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A BEHAVIORAL AND MORPHOLOGICAL ANALYSIS OF AUDITION IN THE MEXICAN BLIND CAVE FISH Astyanax jordani, AND ITS EYED ANCESTOR Astyanax mexicanus

by

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INTRODUCTION

Hearing by fishes involves the use of several structures that may be functionally analogous to the auditory structures found in terrestrial vertebrates. The swim bladder of fishes and the tympanum of terrestrial animals both detect pressure changes in the environment and convert them into displacement energy that stimulates the inner ear. In terrestrial vertebrates, this energy is transduced through one or more middle ear bones. One order of fishes, the Cypriniformes (Ostariophysi), has a series of bones known as the Weberian ossicles that serves to carry acoustical stimuli from the swim bladder to the inner ear of the fish (von Frisch, 1936, 1938a; Tavolga, 1965; Weber, 1820). The inner ear of fishes is homologous to the inner ear of terrestrial vertebrates, but the swim bladder and Weberian ossicles are not related to any terrestrial structure associated with hearing (van Bergeijk, 1966, 1967a).

Considerable morphological variation exists in all parts of the hearing apparatus of terrestrial vertebrates and this is responsible for the wide range of auditory capacities found in different species (von Békésy, 1960). This variation is probably associated with the great range of niches inhabited by the animals and the demands placed upon the auditory apparatus for their survival. Variation in the ear and auditory capacity of fishes is reflected in extensive morphological variation in all parts of the hearing apparatus (see Grassé, 1958; Lowenstein, 1957), including the presence or absence of Weberian ossicles. The great range in the hearing capacity of fishes has been most strikingly shown in the study of nine Bahamian reef fishes by Tavolga and Wodinsky (1963, 1965). Although the hearing capacities of other species of fishes have been investigated, only a few patterns of hearing differences can be ascertained. It is clear that ostariophysines can hear a wider range of sounds and have lower auditory thresholds than fishes without the Weberian ossicles (see reviews by Kleerekoper and Chagnon, 1954; Lowenstein, 1957; Tavolga, 1965). The role of the swim bladder in pressure detection is also partially understood (Alexander, 1966; Harris, 1964).

In addition to having an inner ear and its associated structures for sound detection, most species of fishes have a series of displacement detectors (Harris and van Bergeijk, 1962) in the form of hair cells located on the body. In many species, these neuromasts are enclosed in the lateral line that runs along the body and head region. In addition, other uncovered neuromasts are found in various places on the body. The lateral line and free neuromasts are involved with detection of low frequency sounds (Dijkgraaf, 1963; Harris and van Bergeijk, 1962) that are initiated as displacement stimuli by a sound source (van Bergeijk, 1964).

One of the best ways to determine the role of structural modifications in sound detection is to establish correlations between the structure of the animal's hearing organs and its behavioral and physiological capacity for hearing. Among the fishes, certain basic questions have been answered regarding the significance of the swim bladder

(Kleerekoper and Roggenkamp, 1959; Poggendorf, 1952), the Weberian ossicles (von Frisch, 1938b; Kleerekoper and Roggenkamp, 1959), and the inner ear (Enger, 1963; von Frisch, 1936, 1938b; Furukawa and Ishii, 1967a, b; Lowenstein and Roberts, 1951), but specific details of the functioning of these structures, as well as of their interactions, are still unknown.

Another problem of fish audition has been the difficulty in understanding the stimuli that impinge upon a fish in water. There have been a number of behavioral studies of fish hearing (von Frisch and Dijkgraaf, 1935; Kleerekoper and Chagnon, 1954) but until recently no one has ever precisely defined the sound that was presented to the fish (Banner, 1967; Cahn et al, 1967; Enger, 1966; Jacobs and Tavolga, 1967, 1968; Offutt, 1968). There are still questions about the effects of using a small tank, as has been done in many experiments, on sound in water (van Bergeijk, 1964; Harris and van Bergeijk, 1962; Parvulescu, 1964, 1967a, b). Parvulescu (1964) suggested that sound in a small body of water behaves differently than sound in a large body of water (i.e., the ocean). The air-water interface for a small tank does not attenuate sound as much as it does in the ocean (Parvulescu, 1964) and the walls of a tank may respond to the pressure stimuli setting up secondary acoustic fields that are exceedingly difficult to define (Parvulescu, 1967b, personal communication).

Extrapolations about hearing in fishes can be made from data pertaining to terrestrial vertebrates, but considerable care must be taken since certain structures present in terrestrial vertebrates, such as the cochlea and free floating auditory ossicles, are not present in fishes (von Békésy, 1960; Wever and Lawrence, 1954), and only the inner ear and lateral line of fishes are homologous to structures found in terrestrial vertebrates (van Bergeijk, 1967a). Moreoever, the cochlea of even the primitive tetrapods (von Békésy, 1960; van Bergeijk, 1967a; Frishkopf et al, 1968) makes use of the hair cells in a way that is unavailable for fishes although the cells are very similar in basic structure in fishes and mammals (Flock, 1965, 1967; Flock and Wersäll, 1962; Lowenstein and Wersäll, 1959).

In order to increase our knowledge of audition in fishes, a comparison was made of the hearing capacities of two closely related species of fish. Correlations of behavioral and morphological similarities and differences between the two species provided a means for determining the role of various auditory structures in signal detection and processing. Furthermore, by comparing a blind species of fish, Astyanax jordani, with a closely related species of eyed fish, A. mexicanus, the role of audition in the behavior and survival of blind cave fish could be clarified. While there have been several investigations of other sensory systems in blind cave fish (see below), there has never been a study to determine whether the auditory capacity of these animals changes in association with changes in other sensory structures, such as the eye.

The two species of Astyanax were selected because they are members of the family Characidae and, like all other members of the superorder Ostariophysi, they have Weberian ossicles (Greenwood et al, 1966). While other ostariophysines have been investigated (Dijkgraaf and Verheijen, 1950; von Frisch, 1936; Jacobs and Tavolga, 1967, 1968; Stetter, 1929) there has been only one study of characid fishes (von

Boutteville, 1935) and no studies of this group have been made with a well defined pressure (sound) stimulus. However, investigations by von Boutteville (1935) gave some indication that the characids may have a wider range of frequency detection than the Cypriniformes.

Biology of the Experimental Animal

Astyanax mexicanus is a visually oriented fish that depends on visual stimuli for a considerable amount of information about its environment (Breder, 1943a; John, 1964). Astyanax jordani, along with the closely related A. hubbsi and A. antrobius, are blind cave fish collectively known as the Mexican blind cave fish. The blind fishes are from caves in Mexico that are close to the rivers in which A. mexicanus is found. The first of these blind fishes to be described was called Anoptichthys jordani (Hubbs and Innes, 1936), and two other similar blind species from other caves were subsequently described. The three forms of the blind fish have been interbred with the eyed river fish (Cahn, 1958; Kosswig, 1965; Sadoglu, 1957; Schemmel, 1967). Breder (1942) and Breder and Gresser (1941a) found small-eyed hybrids of crosses between Anoptichthys jordani and Astyanax mexicanus in one of the caves.

In this paper both the blind fish and the river fish will be referred to as members of the genus Astyanax. There is strong evidence for placing the fishes in one genus (Gordon and Rosen, 1962; Greenwood, 1967; Kosswig, 1965; Sadoglu, 1957). There is further evidence that they are members of the same species (Breder, 1942; Gordon and Rosen, 1962; Kosswig, 1965) and, if the species definition of Mayr (1963) and others were applied, the blind fish and river fish would have to be considered one species.

Ecology of the caves

Astyanax mexicanus is found in rivers throughout Mexico and into southern Texas (Vanzolini et al, 1964; Vanzolini and Reboucas, 1965).

Anoptichthys jordani was discovered in La Cueva Chica, a limestone cave in the state of San Luis Potosi, Mexico (Hubbs and Innes, 1936); A. hubbsi was discovered in the nearby Cueva de los Sabinos (Alvarez, 1947); and A. antrobius was discovered in Cueva de la Pachon in Taumalipas (Alvarez, 1946). At least seven other caves are now known to contain the same type of blind cave fish, but these have never been described as species (J. Atz, personal communication; Breder and Rasquin, 1947b).

Only one of these caves has been described in any detail. La Cueva Chica is 0.75 miles from the river Rio Tampaon (Bridges, 1940) and the cave and river are thought to be connected by an underground waterway (Breder, 1942). The cave contains four pools interconnected by small streams and waterfalls. The pools are a maximum of 10 feet deep and the pool furthest into the cave is the one connected to the river (Breder, 1942). The water in the cave is moderately warm, and the water and air temperature are believed to remain fairly constant throughout the year.

The cave is inhabited by a few animals besides A. jordani, but the blind fish is the only aquatic vertebrate. Other aquatic organisms include small invertebrates which may be used as food by the blind fish but none of these organisms are large enough to prey on live blind fish (Breder, 1942). The cave is also inhabited by several species of insectivorous bats which use the cave as a roost (Breder, 1942). The bat droppings cover the surface of several of the pools in the caves, and stomach-content analysis by Breder (1942) indicated that a considerable portion of the food of the Mexican blind fish is made up of nematodes and other small invertebrates found in the droppings of the bats. Food is very abundant in La Cueva Chica as opposed to caves in which other species of blind fishes are found (Heuts, 1951).

Breder (1942, 1943b) and Breder and Gresser (1941a, b, c) studied the populations of blind fish found in La Cueva Chica. They found that all of the pools contained blind fish and, in addition, the pool deepest in the cave had a population of eyed river fish and hybrids between the two forms. The hybrids had small eyes and were typical of the F1 generation (Sadoglu, 1957) while in an F2 or later generation, fish had all different sized eyes and various degrees of body pigmentation (Sadoglu, 1957, 1967).

Genetics

The genetics of the Mexican blind cave fish has been investigated in order to determine the nature of the differences between the Mexican blind fish and the river fish. Sadoglu (1957) and Kosswig (1965) found that the F1 hybrid has small eyes and normal pigmentation and the F2 generation shows various combinations of eye size and pigmentation, indicating independent recombination of the genes associated with these characters. Breder and Gresser (1941a, b) found that fish from La Cueva Chica had a series of different eye abnormalities and some fish had more eye loss than others. This indicated that there have been river fish entering the caves for a long period of time. Sadoglu (1967) attributed this variation to her finding that there were several different, independent factors that affect eye size and development. She found that characters affecting the ultimate eye structure such as lens size, pupillary size, and eye diameter were all controlled independently. Pfeiffer (1967a, b) showed that the eye size in hybrids was correlated directly with the size of the optic lobes.

Vision and light sensitivity

The most obvious changes in *Astyanax* resulting from entry into the cave environment has been the loss of eyes and body pigmentation. Similar losses have been reported in most troglobites (animals that spend their whole lives in caves) both amongst invertebrates and vertebrates (Eigenmann, 1909; Greenwood, 1967; Kosswig, 1965; Poulson, 1963a, b; Vandel, 1965).

Eye loss in the Mexican blind cave fish has been studied and described by Breder (1944), Breder and Gresser (1941a, b), Breder and Rasquin (1947a), and Lüling (1954). Cahn (1958) and Frank (1961) studied the development of the eye in *A. jordani* and *A. hubbsi* and found that although the development of the eye starts at about the same time in the blind fish and the river fish, the rate of development is retarded in the blind form and the eye never reaches full development. After a period of slow development, the eye of the blind fish regresses and becomes covered with skin (Cahn, 1958). The retina in the blind fish contains only a few sensory cells (Cahn, 1958). The optic nerve may be present, but it is poorly developed and may contain no neural tissue (Breder and Gresser, 1941a, b, c).

Astyanax mexicanus schools under extremely low light levels (John, 1964). The Mexican blind cave fish, however, do not move in any pattern with respect to other fish (Breder, 1953), although they do show some response to light. Breder (1944) and Breder and Rasquin (1947b) tested fish in a trough divided into two equal parts, one light and one dark. Most specimens of *Astyanax jordani* showed a slight preference for the dark area, while most specimens of *A. hubbsi* spent more time in the lighted area. River fish had a slight preference for the darkened area and the major proportion of the F1 hybrid between *A. jordani* and *A. hubbsi* were indifferent to the presence or absence of light, spending about 50% of the time in each chamber. In an experiment in a large pond Breder (1944) found that during daylight hours the river fish entered a covered area while in the evening they swam about the whole pond. Strains of blind fish from other caves had varying degrees of light or dark preference (Breder and Rasquin, 1947b), and *A. antrobius* has been studied recently and found to have a preference for a lighted area (Boucquey et al, 1965).

Chemical detection

Breder and Rasquin (1943) reported that *Astyanax jordani* has fewer nasal lamellae than the river fish, and *A. hubbsi* even fewer lamellae than *A. jordani*, but that the structure of the sensory epithelium in the nasal capsule did not differ in the three forms. The number of taste buds on the surface of the head and body of *A. hubbsi* is greater than on the other two fish, and *A. jordani* has more taste buds than *A. mexicanus*. Schemmel (1967) found that *A. antrobius* also has a greater number of the external taste buds than the river fish.

Breder and Rasquin (1943) tested different strains of fish for preference and avoidance of chemical stimuli. They found that *A. hubbsi* shows slightly greater avoidance of noxious stimuli than *A. jordani* and still greater avoidance than the river fish. *A. hubbsi* also showed a greater preference for a sugar solution than the other two forms of *Astyanax*, but Breder and Rasquin concluded that this difference, as with the difference for avoidance of noxious stimuli, was too small to be considered a significant indication of hypertrophy of the chemical sense in the blind fishes. Breder and Rasquin did not believe that greater sensitivity in chemoreceptors would provide any significant selective advantage to the blind fish since there is an abundance of food in the caves. More recently, Glaser (1966, 1968) has found that *A. jordani* learned a task in ten trials when the stimulus was saccharose, while blinded *Gasterosteus aculeatus* needed 21 trials and blinded *Phoxinus laevis* needed 37 trials.

Audition

There has only been a little work on auditory structures and hearing in the Mexican blind cave fish, but there are sufficient data to indicate that the fish can hear and respond to acoustic stimuli. When Breder (1943a) and Bridges (1940) hit the water in one of the pools in La Cueva Chica with their fingers, they attracted numbers of blind fish from the bottom of the pools. John (1957) found that Mexican blind fish congregated around a small jar that contained an air bubbler. The fish were isolated from all but acoustical stimuli from the bubbler.

According to Breder (1943a), Merriman reported that the ear of the blind fish was degenerate, but this was based on preliminary work that was never completed. Schemmel (1967) investigated the inner ear of *A. antrobius* and *A. mexicanus* and found no difference in the pattern of the lateral line canals and free neuromasts of the two fish although he did report the presence of free neuromasts on the orbital region of the blind fish in the area in which the eye would normally be found. John (MS) found that the pattern of free neuromasts does not differ in *A. antrobius* and *A. mexicanus*, but John did find that the number of neuromasts in each area was greater in the blind fish than in the river fish.

Behavior

Mexican blind cave fish swim constantly and only infrequently bump into objects in the tanks (Breder, 1953). Eyed fish in the same tank will frequently attack the blind fish when they do not join the schools of river fish (Breder, 1943a). Blinded river fish swim around in the same random fashion as the blind fish (Breder and Rasquin, 1943; John, 1957). An eyed river fish taken from a cave and placed in the light will continue to show behavior typical of blind fish for several months (Breder, 1943a) during which time the fish will feed like a blind fish and not go directly to food placed in the water. After several months the fish will approach the food in a manner that indicates use of visual information.

Mexican blind cave fish placed into a new tank will initially hit obstructions and walls of the tank, but after a short time the fish will come close to, but not touch, the objects (Breder, 1953; John. 1957). Blind and blinded fish will avoid hitting stable objects in the tanks (Breder, 1943a; John, 1957), but they cannot avoid moving objects in their path. John (1957) showed that the blind and blinded fish will avoid the general area where a noxious object had been in the tank, but they will accurately avoid the object only if the object is present. The fish learns the general position of the object and probably relies on some sort of sensory cues to locate the object precisely. Hahn (1960) reported that A. jordani could detect objects in the water better than could the river fish. He attributed this difference to the lateral line, even though he did not think that the lateral line in the blind fish was more extensive than in the river fish. Walters and Liu (1967) have proposed that the mechanism for object detection by the blind fish and the river fish is detection of water moved by the fish's body in motion and reflected by objects in the water. The detection of the water displacement would then be by the neuromasts on the head of the fish.

The general activity of the river fish and A. antrobius was compared by Thines et al (1965) and Boucquey et al (1965). Thines and his colleagues found that A. mexicanus had a general activity pattern that was correlated with the normal photocycle. The fish showed heights of activity at dawn and at dusk. A. antrobius kept on a similar schedule had a random activity cycle unrelated to the photocycle (Thines et al, 1965). The F1 generation between the river and blind fish exhibited a daily activity cycle similar to that of the river fish, but specimens from the F2 generation had patterns ranging from that of the river fish to the random activity of the blind fish (Boucquey et al, 1965).

Sensory Mechanisms in Other Aquatic Cave Animals

There have been several comparative studies of the sensory systems and behavior of a blind cave animal and an epigean (surface living form) believed to be closely related to the troglobite. The first such study was by Banta (1910a, b) who investigated the sensory systems and behavior of two species of the isopod genus *Asellus*. Kosswig and Kosswig (1940) showed that a European species, *A. aquaticus*, has cave and epigean forms and there is hybridization in nature between the two forms. Banta (1910a, b) used *A. stygius*, an epigean form, and *A. communis*, an obligate cave animal, both from North America. Banta found that the epigean form was less responsive to stimulation by bristles touched to various parts of the body than the cave form. The cave form was sensitive to stimuli emitted by lead shot dropped on to the surface of a piece of wood and a stronger stimulus was necessary to produce a response in *A. stygius*. *A. communis* responded to a much weaker stimulus produced by an electric tuning fork at 100 Hz than did the epigean form.

Poecilia sphenops, a cave fish from Tobasco, Mexico, exhibits a considerable gradation in eye development. Fish found at the entrance to the cave have large eyes and dark body pigmentation but deeper in the cave, the body pigment and eyes are much reduced (Gordon and Rosen, 1962). Hybridization occurs between all forms of the fish (Gordon and Rosen, 1962; Peters and Peters, 1968; Walters and Walters, 1965; Zeiske, 1968) and the eye defects and pigmentation losses are related to genetic differences of different specimens and not to the conditions of light in the various parts of the cave in which the fish are found (Peters and Peters, 1966, 1968).

Walters and Walters (1965) found that Poecilia deep in the cave exhibited hypertrophy of the cephalic lateral line. They found that the preopercular and superorbital canals were not completely covered, resulting in exposure of the canal neuromasts. Other canals had a greater number of pores than in fish from the entrance of the cave. Some specimens of Poecilia from deep in the cave had the head and trunk dotted with large 'pit organs' containing neuromasts.

An increase in the number of free neuromasts has also been reported in several species of amblyopsid cave fishes (Poulson, 1963a). Heuts (1951) compared Caecobarbus geertsi, a blind barb from the Belgian Congo, with Barbus holotaenia, an eyed relative from rivers near the caves in which the fish are found. Heuts reported that the blind fish had a larger number of sensory buds on the head than did the eyed species.

Chardon (1966) studied the South American cave catfish Astroblepus pholeter and compared it to other more or less related eyed catfishes, especially A. grixaluii Humboldt. Chardon found that there was a reduction in the two halves of the anterior swim bladder, and the lateral walls of the swim bladder had an ossification of the outer covering, the tunica externa. Chardon also found that the connection normally present between the two halves of the anterior swim bladder in silurid fishes was absent in A. pholeter. The inner ear and Weberian ossicles of Astroblepus did not differ from other fishes of the family Astroblepidae but the optic lobe was degenerate and the forebrain was enlarged and had several convolutions. Chardon (1966) associated the forebrain with the enlarged barbels he found in A. pholeter. He proposed that the changes in the swim bladder would raise its resonant frequency and aid in detection of higher frequency signals.

Eigenmann (1909) did not find any change in the ear of the blind cave fish *Amblyopsis spelaea* but Poulson (1963a) found that *A. spelaea*, as well as two other amblyopsid cave fishes, *A. rosae* and *Typhlichthys subterraneus*, had enlarged semicircular canals and otoliths as compared with epigean amblyopsids.

There has been a reduction of the optic lobe of the brain in many cave fishes (Poulson, 1963a; Vandel, 1965), but not all of these fishes show hypertrophy of other portions of the brain. Poulson (1963a) found that several cave amblyopsids had enlarged forebrains and Chardon (1966) found the same for *Astroblepus pholeter*. Charlton (1933) found a reduction of the optic lobe in *Troglichthys rosae* and *Typhlichthys eigenmanni* as well as in the blind goby *Trypauchen*. *Pimelodella kronei*, a cave catfish, has reduced optic lobes (Pavan, 1946), and *Typhlogarra widdosoni* is reported to have an enlarged forebrain as well as reduced optic lobes (Marshall and Thines, 1958).

Poulson (1963a) compared the capacity to detect prey of blind cave amblyopsid fishes and found it to be better than that of blinded epigean amblyopsids.

Summary

Investigations of various species of cave fishes, including *Astyanax jordani*, have not revealed changes in any sensory structures that can positively be considered to help the fish survive in the cave environment. However, studies of the cave amblyopsids (Poulson, 1963a) and the blind and river species of *Astyanax* (Rasquin, 1949) have revealed changes in other physiological mechanisms of these cave fish.

This suggests that investigations of other, as yet unexplored, systems might reveal modifications that help the blind fish survive in the caves.

The auditory system is responsive to environmental change (von Békésy, 1960); thus, it is a likely place to look for sensory modifications. The auditory system has not been thoroughly studied in blind cave fishes and, while there is some evidence that suggests the presence of modifications in the auditory capacities of these animals (Chardon, 1966; John, MS; Poulson, 1963a), there is no experimental data indicating that blind cave fish actually make use of these morphological modifications for auditory purposes. In order to determine whether there are adaptive modifications in the auditory system of Astyanax jordani, and if the animals have a different behavioral response to sound than the river fish, their auditory capacity has been investigated.

Sound Detection and Processing by Fish

In addition to providing information about the role of audition in blind cave animals, the comparison between the two species of Astyanax should provide the basis for determining more precisely the roles that the swim bladder, Weberian ossicles, and inner ear play in processing an acoustic signal. Whereas information about hearing in blind cave fishes is minimal, there is a considerable amount of data about hearing in fish and an understanding of this should provide a basis for interpretation of the information obtained from the blind cave fish study.

