Optical and tomographic imaging of a middle ear malformation in the bullfrog (*Rana catesbeiana*)

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Abstract

Using a combination of *in vivo* computerized tomography and histological staining, a middle ear anomaly in two wild-caught American bullfrogs (*Rana catesbeiana*) is characterized. In these animals, the tympanic membrane, extrastapes, and pars media (shaft) of the stapes are absent on one side of the head, with the other side exhibiting normal morphology. The pars interna(footplate) of the stapes and the operculum are present in their normal positions at the entrance of the otic capsule on both the affected and unaffected sides. The pattern of deformity suggests a partial failure of development of tympanic pathway tissues, but with a preservation of theopercularis pathway. While a definitive proximate cause of the condition could not be determined, the anomalies show similarities to developmental defects in mammalian middle ear formation.

I. INTRODUCTION

The unique absorptive characteristics of amphibian skin and egg membranes, combined with their necessary restriction to wetland environments, has made amphibians unique biomarkers for effects of pollutants and parasites on species survival. Occurrences of malformations and mutationsn wild amphibian populations include those affecting the limbs (missing, reduced, or multiple), the head (abnormalities in head or jaw shape, missing or reduced eyes), the spine, and the skin (Meteyer, 2000). The appearance of some of these skeletal and morphological abnormalities, particularly of the limbs, is correlated with exposure to a variety of potential mutagens, including pesticides, pathogens, and increased ultraviolet light, particularly in the larval (tadpole) period (Sessions and Ruth, 1990; Blaustein *et al*., 1997; Johnson *et al*., 1999; Sessions *et al*., 1999). Other abnormalities may be genetic in origin, with no known environmental link.

The auditory system of adult anuran amphibians shares some structural and functional similarities with that of other vertebrates. Two peripheral transduction pathways have been identified in anurans. In the tympanic pathway, vibrations of the external tympanic membrane are transmitted to the inner ear via a cartilaginous extrastapes and bony stapes [also termed the extracolumella and columella (Wever, 1985)]. The stapes is believed to be homologous to that of other vertebrates (Lombard and Bolt, 1988). Although the homology of the extrastapes is unknown (Jaslow *et al*., 1988), the operation of the stapes and extrastapes is functionally analogous to that of the mammalian ossicular chain (Møller, 1963; Mason and Narins, 2002a). The anuran's middle ear apparatus also includes a cartilaginous operculum attached to the caudal end of the oval window and to the shoulder girdle via the opercularis muscle (Wever,

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1985). The operculum and opercularis muscle together make up the opercularis system, the function of which has been debated (Lombard and Straughan, 1974; Wever, 1985; Hetherington, 1994; Mason and Narins, 2002b), but which may be involved in the detection of low-frequency seismic and acoustic signals. In the bullfrog (*Rana catesbeiana*), the opercularis pathway and the tympanic pathway mature at different time points during larval and early postmetamorphic development (Hetherington, 1987; Boatright-Horowitz and Simmons, 1995; Horowitz *et al.*, 2001). The different developmental trajectories of these two pathways may allow specific genetic or environmentally produced abnormalities of one system at specific points in time, leaving the other intact.

Here, we describe an unusual cranial deformity in one adult male and one subadult female American bullfrog collected from the same site in which the left tympanic membrane, tympanic annulus, extrastapes, and pars media of the stapes are missing, but with the corresponding structures on the right appearing normal. Abnormalities appear to be confined to structural elements of the middle ear, with seemingly normal gross features of the skull and otic capsule. The nature of the abnormalities suggests the viability of an amphibian model of developmental hearing disorders, including what the clinical literature refers to in humans as "congenital aural atresia" (Schuknecht, 1989).

