The frog inner ear: picture perfect?

Matthew J. Mason[1]

Johannes M. Segenhout[2]

Ariadna Cobo-Cuan[3]

Patricia M. Quiñones[4]

Pim van Dijk[2,5]

[1] University of Cambridge

Department of Physiology, Development & Neuroscience

Downing Street

Cambridge CB2 3EG

U.K.

Tel. +44 1223 333829

Fax +44 1223 333840

mjm68@cam.ac.uk

[2] University of Groningen

University Medical Center Groningen

Department of Otorhinolaryngology, Head & Neck Surgery

P.O. Box 30.001

9700 RB Groningen

The Netherlands

jmsegenhout@hotmail.com

p.van.dijk@umcg.nl

[3] Department of Animal and Human Biology

Havana University

Street 25 No. 455

Havana CP 10400

Cuba

cobo_cuan@fbio.uh.cu

[4] Department of Physics and Astronomy

1-129 Knudsen Hall, 475 Portola Plaza

University of California Los Angeles

Los Angeles, CA 90095

U.S.A.

yukiq@ucla.edu

[5]University of Groningen

Graduate School of Medical Sciences, Research School of Behavioral and Cognitive Neurosciences

P.O. Box 196

9700 AD Groningen

The Netherlands

p.van.dijk@umcg.nl

Abstract

Many recent accounts of the frog peripheral auditory system have reproduced Wever's (1973) schematic cross-section of the ear of a leopard frog. We sought to investigate to what extent this diagram is an accurate and representative depiction of the anuran inner ear, using three-dimensional reconstructions made from serial sections of *Rana pipiens*, *Eleutherodactylus limbatus* and *Xenopus laevis*.

In *Rana*, three discrete contact membranes were found to separate the posterior otic (= endolymphatic) labyrinth from the periotic (= perilymphatic) system: those of the amphibian and basilar recesses and the contact membrane of the saccule. The amphibian 'tegmentum vasculosum' was distinguishable as a thickened epithelial lining within a posterior recess of the superior saccular chamber. These features were also identified in *Eleutherodactylus*, but in this tiny frog the relative proportions of the semicircular canals and saccule resemble those of ranid tadpoles. There appeared to be a complete fluid pathway between the right and left periotic labyrinths in this species, crossing the cranial cavity. *Xenopus* lacks a tegmentum vasculosum and a contact membrane of the saccule; the *Xenopus* ear is further distinguished by a lateral passage separating stapes from periotic cistern and a more direct connection between periotic cistern and basilar recess. The basilar and lagenar recesses are conjoined in this species.

Wever's diagram of the inner ear of *Rana* retains its value for diagrammatic purposes but it is not anatomically accurate, nor representative of all frogs. Although Wever identified the contact membrane of the saccule, most recent studies of frog inner ear anatomy have overlooked both this and the amphibian tegmentum vasculosum. These structures deserve further attention.

Keywords

Inner ear; frog; amphibian papilla; basilar papilla; tegmentum vasculosum; contact membrane

Introduction

The inner ear structures of ranid frogs have been the subject of detailed anatomical accounts dating back over 150 years, many of which were written in German (see e.g. Deiters, 1862; Hasse, 1868; Retzius, 1881; Gaupp, 1904). Among the best known of the English-language descriptions are those of Ernest Glen Wever. The first figure from Wever's 1973 paper in *Journal of Morphology* (reproduced here as Fig. 1A) shows "A schematic representation of the ear and labyrinth of *Rana pipiens*, in frontal section". An almost identical diagram appeared in two of Wever's later publications including his 1985 book *The Amphibian Ear*, which remains the most comprehensive account of the subject. It has been reproduced, sometimes in a modified form, in review papers (e.g. Fay and Popper, 1985; Lewis and Narins, 1999; Simmons et al., 2007; Gridi-Papp and Narins, 2010), a leading text-book on amphibian biology (Duellman and Trueb, 1986) and several other articles. As such, it must be the world's most widely-consulted scientific illustration of an amphibian peripheral auditory system. However, as explained below, there is some uncertainty as to the species depicted, the orientation and the accuracy of this important diagram.