Underwater sound detection

Sound consists of two forms of energy: pressure and displacement. Pressure waves are produced by alternate compression and rarefraction of the medium, and displacement consists of particle oscillation. Although both pressure and displacement are found in air-borne sounds, the particle oscillation is very small, and pressure is the predominant form of energy (Albers, 1965). Water-borne sounds have strong displacement energy since water is denser than air and particle motion can be transmitted more easily. On the other hand, water is poorly compressible and this presents a high impedance to pressure waves (Albers, 1965).

Particle displacement attenuates as the inverse square of the distance from the source ($1/r^2$) while pressure attenuates as the inverse of the distance ($1/r$) (van Bergeijk, 1964). Harris and van Bergeijk (1962) have calculated that pressure and displacement from the same source have equal amplitude at a distance of $\lambda/2\pi$(wavelength/2π) from the source. The area between the source and the point of equal amplitude is called the near-field and the area beyond this point is the far-field (van Bergeijk, 1964). Displacement is the stronger stimulus in the near-field while pressure predominates in the far-field (van Bergeijk, 1964). More recently, Siler (MS) has argued that there can be no real demarcation point between the near- and far-field but rather that the displacement energy gradually drops off and pressure becomes the predominant signal component.

Harris (1964) used a series of equations to determine the displacement amplitude at any distance from the source. He calculated that for most biological signals the propagation of energy from a sound source most nearly resembles signal propagation from a pulsating sphere. Displacement amplitude (d) at any one point is then calculated as:

$$d = \frac{p}{2\pi f \rho c}\left(1+\frac{1}{(Kr)^2}\right)^{1/2}$$

or

$$d = \frac{p}{2\pi f \rho c}\left(1+\left[\frac{\lambda}{2\pi r}\right]^2\right)^{1/2}$$

(Harris, 1964). The symbols used in the equations are:

c = velocity of sound in water (approximately 150,000 cm/sec for sea water).
d = displacement amplitude in cm.
f = frequency in Hz.
$k = (2\pi f)/c$.
p = pressure.
ρ = density of water (taken as 1 gm per cc).
λ = wavelength (c/f).

The first half of the formula indicates the amount of displacement in the far-field and the right hand portion (in parentheses) indicates the displacement amplitude due to the near-field effect. As the wavelength increases (i.e., frequency decreases), the contribution of the near-field displacement increases, but as the frequency increases, the displacement due to the far-field propagation decreases. It is apparent from the equation that the major source of displacement is at low frequencies and that a displacement detector would not be effective except to detect low frequency sounds unless the fish were next to the sound source.

The lateral line only detects displacement energy (Dijkgraaf, 1963; Harris and van Bergeijk, 1962; Kuiper, 1956), and the major stimulus for the lateral line organ is present when the fish is in the near-field. When the fish is in the far-field, it may still be presented with some displacement (depending upon the frequency), but the major far-field stimulus is pressure, which stimulates the swim bladder (Harris, 1964). If, however, the pressure is extremely intense (p in the formula), there could be some displacement energy present to stimulate the lateral line. It might also be possible for the displacement energy in the near-field to stimulate the swim bladder and thus the inner ear (van Bergeijk, 1964, 1967a; Harris, 1964; Tavolga, 1965).

A major consideration in studies of fish audition is that the displacement energy has both magnitude and direction while pressure is purely a scalar stimulus and has no direction (van Bergeijk, 1964).

Swim bladder

Pressure waves will cause a bubble of air, present in fish as the swim bladder, to pulsate (van Bergeijk, 1967a; Harris, 1964). Moreover, the density of the body of a fish is approximately that of water; consequently, sound travels through the body unless it impinges upon a structure of different density, such as the swim bladder (Harris, 1964). The swim bladder of fishes thus acts as a transducing mechanism (van Bergeijk, 1966, 1967a) and its function is analogous to that of the tympanic membrane of terrestrial vertebrates; i.e., pressure is transduced into displacement which is detected by the inner ear (von Békésy, 1960).

Signals detected by the swim bladder are transmitted to the inner ear via the Weberian ossicles in the ostariophysines or through the skeleton and muscles in non-ostariophysines (Tavolga, 1965).

Experimental studies by Kleerekoper and Roggenkamp (1959) have demonstrated that deflation of the swim bladder in *Ictalurus nebulosus* significantly raised the auditory thresholds. Von Frisch and Stetter (1932) found that the removal of the Weberian ossicles in *Phoxinus laevis* also raised the auditory thresholds.

The wall of the swim bladder is composed of two layers, the inner tunica interna and the outer tunica externa. The two layers are connected by thin connective tissue which allows the two layers of the air-filled bladder to move easily on each other (Chranilov, 1927, 1929; Evans, 1925; Tracy, 1911). There is considerable variation in the shape and make-up of the swim bladder in different species. Some species have a single chamber while others have two or more chambers (Dobrin, 1941; Jones and Marshall, 1953; Marshall, 1960). There is also variation in the connections between the chambers and the mechanism by which the chambers connect to the gut, if they do this at all (Dobrin, 1941; Jones and Marshall, 1953; Qutob, 1962). The physiology of the swim bladder also varies widely (Alexander, 1966; Fange, 1966) and in some bathypelagic species the swim bladder is filled with fat instead of gas (Iwai, 1959).

Several of the structural modofications found in the teleost swim bladder improve the auditory capacity of these animals. The major considerations in determining the role of the swim bladder in aiding audition are the volume displaced by the gas in the swim bladder (Harris, 1964) and the proximity of the organ to the inner ear (Tavolga, 1965). The gas bubble contained in the swim bladder is stimulated by pressure energy from a sound source and the motion of the gas bubble sets up a displacement wave that stimulates the inner ear of the fish (Alexander, 1966). The volume of gas in the swim bladder is affected by a change in pressure as a result of change in depth by the fish and this adjusts the animal's bouyancy (Jones, 1949, 1951, 1952; Qutob, 1962), but sound stimulation does not have this effect on the swim bladder since compressions and rarefractions are more rapid than the changes to which the walls of the swim bladder can mechanically respond (Alexander, 1966). Harris (1964) and Poggendorf (1952) have shown that the swim bladder has a resonant frequency that depends upon the volume of gas in the bubble and that the resonant frequency of the swim bladder increases as the volume decreases. It has been suggested that the maximum amplification of the signal by the swim bladder is at the resonant frequency and as the signal departs from this frequency, the response of the swim bladder drops off (Harris, 1964; Poggendorf, 1952). Alexander (1966) and Tavolga (1964) suggested that the tuning of the swim bladder is not as precise as Harris and Poggendorf believed and that the response of the swim bladder at frequencies other than the resonant frequency is only slightly less intense.

The cellular composition of the swim bladder wall does not affect its role in audition (Alexander, 1966) but the viscosity of the two layers and the connective tissue does play a role since this determines the response of the wall of the swim bladder to a signal.

In many species of fishes, the anterior end of the swim bladder lies beneath the first several vertebrae, and the response of the swim bladder to a sound is transmitted to the inner ear through the muscles and bones between the anterior end of the swim bladder and the inner ear. In a number of species of fish, there are various modifications that aid audition by bringing the signal closer to the ear before it is carried through, and attenuated by, the tissues of the fish. The mormyrids have small bubbles of air within the skull that detect pressure changes and the response of the bubble stimulates the ear which is in close proximity to it (Tavolga, 1965). Diesselhorst (1938) and Stipetić (1939) found that hearing in mormyrids is good and that these fishes can detect sounds up to about 3100 Hz.

The ear of clupeid fishes is also closely associated with an air bubble. In these fishes, the swim bladder has an anterior extension in the form of a thin tube that ends in a terminal vesicle encapsulated in bone and closely associated with the inner ear (O'Connell, 1956; Tracy, 1920; Wolfhardt, 1936). One of the terminal vesicles, the prootic bulla, is divided into two chambers separated by a heavy elastic membrane (O'Connell, 1956). The lower chamber is filled with air and the upper chamber is filled with perilymph (O'Connell, 1956). Sound stimulation of the air in the swim bladders of clupeids is carried close to the inner ear by the capsule in the prootic bulla, and the perilymph in the upper chamber of the bulla leads directly to the utriculus of the ear (Wolfhardt, 1936). Enger (1967) studied hearing in clupeids and found that they respond to a wide range of acoustic signals.

Anabantid, or labyrinth, fishes carry a bubble of air in the buccal cavity that is separated from the sacculus by a thin membrane.

Schneider (1941) found that the auditory range of the fishes without the air bubble reached 1000 Hz while fish with the bubble could hear sounds up to 2500 Hz.

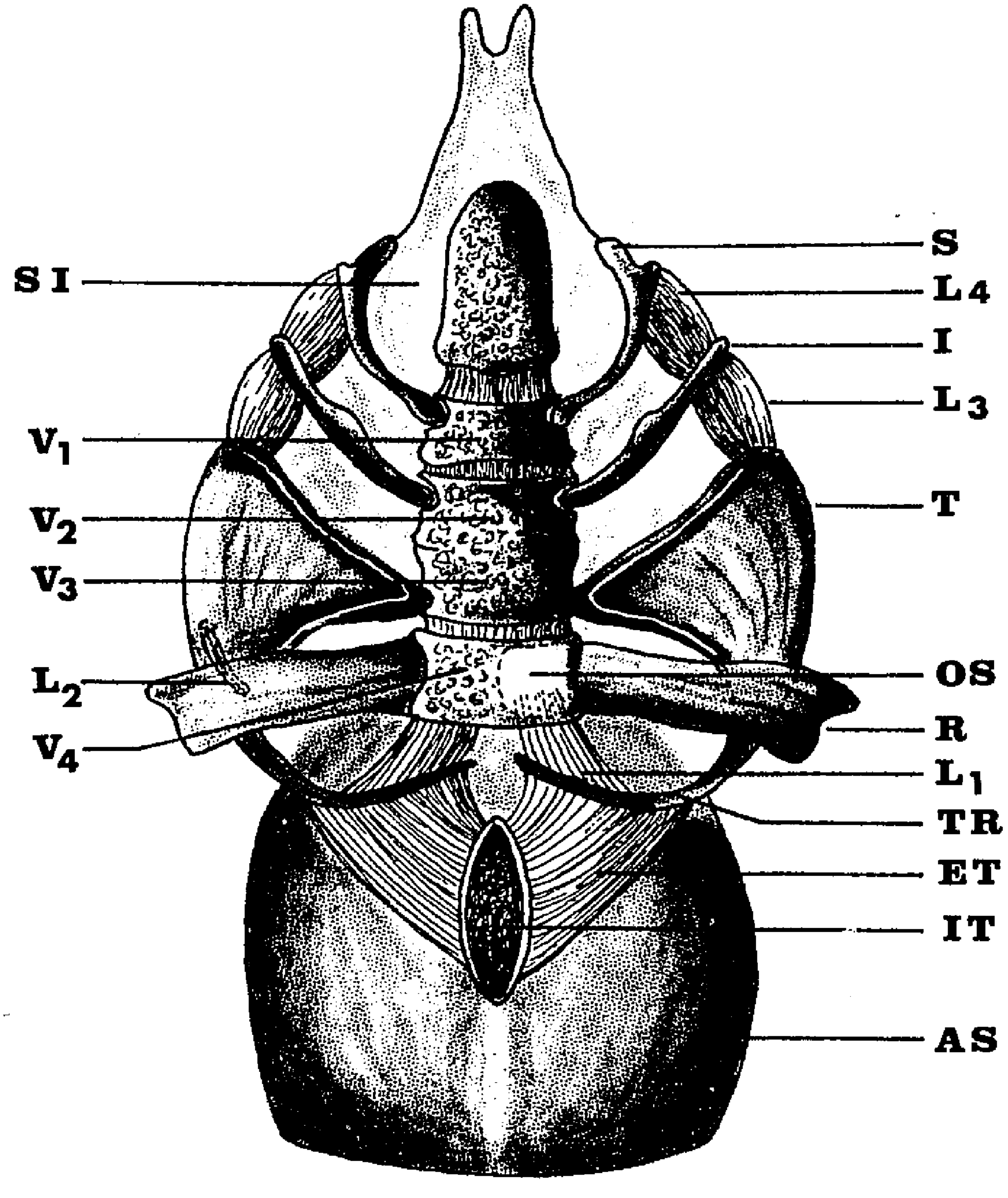
A number of other groups of fishes have anterior diverticula from the anterior chamber of the swim bladder (Greenwood, 1963; Nelson, 1955). Tavolga and Wodinsky (1963) studied hearing capacities in several holocentrids and found a wide variation in the range of frequencies to which different species would respond. Their results are probably related to the wide range of variation in the shape and proximity of the swim bladder to the skull in the holocentrids (Nelson, 1955).

Weberian ossicles and inner ear

The Weberian ossicles (Fig. 1) were first described by Weber (1820) and he suggested that the bones had a role in audition. It was many years, however, before the ossicles were actually shown to have an auditory role (Chranilov, 1929; Dijkgraaf, 1942, 1960; von Frisch and Stetter, 1932; Kleerekoper and Roggenkamp, 1959; Poggendorf, 1952; Sörenson, 1894). Several early workers had suggested that the Weberian ossicles respond to changes in hydrostatic pressure by the swim bladder and that the ossicles provide information about depth to the central nervous system (Bridge and Haddon, 1892, 1893; Nusbaum, 1908).

The tunica externa of the anterior chamber of the swim bladder in ostariophysines is slit on the dorso-anterior surface and the two edges of the slit attach to the posterior sides of the transformator process (Alexander, 1962). The transformator process is the posterior

FIG. 1. A composite drawing of a dorsal view of the Weberian ossicles of the Mexican blind cave fish and the eyed river fish. The differences in the ossicles of the two species are discussed in the text. AS, anterior chamber of the swim bladder; ET, tunica externa; I, intercalarium; IT, tunica interna; L1, 2, 3, 4, ligaments 1 to 4; OS, os suspensorium; T, tripus; TR, transformator process; R, fourth pleural rib; S, scaphium; SI, sinus impar; V1, 2, 3, 4, vertebrae 1 to 4.


S I
V1
V2
V3
L2
V4
S
L4
I
L3
T
OS
R
L1
TR
ET
IT
AS

extension of the third Weberian ossicle, the tripus (Alexander, 1962; Grassé, 1958; Nelson, 1949). Expansion of the tunica interna would cause the tunica externa to expand if the two layers were firmly connected. Alexander (1962), however, found that in ostariophysines the two layers are connected by a thin oily layer of tissue resulting in the tunica externa moving over the surface of the tunica interna when the inner layer expands. If there were no slit in the tunica externa, it would still expand when the inner layer enlarged but the dorsal slit takes up the expansion of the inner layer and only the slit enlarges in response to the expansion of the tunica interna. When the slit widens, the transformator processes are pulled ventrally, and when the tunica interna is compressed, the slit closes and the transformator process moves dorsally (Chranilov, 1929). When the transformator process is pulled ventrally (Fig. 1), the anterior end of the tripus moves anteriorly and the other ossicles also move in the anterior direction. When the swim bladder slit is closed, the transformator process is pulled dorsally and this pulls all of the other ossicles posteriorly (Chranilov, 1929).

The three bones making up the Weberian ossicles in the ostariophysines are attached to the vertebral column by cartilaginous, flexible connections and the three ossicles are connected together by several ligaments (Alexander, 1962). The tripus is connected to the second ossicle, the intercalarium, by ligament 3, and the intercalarium is connected to the most anterior ossicle, the scaphium, by ligament 4 (Chranilov, 1929). Ligaments 1 and 2 are associated with the transformator process and help keep the other ligaments taut so they will respond to movements of the swim bladder. Ligament 1 originates on the os suspensorium, a small bone that is fused to the medial side of the base of the fourth pleural rib. Ligament 1 inserts on the medial side of the transformator process. Ligament 2 originates on the base of the fourth pleural rib and inserts on the lateral side of the transformator. The elastic recoil of ligaments 1 and 2 pulls the transformator dorsally and closes the slit of the tunica externa when the swim bladder is compressed. Ligaments 1 and 2 also maintain tautness in ligaments 3 and 4 so they will not become slack and thus not respond to movements of the more posterior ossicles (Alexander, 1962; Chranilov, 1929).

Inner ear

The inner ear of fishes consists of three semicircular canals and three sensory capsules, each containing a bony otolith lying on a sensory macula made up of hair cells (Lowenstein, 1957). The pars superior (the detector of acceleration and angular motion) consists of the semicircular canals and one sensory capsule, the utriculus (Lowenstein, 1957). The pars inferior, which is associated with hearing, consists of two capsules, the sacculus and the lagena (Lowenstein and Roberts, 1951). The two sacculi are connected by a transverse canal that leads into the sinus endolymphaticus. The sinus endolymphaticus is separated from the posterior atrium, the sinus impar, by a thin membrane that keeps the perilymph of the sinus impar separated from the endolymph of the inner ear (Grassé, 1958). Fluid motions set up in the sinus impar are transmitted to the sinus endolymphaticus and thence to the sacculi. Within the sacculus, the fluid moves the saccular otolith, the sagitta, which has a wing-like extension so that it can be more efficiently moved by the endolymphatic fluid (Lowenstein, 1957; Weber, 1820). Von Frisch (1938b) has also suggested that the fluid movements stimulate the lagenar otolith, the asteriscus, through a thin membrane that separates the sacculus and lagena. Lowenstein and Roberts (1951) obtained neurophysiological evidence to support the auditory role of the lagena in the ray, but Furukawa and Ishii (1967a, b) have recently determined that the lagena in the goldfish may not be stimulated by auditory stimuli.

The perilymph of the atrium sinus impar is moved by the lateral and medial movements of its lateral and posterior walls, which are made up by the scaphium (Chranilov, 1929). When the scaphium moves medially, it would compress the perilymph unless fluid motions are carried through to the inner ear (van Bergeijk, 1967a).

Enger (1963), Furukawa and Ishii (1967a, b), and Lowenstein and Roberts (1951) have made recordings from the sensory maccular and auditory nerves of fishes. Enger (1963) found that there were several classes of nerves in the ear of the tench, and Furukawa and Ishii (1967a, b) have found that there are portions of the auditory nerve of the goldfish that will follow acoustic stimuli up to 1000 Hz.

The sensory macula contains hair cells that are very similar in structure to the hair cells of the tetrapod inner ear and the fish lateral line (Flock, 1965; Flock and Wersäll, 1962). The hair cells in all three systems are stimulated by shearing forces on the hairs (van Bergeijk, 1967a) and there is strong indication of polarization of these hair cells in the bony fish (Furukawa and Ishii, 1967a, b) just as in the ray (Lowenstein and Wersäll, 1959) and terrestrial vertebrates (Wersäll, Flock, and Lundquist, 1965).

METHODS AND MATERIALS

Behavioral measurements of hearing: Fish

Auditory thresholds were determined for Astyanax mexicanus and A. jordani 40 to 50 mm in standard length. This size corresponds to the young adult stage for both the river and blind animals (Breder and Rasquin, 1947b). The blind fish were obtained from the Aquarium Stock Company, New York, N. Y. and the Key Aquarium, Staten Island, N. Y. It was impossible to determine the caves of origin of these fish but it is most likely that they were descended from strains from La Cueva Chica or Cueva de los Sabinos or their hybrids since these were the only blind fishes available when the stocks were originally supplied to the commercial breeders and dealers (C. M. Breder, Jr., personal communication). Alvarez (1946, 1947) has described several morphological differences between the fishes from the two caves but no data are available on the same structures in laboratory-produced hybrids. For this reason, the cave of origin could not be determined by comparing the animal's morphology.

Several river fish, four or five generations removed from the rivers in Texas, were supplied by Dr. Kenneth R. John from stocks that he maintains. Other river fish were collected in the vicinity of San Antonio, Texas by Dr. Clark Hubbs.

The fish were individually housed in 2.5-liter aquaria with continuous aeration and filtration. For training and testing, they were transferred by net to the test tank (shuttlebox) which had neither aeration nor filtration. Feeding, with a dried meal preparation, took place daily in the late afternoon after all of the animals had been tested. Water temperature in the home tank and in the shuttlebox was maintained between 23.8 C and 26.8 C. All testing was done during the day and the fish were kept on a normal photocycle.

Test tank (shuttlebox)

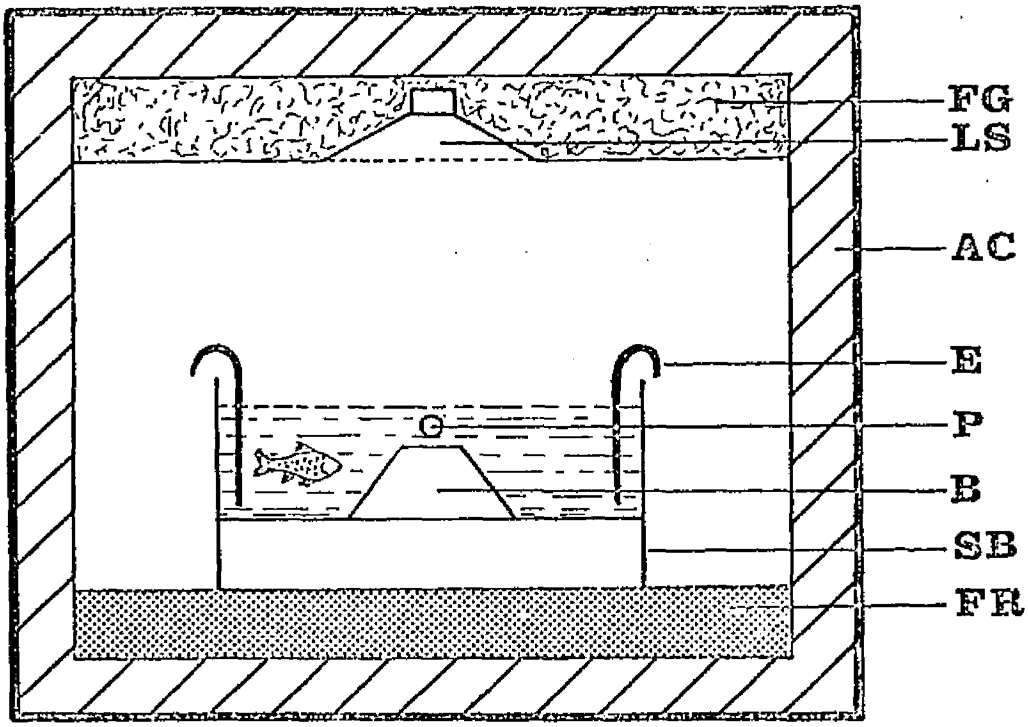
The experimental tank (Fig. 2) was made of $\frac{1}{4}$ inch thick acrylic plastic painted black on the outside. The internal measurements of the tank were 275 mm long, 75 mm wide, and 110 mm deep. A flat-topped barrier, 45 mm high and 25 mm long, was placed in the center of the tank dividing it into two equal compartments. The ramps leading to the top of the barrier were sloped at 45°. A photocell was mounted at the top of the barrier in order to monitor the crossing of the barrier by the fish.