II. METHODS

A. Specimen collection

The first specimen was an adult male bullfrog captured from a small pond in Rhode Island that was host to a modest but healthy bullfrog chorus (typically 6-8 calling males over the course of a season). This particular site was visited for approximately 10 years prior to the capture of the animal as part of a series of field studies on bullfrog chorusing behavior. No other external abnormalities were observed in any other vocalizing males during this period of time, but the pond was not explicitly censused for this purpose. The animal was observed first in early June during playback experiments. He approached a loudspeaker broadcasting male bullfrog advertisement calls, but remained silent although stationed near the loudspeaker for the duration of the playback (approximately 30 min), a behavior not uncommon in the field. The animal was captured by hand and transported to the laboratory where he was measured, photographed, and housed in a 75 l plastic terrarium filled with sterile soil and an internal 4 l water pool. He was fed live adult crickets *ad libitum*. The second animal was collected from the same site 2 years later as a tadpole in metamorphic climax [stage 43 (Gosner, 1960)]. No external abnormalities were noted at the time of collection. The animal was placed in a tadpole colony tank consisting of an aerated 75 l plastic aquarium and fed salt-free cooked spinach *ad libitum*. Upon entering the terminal stages of metamorphic climax, it was transferred to a terrarium similar to that described for the earlier adult. It was not until after completion of climax and some subsequent development to the size of a young subadult (Boatright-Horowitz and Simmons, 1995) that the abnormality of the left tympanic region was noted. Capture and subsequent handling of the animals conformed with relevant state and federal regulations, and research protocols were approved by the Brown University Institutional Animal Care and Use Committee.

B. Computerized tomographic scanning

In order to characterize the extent of the internal deformities *in vivo*, the animals underwent computerized tomographic (CT scan) imaging at the Radiology Department of Massachusetts Eye and Ear Hospital (male adult) and at the Imaging Center of the Woods Hole Oceanographic Institution (both animals). A series of registered CT images was obtained of the entire animalto determine if any abnormalities extended beyond the visible external abnormalities of the head region. The frogs were lightly anesthetized by submersion in 0.6% tricaine methanesulfonat

(MS-222; Sigma) for 20 min and scanned with a Siemens Plus 4 CT unit. Scan images of the adultmale were obtained originally using a 1 mm spiral protocol with 1 mm table increments. These images were formatted in 0.3 and 1 mm slice thicknesses in the transaxial planein both bone and soft tissue kernels. Postscan reformats in coronal and sagittal planes at 0.1 mm were also produced on a Siemens Volume Zoom at the Imaging Center of the Woods Hole Oceanographic Institution. Scan images of the female were obtained in 0.5 and 2 mm thicknesses. Three-dimensional reconstructions of soft and bony elements were obtained using Siemens proprietary software on both of the earlier machines.

C. Histological procedures

Postscanning, the animals were maintained in the laboratory for several weeks. Although the mal's behavior seemed otherwise normal, he never vocalized, either spontaneously, in response to natural vocalizations of other males housed in the laboratory, or in response to playbacks of conspecific advertisement calls. The female's behavior also seemed normal, although she never showed orienting responses to playbacks of natural or recorded advertisement calls. Neither of thesebehaviors is unusual in captive frogs outside of the breeding season. The male was sacrificed by intraperitoneal injection of sodium pentobarbital(100 mg/kg; Abbott), and transcardially perfused with heparinized 0.9%(w/v) saline and 4% formaldehyde in 0.1 M phosphate-buffered saline. The head was removed, embedded in paraffin, sectioned transaxially at 10 μm and stained with Gomori's trichrome to allow better visualization of internal structures. The female was anesthetized with 0.6% MS-222 and the eighth cranial nerve was exposed bilaterally through the roof of the mouth. A fluorescent lipophilic dye (DiI: 1,1′, di-octadecyl-3,3,3′3′-tetramethylindocarbocy anine perchlorate, Molecular Probes) was pressure injected into both the right and left eighth nerve medial to the otic capsule. The animal was allowed to survive for 5 days, then euthanized and perfused as described earlier. The brain and medial portions of the eighth nerves were removed, meninges were cleared, and the brain was sectioned at 50 μm on a vibratome. Both transmitted light and fluorescent sections were viewed using an Olympus BX60 microscope equipped with a fluorescence attachment and images collected on a Pentium 4 computer running MagnaFire software (Optronics).