The species examined

The taxonomy of the North American ranid frogs is currently in a state of flux (Dubois, 2007; Hillis, 2007; Pauly et al., 2009). Some authors place leopard frogs in the genus *Lithobates*, but following Hillis' (2007) recommendation, we retain the genus *Rana* for North American ranids.

Wever (1973) stated that his illustration shows the ear of *Rana pipiens*, but in his 1985 book he distinguished between this species (the northern leopard frog) and the very similar "*Rana utricularia sphenocephala*", now *R. sphenocephala utricularia* (the southern leopard frog). Wever (1985) did not make it clear which of these species is represented in the figure of interest to us here, which appears in slightly modified form as his Fig. 3-17. However, another illustration also reproduced from his 1973 paper (Fig. 3-79 in Wever, 1985) is labelled as *R. sphenocephala*. Wever's 1973 paper may

therefore describe the ear of the southern leopard frog, but it is likely that the ears of these two species are practically identical. The articles that have reproduced Wever's illustration have often implied that a generalized anuran morphology is represented.

The orientation of Wever's illustration

Within his 1973 paper, Wever stated that he sectioned his specimens in "a horizontal plane (dorsal to ventral)...frontally (anterior to posterior) or laterally (right to medial, then continued from medial to left)". This description is somewhat confusing, and perhaps for this reason Wever later redefined the three planes. In Wever's (1985) figure 3-34, a frontal plane is clearly shown dividing the head into dorsal and ventral components. Such a plane would pass through most of the teeth on both left and right maxillae and may be considered horizontal. A sagittal plane vertically divides the head into left and right components. A transverse plane is a vertical plane perpendicular to the frontal and sagittal planes, which divides the head into anterior and posterior components. These definitions agree with the standard veterinary anatomical nomenclature (Blood and Studdert, 1999) and are those used in the present study. In our interpretation, "horizontal" (Wever, 1973) is actually frontal, "lateral" (Wever, 1973) is actually sagittal and "frontal" (Wever, 1973) is actually transverse.

The present study focuses on the first figure from Wever (1973), the caption of which reads "the ear and labyrinth...in frontal section" (Fig. 1A). Was this in fact a transverse section, according to standard nomenclature?

The accuracy of Wever's illustration

When preparing a previous article (van Dijk et al., 2011), two of the current authors had reason to question the accuracy of Wever's 1973 diagram. Wever shows the extrastapes (= extracolumella) as a very short extension of the bony stapes shaft (stapes *pars media*), connecting it to the centre of the tympanic membrane (Fig. 1). It would be natural to assume that this apparatus must operate as a stiff piston, an inflection of the tympanic membrane driving the stapes directly into the inner ear. In reality, the extrastapes is much longer than this and has an angled articulation with the *pars*

media: the stapes/extrastapes system works as a flexible, first-order lever (Jørgensen and Kanneworff, 1998; Mason and Narins, 2002; Werner, 2003).

Turning to the inner ear, Wever shows three pathways for "fluid flow" to pass between the stapes on the right of his diagram to the periotic (= perilymphatic) sac on the left, one via the amphibian papilla, a second via the basilar papilla and a third between the two papillae, each being indicated by arrows in Fig. 1A. The endolymph within the otic (= endolymphatic) labyrinth in frogs is separated from the perilymph within the periotic labyrinth by so-called 'contact membranes' (Fig. 1B), so fluid cannot actually flow between the two systems and the three arrows should instead be taken to indicate three pathways of acoustic energy flow. In their more recent account of energy flow pathways through the ear of the bullfrog (*Rana catesbeiana*), Purgue & Narins (2000a, b) considered the routes passing through the amphibian and basilar recesses but made no mention of Wever's middle pathway. Purgue & Narins regarded the periotic canal as an alternative route for low-frequency energy flow which bypasses the otic system entirely, but this is not labelled in Wever's diagram and there is scant reference to it in his written descriptions. Four potential pathways for sound energy flow between stapes and periotic sac have therefore been described in frogs, but are all four consistently present?