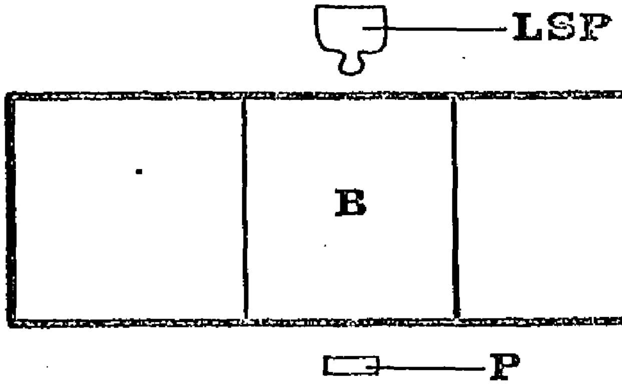
The water level above the barrier was adjusted so that it would permit the fish to cross the barrier but inhibit random crossings between presentations of the stimulus. The water in the shuttlebox was changed after three fish were tested in order to prevent oxygen depletion.

Stainless-steel screen electrodes were placed at either end of the tank. The electrical field presented through these electrodes went through the entire tank, not only the side of the tank with the fish. Shock intensity was selected by observing each fish during its first few trials in the shuttlebox and finding a shock level that would cause the animal to become agitated but did not damage it. No animal

FIG. 2. Shuttlebox and acoustic chamber used in determinations of the auditory thresholds in the two species of Astyanax. The loudspeaker, light source photocell and electrodes are attached to the control apparatus through jacks on the side of the chamber. The chamber was darkened and rested on double deflection rails (not shown) to help eliminate low frequency sounds. AC, acoustic chamber (4 inch walls); B, barrier; E, electrodes; FG, fiberglass; FR, foam rubber; LS, loudspeaker; LSP, photocell light source; P, photocell; SB, shuttlebox.

Shuttlebox and Acoustic Chamber





Top View of Shuttlebox

was ever lost from shock. Shock was continued throughout the testing sessions and was presented whenever the animal did not respond to the sound stimulus by crossing the barrier.

Training procedure

Thresholds were determined by using the avoidance conditioning technique developed by Behrend and Bitterman (1962) and modified by Tavolga and Wodinsky (1963). Animals were trained to cross the barrier in response to the onset of the sound stimulus in order to avoid receiving an electric shock. New animals were placed into the shuttlebox and allowed to become acclimated for several minutes after which they were presented with a 500 Hz pure sine wave at +20 dB (re 1 microbar) for 10 seconds. If the fish did not respond to the sound (the conditioned stimulus) within 10 seconds, it received an 8 millisecond 60 Hz pulsed shock (the unconditioned stimulus) each second until it crossed the barrier. When the fish crossed the barrier, it broke the beam of light to the photocell and both the conditioned stimulus (CS) and unconditioned stimulus (UCS) were stopped.

The reaction of naive animals to the shock consisted of an increase in random movement and this was often accompanied by barrier crossing. After several shock presentations, the fish learned to cross the barrier after one or two shocks. Within several days the fish started to show the avoidance response which consisted of crossing the barrier during the initial 10 seconds during which only the sound was presented. When the fish achieved a 90% avoidance level and maintained it for three consecutive days, threshold determinations were started. An 'avoidance response' was recorded when the fish crossed the barrier during the presentation of the sound and before the onset of shock. Crossing of the barrier as a response to the shock stimulus constituted an 'escape response'.

The blind fish had some problems finding the barrier although it was clear that they learned to associate the shock with the sound. After several days of training, they exhibited increased activity at the onset of the sound. In order to help orient to the barrier, an acrylic plastic paddle was used to push the blind fish in the correct direction for crossing of the barrier. The paddle was used after the fish had received four or five shocks during a trial. After about 7 days training with the paddle, the blind fish consistently found the barrier and thereafter they responded to shock or sound as rapidly as did the eyed river fish.

The river fish learned the escape response rapidly but did not develop a reliable avoidance response. A few achieved the 90% level in an open training chamber but they stopped avoiding or escaping when they were moved to the closed acoustic chamber for threshold determinations. After training the first several river fish in the open chamber, subsequent animals were trained in the closed acoustic chamber. These fish reached the criterion rapidly and no further training problems were encountered.

Threshold determination

Six blind fish (three male and three female) and eleven river fish (five male and six female) were used in the threshold determinations. Testing was done in a shuttlebox placed inside of an acoustic chamber. Each day's threshold determination was not started until an animal had given the avoidance response to a pure tone of at least

30 dB above the threshold range for five successive trials. Threshold determinations used the up-down staircase method (von Békésy, 1960; Dixon and Massey, 1951). If the fish responded to the sound and crossed the barrier during the 10-second CS period, the sound level for the next trial was lowered by 5 dB. If the animal did not respond to the sound, it received a shock and in the next trial the sound pressure level was increased by 5 dB. At the beginning of a day's testing, the fish generally avoided the shock until the sound level was lowered to the vicinity of the threshold for that frequency. There would then be a series of avoidances alternating with escapes. If a fish avoided a sound at -25 dBμb and then did not avoid at -30 dBμb, it was considered to have made one alternation. Twenty of these changes constituted the basis for the threshold for that animal for that day. The actual threshold was calculated by summating the mean sound pressure levels of successive escape-avoidance or avoidance-escape steps and dividing by the total number of changes for that day (Dixon and Massey, 1951).

After each trial there was a 1-minute intertrial interval (ITI) when no signal was presented. Barrier crossings during the intertrial interval (intertrial responses) were continuously monitored, and if the fish crossed the barrier three or more times during the intertrial interval, the water level over the barrier was lowered. This usually served to lower the intertrial responses. If intertrial crossings were high, responses during the CS period might, of course, have resulted from the random crossings rather than response to the sound.

Thresholds were sometimes determined with intertrial intervals that were randomly changed among 1, 2, 3, 4, and 5 minutes. If these thresholds were different from those with the 1-minute intertrial interval, the fish probably had developed a temporal sense and were responding to the 1-minute ITI rather than the sound stimulus. Fish were also tested with no sound in order to determine if the animals were responding to extraneous sounds that might have existed in the system.

After determining the threshold at the training frequency, the testing was transferred to another frequency for threshold determination. The fish were given one day's training without sound attenuation. Transfer steps of 100 or 200 Hz were initially used, but after the fish had had experience at several frequencies, transfers in 500-Hz steps were used. The blind cave fish had no difficulty in the transfer of stimulus response from one frequency to another, but the river fish trained at 500 Hz did not respond well to frequencies above 3000 Hz, even when 100-Hz steps were used in the transfer from 2500 Hz. Several new animals were given initial training at 3000 Hz, and these animals transferred their responses to frequencies up to 7500 Hz, the highest tested. During the course of the study, several fish were returned to previously tested frequencies after being tested at other frequencies, in order to determine if thresholds were different after additional experience with the avoidance procedure.

Frequencies below 3000 Hz were selected to correspond to the frequencies used in earlier studies on ostariophysine fishes (Enger, 1966; Jacobs and Tavolga, 1967; Weiss, 1967). Quantitative studies above 3000 Hz have never been made; frequencies above 3000 Hz were therefore selected so that they were far enough apart to prevent getting the redundant data that would accumulate if the test tones were too close to one another. The tones, however, were close enough together to show any trends in the audiogram.

Acoustic equipment

The stimulus was a pure tone sine wave produced by a Hewlett-Packard 200 CD or 202 C signal generator and amplified by a MacIntosh 75 audio amplifier or a Dyna Kit 35 Watt audio amplifier. Attenuation in the river fish study was done using a Hewlett-Packard attenuator set (model 350D) and in the blind fish study by an attenuator built into the control apparatus. Attenuation steps of 5 dB were used in all of the threshold determinations.

The test signal was presented through a 20.3-cm loudspeaker suspended 20 cm above the surface of the water in the shuttlebox. The frequency response of the speaker accurately followed the input signal over the range of stimuli used.

During training of the blind fish, the shuttlebox was placed into a container that lowered ambient noise levels by about 40 dB. Testing of the blind and river fish was in an acoustic chamber (Industrial Acoustics Corporation) that lowered ambient noise by 60 dB when measured with a band pass filter set for wide band noise. The chamber was double walled and RF shielded. It rested on double deflection railings to reduce low frequency pickup, and the floor of the chamber was lined with a 5-cm thick pad of foam rubber. The loudspeaker was suspended in the air from the ceiling of the chamber and was surrounded by fiber-glass and styrofoam to dampen backwaves and produce an infinite baffle. In the river fish study, an incandescent lamp was left on in the chamber since the fish stopped responding when placed in complete darkness.

Control apparatus

The solid-state control apparatus used in the threshold determinations has been described in detail by Tavolga (1966) and only a brief description is included here. The training of both kinds of fishes, and the testing of the river fish, was done by use of the Audio-Ichthyotron Mk IV. This is a semi-automatic apparatus controlling six tanks, any one of which can be selected by the experimentor for a trial. The presentation of the signal, timing of the CS period, shocking, and monitoring of the barrier crossing were all automatically carried out and recorded. Attenuator settings were done manually and, during the testing of the river fish, one of the channels from the Mk IV was wired to control the shuttlebox inside the acoustic chamber. Intertrial responses from all six tanks were continuously monitored.

Threshold determinations during the study of the blind fish were done using the Audio-Ichthyotron Mk V. The Mk V is a fully automatic device modeled after the audiometer designed by von Békésy (1960) for determining auditory thresholds using the staircase method. The Mk V controls only one tank, but it completely controls threshold determinations. Intertrial responses, latency of response, and attenuator settings are recorded on a seven-channel printer.

The signal from the audio-oscillator was put into the control apparatus and the signal was turned on by means of a photoconductive switch with a rise time of 10 milliseconds. This prevented sound transients from entering the system at the onset of the signal.

The unconditioned stimulus (shock) was produced by an internal source in the Mk IV and by a variable transformer (Fisher Powerstat model 136) attached to the Mk V. Shock pulsing and duration were controlled by the control apparatus. The voltage was measured at the source, and no attempts were made to determine the shock intensity received by the fish.

Sound calibration

Sound calibration in the acoustic chamber was done by placing a hydrophone (Chesapeake Instrument Company model SB 154C) into the wells of the test tank. The sound level was measured by the hydrophone and amplified through a transistorized pre-amplifier with a flat gain of 51 dB. The voltage to the loudspeaker corresponding to 0 dB (re 1 μb) was measured at each frequency and the voltage to the speaker was set each day to correspond to this level. The tank was re-calibrated several times during the course of the study; no significant changes were found in the voltage levels needed to produce a signal of 0 dB. Sound input and output were continuously monitored on a Tektronix 502A oscilloscope to insure signal purity. Signal intensity varied one or two dB in different parts of the shuttlebox, but the random movement of the fish compensated for this variation (Tavolga and Wodinsky, 1963).

Ambient noise levels were measured using a Krone-Hite band pass filter (model 310AB) and a General Radio sound level meter (model 1551-C) set at the recommended weighting network for each frequency.

Statistical analysis of data

The thresholds for each frequency were calculated by taking the mean of the individual thresholds at that frequency. A Student's t-test was used to compare thresholds at the same frequency between the two kinds of fishes. Analysis of variance was used in comparing three or more adjacent thresholds for each kind. Comparisons between the two complete audiograms, and between portions of the two audiograms, were done with the Mann-Whitney U test.

Morphological investigations - general methods

The fish used in the histological and anatomical investigations were from the same sources as the animals used in the behavioral studies. In some instances, animals that had been used in the behavioral studies were preserved and used for dissection.

Histological sections were prepared with Delafield's hemotoxylin and Eosin Y as described by Lillie (1965). Decalcification was accomplished in a modified Bouin's solution for a minimum of 7 days. The Bouin's solution contained 90-95% formic acid instead of acetic acid, since formic acid is supposed to provide better decalcification (Dr. Albert Stenger, personal communication). Cross and sagittal serial sections at 10 microns were made of whole animals as well as of separate preparations of the swim bladder, Weberian ossicles, and inner ear.

Alizarin preparations, using an enzymatic maceration procedure (Taylor, 1967), were used for studies of the gross structure of the Weberian ossicles and inner ear. Dissections were made of specimens preserved in 10% buffered formalin.

Measurements were made with a vernier caliper and a Bausch and Lomb optical micrometer.

RESULTS

Auditory Thresholds

Behavior of the fish during training and testing

The Mexican blind cave fish and the eyed river fish responded differently to the avoidance training.

Many visually dependent species of fishes learn to respond to visual stimuli much more rapidly than to auditory stimuli (Jacobs, MS; Jacobs and Popper, 1968); thus Astyanax mexicanus probably learned to escape by associating the easily seen barrier with shock termination. The blind cave fish had considerably more difficulty in learning to escape since they could not orient to the barrier. They would only cross the hurdle if they moved in the correct direction as a random response to the shock. In order to facilitate escape training, the fish were pushed with a paddle toward or over the barrier, if they had not crossed after receiving five shocks in a given trial. After approximately 7 days, in which assistance was needed in about 25% of the trials, the fish learned to escape consistently after receiving one or two shocks. During the intertrial interval, trained blind fish would face the barrier and remain fairly still in the water, whereas trained river fish were active and did not appear to show a specific orientation response.

After learning to escape, the blind fish started to develop the avoidance response, and the avoidance latency (time between onset of the sound and crossing of the barrier) became very similar to the avoidance latency of the river fish. Behavior of the blind fish during the avoidance portion of the training was the same as the behavior of other species of fishes trained in the same manner (Jacobs and Popper, 1968; Jacobs and Tavolga, 1967, 1968; Tavolga and Wodinsky, 1963, 1965).

The river fish learned to escape within the first 10 trials in the shuttlebox on the first day of training, but they seemed to have considerable trouble in reaching the avoidance criterion even after 3 months of training. During training their avoidance responses were not consistent from day to day, and they appeared to be easily distracted by ambient visual stimuli. In order to minimize such visual stimuli, the training was shifted to the closed acoustic chamber. New river fish, and river fish unsuccessfully trained in the open tank, reached the 90% criterion level within two weeks after being placed into the closed chamber (N = 12). Blind fish took 4 weeks to learn the avoidance problem.

More river fish than cave fish were used for threshold determinations since the river fish had great difficulty in the transfer of the avoidance response from the training tone, 500 Hz, to other frequencies. Consequently, none of the river fish could be used at all of the frequencies for which data are reported. The blind fish had no difficulty in response transfer and they were used at all of the test frequencies.

The first four river fish to reach criterion in the acoustic chamber were transferred to 400 Hz at which thresholds were successfully obtained. The fish were then transferred to 300 Hz but they stopped avoiding or escaping after one or two days of testing. The fish were transferred back to 500 Hz and started avoiding again almost immediately. Blind fish were initially transferred to 300 Hz and below without any transfer problems.

All other river fish were transferred initially to frequencies above the training tone and no difficulty was encountered until the fish were tested at 2500 or 3000 Hz. The fish would not avoid above 50% of the time at these frequencies even after being transferred in 100-Hz steps from the highest frequency at which the fish would respond and give thresholds. In order to obtain data above 2500 Hz, several new fish were trained at 3000 Hz and these animals were successfully transferred up to 7500 Hz. Training at 3000 Hz took no longer than training at 500 Hz and thresholds taken on these fish at frequencies below 3000 Hz were the same as the thresholds determined with the fish trained at 500 Hz.

Data were obtained at 300 Hz and below by shifting the river fish to these frequencies after threshold determinations at higher frequencies had been made for 1 month or more. The fish were transferred to 300 Hz in 500-Hz steps from higher frequencies. There was no difficulty obtaining data at the low frequencies after the fish had been tested at several higher frequencies.

Threshold determinations - River fish

Auditory thresholds for the river fish were determined at 17 frequencies from 50 to 7500 Hz. Frequencies below 50 Hz were not used in either study since the speakers did not reproduce low frequency signals without considerable distortion and loss of signal stability.

Thresholds for the river fish are shown in Table 1 and are represented graphically in Fig. 3. At 50 Hz, the mean threshold for four fish was -14.9 dBμb with a range of -8.8 to -28.0 dBμb. The threshold at 7500 Hz was +2.8 dBμb and the lowest threshold for the river fish was at 1000 Hz where the threshold was -40.5 dBμb.

Statistical comparisons of the river fish thresholds are shown in Table 2A. The thresholds from 100 Hz (-32.7 dBμb) to 500 Hz (-30.6 dBμb) varied significantly (P = 0.005), while the thresholds from 800 Hz to 1500 Hz varied insignificantly (P = 0.1). The thresholds from 2000 to 3000 Hz were alike (P = 0.1), but there was significant difference between the thresholds from 4000 to 5200 Hz (P = 0.05).

The threshold determinations at 1000 Hz for the river fish are shown in Table 3. An analysis of variance of the thresholds of the four fish tested at 1000 Hz showed that the mean thresholds were all similar to one another (P = 0.1) and similar results were obtained at 500 Hz (P = 0.1). A comparison between a single river fish and the audiogram for all of the river fish was not possible since a single animal was not tested at all of the frequencies. Instead, a composite audiogram is shown in Fig. 4A. The composite is made up of the thresholds for three river fish: R17 from 50 to 1500 Hz, R20 from 2000 to 4500 Hz, and R26 from 5200 to 7500 Hz. The similarity between the individual thresholds and those for all of the animals is apparent from Fig. 4A. The thresholds among the three fish used in the composite did not vary significantly from one another at the frequencies where there was overlap of thresholds determined.

TABLE 1. Auditory thresholds in the river fish, <u>Astyanax</u> <u>mexicanus</u>

Frequency (in Hz)	Mean threshold (in dB re 1 μb)	Standard deviation	Number of determinations	Number of animals
50	-14.95 (-8.8 to -28.0)*	6.41	14	4
100	-32.7 (-27.3 to -37.8)	8.88	12	4
200	-39.1 (-35.5 to -46.5)	6.10	9	3
300	-31.2 (-21.0 to -40.0)	6.61	13	4
500	-30.6 (-23.2 to -43.8)	5.85	16	5
800	-37.0 (-30.5 to -44.5)	3.75	12	4
1000	-40.47 (-32.1 to -46.6)	5.13	12	4
1500	-37.13 (-27.7 to -53.3)	7.49	13	4
2000	-19.8 (-7.9 to -34.0)	8.52	12	4
2500	-20.3 (-14.9 to -26.1)	5.05	15	5
3000	-23.8 (-15.8 to -39.0)	6.10	12	4
3500	-32.4 (-18.5 to -42.5)	8.58	18	7
4000	-22.9 (-17.0 to -28.0)	3.29	8	3
4500	-29.4 (-15.8 to -42.8)	6.98	11	5

continued on next page

TABLE 1. (continued)

5200	-23.9 (-19.1 to -30.8)	3.75	10	4
6400	-10.3 (-8.3 to -14.5)	2.32	9	3
7500	+2.82 (-6.8 to +9.7)	3.67	9	3

*range

FIG. 3. Graph of the auditory thresholds of <u>Astyanax</u> <u>mexicanus</u>. The outer limits on either side of the mean represent the standard deviation and the inner rectangles show the range of the 95 per cent confidence values. The figure is based on data in Table 1.