III. RESULTS

At capture, the male measured 10.8 cm snout-vent length. A dorsal view of his head is shown in Fig. 1(a). The tympanic membrane is absent on the left side [Fig. 1(b)]. The right tympanic membrane [Fig. 1(c)] measured 1.9 cm diameter, which is in the range of tympanic membrane diameters observed in normal adult males (Boatright-Horowitz and Simmons, 1995). The dorsal aspect of thenormal (right) tympanic membrane is bounded by a distinct, semilunate supratympanic ridge of connective tissue, extending from the retro-orbital region to the caudal edge of the tympanic membrane itself. The tympanic membrane is attached superiorly to this supratympanic ridgeand is normal in appearance with the typical large central patch (the thickened region overlying the attachment region for the extrastapes). A ridge is also present on the abnormal (left) side [Fig. 1(b)], but is reduced in extent and extends laterally rather than dorsally [Figs. 2(a)-2(c)]. The outermost region on the left side of the head is occupied by conventional, smooth epidermis. Neither the central patch of the tympanic membrane nor a tympanic annulus is present.

Figure 1(d) shows the CT-derived skeletal structure of the frog, which largely displays normal form and structure. There is no apparent postcranial skeletal abnormality anywhere in the body; individual skull bones are all present and symmetric. The only anomalies visible in this section are the lack of the tympanic annulus on the left (overlying the squamosal and pterygoid bones, which are present bilaterally).

Figure 2 shows histological sections through the head region, cut in the transaxial (coronal) plane, to show internal morphology [Figs. 2(a)-2(c)], and corresponding tomographic sections through the head in the living animal [Figs. 2(d)-2(f)]. The absence of the middle ear structures is quite obvious on the left side of the images in both sets of figures. The large white ovals in the otic capsule in the CT sections are the otoliths, which are very dense [Figs. 2 (e) and 2(f)]. From these figures, it is apparent that the basic skull anatomy is largely symmetrical, although there may be a slight depression of the dorsal process of the prootic bone on the deformed side. The internal structure of the otic capsule and bony labyrinth also appear tobe normal. Figures 2(b) and 2(e) show the presence of a properly formed stapes on the right, and the lack of most of the stapedial shaft (pars media) on the left. The pars interna (footplate) cartilage is present on both sides [Fig. 2(b) right side, Fig. 2(c) left side due to slight bias in section slicing]. The operculum cartilage was observed bilaterally overlying the oval window, visible in more caudal sections (data not shown).

Figure 3 shows a close-up of the area of the tympanic membrane on both the deformed [Fig. 3(a), left] and normal [Fig. 3(b), right] sides, with the thick, filled-in connective tissue on the left and laminated structure of a normal tympanic membrane clear on the right. The lackof the tympanic annulus is also clear in this figure. Figures 3(c) and 3(d) shows sections of the oval window area showing the anterior margin of the pars interna, similar in size and positionon both sides of the head. Note that the pars interna is free medially, and fused to bone laterally. It is visible bilaterally in the CT scans as well [Fig. 2(e)].