In this study, histological sections were made from the inner ears of three species of frogs, including leopard frogs (these were believed to be *Rana pipiens* rather than *R. sphenocephala*, but this could not be confirmed beyond doubt by the suppliers). Photomicrographs and three-dimensional reconstructions were used to assess the accuracy of Wever's accounts and other recent descriptions of ranid inner ear morphology.

While leopard frogs are in the family Ranidae, within the Ranoidea clade of the Neobatrachia, *Eleutherodactylus limbatus* (Eleutherodactylidae) is placed within the other major neobatrachian clade, the Hyloidea (Hoegg et al., 2004). *Xenopus laevis*, family Pipidae, is an aquatic 'archaeobatrachian', the 'Archaeobatrachia' being a paraphyletic assemblage of frogs which

diverged before the Neobatrachia (Hoegg et al., 2004). In order to assess whether Wever's diagram is representative of a more diverse range of frogs, the leopard frog ear was compared here with the ears of *E. limbatus*, one of the world's smallest frogs, and with those of *X. laevis*.

Materials and methods

Twelve frogs from three different species were used in this study. Three leopard frogs believed to be *Rana pipiens* (40-50 g body mass) were obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, U.S.A.) via Exoterra Schaudi GmbH, Holzheim, Germany. They were housed at the University of Groningen laboratory animal facilities. The frogs were euthanized using the double pith procedure and then decapitated. The lower jaw was removed and the remaining part of the head was divided sagittally. Skin was removed and small holes were made in various places in the skull, away from the structures of interest, to improve fluid impregnation. The ears were fixed by immersion in a 10% neutral buffered formalin solution (pH 7.4) for at least 24 hours at 4°C. The fresh corpse of a male *Rana pipiens*, originating from Nasco (Fort Atkinson, WI, U.S.A.), was used for micro-computed tomography (micro-CT) scanning, as described below.

Two *Eleutherodactylus limbatus* specimens (each around 0.2 g body mass) were captured at Las Terrazas, Artemisa province, Cuba. They were euthanized by double pithing and decapitation, the palatal skin was removed and their heads were preserved in 10% formalin and sent to Groningen for further processing. The head of one specimen was halved prior to sectioning, while the other was sectioned whole.

Six *Xenopus laevis* specimens (males 55-60 g, females 120-220 g, all gonadectomized body masses) were obtained as fresh corpses from a breeding colony in the Wellcome Trust/Cancer Research U.K. Gurdon Institute, Cambridge, U.K. They had been euthanized via tricaine overdose followed by cooling, as part of another study. The otic capsules of one male and one female specimen were cut

out and placed in 4% buffered formaldehyde solution within two hours of euthanasia. They were then sent to the University of Groningen where they were processed as the *Rana* specimens. A micro-CT scan was made of the head of another male specimen at the University of Cambridge, and the head was then dissected under light microscopy. The remaining three specimens, two females and a male, were also dissected.

Animal care and euthanasia procedures conformed to local and national regulations and were approved by the appropriate institutional Animal Care and Use Committees.