Auditory Thresholds of the Eyed River Fish *Astyanax mexicanus*

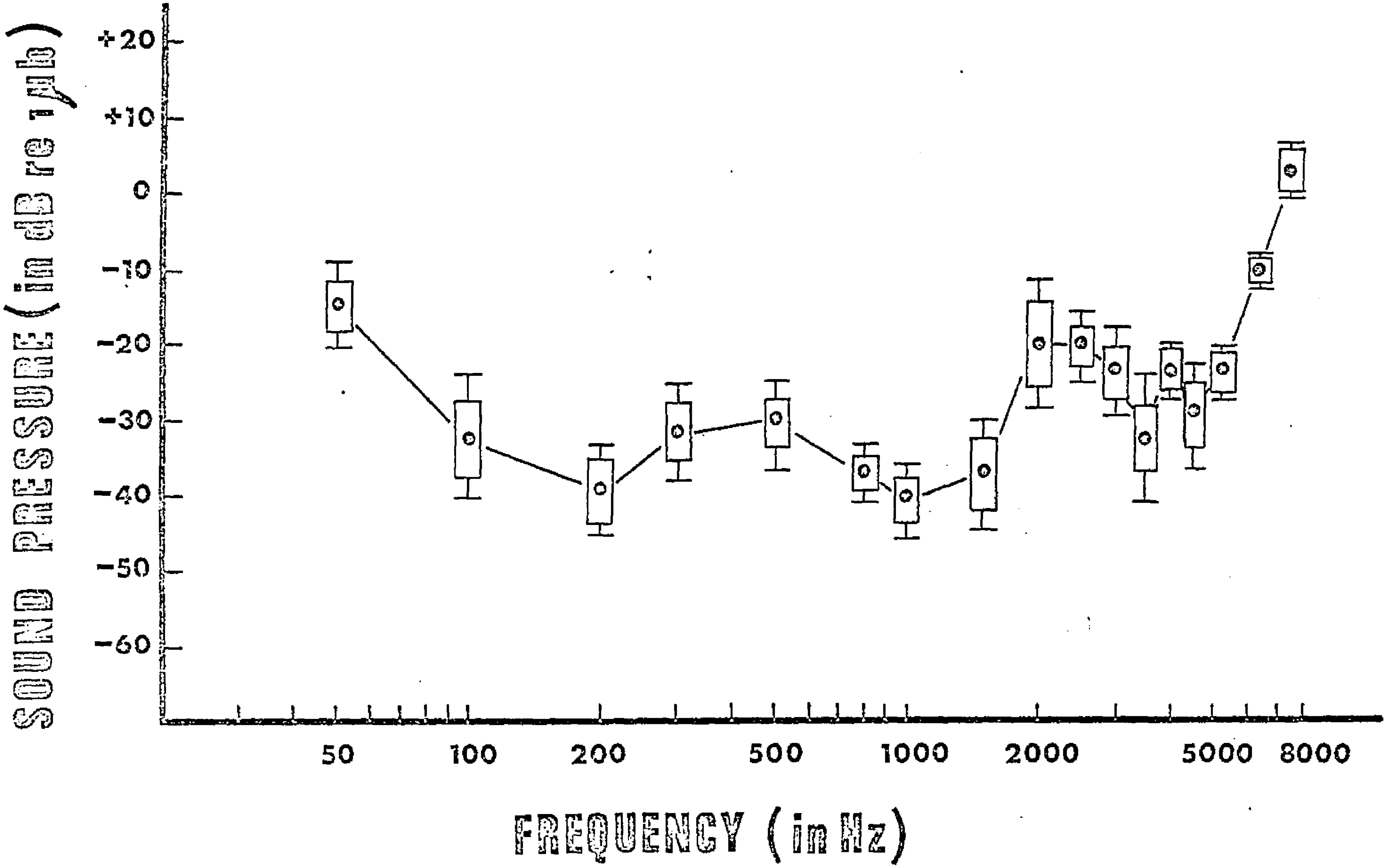


TABLE 2. Results of statistical tests between auditory thresholds at different frequencies

A. River fish

Frequency (in Hz)	P =*	
50	0.05	
100		
200	0.1	0.05
300		
500		
800	0.1	
1000		
2000	0.05	
2500		
3000		
3500		
4000	0.01	
4500		
5200		

B. Blind fish

Frequency (in Hz)	P =*	
50	0.005	
100		
100	0.005	0.005
200		
300		
500		
800	0.1	
1000		
1500		
2000	0.01	0.005
2500		
3000		
4000	0.05	
4500		
5200		

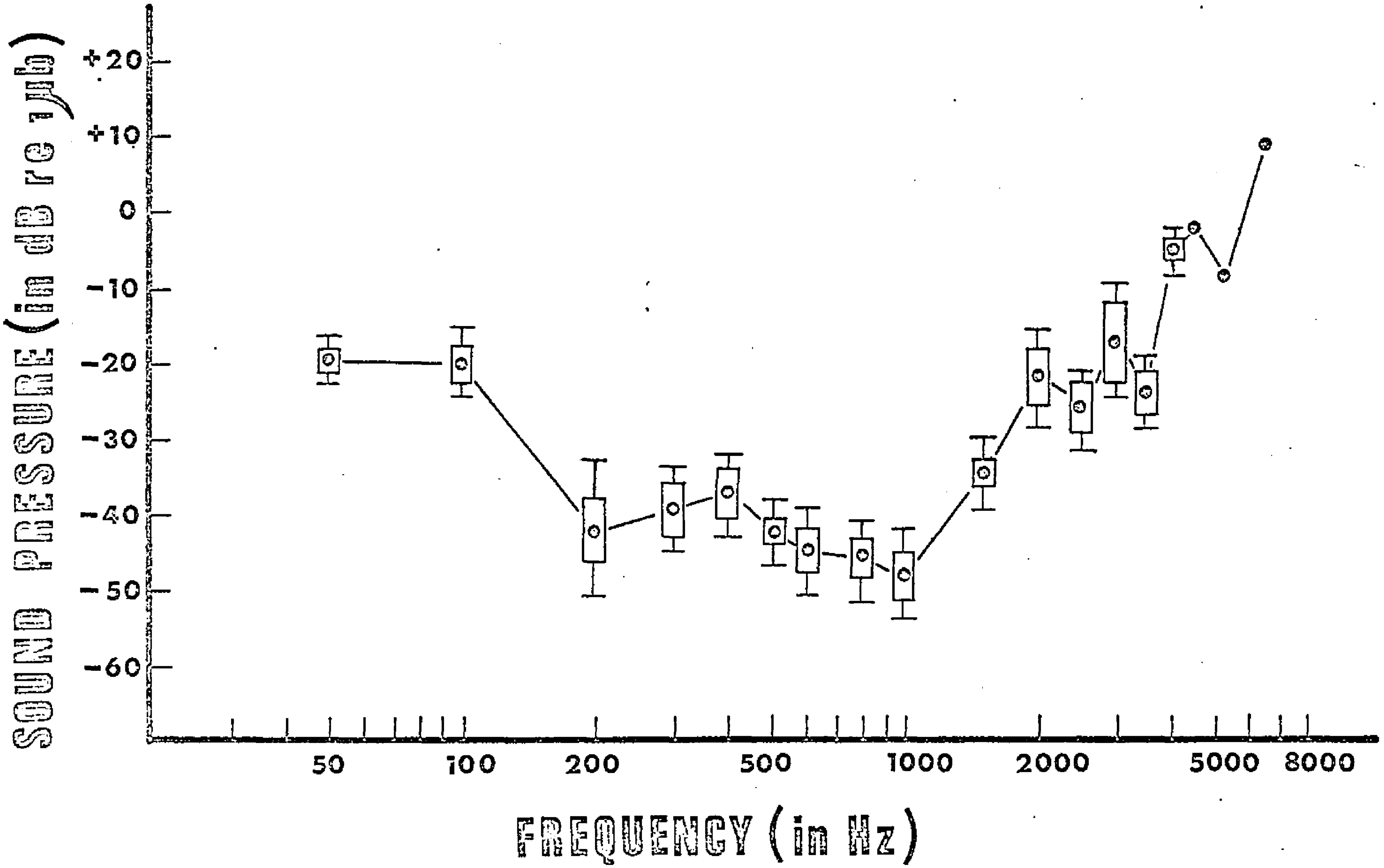
*Student's t-test used for testing significance between two frequencies and a one-way analysis of variance used for testing significance between three or more thresholds.

TABLE 3. Daily threshold determinations for all river fish tested at 1000 Hz

Fish #	Date	Threshold (dB re 1 µb)
R17	Feb. 3, 1968	-35.0
	March 1, 1968	-38.0
	March 9, 1968	-34.8
		-35.9 Mean threshold
R15	March 2, 1968	-39.5
	March 4, 1968	-45.0
	March 5, 1968	-46.6
		-43.7 Mean threshold
R12	March 8, 1968	-45.5
	March 9, 1968	-36.8
	March 11, 1968	-32.1
		-38.1 Mean threshold
R20	March 15, 1968	-41.3
	March 18, 1968	-44.8
	March 19, 1968	-46.3
		-44.1 Mean threshold

FIG. 4. Graph of the auditory thresholds of the Mexican blind cave fish, <u>Astyanax jordani</u> using data presented in Table 4. The outer limits on either side of the mean show the standard deviations and the inner rectangles represent the range of the 95 per cent confidence values. The standard deviation and confidence limits at 4500, 5200 and 6400 Hz could not be calculated since there were not enough threshold determinations made at these points.

Auditory Thresholds of the Mexican Blind Cave Fish



Auditory thresholds - Blind cave fish

Auditory thresholds for the blind cave fish were determined at 16 frequencies from 50 to 6400 Hz. Table 4 shows the thresholds, standard deviations, range, and number of determinations at each frequency. At 50 Hz, the mean threshold was -19.7 dB (re 1 µb) for 10 determinations on three animals. The threshold at 6400 Hz was +1.5 dBµb for one determination. The greatest auditory sensitivity in the cave fish was at 1000 Hz at which the threshold was -48.2 dBµb with a range of -35.1 to -56.1 dBµb. The thresholds at 1000 Hz and at 800 Hz did not differ significantly ($P = 0.1$).

The auditory thresholds of the blind cave fish are represented graphically in Fig. 5. Statistical analyses of the thresholds were made to determine if the thresholds at frequencies close to one another were statistically similar. Table 2B summarizes the comparisons of the t-tests and analyses of variance. The thresholds at 50 Hz (-19.7 dBµb) and 100 Hz (-20.1 dBµb) were similar ($P = 0.5$) as were the thresholds from 200 to 500 Hz ($P = 0.3$) and 4000 Hz to 5200 Hz ($P = 0.1$) There was significant variation in the thresholds from 2000 to 3500 Hz ($P = 0.01$).

The auditory thresholds did not vary significantly between individual blind fish. Mean thresholds were calculated for individual fish at 500 Hz (Table 5) and the thresholds were found to be insignificantly different from one another ($P = 0.1$). Similar analysis of the data at 1000 Hz also showed the similarity between the mean thresholds of different animals ($P = 0.1$).

TABLE 4. Auditory thresholds in the Mexican blind cave fish, *Astyanax jordani*

Frequency (in Hz)	Mean threshold (in dB re 1 µb)	Standard deviation	Number of determinations	Number of animals
50	-19.7 (-15.9 to -26.3)*	3.31	10	3
100	-20.1 (-12.8 to -28.7)	4.45	12	3
200	-42.3 (-27.1 to -58.2)	9.33	16	3
300	-39.2 (-28.7 to -46.6)	4.93	12	3
500	-42.1 (-35.9 to -51.2)	3.54	27	5
800	-45.5 (-35.8 to -60.2)	5.76	21	4
1000	-48.2 (-35.1 to -56.1)	6.55	19	5
1500	-34.8 (-30.2 to -43.7)	3.82	16	4
2000	-21.8 (-14.3 to -30.5)	6.18	14	4
2500	-25.9 (-19.5 to -36.1)	5.10	9	3
3000	-17.3 (-10.5 to -35.8)	7.48	9	3
3500	-24.0 (-15.5 to -30.9)	4.90	8	3
4000	-5.76 (-2.2 to -8.8)	2.75	6	3
4500	-2.2 (-9.2 to +6.5)	-	4	2

continued on next page

TABLE 4. (continued)

5200	-9.0 (-8.1 to -9.1)	-	2	1
6400	+1.5	-	1	1

*range

FIG. 5. A. Comparisons between the auditory thresholds for individual river fish and the composite threshold data for all of the river fish. Different animals were used for the thresholds at different points since no single river fish could be tested at all frequencies due to behavioral problems (see text). The auditory thresholds for the three fish used in the graph were virtually identical when they were determined at the same frequency.

B. Auditory thresholds for the Mexican blind cave fish A23 compared to the composite thresholds for all of the blind cave fish. A23 was tested at all frequencies, as were several other blind cave fish.

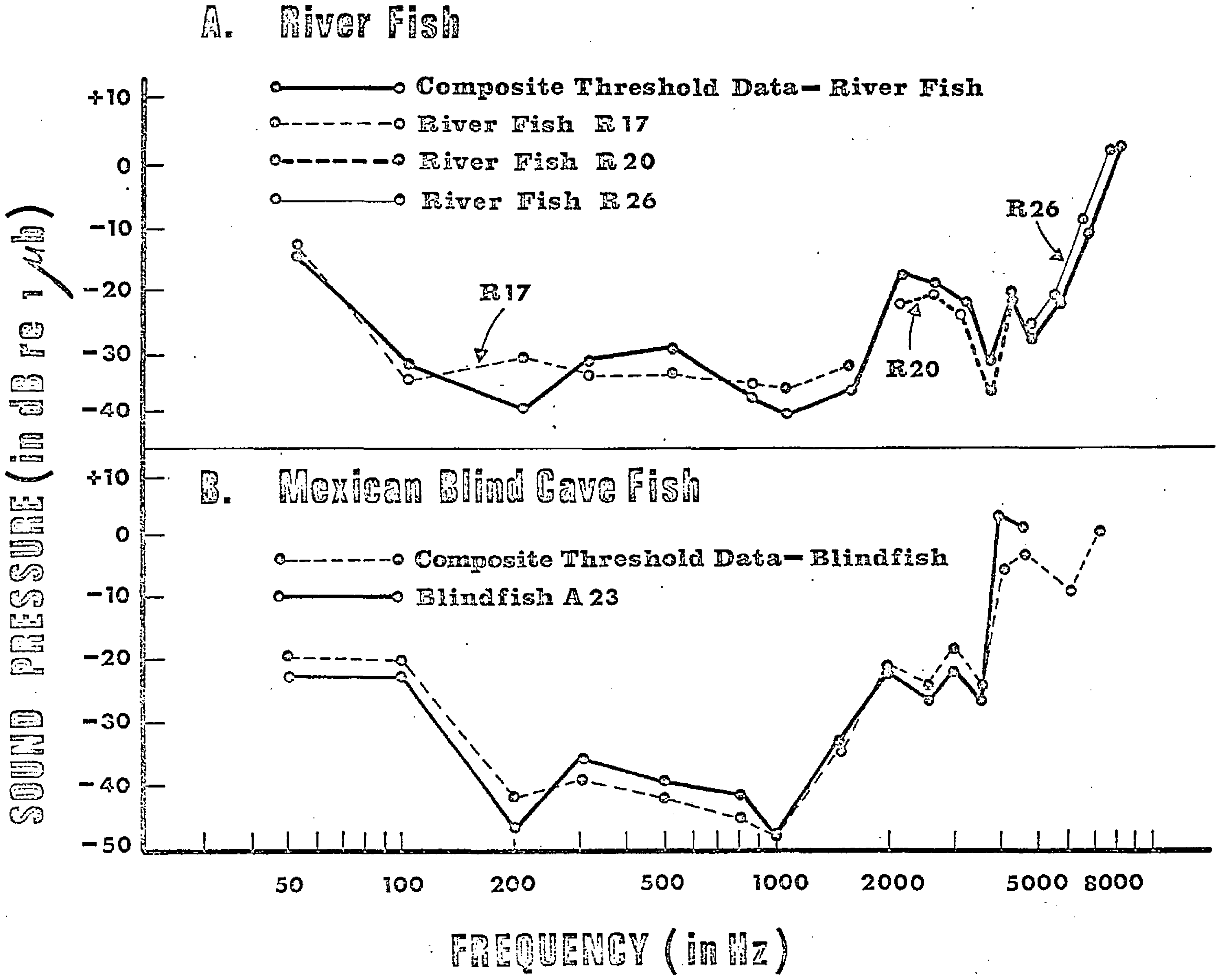

A. River Fish
Composite Threshold Data — River Fish
River Fish R 17
River Fish R 20
River Fish R 26
R 17
R 20
R 26
B. Mexican Blind Cave Fish
Composite Threshold Data — Blindfish
Blindfish A 23
SOUND PRESSURE (in dB re 1 μb)
+10
0
−10
−20
−30
−40
+10
0
−10
−20
−30
−40
−50
50
100
200
500
1000
2000
5000
8000
FREQUENCY (in Hz)

TABLE 5. Daily threshold determinations for all blind cave fish tested at 500 Hz

Fish #	Date	Threshold (dB re 1 μb)
A7	Nov. 10, 1966	-39.5
	Nov. 11, 1966	-40.8
	Nov. 12, 1966	-42.0
	Nov. 13, 1966	-40.9
	Jan. 3, 1967	-38.9
		-40.0 Mean threshold
A16	Nov. 15, 1966	-38.4
	Nov. 16, 1966	-40.7
	Nov. 17, 1966	-37.8
		-38.9 Mean threshold
A18	Jan. 15, 1967	-35.8
	Jan. 16, 1967	-40.3
	Jan. 17, 1967	-45.0
	Jan. 18, 1967	-43.5
	Jan. 20, 1967	-47.5
	Jan. 21, 1967	-40.8
	Jan. 22, 1967	-40.8
	Jan. 23, 1967	-45.8
	Feb. 4, 1967	-39.1
		-42.1 Mean threshold
A23	Jan. 24, 1967	-40.6
	Jan. 25, 1967	-39.8
	Jan. 26, 1967	-43.0
	Jan. 28, 1967	-36.5
	Feb. 14, 1967	-38.5
		-39.7 Mean threshold

continued on next page

TABLE 5. (continued)

A25	Jan. 26, 1967	-44.8
	Feb. 2, 1967	-39.8
	Feb. 3, 1967	-45.0
	Feb. 4, 1967	-51.2
	Feb. 6, 1967	-37.1
		-43.6 Mean threshold

The thresholds for blind cave fish number A23 are plotted, along with the thresholds for all of the blind fish, in Fig. 4B. A23 was selected as the representative animal since it was tested at all frequencies used in the study. It is clear from Fig. 4B that the threshold data for A23 are very similar to the combined data for all of the blind fish.

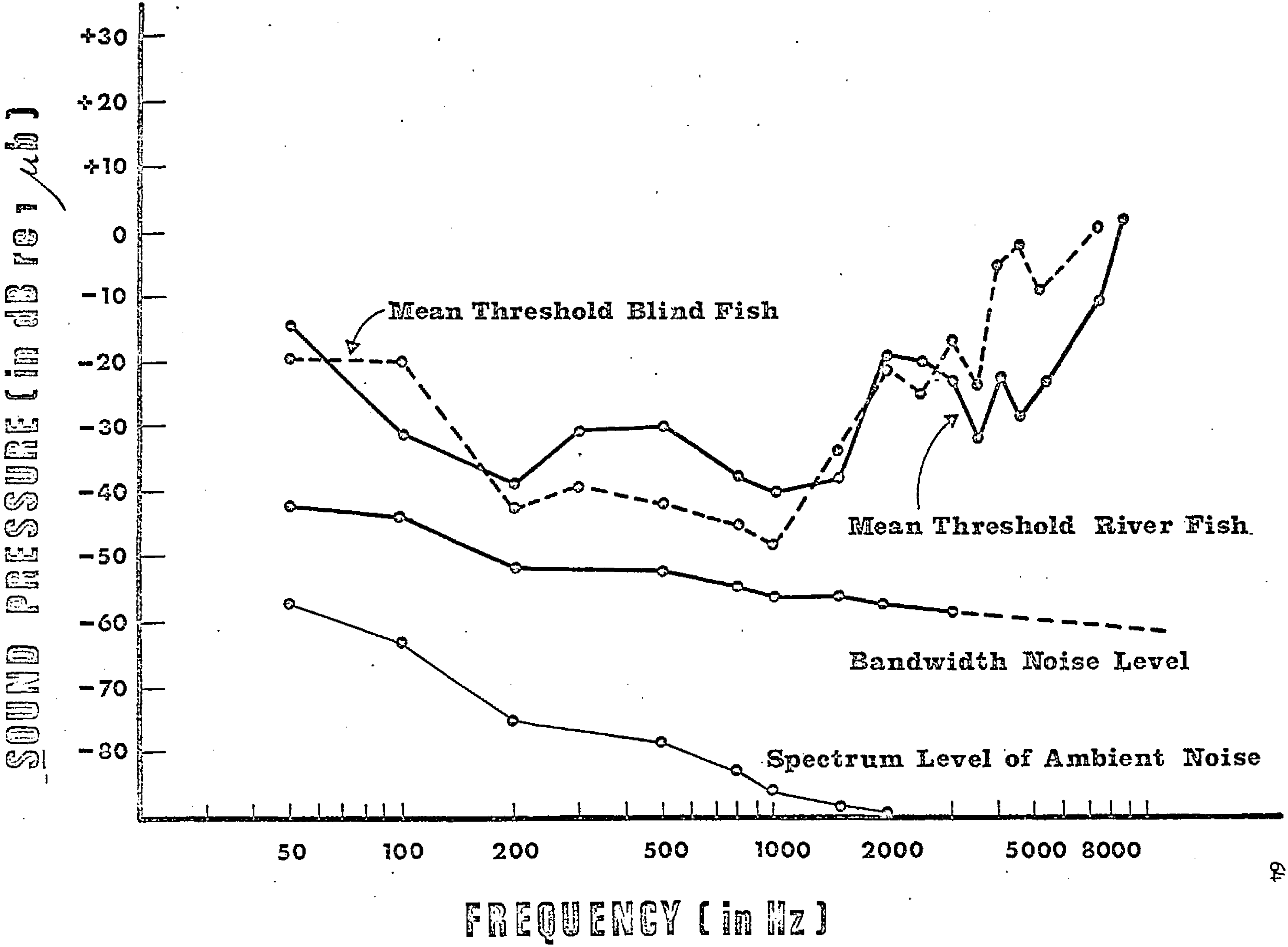
Audiogram comparisons

A comparison of the audiograms of the blind fish and river fish was made using the Mann-Whitney U test. The differences between the two complete audiograms (Fig. 6) were not significant ($P = 0.1$). Comparisons between individual points on the two audiograms were made using the Student's t-test, and significant variation was found at individual frequencies from 100 to 1000 Hz and from 3000 to 6400 Hz. The Mann-Whitney U test was used to compare the portions of the two audiograms from 50 to 1500 Hz and from 2000 to 6400 Hz; the differences indicated by the t-test were shown to be not significant ($P = 0.097$ and $P = 0.052$, respectively) when the segments of the audiogram were considered together.

Behavioral controls

Thresholds at 500 Hz were determined for several blind fish both immediately after training and after a 4-month period, during which time the fish were tested at a variety of other frequencies. The thresholds for the blind fish before and after the additional avoidance experience were compared in order to determine whether experience in any way affected the auditory thresholds. No differences

FIG. 6. A comparison between the auditory thresholds for the two species of Astyanax and the ambient noise levels in the acoustic chamber. Bandwidth noise levels were determined using a band-pass filter up to 2500 Hz. Above 2500 Hz the ambient noise levels could not be determined due to electrical noise in the system. Spectrum levels of ambient noise (noise per cycle) was calculated from the bandwidth noise level.



+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
SOUND PRESSURE (in dB re 1 μb)
Mean Threshold Blind Fish
Mean Threshold River Fish
Bandwidth Noise Level
Spectrum Level of Ambient Noise
50
100
200
500
1000
2000
5000
8000
FREQUENCY (in Hz)

were found that could be attributed to the additional experience.

Buerkle (1967) found some evidence that larger codfish have lower auditory sensitivity at some frequencies than smaller ones. Harris (1964) predicted that as the swim bladder of a fish enlarges, the resonant frequency would become lower and this may affect audition. All of the fish used in the experiments described here were of similar length so that no variation in threshold could be attributed to differences in size.

The temperature varied in the home and test tanks from 23.8 C to 26.8 C. There is some suggestion in the literature that fish will give different thresholds at different temperatures (Dudok van Heel, 1953; Offutt, 1968, personal communication). In order to determine whether the auditory thresholds of Astyanax would vary within the 3-degree temperature range used, river fish R15 was maintained and tested at both ends of the range with carefully controlled temperatures. Three determinations were made at 23.8 C and three determinations at 26.8 C. The mean thresholds were -33.5 dBµb and -34.5 dBµb, respectively. The variation resulting from temperature was not significant.