At the time of examination, the female's snout-vent length was 7.8 cm, with a 0.75 cm tympanic membrane clearly visible on the right side of her head [Figs. 4(a) and 4(c)], consistent with normal measurements for females at this developmental stage (Boatright-Horowitz and Simmons, 1995). As with the male, on the normal (right) side, there was a clear supratympanic ridge dorsal and caudal to the tympanic membrane, and the tympanic membrane itself had a large central patch. The abnormal side showed striking similarities to the malformation observed in the male, with a complete lack of tympanic membrane, reduced supratympanic ridge, and smooth epidermal covering overlying the region the tympanic membrane should occupy [Fig. 4(b)]. Transaxial CT scans of the animal's otic region [Fig. 4(d)] also showed great similarity to that observed in the male, with normally placed otoliths, and normal skull and postcranial skeletal anatomy, but with no stapedial shaft, extrastapes or tympanic membrane structures on the abnormal side. The footplate of the stapes and the operculum were present on both normal and abnormal sides. Transport of DiI from the eighth nerve to the medulla (dorsal lateral nucleus and vestibular nucleus complex) was qualitatively similar, showing extensive fiber and soma label, on both the normal and deformed sides.

IV. DISCUSSION

The proximate cause for the lack of tympanic membrane, tympanic annulus, extrastapes and pars media of the stapes combined with otherwise normal skeletal anatomy is unknown. Our inability to obtain reliable laboratory data on the animals' hearing sensitivity did not allow us to quantify the extent of any hearing loss. Even though the male animal was not observed to vocalize either in the field or in the laboratory, his behavior in the field, approaching playbacks of conspecific advertisement calls, indicated that he probably had some auditory function and at least some rudimentary ability to localize sound sources. The extent of the female's auditory functionis unknown, but any auditory function in each animal could arise from several sources. First, unilateral tympanic input was clearly possible given the normal anatomy on the right side of the animals' heads. Second, the observation that the operculum cartilages were present on both the affected and unaffected sides of the head implies that the animals possessed a functional opercularis system, which could convey sensitivity to low-frequency signals (Lombard and Straughan, 1974). Third, in some, typically smaller, anurans, the lungs and body

wall serve as an input pathway to the inner ear (Narins *et al*., 1988). Several extant species of anurans have reduced or missing tympani along with well-developed inner ears and intact operculum cartilages (Jaslow *et al*., 1988). Vibrations of the body wall transmitted to the inner ear are important in mediating low frequency sensitivity in these "earless" anurans (Hetherington and Lindquist, 1999). Comparisons of auditory sensitivity in harlequin frogs (*Atelopus*) with and without a tympanic ear indicate that the tympanic pathway conveys greater sensitivities to high frequencies, but that the absence of a tympanic ear is associated with greater sensitivities to low frequencies (Lindquist *et al*., 1998). It is unclear if a lung/body wall input pathway mediates hearing in larger bullfrogs with a normal tympanic system, but might be important in our specimen animals.

Because the male animal was captured as an adult, it was not possible to ascertain with certainty whether he was hatched in the same pond from which he was collected, or whether he migrated there as an adult from neighboring bodies of water. The presence of a second animal, captured as a tadpole and so bred in the same site, suggests that the presence of some agent within the aquatic environment may be the causative factor in expression of the deformity. It is interesting to note that the animals were collected several years after the onset of treatment of sewage catchbasins in the area with methoprene as a mosquito control measure. Methoprene, a commonly used domestic and agricultural pesticide, breaks down in the presence of ultraviolet light into compounds with the same structure and receptor binding properties as retinoic acids, which induce middle and external ear anomalies in mammals (Lammer, 1991; Mallo, 1997; Moerike *et al*., 2002), and that have been implicated in the formation of craniofacial deformities in leopardfrogs (*Rana pipiens*) (Ankley *et al*., 1998). Given the homology between the mammalian and anuran stapes (Lombard and Bolt, 1988) and the functional analogy between the mammalian ossicular chain and the frog stapes/extrastapes system (Møller, 1963; Mason and Narins, 2002a), one possibility is that exposure to retinoic acid or its derivatives may have led to the frog's unilateral deformity. The presence of the female, captured as a tadpole, rules out the possibility that direct injury produced the deformity.