Histological procedures

After fixation the *Rana* and *Xenopus* specimens were rinsed in distilled water, refreshed several times. All subsequent steps were performed on a rolling bank to keep the specimens moving in the experimental solutions. Decalcification took place in a 10% EDTA solution (Sigma, ED5SS, pH 7.34) at a temperature of 50°C in a microwave oven (T/T MEGA microwave histo-processor, Milestone), in four sessions of twelve hours. After decalcification the specimens were rinsed again in distilled water and dehydrated in a graded, seven-step ethanol series (30%, 50%, 70%, 90%, 96%, 100%, 100%) where each step took one hour and solutions were refreshed three times. If necessary, specimens were stored overnight in 70% ethanol. Next, specimens were placed in a 100% ethanol/ hydroxypropyl methacrylate (HPMA) solution (50:50) for 4-8 hours and then put in pure HPMA solution for 24-48 hours. The specimens were then embedded in pure HPMA solution with addition of a plasticizer (around 25:1). The HPMA solution contained 45 ml HPMA, 5 ml ethylene glycol monobutyl ether, 0.5 g benzoyl peroxide, 1.25 ml glycerol and 0.25 ml ethylene glycol dimethacrylate. The plasticizer consisted of 1 ml n,n-dimethylaniline and 10 ml polyethylene glycol 400.

The *Eleutherodactylus* sections were prepared using a faster procedure owing to time constraints. The decalcification was performed in only two steps of 7 and 12 hours and the ethanol dehydration series was also slightly altered (30% for 30 minutes, three times; 70% for 30 minutes, three times;

90% for 10 minutes, three times; 96% for 10 minutes, three times and 100% for 15 minutes, twice). The specimens were placed in the ethanol/HPMA solution for two sessions of 1 hour, then overnight.

After polymerization, transverse sections of 4 µm thickness were cut using a motorized microtome (HM350S, Microm, Heidelberg, Germany). In some cases, the otoconial mass of the saccule, if identified during the sectioning procedure, was removed from the embedded specimen using a fine needle so as to avoid damaging the microtome. A subset of sections was stained with toluidine blue 1% (10 min) and contrast-stained with basic fuchsin (15-20 s).

3-D reconstruction from serial sections

Digital photographs of *Rana* and *Xenopus* sections were made with an Olympus Camedia C-5050 digital camera and stored as tiff files. Digital photographs of the smaller *Eleutherodactylus* specimens were made using a Leica DM RXA microscope fitted with a Colorview 1 MP camera (Soft Imaging System), working with AnalySiS software (Olympus). Individual files, in some cases reduced in size by cropping and/or conversion to greyscale, were then loaded into ImageJ 1.45s (W. Rasband, 2011, National Institutes of Health) and autoregistered using the StackReg plug-in (P. Thévenaz, 2011, Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne; see Thévenaz et al., 1998). StackReg uses a recursive procedure based on rigid-body translation and rotation to align each consecutive section. WinSurf 4.0 (S. Lozanoff & D. Moody, 2001) was then used to construct three-dimensional images, following visual identification of relevant structures. Where wall thickness was significant, the internal rather than external walls of the otic and periotic labyrinths were traced and modelled. The choice of interval between sections used to make the final reconstruction depended upon the size of the structure being reconstructed and the level of detail required. In the production of Fig. 12, MicroView 2.1.2 (GE Healthcare, 2006) was used to reorient the registered image stacks.

One potential problem with 3D reconstruction from serial sections is systematic misalignment of the sections, resulting in a distorted (skewed or twisted) representation, and it can also be difficult to determine orientation. *Eleutherodactylus* was small enough that a whole head could be sectioned and reconstructions from right and left ears compared. In the case of *Rana* and *Xenopus*, the reconstructions from serial sections were compared with micro-CT reconstructions of the whole skull and the ear regions within it (see below). Although soft tissue could not be visialized in our CT scans, hard-tissue structures including otic capsule walls and stapes shaft provided sufficient landmarks for comparison with the serial section reconstructions.

Photomicrographs and reconstructions were laterally inverted where necessary, to facilitate comparison.