No differences were found in the auditory thresholds of male and female fish.

Ambient noise levels

Ambient noise levels in the acoustic chamber were measured, and the sound spectrum level (noise level per cycle) was calculated. The bandwidth noise level and spectrum level of the ambient noise is shown in Fig. 6, along with the thresholds for the river and the blind cave fish. Bandwidth noise levels had to be extrapolated from the values measured above 3000 Hz since electrical noise would have masked the ambient noise levels at those frequencies. The noise level in the chamber decreases up to 3000 Hz and should be expected to decrease even further since the greater portion of the noise in the laboratory consisted of low-frequency sounds produced by movement in the laboratory, street noises, and sounds from the subway seven floors below.

Bandwidth noise levels were obtained by placing a microphone in the acoustic chamber and passing the signal through the band pass filter and into a sound level meter. The high and low filter settings on the band pass filter were set at the same frequency, allowing a band of noise to pass through the filter around this center frequency. The bandwidth of the passed noise was found by multiplying the center frequency by 1.30 and 0.77, specifications supplied with the filter. The bandwidth included all frequencies at which the noise level per cycle was within 3 dB of the noise level at the center frequency. The noise passed drops off on either side of the center frequency in a normal curve. Once these 3-dB down points were passed, the noise dropped off at 24 dB per octave and it was so minimal that it could be ignored in the calculations. The sound spectrum level was calculated by converting the bandwidth of passed noise to dB (= 10 log bandwidth) and subtracting this from the bandwidth noise level (Scharf, 1966).

The noise levels in the acoustic chamber were at least 20 dB below the thresholds for both animals and the spectrum level was over 30 dB below the threshold levels.

The bandwidth noise level was determined several times during the course of these studies and there was no significant variation in the bandwidth noise levels.

Morphology of the Auditory Apparatus

Inner ear

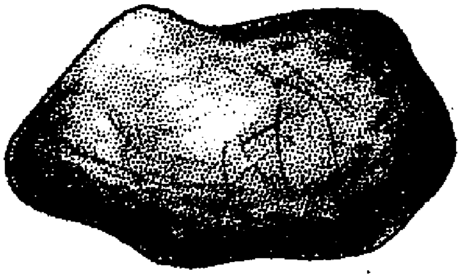
Schemmel (1967) found no difference between the inner ears of Astyanax mexicanus and A. antrobius from Cueva de la Pachon. No differences were found in the ears of the two forms of Astyanax used in the experiments described here.

The most variable portions of the inner ear were the otoliths. The lagenar otolith, the asteriscus, exhibited the greatest variation in shape, and the utricular otolith, the lapillus, also showed some variation. The asteriscus and lapillus are relatively flat, ovate calcareous concentrations with pitted edges. The lateral surfaces of both stones are slightly convex and the dorsal and ventral margins of the lapillus are somewhat flattened (Fig. 7). The dorsal margin of the asteriscus is flat and has a V-shaped notch that varied in depth in different specimens (Fig. 8). The ventral margin of several of the asteriscus specimens had serrated edges. The intra-specific variation in the shape of the otolith was as great as the inter-specific variation.

In order to determine if there was significant variation in the length, depth, and width of the two otoliths, they were removed from five blind fish and four river fish stained in Alizarin red, and measured. All of the fish were between 40 and 44 mm in standard length. The asteriscus had a mean diameter of 1.10 mm in the blind

FIG. 7. Drawings of the lateral view of the lapillus, the utricular otolith. The three river fish and three blind fish stones are representative of the otoliths found in all of the fish.

1mm



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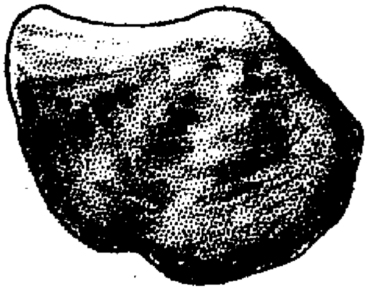
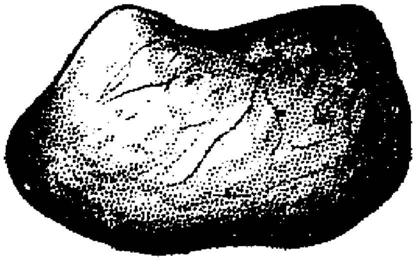


RA5



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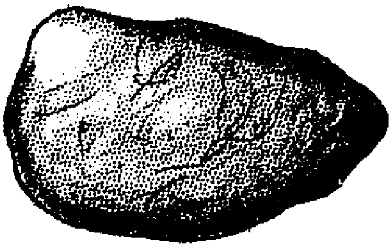
RA11



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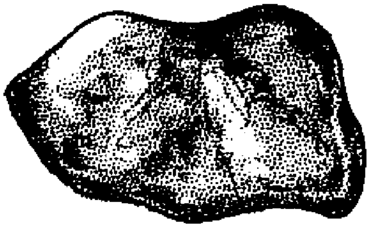
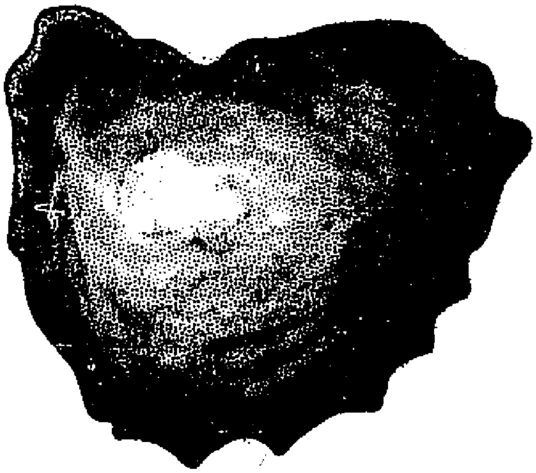


RA15

FIG. 8. Drawings of the lateral view of the lagenar otoliths, the asteriscus, of the river and blind fish. The otoliths shown are representative of all of the stones used in the study.



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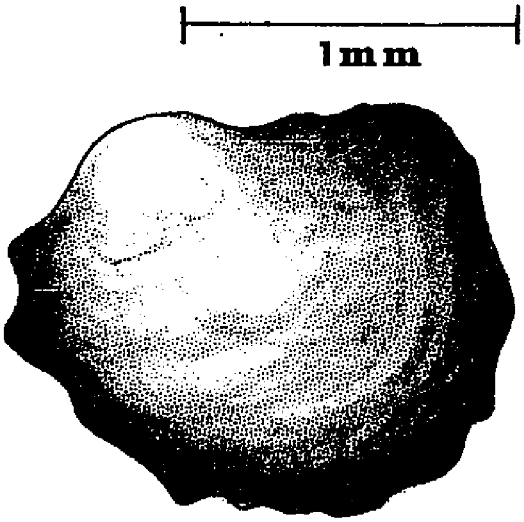
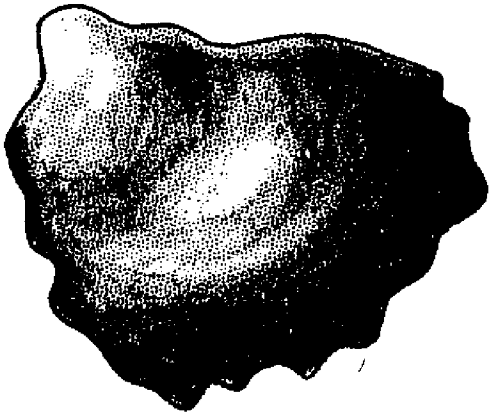


RA5



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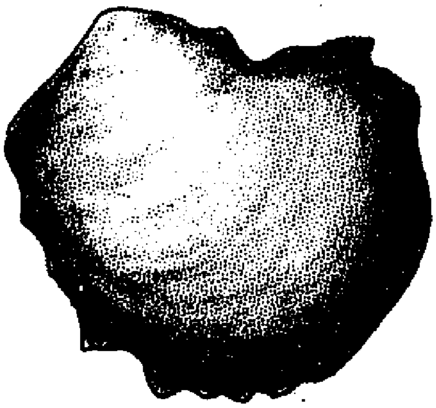

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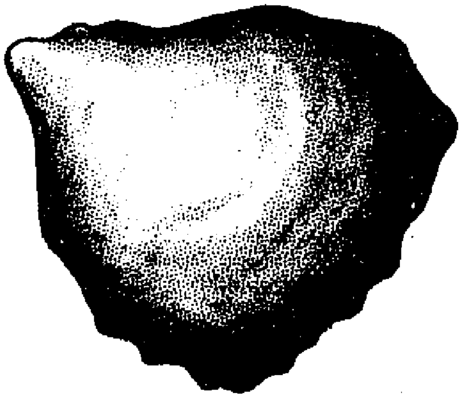

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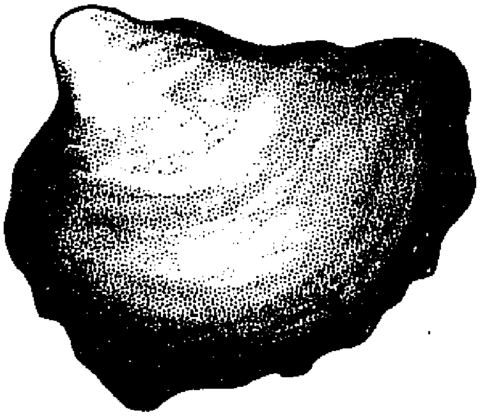


RA15

fish and 1.9 mm in the river fish. The difference was not significant according to the Student's t-test ($P = 0.7$). The maximum width of the asteriscus was 0.97 mm in the blind fish and 0.94 mm in the river fish ($P = 0.7$), and the mean depth of the two stones also varied insignificantly ($P = 0.6$). Similar measurements made of the lapillus indicated no inter-specific variation in these otoliths.

Serial sections of the sagitta were examined since the bone is very small and difficult to dissect out without destroying it. The sagitta does not appear to vary significantly between the two fish and projection into the endolymph (see Fig. 9) appears to be of the same length in the blind and river fishes. The connection with the saccular macula, upon which the sagitta lies, is the same in both kinds of fishes, and no differences could be ascertained in the shape of the sacculus in either form. The relationship of the sagitta to the transverse canal, through which the fluid movements pass to move the sagitta, are the same in both fishes.

Comparisons were made between the ear structures of males and females to determine if any sexual difference could be found. In the ear, as in all of the other structures that are discussed below, no sexual differences were apparent.

Swim bladder

Comparisons of the swim bladder were made using specimens that had been preserved in formalin for approximately 4 months. The five male and six female river fish were from 38.2 to 83.5 mm in standard length. The four male and four female blind fish were from 34.5 to 57.5 mm in standard length. Since the variation in standard length

FIG. 9. A. Photograph of a cross-section of the two sacculi of river fish SR20. The two otoliths are at slightly different levels and they are lying on the sensory macula. The photograph shows the transverse canal that connects the two sacculi and allows the movements of the endolymphatic fluid in one sacculus to be carried through to the other. (100 X).

B. Enlargement of one of the saccular otoliths of river fish R20 shown in Fig. 9A. The connection between the sagitta and the sensory macula at the edge of the otolith can be seen. (430 X).

S, sagitta; W, wing of sagitta, C, transverse canal; H, sensory hair cells.


A
W
S
C
B
W

was large, it was not possible to compare the absolute values of the various measures that were made. Instead, comparisons were made using a ratio derived by dividing the absolute value into the standard length of the fish or into the maximum depth of the fish, whichever was appropriate.

Although the standard lengths varied considerably, there is little variation in the inter-specific ratio and this indicates that the growth rate of the swim bladder is linear with respect to the total growth rate of the fishes.

The ratio of the mean length of the anterior chamber of the two-chambered swim bladder is 14.3 in the river fish and 13.6 in the blind cave fish. Variation in the length ($P = 0.6$), maximum depth ($P = 0.3$), and maximum width ($P = 0.4$) of the anterior chamber is not significant. Similar measurements were made on the posterior chamber, although it is probably not involved in audition (Alexander, 1966), and there were insignificant differences between the two species.

The shape of the swim bladder is the same in both species and the connection to the Weberian ossicles (see below) is identical in the two forms. Histological examination was not extensively undertaken since the cellular structure of the swim bladder does not appear to have a significant effect on its auditory role (Alexander, 1966).

Weberian ossicles

The investigations of the Weberian apparatus were limited to the structures that actually play a role in transmission of acoustic energy (Fig. 1). The first four vertebrae are generally included in discussions of the Weberian apparatus (see Nelson, 1949; Niazi, 1967;

Weitzman, 1962), but since they have no role in energy transmission, only the areas of actual contact with the functional ossicles were examined extensively. Cursory examination of the first four vertebrae did not show any significant differences between the blind cave fish and the river fish. Some authors consider the first ossicle to be the claustrum (Weitzman, 1962), but since it plays no role in audition (Alexander, 1966; Grassé, 1958) it is not included in this discussion.

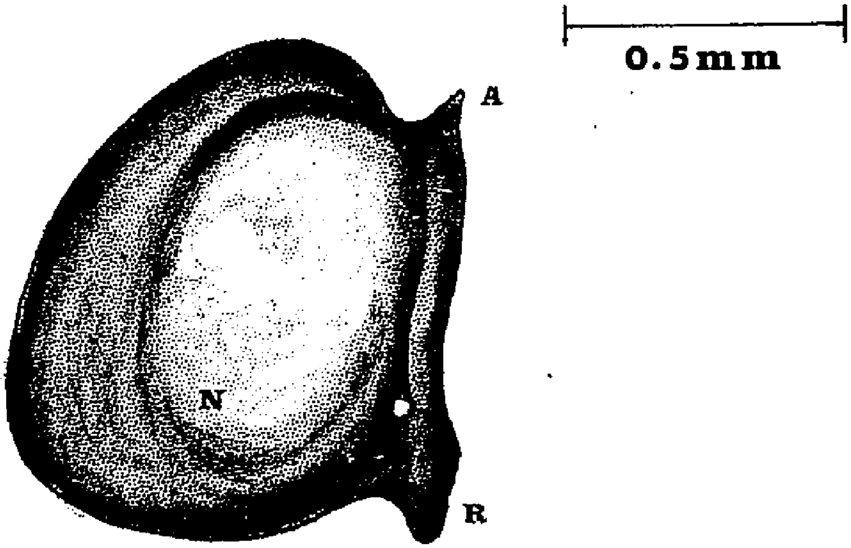
Several inter-specific differences were found in the Weberian ossicles. Alizarin-stained fish, 40 to 44 mm in standard length, were used for gross examinations of the ossicles and of the two anterior ligaments. Serial sections of fish, 35 to 40 mm in standard length, were used in the investigations of the connections of the ossicles to the vertebral column and of the connections of the swim bladder to the ossicles. Dissections of preserved materials were also used for gross analysis of the structure of the ossicles. The investigations were usually made on material from the left side of the fish, and comparisons with the structures from the right side revealed little intra-animal variation.

Scaphium

The most anterior ossicle actively involved in energy transduction is the scaphium. This oval bone has a straight posterior margin made up of dorsal and ventral processes one above the other (Fig. 10). Ligament 4, which connects the scaphium to the second ossicle (Fig. 11), inserts just ventral to the center of the convex lateral side of the bone. The concave medial side of the scaphium comprises the lateral, ventral, and posterior walls of the atrium

FIG. 10. Drawing of the scaphium of three blind fish (A.) and three river fish (B.). The ascending arm of the scaphium of the blind fish is shorter than in the river fish but all of the other characteristics of the bones are of of equal proportions in both species. A, ascending process; N, insertion of ligament 4; R, articulating process.

A



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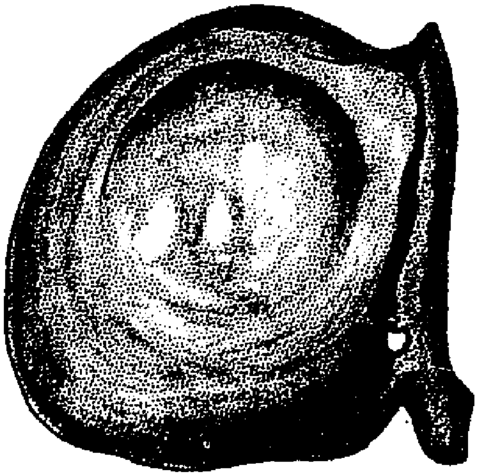

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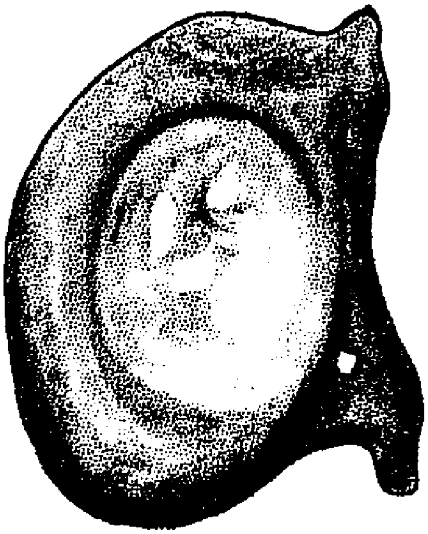
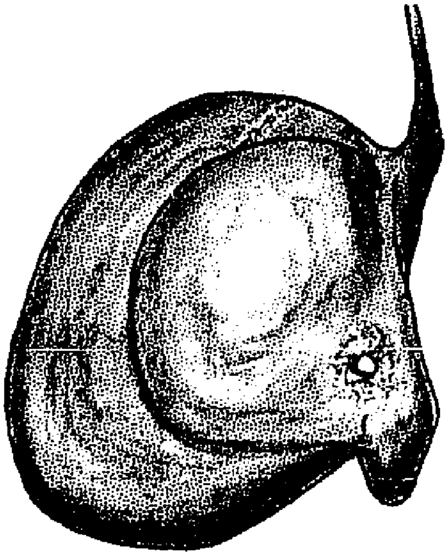
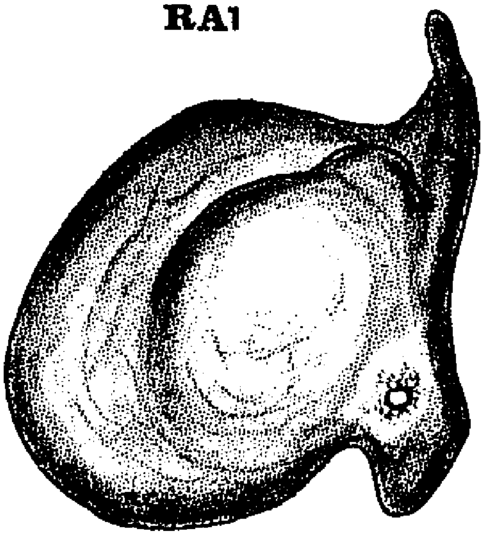


BA4

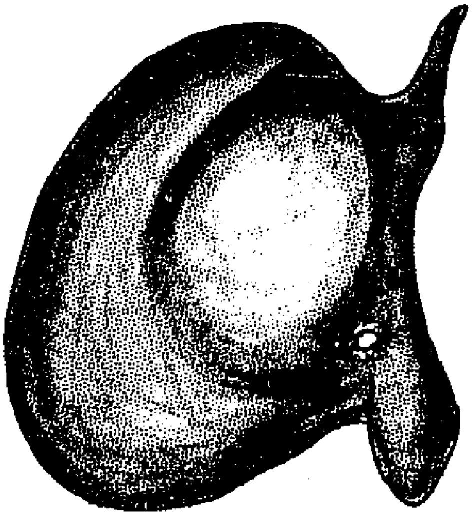
B



RA1



RA4



RA5

FIG. 11. Photograph of a frontal section of the scaphium of river fish SR29. The photograph shows the sinus impar and the connective tissue that closes it off and prevents the loss of perilymphatic fluid. C, cartilaginous base of scaphium; M, muscle from scaphium to dorsal musculature of the fish; O, connective tissue closing off sinus impar; S, scaphium; V, vertebra 1; Y, sinus impar. (100 X).


A
S
Y

sinus impar. The sinus impar is filled with perilymphatic fluid and the volume of the sinus changes as the scaphium moves in and out in response to movement transmitted to it from the other ossicles (Chranilov, 1929).

The scaphium is supported on its dorsal, ventral, and anterior edges by a continuous sheet of connective tissue that holds the scaphium to the body musculature and keeps it from moving too far laterally (Fig. 11). The connective tissue also seals the sinus impar and prevents loss of the perilymph when the volume of the sinus changes.

The scaphium connects with the centrum of the first vertebra through the ventral articulating process on the posterior margin of the bone. The tip of the articulating process fits into a pit on the dorso-lateral side of the centrum, the connection consisting of a cartilaginous area within the centrum (Fig. 11).

There is considerable inter-specific overlap in the various characteristics of the scaphium and its length, width, and depth do not differ significantly between the two species. One characteristic, however, the length of the ascending process, varies significantly between the two forms (Fig. 8).

The ascending process in the river fish is longer than in the blind cave fish (Table 6A). Quantitative comparisons were made since the difference between the lengths of the bones, while clearly present, was small. The mean length of the ascending process was 0.196 mm in the blind fish (N = 4) and 0.253 mm in the river fish (N = 4). The difference is statistically significant (P = 0.05). The top of the ascending process in the river fish is 0.174 mm above the highest point of the scaphium but it is 0.079 mm below the top of the scaphium

TABLE 6. Statistical analysis of morphological differences between the two species of <u>Astyanax</u>

		Blind cave fish			River fish	
A.	Length of ascending arm of the scaphium	BA1	0.253 mm		RA4	0.248 mm
		BA3	0.170		RA5	0.225
		BA4	0.182		RA11	0.253
		BA9	0.182		RA15	0.284
	Mean		0.196			0.253
	Standard deviation		0.372			0.024
				T = 2.478		
				P = 0.05		
B.	Length of distal arm of intercalarium	BA2	0.542 mm		RA4	0.480 mm
		BA3	0.592		RA5	0.446
		BA4	0.558		RA11	0.454
		BA9	0.515		RA15	0.464
	Mean		0.552			0.461
	Standard deviation		0.032			0.146
				T = 5.164		
				P = 0.001		
C.	Length of intercalarium	BA2	0.589 mm		RA4	0.662 mm
		BA3	0.677		RA5	0.624
		BA4	0.688		RA11	0.648
		BA9	0.646		RA15	0.580
	Mean		0.650			0.629
	Standard deviation		0.044			0.036
				T = 0.753		
				P = Not significant		
D.	Length of ascending arm of intercalarium	BA2	0.250 mm		RA4	0.345 mm
		BA3	0.254		RA5	0.294
		BA4	0.293		RA11	0.366
		BA9	0.267		RA15	0.275
	Mean		0.266			0.320
	Standard deviation		0.019			0.043
				T = 2.309		
				P = 0.05		

in the blind fish (P = 0.02). These differences were first noticed and compared quantitatively in the alizarin preparations but the differences were corroborated in examination of more than 20 other dissections of fish from 30 to 83 mm in standard length.