It is possible that some genetic anomaly produced the observed deformities. In mammals, components of the tympanic pathway arise via differentiation of neural-crest derived mesenchymal tissue, from the first (malleus, incus) and second (stapes) branchial arches, respectively, with the tympanic membrane derived from interactions between tissue from the first branchial cleft and branchial pouch. These developmental pathways are under the control of a wide variety of genes which are expressed at different time points in development (Mallo, 2001). These differential time courses controlling development of elements of the auditory periphery can lead to various types of anomalies, each of which can seriously impact auditory function. In many instances, malformations are restricted to developmentally related structures, which allows estimates of the time of damage. For example, in mice, exposure to retinoic acid yields differential malformations of middle ear elements depending on gestational time of exposure and hence the temporal sequence of migration of their precursors from the neural crest (Mallo, 1997). Furthermore, in many cases, both sides of the same embryo reached different stages of development, suggesting that theprogram regulating differentiation of the parts of the middle ear can act independently on eachside (Mallo, 1997).

The observed pattern of deformities in the two frogs probably reflects the common embryological derivatives of the affected structures. Early induction studies in ranids demonstrate that transplanted portions of the tympanic annulus could induce formation of a tympanic membrane in anomalous locations (Helff, 1928). In bullfrogs, the tympanic membrane forms at the conclusion of metamorphic climax, and is often first evident by a thinning and darkening of the epidermis overlying the tympanic cavity at approximately Gosner stage 44 (Boatright-Horowitz and Simmons, 1995). The stapes forms at approximately this time as well, with the pars interna at the rostral end of the oval window (Horowitz *et al*.,

2001). The tympanic annulus appears to condense slightly before this, at about stage 43 (unpublished data). The opercularis system develops and reaches maturity earlier than the tympanic system, with the operculum cartilage forming over the oval window by about larval stage 40, and the opercularis muscle connection completed by stage 42 (Hetherington, 1987), coincident with the onset of metamorphic climax. This normal pattern of development suggests that the deformity described here most likely arose during metamorphic climax stages.

Certain human otolaryngological pathologies, such as congenital auralatresia, are often presented in pediatric patients with gradations ranging from minor external ear malformation to severe disruption of the anatomy of the entire tympanic pathway and inner ear. Aural atresia (absence or incomplete formation of the ear canal and external pinna) is a congenital syndrome seen in humans (Schuknecht, 1989; Shah and Shah, 2002) of poorly understood etiology. Usually, itinvolves abnormalities of the outer and middle ears, but with no involvement of inner ear structures. The incidence of abnormalities in the tympanic membrane varies; in some cases, it is abnormally small, while in more severe cases, it is totally absent. The syndrome can be unilateral or bilateral, is more common in males than females, and may occur in conjunction with other craniofacial syndromes (Llano-Rivas *et al*., 1995; Kountakis *et al*., 1995). Further study of the mechanism producing the absence of the tympanic membrane in anurans, with its qualitative similarity to aural atresia, may shed light on the etiology of this otolaryngological syndrome.

