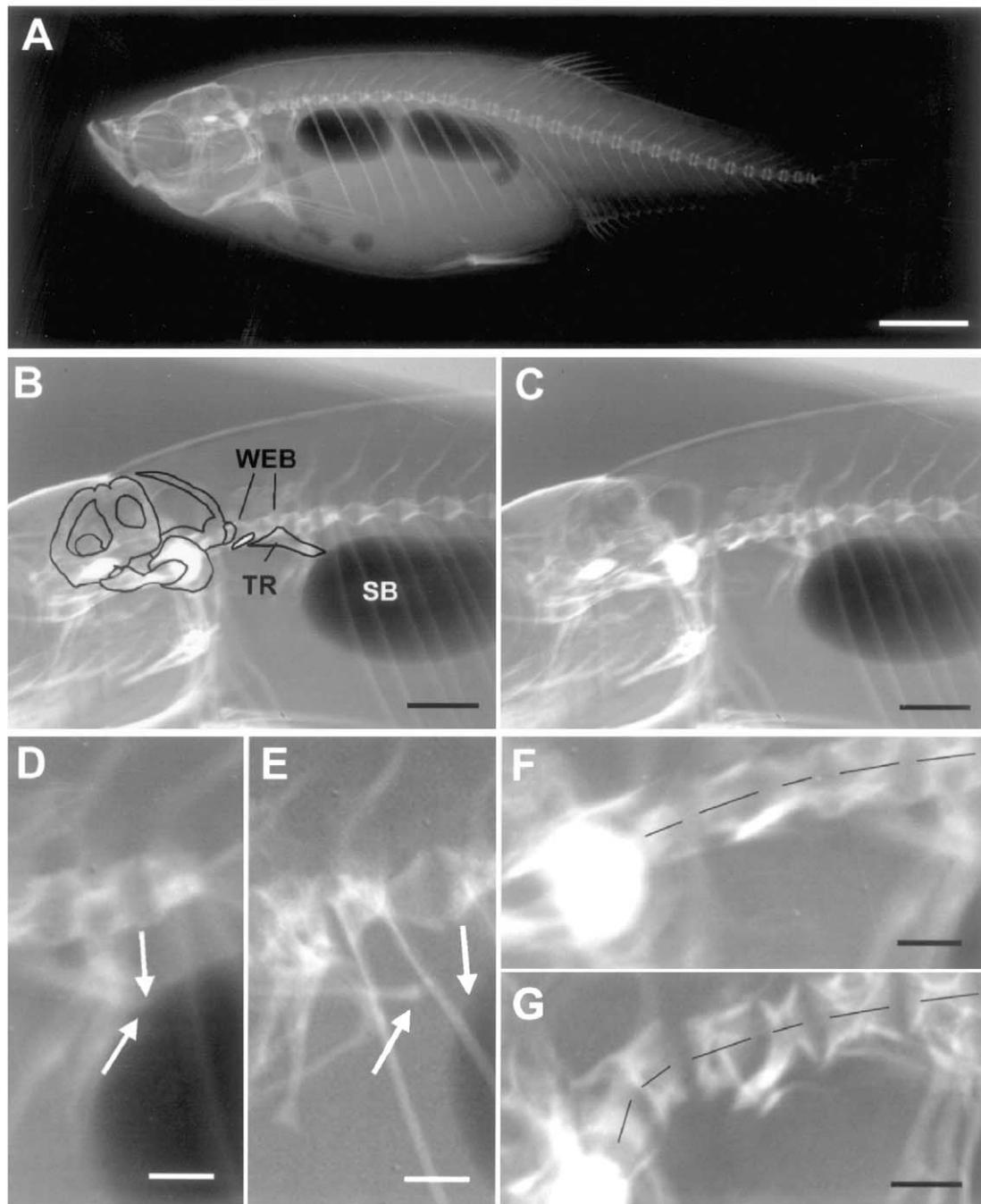


Fig. 5. (A) Radiograph of a normal 7-month zebrafish. Lateral view. The air-filled swim bladder (SB) is seen as two dark regions beneath the vertebral column. (B and C) Magnified views of the inner ear and the Weberian apparatus in a normally responding fish. Panels B and C show the same image. The inner ear and the Weberian ossicles are outlined in panel B. Calciferous otoliths of the inner ear appear as dense white bodies. The SB is coupled to the inner ear via a set of four Weberian ossicles (WEB). The largest and most caudal ossicle, the tripus (TR), is located nearest to the SB. (D and E) Magnified views of the contact area (arrows) between the TR and the SB. (E) This panel shows a fish with the SB malformation, where the SB comprises only one chamber (not shown) and the TR is separated from the SB. For comparison, the normal relationship between the TR and the SB is shown in (D). (F and G) Radiograph, lateral view, of the rostral vertebrae in a phenotype showing abnormal alignment (G) is compared with that of a normal fish (F). Note the change in the position of the lagenar otolith relative to the vertebral column (indicated with black lines) in the abnormal fish. Scale bars = 5 mm in (A); 1 mm in (B and C); 300 μ m in (D–G).

respond to the sound is mediated by the saccule (Fay, 1995).