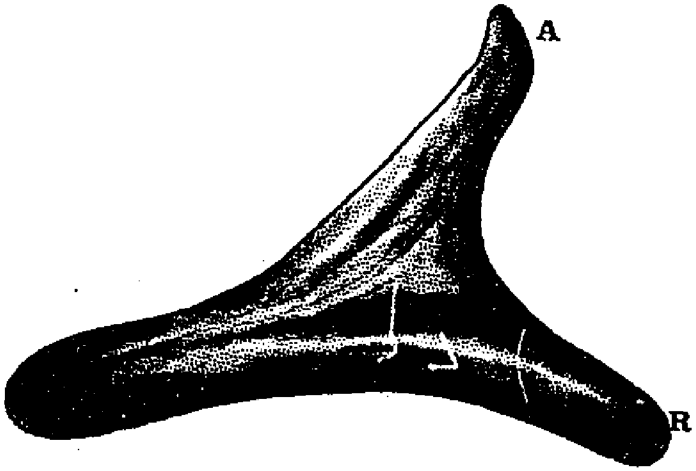
The ascending arm is located in a sheath of connective tissue but no tissue inserts on the process. A thin muscle inserts on the scaphium, just dorsal to the insertion of ligament 4 (Fig. 11). The origin of the muscles seems to be within the body musculature, just lateral to the midline of the fish and 5 mm below the dorsal margin of the fish.

Intercalarium

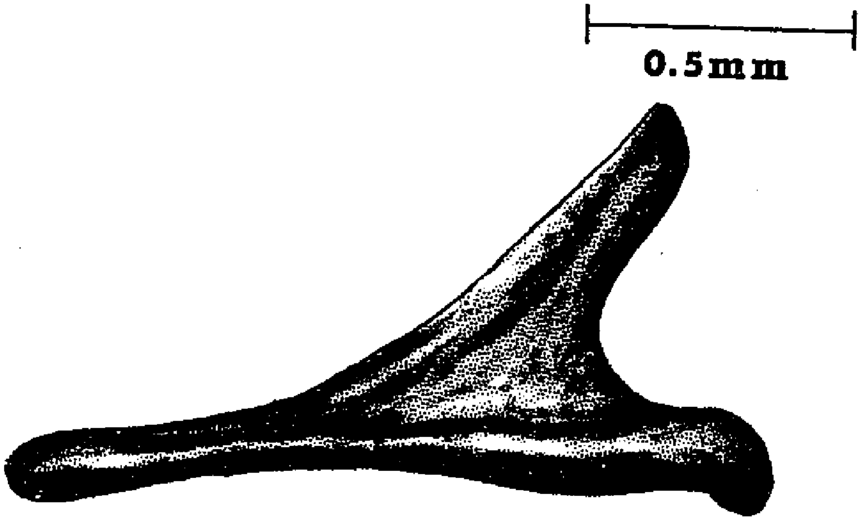
The intercalarium is the second active bone in the ossicle chain. It is shaped like a forked stick (Fig. 12) with the fork of the stick pointing medially. The long axis, or manubrium incudus (Niazi, 1967), is almost perpendicular to the vertebral column. The articulating arm of the fork forms a proximal continuation of the manubrium incudus and articulates with the dorsolateral pit on the centrum of the second vertebra. The connection between the intercalarium and the centrum is cartilaginous and very similar to the connection between the scaphium and first vertebra (Fig. 13). The base of the dorsal ascending arm is along the manubrium incudus. No muscle or ligament inserts on the arm, but Niazi (1967) has reported that there is connective tissue between the ascending process and the third neural pedicle in several species of Barbus.

Ligament 4 has its origin on the anterior side of the distal tip of the intercalarium and ligament 3, from the tripus, inserts on

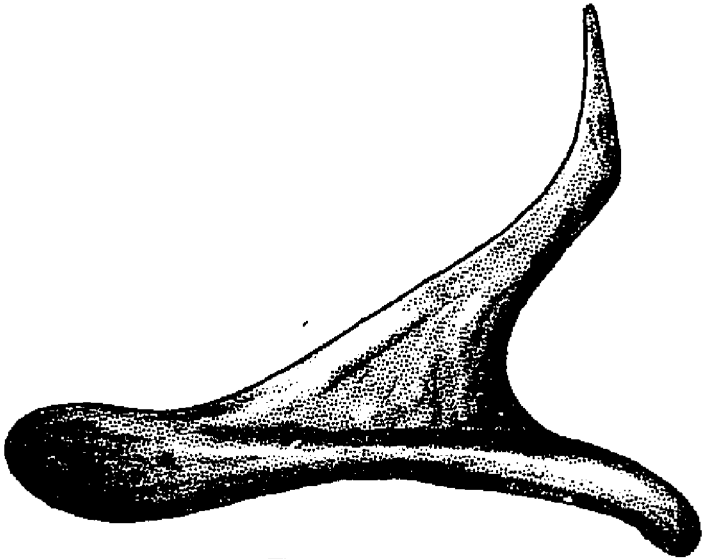
FIG. 12. Drawings of the intercalaria of two blind cave fish and two river fish. The intercalarium in *Astyanax mexicanus* is curved on two planes (see text) while the bone is only curved on one plane, if at all, in the blind cave fish. The ascending arm of the intercalarium is longer in the river fish than it is in the blind fish. A, ascending arm; R, articulating arm.



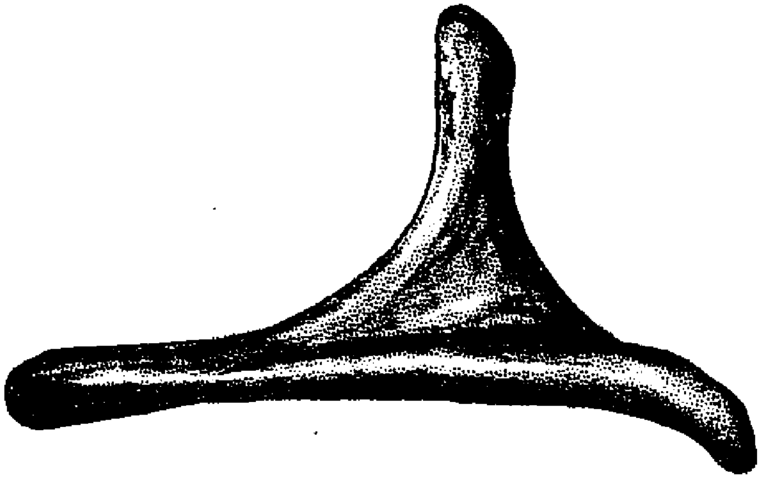
RA5



BA3



RA11



BA4

FIG. 13. Photographs of cross-sections of the cartilaginous connection between the intercalaria and second vertebrae in river fish SR28 (A) and blind cave fish SB41 (B). The connections are very similar to the one between the scaphium and first vertebra shown in Fig. 11. I, intercalaria; V, second vertebra. (430 X).


V
I

the posterior side of the proximal tip (Fig. 14).

The base of the ascending arm of the river fish intercalarium is closer to the center of the manubrium than it is in the blind cave fish (Table 6B). There was no overlap between the two species and quantitative comparisons showed the difference to be significant. The mean length of the intercalarium (Table 6C) was 0.650 mm in the blind fish (N = 4) and 0.629 mm in the river fish (N = 4). The difference between the length of the bones was not significant (P = 0.5), but the distance from the distal end of the intercalarium to the base of the ascending arm (Table 6B) was significantly different in the two species (P = 0.001). The ascending arm was 0.552 mm from the distal end of the intercalarium in the blind fish and 0.461 mm in the river fish. The ascending arm (Table 6D) was 0.266 mm long in the blind fish and 0.320 mm long in the river fish (P = 0.05).

The main axis of the intercalarium is curved in all of the river fish and in several of the blind cave fish but the extent of the curvature differs in the two forms. The intercalarium in the blind fish is straight, or curved slightly, with the distal tip slightly more anterior than the proximal tip when the bone was perpendicular to the vertebral column. The intercalarium in the river fish is strongly curved horizontally as well as vertically. This results in the distal tip of the intercalarium in the river fish being both anterior and ventral to the proximal tip of the manubrium. In effect, the intercalarium of the river fish resembles an incomplete spiral. These differences were corroborated in dissections of formalin-preserved material, as were those concerning the scaphium.

FIG. 14. Photograph of a frontal section of an intercalarium of Mexican blind cave fish SB32. The insertions of ligaments 3 and 4 on the intercalarium are shown in the photograph. F, ligament 4; I, intercalarium; L, ligament 3; S, scaphium. (430 X).

The angle between the ascending and articulating arm of the intercalarium varies considerably in different specimens, but there is extensive overlap in the variation between the two species. One intercalarium may have an ascending process oriented at 45 degrees with the articulating process and in another fish the two arms may be almost perpendicular to each other (Fig. 12).

Tripus

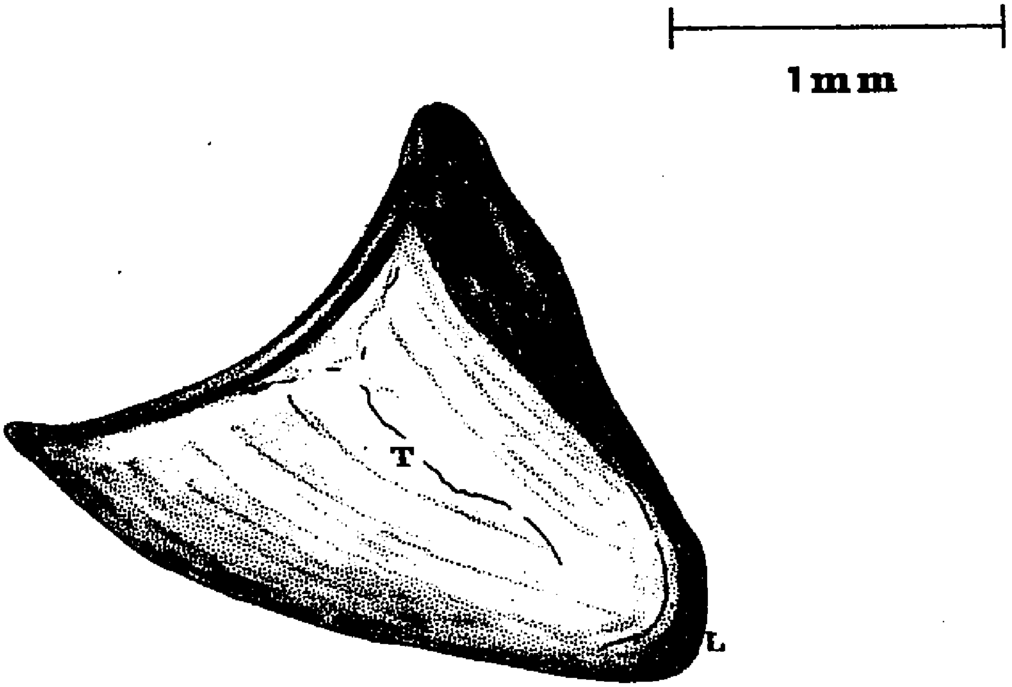
The third and largest ossicle involved in acoustic transmission is the tripus. As in the other ossicles, there is some variation in the shape of the tripus between different specimens but none of the differences is significant (Fig. 15).

Ligament 3 arises on the anterior articulating process of the tripus, and the posterior extension of the bone is in the form of a transformator process that connects with the tunica externa of the anterior chamber of the swim bladder.

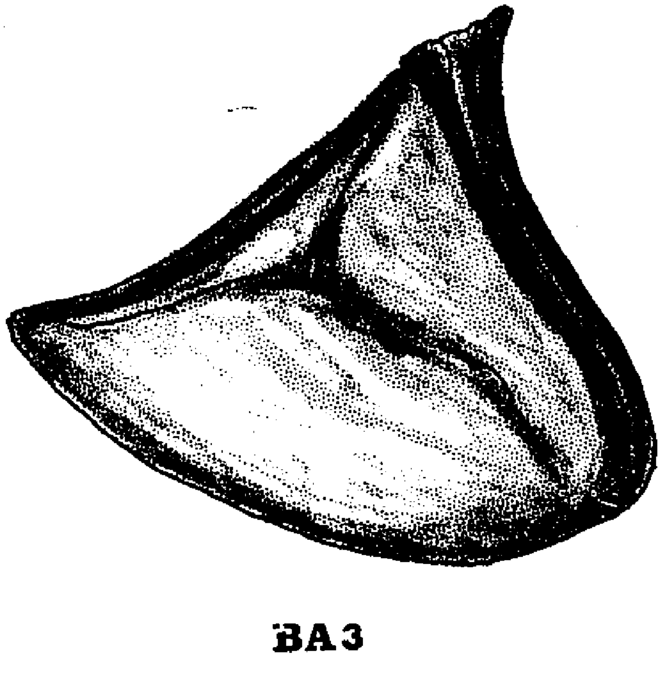
The medial side of the tripus is concave and the ventrolateral margin is convex. The tripus articulates with the ventrolateral side of the third centrum through a cartilaginous articulating surface on the dorsomedial side of the bone. This articulation is longer than the articulations between the other ossicles and their respective centra. The articulation is thin enough to act as a spring for the anterior-posterior movement of the bone while keeping it from moving in any other direction.

The transformator is a long thin extension of the posterior end of the tripus that curves 180° and ends under the centrum of the

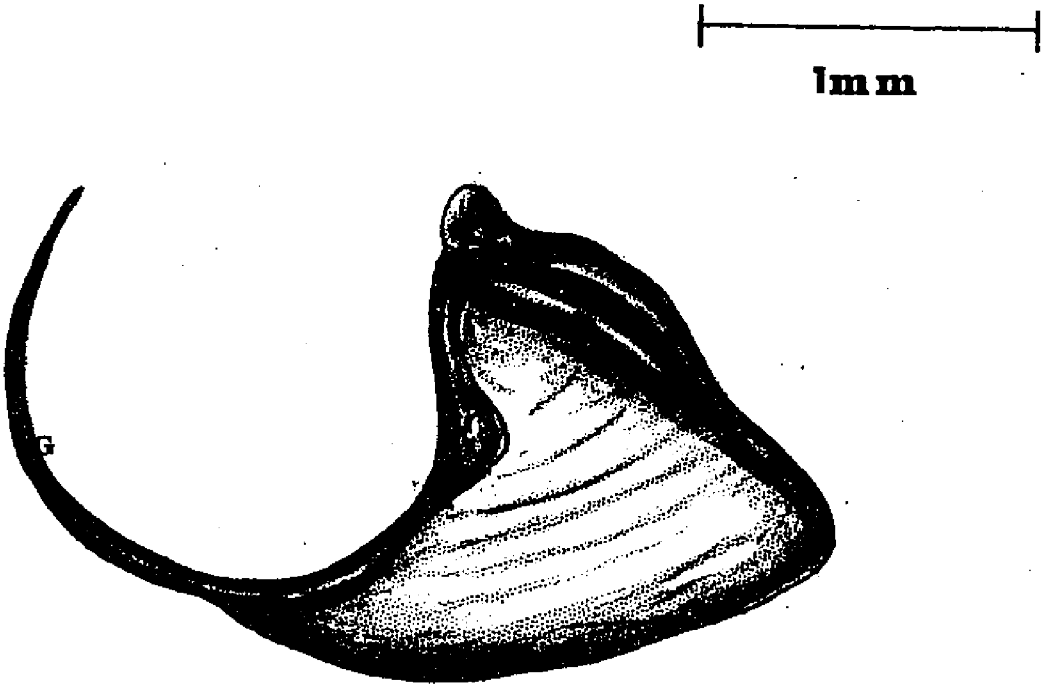
FIG. 15. Drawings of the tripus of two blind cave fish (A.) and two river fish (B.). The drawings were prepared from alizarin preparations and the transformator process could only be retained in the river fish preparations. The transformator process in both species has the same appearance. G, transformator process; L, point of insertion of ligament 3 on the tripus; T, body of tripus.



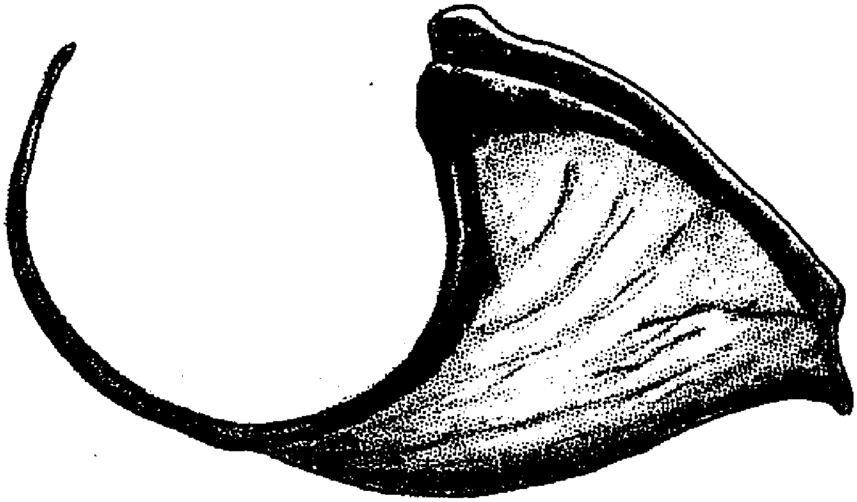
BA1



BA3



RA5



RA11

fourth vertebra. The tunica externa, from the slit on the anterio-medial margin of the swim bladder, inserts along the posterior margin of the transformator, covering about 130° of its surface. Ligament 1 arises on the ventro-lateral side of the os suspensorium and inserts over 100° of the anterior side of the transformator. Ligament 2 inserts on the medial side of the transformator process, just posterior to the end of the body of the tripus and the anterior end of the transformator. This ligament arises on the medial side of the base of the fourth pleural rib.

The os suspensorium is a medial extension of the base of the fourth pleural rib. It is a small, flat bone that lies just under the vertebral column and its posterior end slopes ventrally about 25° from the horizontal. The os is a separate bone from the basal portion of the rib but it is firmly fused with the rib and, consequently, the os is an immobile fulcrum for the transformator process.

DISCUSSION

Comparisons of Hearing in the Two Species of Astyanax

There are several factors that must be considered when determining whether the auditory thresholds differ significantly between the two species of Astyanax. Some variation is to be expected since the concept of a threshold is based upon the probability of signal detection by an organism (Green and Swets, 1966). The threshold is only a statistical value and it is considered to be the point at which the animal responds to a signal 50% of the time (Dixon and Massey, 1951). The variation in the thresholds can be caused by many factors, including the health of the animal, the shifting from one tank to another, and probably its state of 'hunger' at the time of testing. Although attempts were made to test each animal at the same time of day and under constant conditions, uncontrollable factors undoubtedly acted to produce variation in the thresholds.

Comparisons of the audiograms of the two fishes frequency by frequency do not tell us about the total trend of the thresholds of the animals. One comparison of the auditory thresholds at all frequencies, taken together, is the Mann-Whitney U test, a rank sum test for non-parametric statistics. The Mann-Whitney U test indicated that the differences between the two audiograms were not significant and that the variation was random. The reasons for the statistical insignificance is clear from a consideration of the two audiograms (Fig. 6). If the threshold curves were consistently different, the relationship between the two audiograms should remain relatively constant and, at most, a single crossing of the two curves might be expected. In the case of the audiograms for the two species of Astyanax, the overlap occurs three times, indicating randomness in the variation.

Although there were only a small number of animals used in the experiments, the threshold points are valid and consistent. Variation was not significant in the individual threshold determinations for each animal and among different animals of a single species. Standard deviations were usually less than 5 dB, which was the size of the steps used in the staircase method of threshold determinations. Precise determinations of the threshold were not made, but it has been found (Jacobs and Tavolga, 1967) that using 2-dB steps gave standard deviations in the order of 2 dB and thresholds identical to those determined with 5-dB steps.

Morphological Comparison of the Weberian Ossicles

The morphological variation found in the Weberian ossicles may provide some insight into the way the bones carry acoustic information between the swim bladder and the inner ear. Although the variation in the ossicles is not reflected in the thresholds for pure tone sine waves used in these experiments, the variation may affect the more complex signals that impinge upon the swim bladder.

Some morphological differences in the Weberian ossicles of the two forms are statistically significant while others vary as much intra-specifically as inter-specifically. The morphological differences in the ossicles may alter the centers of gravity of the bones and thereby affect the way in which the Weberian ossicles respond to a signal. The shortening of the arm of the scaphium of the Mexican blind cave fish may shift the weight of the bone forward and thus put more of a strain on the articulation with the vertebra. The ascending process lies in a sheath of connective tissue which may help support the weight of the bone, and a shortened process in the blind fish would provide less support to the bone.

Similarly, the ascending arm of the intercalarium in the river fish is larger and more centrally located along the major axis of the bone than in the blind fish. The shift in position may affect the balance of the bone since the distal tip would be lighter in the blind fish than in the river fish. The effective weight of the ascending arm would also be different in the two species since the distance from the fulcrum is not the same in the two species.

Morphological variation and the response of the Weberian ossicles in energy transduction

The intra- and inter-specific variation of the Weberian ossicles indicates that the response of the ossicles to an acoustic signal may vary from individual to individual. This variation would mean that two fish do not have precisely the same signal arriving at their central nervous systems, even though the signal impinging upon their swim bladders is the same for both animals.

There is evidence that the Weberian ossicles, like the middle ear bones of mammals (von Békésy, 1960) are damped. A damped system is one that will respond with considerable accuracy and with little attenuation to signals a considerable distance from the resonant frequency of the structure. The structure will respond quickly to the onset of a signal (De Vries, 1950), stop responding as soon as the signal stops, and follow the signal accurately until it is considerably different from the resonant frequency.