Micro-CT reconstructions

Micro-CT images were obtained of the head of one male *Xenopus* specimen at the University of Cambridge. The posterior part of the head was skinned and tissues between the mandibles were removed. The head was then wrapped in cellophane to reduce the rate of drying, and the head was scanned using a Metris X-Tek HMX 160 micro-CT scanner operating at 50 kV and 50 µA with no prefilter. The stepping rotational angle was 0.5 degrees. The software used in the processing of the scan data included iXS Integrated X-ray System Control version 4.1.29 (X-Tek Systems Ltd., 2002), NGI CT Control version 1.5.4 (X-Tek Systems Ltd., 2005) and CT-Pro 2.0 (Metris, 2008). At UCLA, a micro-CT scan was made of the head of one male *Rana pipiens* specimen, immersed in a buffered salt solution within a sample holder. A desktop micro-CT machine was used (MicroCT 40; Scanco Medical, Bassersdorf, Switzerland), operating at 55 kV and 145 µA with a 0.5 mm Al prefilter. The stepping rotational angle was 0.36 degrees. The image was processed using Scanco proprietary software. For both animals, the voxels in the scan images were of 30 µm side length.

VGStudio Max 2.0.1 (Volume Graphics GmbH, 2008), MicroView 2.1.2 and WinSurf 4.0 were used to construct 3D images from the CT data obtained. The CT reconstructions were used to verify that the

reconstructions made from serial sections of *Rana* and *Xenopus* were not distorted, and to determine their orientation relative to the skull.

Results

WinSurf reconstructions of the inner ears of the three anuran species are presented for comparison in Fig. 2. There was no evidence of systematic distortion of the reconstructions made from serial sections, as determined by comparison between different ears and/or comparison with micro-CT reconstructions. Histological artefacts inevitably affected the reconstructions, however, as described below.

Rana pipiens

Reconstructions of the inner ear of *Rana* are shown in Figs. 2 and 3, and photomicrographs of sections of particular interest are presented as Figs. 4 and 5.

Considering first the otic labyrinth, the saccule is partially divided by a central constriction into inferior and superior compartments (Fig. 3). The **inferior saccule** is an ovoid chamber, flattened rostromedially. The saccular macula (sensory epithelium) is at the centre of the flattened surface. The **superior saccule** has an expanded dorsal chamber and four relatively small, posterior diverticula:

1) The prominent **amphibian recess** (Figs. 3, 4A, B, 5B) extends medially from the dorsomedial part of the superior saccule before turning caudally. The sensory epithelium on its dorsal wall is known as the **amphibian papilla**, although this term is sometimes used to refer to the whole chamber and its contents.
2) The **lagenar recess** (Figs. 3, 4A) extends medially from the caudoventral part of the superior saccule, below the amphibian recess. Its sensory epithelium covers its medial wall.

3) A third small diverticulum, the only one to lack a sensory end-organ, extends caudally from the dorsolateral part of the superior saccule (Fig. 3). The thick epithelium forming the internal lining of this diverticulum is known as the **tegmentum vasculosum** (Figs. 4A, 5D). This lining extends rostrally into the posterior part of the superior saccular chamber.

4) The **basilar recess** (Figs. 3, 4A, 5A) is located between the cavity of the tegmentum vasculosum and the lagenar recess. Its sensory epithelium (**basilar papilla**) lies on its medial wall.

Rostral to the amphibian recess, the superior saccule communicates via a constricted region, the utriculo-saccular foramen, with the elongated **utricular chamber**. The sensory epithelium of the utricle is on the ventral wall of the free, rostral portion of this chamber, which then divides to form the ampullae of the anterior and lateral **semicircular canals** (Fig. 2). From the caudal end of the utricular chamber arise the other end of the lateral semicircular canal and the ***crus commune***, a short, vertical segment representing the convergence of the anterior and posterior semicircular canals (Fig. 3). The ampulla of the posterior semicircular canal is located just underneath the caudal-most part of the lateral semicircular canal; the two are not in contact.