ACKNOWLEDGMENTS

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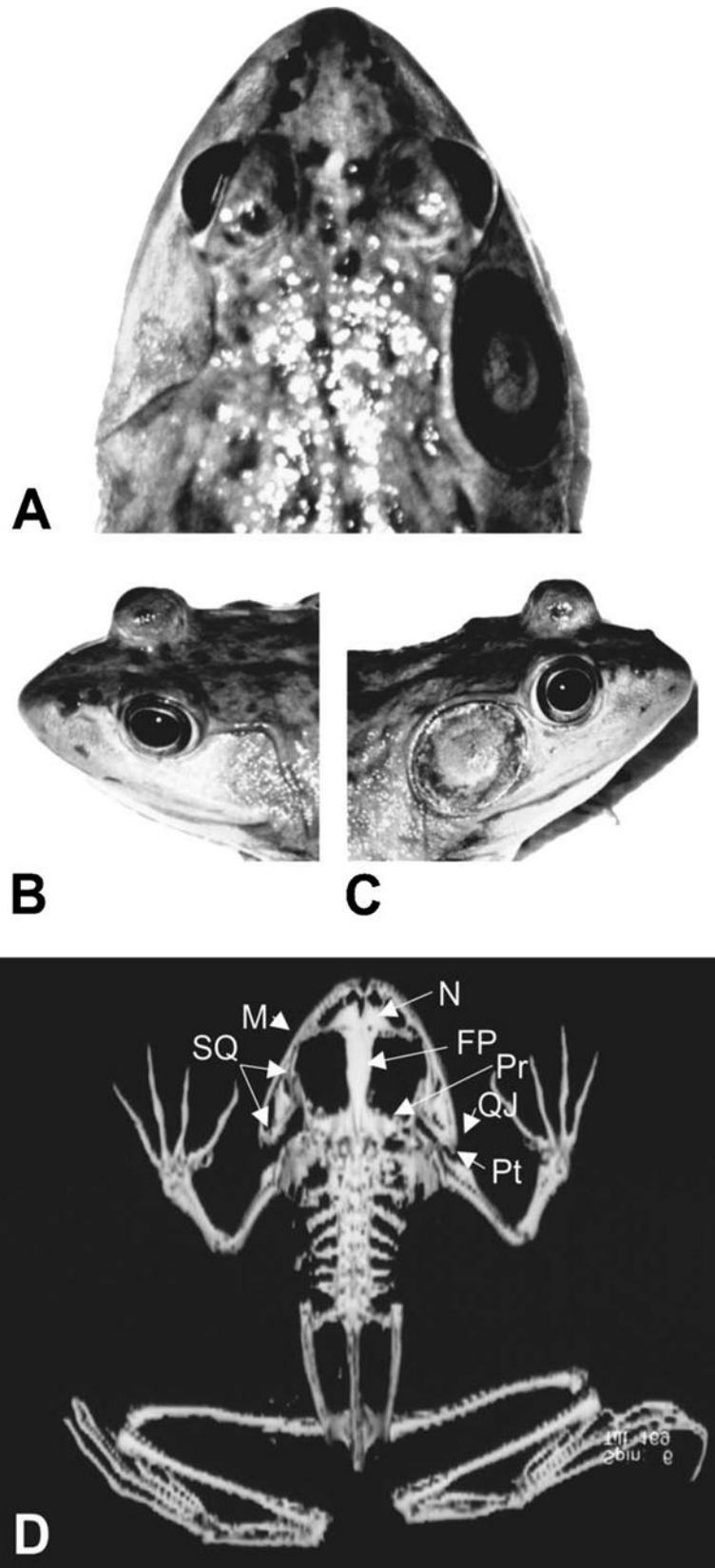
FIG 1.
(a) Dorsal view of adult male frog. The large, normal looking tympanic membrane is on the animal's right. (b) The abnormal side, lacking any tympanic membrane and with a reduced supratympanic ridge, whose caudal aspect passes dorsal-ventrally through what would be the caudal extent of a normal tympanic membrane's central patch. (c) The normal tympanic membrane, showing a robust medial thickening of the central patch. (d) CT scan of skeleton, showing normal, axialskeleton, and normal, symmetric skull bones. Abbreviations: **FP**: frontoparietal; **M**: maxillary; **N**: nasal; **Pr**: Prootic; **Pt**: pterygoid; **SQ**: squamosal; **QJ**: quadrojugatal bone.
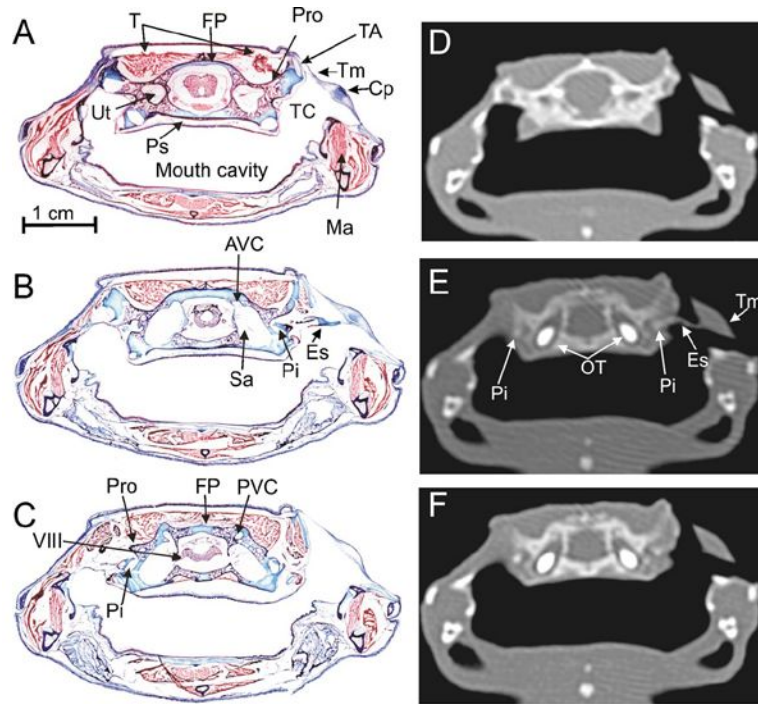
FIG 2.