The Weberian ossicles are firmly attached to the vertebrae by cartilaginous connective tissue and they are surrounded by various tissues including body musculature (Alexander, 1962) which could produce damping. The highly damped source for signal input to the ossicles, the swim bladder (Alexander, 1966; Tavolga, 1964), would also contribute to creating a rapid response by the Weberian ossicles since the ossicles cannot respond, and would be impeded from responding, if the swim bladder were not responding to a signal (see below). There is also some experimental evidence that the ossicles are highly damped (De Vries, 1950; Furukawa and Ishii, 1967a, b). Furukawa and Ishii (1967b) found that the response to a signal in the inner ear of the goldfish is very rapid, indicating that there is little, if any, lag in response between the swim bladder and inner ear.

Although there is evidence that the Weberian ossicles are damped, several factors indicate that the damping is not too great.

The middle ear bones of mammals are very precisely balanced and if the balance is disturbed, the acoustic transmission may be altered (von Békésy, 1960, p. 102). The bones are constructed in a manner which lets them revolve around their own centers of gravity and they are effectively free floating instead of dependent upon other structures for their support. The few muscles that are associated with the middle ear bones can be removed without disturbing the normal response of the bones (von Békésy, 1960, p. 102). The wide variation in the structure of the Weberian ossicles suggests that they are not precisely balanced.

Another potential source for alteration of the signal content by the ossicles is the presence of various ligaments and muscles associated with the Weberian ossicles. Von Békésy (1960, p. 114) believed that the muscles and ligaments associated with the mammalian inner ear could produce harmonics and introduce them into the sound being carried through the ossicles. To prevent this, they are encased in bony capsules. The muscles and ligaments associated with the Weberian ossicles may not be a source for harmonics but they could provide some signal distortion by vibrating independently as the ossicles move in response to stimulation from the swim bladder. If the ossicles were not firmly attached to the vertebrae in a manner that only permits them to move in one direction, the vibrations produced by the ligaments could alter their path of movement and affect the energy carried to the inner ear.

If the ossicles are not highly damped, the signal response of the ossicles would be relatively crude and the rapid changes occurring in fish sounds (Tavolga, 1965) might not be followed. Enger (1963) and Furukawa and Ishii (1967a, b) have presented evidence that the hair cells of the inner ear of fish will accurately follow a sound up to about 1000 Hz. This suggests that the swim bladders and ossicles are able to follow a signal up to at least 1000 Hz but it is not possible to predict the type of response in these structures to higher frequencies than this.

This suggests that below a certain frequency the ossicles are highly damped and above that frequency some of the damping is lost. Above an undetermined frequency, the Weberian ossicles may follow an incoming signal differently than with a 1 to 1 signal-response ratio. The ossicles may lag behind the swim bladder response at the higher frequencies and thus inaccurately follow the incoming signal. The ligaments associated with the ossicle-swim bladder connections are elastic (Alexander, 1962) and this elasticity would act as an interface between the swim bladder and ossicles allowing them to respond at different frequencies.

As the frequency of the acoustic signal is raised, the response of the Weberian ossicles may vary. If the response varied for every different signal, the ossicles would provide an alternate method for signal following. It is more likely that as the signal frequency rises there is a group of frequencies that all cause a similar response in the ossicles. As an example, the ossicles may respond in the same way to signals at 2500 and 3000 Hz. If all of the frequencies were of the same acoustic energy, the ossicles would respond in a similar way to all of the signals, and the output from the ossicles to the inner ear would be the same. Consequently, all of the pure tone thresholds for this group of frequencies would be approximately the same, assuming that all of the hair cells of the sensory macula of the inner ear respond to an incoming signal above 1000 Hz. There is no opportunity for various 'places' on the sensory macula (van Bergeijk, 1967b) and different frequencies probably do not stimulate different groups of sensory cells as in the cochlea of terrestrial vertebrates (von Békésy, 1960). If the intensity of the stimulus input to the inner ear changes for each group of thresholds, as would be expected in the hypothesized response mechanism, the thresholds of the sensory cells would play an insignificant role in threshold determinations, and the actual threshold would be determined by the response of the Weberian ossicles to the incoming signal. Von Békésy (1960, p. 103) has found that as the frequency is raised, with the acoustic energy of the signal to the tympanum kept constant, the displacement output from the middle ear bones drops. If a similar mechanism were functioning in the Weberian ossicles, an attenuation would be expected as the ossicles respond at higher frequencies. Thus, two different signals could have the same energy as they impinge upon the swim bladder, but the energy that reached the inner ear would be different. This would account for different auditory thresholds for the two signals even though the same hair cells had responded to both signals.

Effects of ossicle variation on sound detection in fishes

The difference in the auditory capacity of *Astyanax* and the goldfish, *Carassius auratus*, may be explained by a change between a damped and non-damped response by the Weberian ossicles and, in turn, the variation in the thresholds between these two species may indicate the point at which the damping is lost. Comparisons using the Mann-Whitney U test between the absolute thresholds of *Astyanax jordani* and *Carassius auratus*, as determined by Jacobs and Tavolga (1967), show that the auditory thresholds for the two species are almost identical at all frequencies up to 1500 Hz ($P = 0.5$). Above 1500 Hz, the thresholds for the goldfish increase rapidly and the animal can only hear signals up to 3000 Hz. Above 1500 Hz, the thresholds for the Mexican blind cave fish, and also for the river fish, still remain relatively low. There are no major differences between the inner ears and swim bladders of the characids and cyprinids (Alexander, 1962), but Alexander has found that the connection of the tripus to the vertebra is thicker in the cyprinids than in the characids. There may be additional differences that would be found in a detailed comparison of the Weberian ossicles of the goldfish and Astyanax, and the difference in auditory thresholds above 1500 Hz may be related to the difference in the Weberian ossicles.

If the variation in the structure of the ossicles affects the way the bones respond to a stimulus, aspects of biological signals may be changed during transduction by the bones. In addition, the ossicles and their ligaments may set up harmonics and distort the signals to some extent. The sounds produced by non-ostariophysine fishes are composed of complex alternations in frequency and intensity components (Fish, 1954; Griffin, 1950, 1955; Moulton, 1963; Tavolga, 1965; Winn, 1964, 1967). The sounds produced by the few species of ostariophysines that have been investigated are equally as complex as the sounds produced by the non-ostariophysines (Moulton, 1963; Nelson, 1965; Tavolga, 1962). However, the variations in the ossicles, discussed above, could mean that the ostariophysines do not make use of many of the changes in the signal since they would be lost in the transduction process. This would be especially true if the signals were composed of rapidly changing high frequency components. The sounds produced by the characid fish Glandulocauda inequalis consist of rapidly repeating pulses with the major portion of the acoustic energy between 2000 and 4000 Hz (Nelson, 1965). Pulsing of the signal of Glandulocauda would compensate for poorly damped ossicles.

Whereas pulses may be easy for the Weberian ossicles to transduce with little distortion, the sounds produced by the marine catfish, Galeichthys felis, is a continuous sound with the major portion of the acoustic energy below 1000 Hz (Tavolga, 1960, 1962). Until considerably more data are available about sound production in ostariophysines, it shall be difficult to determine whether the Weberian ossicles have any significant role in alteration of sounds detected by these fish. The Weberian ossicles may act as a signal filter in ostariophysines that produce complex high frequency tones.

Many other ostariophysines, such as Astyanax, may not be sound producers and the behavioral significance of the wide auditory capacity and the development and maintenance of the Weberian ossicles to aid audition is unknown. It would be of considerable interest to investigate the actual effects on the Weberian ossicles of pure tones and, more significantly, actual sounds produced by fishes. The necessity and extent of damping would provide a good deal of information about the use that fishes make of the various signals making up a complex tone. It is possible that a considerable portion of the signal is produced because of the nature of the sound-producing structure, but the portions of the signal that are actually of significance to the fish may be limited.

Movement of the Weberian ossicles

The connective tissue between the scaphium and the sinus impar, along with several other morphological features of the Weberian ossicles, may help explain the movements of the ossicles in response to a signal. It is relatively easy to explain how the ossicles move posteriorly in response to swim bladder compression but anterior motion of the ossicles has never been satisfactorily explained.

When the swim bladder is compressed, the ossicles are pulled posteriorly and when the swim bladder expands, the ossicles move forwards (Chranilov, 1929). Ventrad movement of the posterior portion of the tripus is accomplished by a direct connection of the tripus to the tunica externa. The tripus rotates on the connection to the centrum of the third vertebra. The intercalarium and the scaphium move posteriorly when the tripus pulls them through ligaments 3 and 4. When the ossicles are in the posterior position and the swim bladder expands, the tripus changes position but the scaphium and intercalarium do not move forward solely through the movement of the tripus. As the tripus changes position, ligament 3 relaxes but it provides no motive force to the other two ossicles as it had when it pulled them posteriorly. Unless there was something pulling the ossicles anteriorly, they would stay in the posterior position and thus only respond to the first rarefraction of an incoming signal and then no longer respond to the sound.

There are several mechanisms that could help the ossicles return to the anterior position. The connections of all three of the ossicles to the vertebrae act as springs upon which the ossicles place tension when they are pulled posteriorly in response to compression of the swim bladder. If one assumes that the ossicles are in a normal resting position when they are in the anterior position, then, when the tension on ligament 3 is relieved, the thin cartilaginous connections would return to the normal resting position and move the ossicles forward again. If the ossicles were not highly damped, as discussed in the previous section, they would set up a vibratory pattern due to the spring-like behavior of the connections to the vertebrae.

Another mechanism that may help return the ossicles to the anterior position is the connective tissue that surrounds the scaphium and which appears to help retain the perilymphatic fluids within the sinus impar. This connection is analogous to the connection between the stapes and oval window in the mammalian inner ear (von Békésy, 1960, p. 103). Von Békésy (1960) has reported that the mammalian connective tissue surrounds the stapes and prevents the endolymphatic fluid from flowing out of the oval window. Von Békésy did not believe that this connection played any significant role in mammailian audition. The connective tissue may be elastic enough in <u>Astyanax</u> to permit the ossicles to move posteriorly in response to the swim bladder compression. When ligament 3 is relaxed, the connective tissue will pull the ossicles anteriorly.

The tissue that was found to surround the scaphium may have a third function. If the fish were subjected to a very intense signal, the swim bladder would expand more than usual and the signal transmitted through the ossicles would be very intense. A strong signal could damage the inner ear of the fish. In mammals there are several muscles associated with the middle ear bones that can alter their response to a signal and prevent a strong signal from harming the inner ear (von Békésy, 1960). The connective tissue around the scaphium may provide a similar function for the Weberian ossicles and the inner ear of fishes. A large posterior movement of the ossicles would be limited by the distance the connective tissue allows the scaphium to move. In a signal of 'normal' intensity, the ossicles would not move a greater distance posteriorly than the extent of the elasticity of the connective tissue. In an abnormally loud signal, the ossicles would be stopped before going too far and possibly damaging the ear. If the ligaments connecting the ossicles are elastic enough, they will absorb the excess motion of the swim bladder and allow the swim bladder itself to respond to the full magnitude of the sound. If the ligaments are not elastic enough, the swim bladder would be impeded in some complex way that would depend upon the lines of tension on the swim bladder by the connection of the tunica externa to the transformator process. The transformator process is very thin and it too may provide some absorption of excessively large sounds when the signal is too strong.

Significance of Sounds to the Mexican Blind Cave Fish

We do not at present know what use the Mexican blind cave fish make of their extensive auditory capacity in their natural environment. Breder (1942) and Bridges (1940) did not mention any source of high frequency (above 500 Hz) sounds in La Cueva Chica that would be of significance to the blind fish and the ambient noise in the caves has never been described. The predominant sounds in the caves are probably produced by the movement of the water over waterfalls and rocks between the different pools in La Cueva Chica, and these sounds would have most of their acoustic energy below 1000 Hz (Wenz, 1964).

Other aquatic organisms in the caves may produce sounds but there have been no investigations of these organisms, and it is questionable whether the sounds would be of significance to _A. jordani_ since these other kinds of organisms are not known to interact with the fish. Two species of crayfish are found in the pools (Breder, 1942). They may produce a broad-band sound when they close their large chelipeds (Frings, 1964; Tavolga, 1965) but crayfish are not known to produce any other sounds (Dumortier, 1963). The other smaller aquatic organisms in the caves do not produce significant pressure energy but, as shall be seen in the next section, they may produce displacement energy that is significant to the Mexican blind cave fish.

The only other biologically produced sounds that may be within the auditory range of the blind cave fish would be produced by the bats inhabiting the caves. Bats are known to produce sounds other than echolocating sounds and these sounds are frequently below 7000 Hz (Möhres, 1966). If the low frequency sounds were of sufficient energy in the water, they would be audible to the fish. The significance of the sounds of this sort to the fish is unknown, and Breder (1942) did not believe that the fish served as food for the bats. If the bats did use the fish as food, the echolocating sounds used to detect the ripples on the water produced by the fish would be well beyond the auditory range of the fish (Suthers, 1967). Therefore, it is unlikely that low frequency sounds produced by the bats would provide warning to the fish.

Sounds below 7000 Hz produced by the bats or other organisms entering the cave may be detectable in the water. If the sounds were produced on the ground, they would be carried to the water through the substrate. Sounds produced in air are attenuated by approximately 40 dB when they enter a large body of water (Albers, 1965), but Parvulescu (1964, 1967a, b) has suggested that a small body of water, such as in the shuttlebox used in the behavioral experiments or the small pools in La Cueva Chica, would respond differently to air-borne sounds than would a large body of water.

Astyanax mexicanus, the ancestral form of the Mexican blind cave fish (Breder, 1943a, 1953; Hubbs and Innes, 1936; Kosswig, 1965; Sadoglu, 1957, 1967), has as wide an auditory sensitivity as its blind derivative. The natural history of the river fish is not known, and the significance of sounds to these animals can only be guessed at. There are ambient sounds present in the rivers in which the fish are found, but it is unlikely that the fish evolved an extensive auditory capacity to deal with ambient sounds. It is possible that there are other organisms in the same environment in which the river fish are found that produce sounds of significance to the river fish. Even this is pure speculation, however, since there have been no investigations of sounds in the rivers of tropical climates.

It has been suggested that A. jordani and the other species of Mexican blind cave fish have entered the caves and become cave obligates only recently (Kosswig, 1965; Poulson, 1963a, b). This could explain why they do not exhibit the hypertrophy or degeneration of non-sensory structures that are found in other cave fishes (Poulson, 1963a, b; Vandel, 1965).

Low frequency sound detection by the Mexican blind cave fish

River fish maintained on a normal photocycle obtain food through use of visual stimuli (Breder, 1943a). Prey detection in fishes is also possible through use of the lateral line (Schwartz, 1967; Schwartz and Hasler, 1966) since moving objects produce low frequency water displacements that continue for a substantial distance from the source (van Bergeijk, 1964, 1967a; Harris and van Bergeijk, 1962). If any portion of the auditory system of *A. jordani* were to be hypertrophied, it would probably be the lateral line or free neuromasts. Schemmel (1967), however, found no difference between the lateral lines of *A. mexicanus* and *A. antrobius* and in the pattern of free neuromasts on their bodies. Kenneth R. John (MS) also found that the pattern of free neuromasts was the same in both species, but he also determined that the number of neuromasts, in any area where they were present, was greater in the blind fish than in the river fish. An increase in the number of neuromasts innervating the same lateral line neuron would increase the probability of detection of low frequency sounds. A greater number of receptors would also help the neuron reach its firing threshold at a lower sound intensity since there would be stimulus summation.

Low frequency water displacement would be produced by the major food sources of the Mexican blind cave fish. The nematodes making up the bulk of the diet of the blind fish (Breder, 1942) move in the water, and the movement sets up the water displacement which would be detectable by a blind fish. Although the Mexican blind cave fish in an aquarium find food predominantly by detecting chemicals or rapid movements of other fish around the food, the chemical stimuli are not present in the water of the caves since any odor produced by nematodes would probably be masked by the odor of the bat droppings covering the water.

Comparisons between Astyanax and other cave fish

The comparison of the two forms of Astyanax suggests that pressure detection in other cave fishes may not differ greatly from pressure detection in their epigean ancestor stock. The only reason there may be a change in the auditory capacity of the cave form is that the ear has become hypertrophied for a reason not directly associated with hearing. Poulson (1963a) found that the inner ear of several species of cave amblyopsids was larger than the ear of epigean relatives, and he believed that an enlarged ear would help the fish maintain equilibrium in the absence of eyes. Enlargement or other changes have not been reported in the ears of any other cave fishes although most of the 32 cave forms have not been investigated. Hypertrophy of the ears of caves fishes may not occur if the ears of the epigean ancestors were sufficiently large to provide the blind cave derivatives with the equilibrium information necessary to compensate for eye loss. Likewise, the ear of the blind fish may not degenerate if it is supplying the necessary equilibrium information to the fish.

The acoustic conditions present in La Cueva Chica are also probably present in the other caves in which blind fish are found. Heuts (1951) described the caves in which Caecobarbus geertsi was found and he reported that there was less food in these caves than in the Mexican caves. This would indicate that there was even less animal life in these caves, other than the blind fish, than in La Cueva Chica and so at least one other species besides Astyanax probably does not have any predators. Cave populations are usually sparse and only a few species, eyed or eyeless, inhabit one cave (Barr, 1967). This would make the likelihood of predation, especially by a sound producing organism, rather unlikely.

The most likely source of acoustic stimulation for cave fishes is in the form of displacement energy. Several species of fish besides Astyanax have an hypertrophied series of displacement detectors (Heuts, 1951; Poulson, 1963a, b; Walters and Walters, 1965). The types of stimuli present in the caves are likely to be low frequency, as has been discussed for the Mexican cave fish, and an hypertrophied system for detecting these stimuli would aid the fish in finding food and detecting objects in the water.

Significance of Ambient Sounds During Threshold Determinations

The presence of ambient sounds during the determination of auditory thresholds in fishes may affect the threshold level (Buerkle, 1968; Tavolga, 1967). Tavolga (1967) presented evidence that can be interpreted to indicate that, in some species, two simultaneously presented sounds will interfere with each other and possibly alter the threshold for these sounds. Buerkle (1968) showed that background noise with frequency characteristics similar to those of the test signal can raise the apparent threshold of the fish by a significant amount. Since background sounds can alter the threshold of fish, the ambient noise levels and the sound spectrum level have been presented along with the threshold determinations for Astyanax (Fig. 6).

It is unclear whether the significant factor affecting the threshold would be the total noise level or the noise energy per cycle. The significance of either measure of the ambient sound level would depend upon the auditory mechanism of the fish and, more specifically, the way that the inner ear of the fish functions.

The significance of background sounds during threshold determinations has been demonstrated by studies on hearing in mammals. Different frequencies stimulating the mammalian inner ear stimulate different portions of the cachlea depending upon the wavelength of the signal. When two signals have 'places' on the cochlea that are close to each other, the signals can interfere with one another and the observer may respond as if hearing only a single signal, or the threshold of both signals may be considerably raised (Scharf, 1966). This interference is known as 'masking' and it only occurs when the signals are within the same 'critical band'. When one of the signals moves outside the critical band around the other, they will be heard separately (Scharf, 1966). The width of the critical band varies with different frequencies but the bands seem to be related to the places on the cochlea which different frequencies stimulate (Scharf, 1966).

The ability to make frequency discriminations is partially based upon the existence of places on the cochlea. If two different places are stimulated, the organism can discriminate between the signals (von Békésy, 1960). Fishes do not have a cochlea and there is no structure that provides an anatomical basis for a place theory in fish (van Bergeijk, 1967b), but fish can perform frequency discrimination (Dijkgraaf and Verheijen, 1950; Stetter, 1929; Stipetić, 1939), and Jacobs and Tavolga (1968) have shown that the goldfish could discriminate tones as close to each other as 3%.

If a critical band mechanism is present in fish, ambient sounds could play a role in masking the absolute threshold. It is also possible that while specific masking is not present, the presence of noise in the environment at any frequency can interfere with, and mask, the absolute threshold. Recent evidence indicates that some sort of masking may occur in fishes (P. Cahn, personal communication; Tavolga, 1967, personal communication). This would mean that ambient sounds at and around the critical band would have to be kept very low in order to prevent masking.

Buerkle (1968) showed that the thresholds of the codfish could be raised by having a background noise that was 20 dB lower than the stimulus intensity. He raised the level of the background noise and simultaneously raised the thresholds of the fish. If the fish had a mechanism similar to the critical band, then the significant background noise would be in a band around the center frequency. If a mechanism similar to the critical band does not exist in fishes, the thresholds of the fish would be masked by any sound in the environment. Whereas, if a critical band is present, only ambient sounds with frequency components close to the frequency of the test signal would do the masking. It is necessary to keep the sound levels as low as possible during testing in order to prevent any interference with the threshold determination. Both the bandwidth noise level and the sound spectrum level of the sound are presented in Fig. 6 but the significant measure is not known. The bandwidth noise level and the sound spectrum level are 20 dB or more below the thresholds at all points and it is unlikely that the ambient noise had any effect on the measurements of the absolute thresholds of the fish.

Nature of the Stimuli Represented in the Audiograms

It is still necessary to determine the type of stimulus to which *Astyanax* was responding during threshold determinations. Parvulescu (1964, 1967a, b) has determined that a small body of water in a closed area stimulated by a speaker in the air will have a pure pressure field (Fig. 2). The sound pressure level measured inside the shuttlebox with a hydrophone and outside the shuttlebox with a sound level meter were identical, indicating that the sound stimulus was not attenuated by the air-water interface which normally disturbs sound entering water when the water is a free field (Parvulescu, 1964). Cahn et al. (1967) have found that the speed of sound in a small body of water is much closer to the speed of sound in air than the speed of sound in a large body of water. These findings indicate that the sound stimulus above 400 Hz that most probably affects the fish in the avoidance conditioning apparatus is mainly a pressure field. There is some evidence that at 300 Hz and below there is a strong displacement stimulus present (see below) but the thresholds for 500 Hz and above are probably entirely pressure stimuli. From the appearance of the thresholds at 300 Hz and below, these thresholds are also pressure thresholds. Tavolga and Wodinsky (1963) found double thresholds in some of the species they worked with and the displacement thresholds at the low frequencies, plotted on a pressure scale, were below the levels for the pressure thresholds. Although the sensitivity to pressure stimuli in Astyanax is considerably greater than the pressure sensitivity of non-ostariophysines, it is likely that the threshold points for displacement stimuli, if present, would be different from the points shown for the two species of Astyanax. The points plotted for 300 Hz and below follow the general shape of the rest of the audiogram and, in the blind fish, the thresholds at 200 Hz and 300 Hz are insignificantly different from the threshold at 500 Hz.