It is unlikely that the screen is detecting lateral line organ defects. First, there is relatively little energy in the

frequency range to which the lateral line organ is most sensitive (50–100 Hz) (Coombs and Janssen, 1990; Coombs, 1994). Second, as described above, the ability of the screen to identify fish with swim bladder and

Weberian ossicle defects suggests the lateral line organ is not playing a major role in the response, because these structures should have no influence on reception of motion by the lateral line organ.

The three different strains of fish tested required different levels of acoustic stimulation to produce a response. We attribute these differences to diversity in the genetic backgrounds of these strains. Evidence for this diversity arises, for example, from the analysis of simple sequence length polymorphism (SSLP) alleles, which are known to be significantly different in these strains (Knapik et al., 1996). Similar differences in hearing function exist between mouse strains (Ahituv and Avraham, 2000). These differences are also thought to be genetically based and are being explored to reveal the genetic bases of hearing in mammals.

3.2. Other applications for the automated image analysis of the startle response

We developed our automated objective screen for use with an acoustic stimulus, but this approach should be easily applied to any other stimulus that can be triggered by a computer so long as it generates a startle response in the fish. Fish also produce startle responses to visual, olfactory and proprioceptive stimuli (Eaton et al., 1977a,b; Zottoli et al., 1995; Hamdani et al., 2000). This screening method is also suitable for testing responses in zebrafish larvae. We have used this method to test startle responses at 6 days post fertilization (dpf) in larvae. These tests required the interposition of a dissection microscope between the fish and the camera and the modification of the fish holder to be much smaller. In pilot studies, we have used a 96-well plate to contain the larvae. The testing of fish at early stages would make it feasible to screen for recessive mutations, which often preclude survival of the animal to adulthood.

The screening approach described here could detect defects in any component of the auditory system including the swim bladder, the Weberian ossicles, perilymphatic spaces, sensory maculae, or the innervation thereof. One of the unquestionable strengths of using behavioral criteria is that they allow one to detect both gross morphological abnormalities and very subtle functional deficits that would escape even a thorough anatomical investigation (reviewed in Malicki, 2000). Although our screen is primarily aimed at the detection of auditory defects, phenotypes that originate outside of the auditory system can also be detected. The most likely site of such defects are neurons of the reticulospinal formation that receive input from sense organs and project to the spinal cord, where they synapse on motor neurons (Kimmel et al., 1982; Metcalfe et al., 1986; Lee and Eaton, 1991). Studies in zebrafish larvae have demonstrated that unilateral ablation of three neurons

of the reticulospinal system, the Mauthner cell and its two serial homologues, MiD2cm and MiD3cm, results in a loss of the fast component of the startle response (Liu and Fetcho, 1999). These studies provide an example of a deficit outside the auditory system that would also be identified by our screening method.

Automation of the behavioral test offers many advantages, given that thousands of fish must be tested in a typical mutagenesis screen and that each fish must be tested multiple times. The duration of the escape response is very short, on the order of 0.1 s (see Fig. 1). This strains the temporal resolution of human perception and makes it virtually impossible to detect whether or not a response occurred, especially when multiple fish are observed at once. While a human observer can distinguish a robust response from the lack of response, weak responses may be difficult to distinguish from normal swimming behavior. In addition, it is very difficult for an individual to observe more than one fish at a time. Given that the response is probabilistic and each fish needs to be tested at least ten times, the inspection by a human observer is far less efficient than an automated screening method. Additionally, an automated protocol uses a more consistent set of criteria than would a human observer.

Approximately 1% of the adult wild-type zebrafish population did not respond to the stimulus and almost all of those showed defects in the swim bladder or Weberian ossicles in radiological analysis. These findings attest to the importance of the swim bladder and Weberian ossicles in sound reception. The non-responders did not show genetic transmittance of these traits suggesting an environmentally induced anomaly. By combining the radiological screen with the behavioral test, this assay should prove useful both in the gross analysis of inner ear function and may also serve in experiments designed to better understand the function of the Weberian ossicles and the swim bladder in hearing. The capability of screening large numbers of animals makes the assay very well suited for a mutagenesis screen for deafness genes. Its applicability to other sensory modalities that produce a startle response may make its use widely applicable to other sensory systems.

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