(Color online). (a-c, left). 10 micron Gomori trichrome stained transaxial (coronal) section at levels of (a) auditory midbrain, (b) cerebellum, and (c) auditory medulla/VIII nerve from adult male frog. (d-f, right): 1.0 mm CT transaxial scans from living frog at levels corresponding to histological sections on left. Saccular (Sa) and Utricular (Ut) spaces are labeled, although at higher resolution, the organs themselves are plainly visible and appear normal in both orientation and extent. Abbreviations: **AVC**: anterior vertical canal; **CP**: central patch; **Es**: extrastapes; **Ma**: masseter muscle; **OT**: otoliths; Pi: tympanic columella pars interna;**Ps**: parasphenoid; **PVC**: posterior vertical canal; **Sa**: saccular space; **T**: temporalis muscle; **TA**: tym-panic annulus; **TC**: tympanic cavity; **Tm**: tympanic membrane; **Ut**: utricular space; **VIII**: eighth cranial nerve.
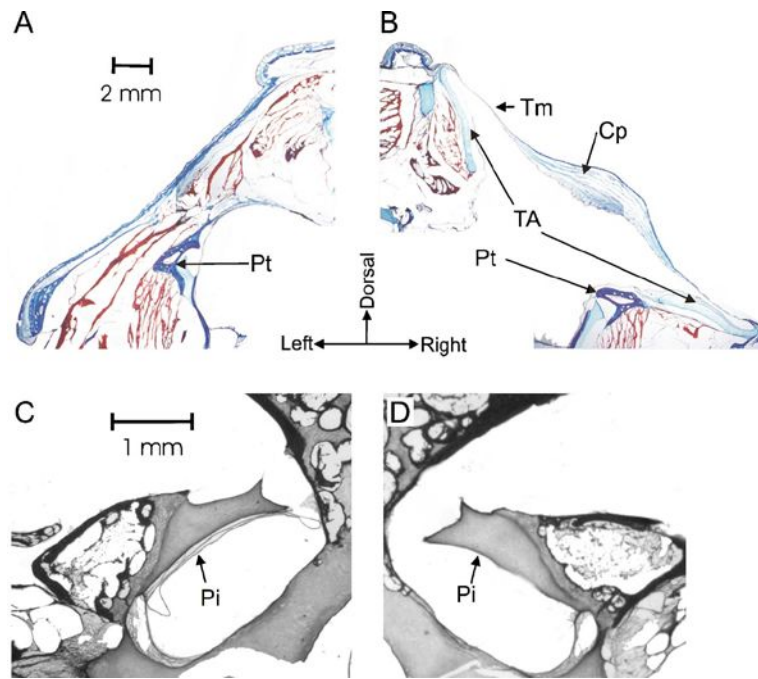
FIG 3.