Behavior of the Fish: Response to Different Stimuli

The two species of Astyanax behaved differently to the transfers of test frequencies and the behavioral difference may have been related to how the species perceived the acoustical stimuli. Initial training of Astyanax jordani and A. mexicanus was at 500 Hz and, after the fish were well trained, the fish were transferred to other frequencies to determine the auditory thresholds. The Mexican blind cave fish could easily transfer to other frequencies but the river fish had difficulty transferring to 300 Hz and 3000 Hz. The blind fish are normally oriented to acoustical stimuli while the river fish are visually oriented and they may have had difficulty orienting to different stimuli. The break-down of the river fish behavior at

300 Hz probably occurred because the fish were trained to respond to the pressure stimulus present at 500 Hz and they did not transfer to the predominant displacement stimulus present at 300 Hz and below. The 3000 Hz stimulus may also be qualitatively different from the 500 Hz signal. The blind cave fish may normally equate pressure and displacement or they may detect and learn to respond to both stimuli at 500 Hz. The displacement stimulus at 500 Hz is considerably weaker than the pressure stimulus and the river fish, having fewer displacement detectors than the blind fish, may not have detected it.

Tavolga and Wodinsky (1963) found evidence for two different low frequency threshold curves for several species of marine fishes. On some days the thresholds were considerably higher than on other days and they theorized that the fish were responding to pressure and displacement stimuli during different tests. Cahn et al (1967) reported auditory thresholds for _Haemulon sciurus_ using a technique that isolates the pressure and displacement stimuli and lets thresholds be determined for each of the two forms of acoustic energy. They were able to confirm the existence of double thresholds in the grunt and they showed that the shift that Tavolga and Wodinsky (1963) had reported was from a pressure response to one of displacement.

When the river fish were transferred to 300 Hz, they initially responded to the pressure portion of the stimulus, but after several trials or several days they may have also begun responding to the strong displacement. The river fish tend to stop responding when there is any sort of change in the stimulus, and the presence of a strong displacement energy would have been a sufficient stimulus to cause this disjunction in their behavior.

The fact that the curves follow the higher frequency pressure thresholds for both species also indicates that the thresholds below 300 Hz are probably responses to pressure stimuli. In the study by Tavolga and Wodinsky (1963), the displacement thresholds were considerably lower than the pressure thresholds when the two different thresholds were plotted on a scale for pressure thresholds. The pressure sensitivity of Astyanax is lower than any of the species studied by Tavolga and Wodinsky; therefore, comparisons may be invalid.

The transfer difficulty encountered with the river fish at frequencies between 2500 and 3000 Hz may have resulted from either a change in perceived stimuli at 2500 to 3000 Hz or the inability of the fish to transfer their response from the training frequency to certain other frequencies. The possibility of a change in perceived stimulus at 3000 Hz has already been discussed. The Weberian ossicles may have had a frequency response below 300 Hz while above 3000 Hz the ossicles may have responded to signals at less than a 1 to 1 ratio, and this may have confused the fish. It is also possible that the response of the inner ear hair cells shifted from a frequency to an intensity response, and this confused the fish.

Kimble (1951, p. 344) discussed stimulus generalization in mammals and he showed that as the stimulus becomes less and less like the training stimulus, the animal's response gets poorer. The investigations of Astyanax were not treated as a problem in stimulus generalization, but if the break-down at 3000 Hz was a result of a lack of stimulus generalization, it may be the first time such a phenomenon has been encountered with fish. The fact that the fish avoided between 25 and 50% of the time at 3000 Hz, but never improved beyond this, suggests that the difficulty here might have been associated with generalization.

The blind fish had no difficulty between 2500 and 3000 Hz, and this indicates that the blind fish and the river fish had learned to respond to different aspects of the stimuli. The blind fish may have learned to respond to 'sound' stimuli in general, while the river fish may have learned to a 500 Hz signal or the specific qualitative composition of the 500 Hz tone. This suggests an improved method for training fish for threshold determinations. Rather than train a fish initially to a single frequency, it should be trained to a variety of different signals so that it learns to respond to 'sound' and not to a specific signal or specific signal content. Although this method may not make training faster, it may make stimulus transfer easier, and retraining during testing would no longer be necessary.

Fish trained at 3000 Hz had no difficulty in stimulus transfer to low frequencies; this may have resulted from the training at a central frequency in the fish's detectable range.

Once the river fish had received training at higher frequencies (up to 2500 Hz), they had no difficulty shifting to 300 Hz and below. This may indicate that they were so overtrained to the pressure stimulus that the displacement stimulus was effectively 'ignored'.

At several points during testing of the Mexican blind cave fish below 300 Hz, there were indications that the fish were shifting from one modality to another. During the course of a day's threshold determinations, the threshold would suddenly drop after 10 or 15 changes or steps. This did not occur consistently since it was observed only about a half dozen times at several frequencies.

Behavior in the shuttlebox

The shuttlebox used in avoidance training was originally designed and used with visually oriented animals (Behrend and Bitterman, 1962; Tavolga and Wodinsky, 1963). Several aspects of the tank helped visually oriented fish learn to cross the barrier but the blind fish had to make use of other cues that they could perceive. The barrier was painted in contrasting colors to the rest of the tank in the studies by Tavolga and Wodinsky (1963, 1965) and Jacobs and Tavolga (1967, 1968). There was a beam of visible light above the barrier that excited the photocell in these investigations. The light may have helped the fish to orient to the barrier. Astyanax mexicanus rapidly learned to cross the barrier in response to almost any visual cue. The photocell light and the barrier color provided a continuous cue that the fish probably learned to discriminate from the rest of the tank. Whether fish will learn the initial escape response any differently with and without such cues is not known because the same species of fish has never been tested under these two conditions. Goldfish and Astyanax mexicanus, trained with the two cues present, learned the escape response more rapidly than the pinfish, Lagodon rhomboides, trained in a black tank with infrared light to excite the photocell (Jacobs and Popper, 1968).

Presumably the pinfish could not see the light since it was outside the visual range of fish (W. N. Tavolga, personal communication; Yager, 1967). Escape-trained pinfish and goldfish learned the sound avoidance problem in about the same amount of time, but the escape response took several days for the pinfish to learn and only several trials for the goldfish and Astyanax. While this is not clear-cut evidence, it does seem to indicate that specific highlighting of the barrier would aid a fish to learn a portion of the escape response.

Blind cave fishes cannot use the visual cues built into the shuttlebox to learn to make the escape response. Consequently, it takes a considerable amount of time for the blind fish to learn to escape. Once they have learned the escape response, the latency of response is the same as that of the eyed river fish, and their avoidance response also has the same latency as that of the river fish. It is possible that a blind fish learns the shape of the test tank and orients itself in the correct direction, or keeps track of its orientation and turns towards the barrier whenever the stimulus starts. During preliminary experiments on hearing in the Mexican blind cave fish, they were tested in open tanks and could be observed closely. The blind fish swam slowly or took up positions in the tank and remained there until the stimulus started. The fish then swam directly over the barrier. The fish selected the same position consistently and kept returning to it during the intertrial interval. Even when the fish did not remain in one position, they often crossed the barrier rapidly when the stimulus started.

John (1957) found that both the Mexican cave fish and blinded river fish avoided noxious stimuli. When the stimulus was removed from the tank, the fish still made the avoidance response in the general area where the noxious stimulus had been. It appeared that the fish learned the general area of the stimulus and then relied on sensory information to determine its precise location.

The blind cave fish may learn the general position of the barrier in the shuttlebox. If the fish can keep track of their position relative to the hurdle, they would only have to swim in the correct direction to eventually cross the barrier. If the fish remained motionless during the intertrial interval, they would then have no difficulty orienting to the barrier. When the fish move around, they can obtain additional orientation information through their lateral lines and free neuromasts which will respond differently to the hurdle and the other walls of the tank. The wall of the barrier is sloped while the other walls of the tank are perpendicular to the bottom of the tank (Fig. 2). Dijkgraaf (1963) has found that as fish swim they push water ahead of themselves. If there is an object in the path of the fish, the water reflections are detected by the lateral line canals and free neuromasts on the head of the fish. Walters and Liu (1967) observed that Astyanax jordani detects such water displacements. This probably played a role in the stimulus detection discussed by John (1957). The reflected displacement in the shuttlebox differs for the barrier and the walls of the tank. If the fish were swimming directly towards a wall of the tank, all of the sensory cells equidistant from the wall would be stimulated with the same amount of energy. All cells receiving the same intensity stimulus would be on the same vertical plane. If the fish were swimming towards the sloped hurdle, the cells on the top of the head of the fish would be farther from the barrier than the cells on the ventral surface of the head. If the information interpretation system is not very sophisticated, it would take the fish a long time to learn to discriminate between the sloped and angled wall.

A more sophisticated capacity to interpret this type of data would mean that the fish could discriminate between small angles on vertical and horizontal planes. The fish would also be able to discriminate between horizontal and vertical surfaces if they could keep track of their body position. A highly integrated receptor system would let the fish discriminate between multi-angled objects where each neuromast or small group of neuromasts would receive different intensity stimuli.

Comparisons of and Techniques for Obtaining Thresholds for Astyanax and Other Species of Fish

Astyanax mexicanus was able to hear sounds up to 7500 Hz and *A. jordani* up to 6400 Hz. It is likely that the Mexican blind cave fish would have heard signals at least as high as 7500 Hz and that both species can probably detect pure tones up to 8000 or 9000 Hz. The threshold at the highest frequency used in the river fish study was +2.8 dBµb. In contrast, other experimentors have usually ended their threshold determinations when the threshold of the fish was +30 dBµb (Enger, 1966; Jacobs and Tavolga, 1967; Weiss, 1967). It was not possible to determine thresholds at intensities higher than the threshold at 7500 Hz. In order to obtain a threshold, the starting intensity before attenuation must be at least 20 dB above the threshold level. At these high frequencies and intensities, the loudspeaker used did not accurately reproduce pure tones.

Earlier studies with other ostariophysines have indicated that the highest frequency that different species could hear varied considerably. The highest frequencies were 6000 Hz in the cyprinid *Phoxinus laevis* (von Frisch, 1938a; von Frisch and Stetter, 1932; Stetter, 1929), 6960 Hz in the characids *Hyphessobrycon flammeus*, *Hemmigrammus caudovittatus*, and *Pyrrhulina rachoviana* (von Boutteville, 1935), 870 to 1035 Hz in the gymnotid *Electrophorus electricus* (von Boutteville, 1935), and more than 10,000 Hz in the silurid *Ictalurus nebulosus* (Autrum and Poggendorf, 1951; Poggendorf, 1952).

The highest frequencies detectable by non-ostariophysines were 2068 Hz in *Poecilia reticulatus* (Farkas, 1936), 2500 Hz in *Mugil cephalus* (Maliukina, 1960), 488 to 650 Hz in *Anguilla anguilla* (Diesselhorst, 1938), 2637 to 4699 Hz in the anabantid *Macropodus opercularis* (Schneider, 1941), up to 800 Hz in several species of *Gobius* (Dijkgraaf, 1952), 2800 Hz in *Holocentrus ascensionus*, and 1200 Hz in *Haemulon sciurus* (Tavolga and Wodinsky, 1963, 1965).

Generally, the minimum auditory thresholds for both ostariophysines and non-ostariophysines are found between 200 and 1500 Hz (Tavolga, 1965), but the thresholds in the ostariophysines are considerably lower than in fish without Weberian ossicles. None of the early studies on hearing in fish has differentiated between pressure and displacement thresholds. In many of the early experiments, the sound source, often a tuning fork, was placed directly on the tank (Parker and van Heussen, 1917) and the tanks were made of glass or other material that responded to acoustical stimuli and set up displacement waves that may have been independent of the stimulating pressure energy (Parvulescu, 1964). In such uncontrolled acoustical situations, the fish tanks may have acted like the swim bladder of fishes and set up near-field displacements that stimulated the fish.

More recent experiments have made use of precision audio oscillators, hydrophones, sound measuring equipment, etc., and yet there are still discrepancies between the results from different laboratories when the same species of fish is used. For example, different workers have obtained different results with the common goldfish, Carassius auratus. How valid then are the data obtained for the two species of Astyanax as compared with other thresholds and, under other conditions, would these thresholds be any different from those already obtained?

Enger (1966), Jacobs and Tavolga (1967), and Weiss (1967) found that the goldfish can hear sounds up to 3000 Hz. The thresholds found by Jacobs and Tavolga (1967), however, were significantly lower than the others. Using a speaker in the air, Enger (1966) found thresholds that were slightly higher than those found by Jacobs and Tavolga, and Weiss (1967), using a shuttlebox whose ends were made up by the loudspeakers, found thresholds that were almost consistently 20 dB above those in the determinations by Jacobs and Tavolga. Enger (1968) suggested that the pressure thresholds found by Jacobs and Tavolga were lower than his determinations (Enger, 1966) because Jacobs and Tavolga were able to produce better pressure stimuli than he could. Weiss used avoidance conditioning, but he did not present shock after training whenever the fish did not avoid the sound. He contended that shock during testing would give the fish "neuroses". Wodinsky (personal communication) has recently determined that fish tested without shock will consistently exhibit higher thresholds than fish tested with shock, the difference being about 15 to 20 dB.

Cahn et al (1967) worked with the grunt, Haemulon sciurus, and they used a new acoustic apparatus in which they were able to isolate pressure and displacement stimuli from each other and thus test the fish in relatively pure pressure or displacement fields. The thresholds they obtained were almost identical to the thresholds for the grunt determined by Tavolga and Wodinsky (1963), although Tavolga and Wodinsky used an undefined stimulus produced by a small underwater speaker.

The technique used for the investigations of hearing in Astyanax was very similar to the one used by Jacobs and Tavolga (1967, 1968). The major difference between the Jacobs and Tavolga (1967, 1968) studies and those of Tavolga and Wodinsky (1963) was that Jacobs and Tavolga used a speaker in air, which is supposed to produce a pure far-field (Parvulescu, 1964), while Tavolga and Wodinsky (1963) used an underwater speaker which produces an undefined stimulus.

After comparing the thresholds obtained under different acoustical conditions, it can be concluded that the thresholds presented for the two species of Astyanax are reliable as absolute thresholds. The absolute threshold, as determined in these experiments, is the minimum signal to which the fish responds 50% of the time. It is possible that the auditory system of the fish detects lower intensity stimuli, but this may be filtered out in the peripheral or central nervous system and the animal would not, therefore, respond to it. The presence of shock provides a stimulus to which the fish can respond as long as it can detect the signal. Although observations were not made of the behavior of the fish during threshold determinations, they probably maximize their chances of detecting the signal at its lowest intensities. Tavolga (personal communication) found that fish trained to a visual stimulus will take up a position facing the place at which the stimulus will be presented and, C. S. Johnson (personal communication) observed that the bottlenose porpoise, Tursiops truncatus, waited for the onset of the conditioned stimulus in the area of the test tank where the stimulus was the loudest. Wodinsky's findings that the presence of shock during testing also results in a lower threshold supports the contention that the fish are responding to the minimum possible threshold in the avoidance conditioning apparatus.

It would be of considerable interest to determine whether the auditory thresholds obtained with avoidance conditioning techniques would be similar to thresholds obtained using other testing methods. Only one comparative threshold study has been reported in the literature. Dalton (1968) determined that the auditory thresholds of several species of monkeys were lower when an operant technique was used to test the animal and higher when evoked cortical responses were used. Thresholds using a galvanic skin response were between the other two.

The data on the auditory capacity for Astyanax adds to our knowledge of the auditory capacity of the ostariophysines by indicating that the characids may indeed have a broader frequency response than any other fish. Before generalizations can be made, it will be necessary to determine the auditory capacity, under well-defined acoustical situations, for other ostariophysines. The animals selected for study should be fishes with well-known natural histories. Although the few species of characids that have been investigated all have the same range of auditory detection, there is considerable structural and ecological diversity within this large family (Weitzman, 1962) and this must be kept in mind whenever generalizations are made about their hearing capacity.

General Discussion and Conclusions

A substantial portion of the auditory capacity of many bony fishes depends upon the mechanism by which sound is transmitted from the swim bladder to the inner ear. The Weberian ossicles are analogous in many ways to the middle ear bones of mammals (Weber, 1820) and although the energy transduction by the ossicles is probably very crude, especially above 1500 Hz, they consist of certain structures that probably function in ways analogous to the middle ear bones. The signals passed by the Weberian ossicles are possibly only a small portion of the total acoustic energy impinging upon the swim bladder. Actual acoustic thresholds to pure tone sine waves may be representative of the limitations of the hair cells of the inner ear, the characteristics of the central nervous system, and the functioning of the swim bladder. Another, previously overlooked, factor that may have a significant role in determining what a fish can and cannot hear is the Weberian ossicles. Because of the various limitations imposed upon the ossicles by their construction, they may eliminate considerable portions of the acoustic energy that impinges on the fish. This filtering process may be important to the fish since it would immediately eliminate some portions of signals that are present due to the nature of the sound producing apparatus. By filtering the incoming signals, the Weberian ossicles may be enhancing the capacity of fish to discriminate signals by increasing the perceived differences between the sounds.

The auditory capacity of the Mexican blind cave fish indicates that cave fishes are not necessarily equipped with either an hypertrophied or degenerated auditory system. It is necessary, in investigations of cave organisms, to look for other than the most obvious reasons for the maintenance of a structure that seems not to be used by the animal. It is also possible that the interrelationships between the ear and other structures that start to develop early in the developmental process of the fish are so complex that a gene that would alter the ear of the fish would disasterously alter other organ systems of the fish.

The similarity between the auditory capacity of Astyanax mexicanus and the Mexican blind cave fish furthermore points up the usefulness of animals in which comparisons between two closely related species are possible.

There are other questions about audition in fish that may be answered by further use of the two species of Astyanax. Comparisons of low frequency audition in fish trained in displacement detection, using methods similar to those described by Cahn and her colleagues

(Cahn et al, 1967) and Banner (1967), would help clarify the significance of the hypertrophied system of free neuromasts found in the blind cave fish. Trained fish tested before and after destruction of all or portions of the displacement detectors would provide information about the auditory function of specific receptors to different stimuli.

Pure tone thresholds in fish have been extensively studied (see introduction and discussion) but little work has been done using other types of sound stimuli. Specifically, investigations using sound pulses and clicks could provide important data on the capabilities of the auditory system of fish. Information about the types and duration of pulses that fish can discriminate between would give some indication of the method of signal analysis performed by the Weberian ossicles. Thresholds for pure tone and broad-band pulses should be different from the pure tone thresholds if the auditory system of the fish uses signal change rather than a continuous signal for the major portion of its acoustic information.

The work of Furukawa and Ishii (1967a, b) has suggested a method for determining the effecta of the Weberian ossicles on the detection of pressure stimuli. Recordings made in the sacculus both before and after removal of portions of the Weberian ossicles, or experimental inhibition of the ossicles, would help clarify the relationship they hold to signal input and signals detected in the inner ear. If a suitable technique can be developed for exposing the ossicles while the fish is still kept alive, the response of the ossicles to different types of stimuli could be observed using techniques similar to those developed by von Békésy (1960) in his studies of the inner ear of mammals.

An extensive amount of work still remains to be done on hearing in fish. Although extrapolations to fish audition can be made from the work on mammals by such people as von Békésy (1960) and his many colleagues, there are many aspects of the fish auditory system that are enough unlike the mammalian system to warrant further investigations. This type of data may not only help to elucidate the nature of fish audition but it may yet clarify questions about mammalian audition that would best be studied in a relatively 'simpler' system.

SUMMARY

1. Using avoidance conditioning techniques, comparisons between the auditory capacity of <u>Astyanax</u> <u>mexicanus</u> and its blind cave derivative, the Mexican blind cave fish, were made.
2. <u>Astyanax</u> <u>mexicanus</u>, the river fish, can hear pure tone sine waves from 50 to 7500 Hz, and thresholds were determined for <u>A.</u> <u>jordani</u> from 50 to 6400 Hz. The audiograms for the two species were insignificantly different as shown by the Mann-Whitney U test.
3. Evidence has been presented that the thresholds measured were for pressure stimuli rather than for displacement.
4. The auditory thresholds for both species were lowest from 200 to 1500 Hz with the minimum threshold at 1000 Hz.
5. The behavior of the two species in the training apparatus was strikingly different. The blind fish had difficulty learning the escape response but once this was learned, the fish responded quickly to the sound stimulus. The river fish learned to escape quickly but they had difficulty learning to avoid the shock since they were easily distracted by ambient visual stimuli. Training of the river fish was shifted to an isolated acoustic chamber to prevent ambient visual stimuli.
6. The river fish learned to respond to the sound stimulus in 2 weeks of training while the blind fish needed 4 weeks.

7. The blind fish had no difficulty in transferring the response from one signal to a sound at another frequency. The river fish had considerable difficulty transferring from 500 to 300 Hz and from 2500 to 3000 Hz.
8. It is suggested that in future behavioral studies of this type, the fish be trained at a different frequency during each training period so that the fish would learn to respond to 'sound' rather than to a single frequency.
9. Comparisons of the Weberian ossicles between the two forms revealed several significant differences.
10. The ascending arm of the scaphium was longer in the river fish than in the cave fish and the ascending arm of the intercalarium was more centrally located on the bone in the river fish than in the cave fish.
11. The other characteristics of the Weberian ossicles varied considerably in the two species but, except the characteristics referred to in 10, the variation was as great between the species as it was within each species.
12. There is evidence that the Weberian ossicles are damped but that various morphological characteristics limit the damping so that the ossicles do not follow the signal to the swim bladder with complete accuracy.
13. It is suggested that the Weberian ossicles respond with a following response below 1500 Hz but with a generalized response above 1500 Hz.
14. A previously unreported connection was found between the margin

of the scaphium and the tissue around the sinus atrium impar. This connection is thought to prevent the perilymphatic fluid from flowing out of the sinus impar when the scaphium moves posteriorly.

15. The connective tissue between the scaphium and the sinus impar may help move the ossicles anteriorly after they have been pulled posteriorly by the compression of the swim bladder. This tissue may also prevent the ossicles from moving too far posteriorly in response to a very strong pressure stimulus and thus help to protect the fish's inner ear from stimulation that could damage it.

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