(Color online). (a,b) 10 micron Gomori trichrome stained sections showing detail of tympanic region in malformed (a, left) and normal (b, right) sides of adult male frog. (c,d) Grayscale image of 10 micron Gomori trichrome stained sections showing detail of the pars interna of the stapes in oval window region on left (c) and right (d) sides, demonstrating presence of normal cartilage bilaterally. Abbreviations as in Fig. 2.
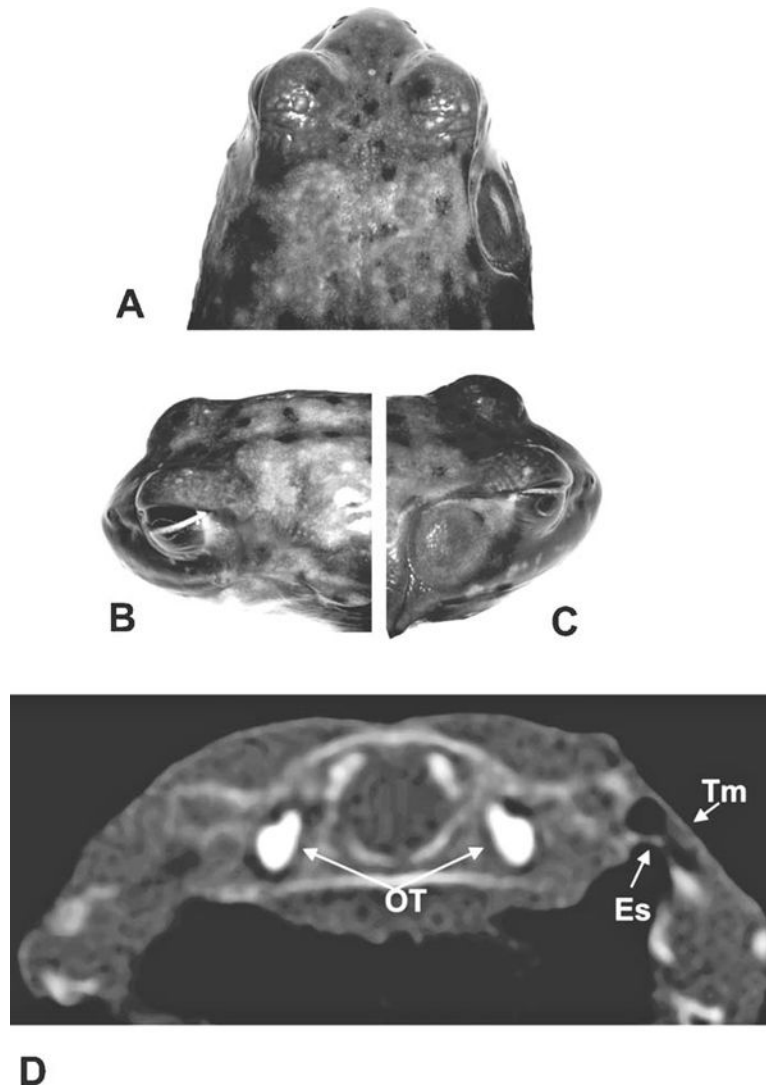
FIG 4.
(a) Dorsal view of female subadult frog. The normal looking tympanic membrane is on the animal's right. (b) The abnormal side, lacking any tympanic membrane and with a reduced supratympanic ridge. (c) The normal tympanic membrane, showing a robust central patch and supratympanic ridge. (d) **CT** scan of otic region showing normal middle ear structures on the right and loss of tympanic membrane, extrastapes, and parts of the stapes on the left. Abbreviations:**OT**: Otoliths; **St**: stapes; **Tm**: tympanic membrane.