We turn now to the periotic system, which may be divided (after Lombard, 1977) into periotic tissue and the periotic labyrinth proper. **Periotic tissue** is the connective tissue found separating both otic and periotic labyrinths from the walls of the otic capsule. In places, it takes the form of condensed and cartilage-like 'limbic tissue' (Wever, 1973). Limbic tissue forms a thin layer around the membranes of the semi-circular canals and utriculus, but it is much thicker around the amphibian and basilar recesses (Figs. 5A, B). The lagenar recess and part of the tegmentum vasculosum are also supported by limbic tissue. Elsewhere, the periotic tissue consists of little more than a diffuse collection of fibres within a fluid space. The semicircular canals, within their thin shells of limbic tissue, are separated from the otic capsule walls by such a fluid space, as is much of the superior saccule (Figs. 4A, B).

The other component of the periotic system, the **periotic labyrinth**, is a membranous sac of complex shape containing apparently acellular fluid. Its three main subdivisions are the periotic cistern, the periotic canal and the periotic sac. The capacious **periotic cistern** (Figs. 3, 4A, B) almost completely surrounds the inferior saccule, extending around it on the medial side as far dorsally as the utricular chamber. A diverticulum of the lateral part of the periotic cistern extends through a narrow, oval-shaped foramen in the wall of the otic capsule and turns sharply rostrally to expand into a **lateral chamber** (Figs. 2-4). The cartilaginous **operculum** lies immediately over the foramen (Figs. 4A, B), while the **stapes footplate** is rostral to this. The footplate comprises the expanded medial part of the bony *pars media* and, around its periphery, the U-shaped, cartilaginous *pars interna*. The operculum and stapes footplate interlock: a flange of the *pars interna* extends a short distance medial to the operculum, while the rostrolateral corner of the operculum fills the gap between the *pars interna* and a ventral process of the *pars media* which articulates with the otic capsule.

The **periotic canal** is a long, narrow tube which ascends dorsally from the lateral part of the periotic cistern and wraps closely around the anterior aspect of the superior saccule (Figs. 2, 3). There is a thin, shared membrane between periotic and otic labyrinths throughout this course. The periotic canal then parts from the saccule near the *crus commune*, turns sharply caudolaterally and bends down around the lateral semicircular canal to meet the superior saccule again between the amphibian recess and the diverticulum of the tegmentum vasculosum. The oval region of apposition found here between otic and periotic labyrinths is the **contact membrane of the saccule** (Figs. 3, 5C), identified in all three specimens of *Rana pipiens* just lateral to the contact membrane of the amphibian recess. The contact membrane of the saccule has 33-76% (n=3 ears) of the area of the contact membrane of the amphibian recess and it is more than twice as thick, but it still represents a relatively thin window between otic and periotic labyrinths, in a region where much of the otic system is surrounded by thick limbic tissue.

The periotic canal then turns ventromedially to form an elongated, curved contact membrane with the lateral wall of the amphibian recess (Fig. 5B). Leaving the otic capsule, the canal runs for a short distance parallel to the ***recessus partis basilaris***, a blind-ending periotic diverticulum heading rostrally towards the basilar recess (Figs. 3, 5A). There is a small contact membrane between the apposed tips of the *recessus partis basilaris* and the basilar recess (Fig. 5A), which is 6-14% (n=3 ears) of the area of the contact membrane of the amphibian recess. The sections of the three *Rana* specimens stopped at this point, so the relationship between the *recessus partis basilaris* and the rest of the periotic system could not be examined. From the literature (see e.g. Lewis and Narins, 1999), the *recessus partis basilaris* and the periotic canal are expected to communicate with each other via the **periotic sac**, a caudal expansion of the periotic canal which projects out of the otic capsule.

Eleutherodactylus limbatus

Reconstructions of the inner ear of *Eleutherodactylus* are shown in Figs. 2 and 6, and photomicrographs of sections of particular interest are presented as Figs. 7-9.

There is no distinct lateral chamber in *Eleutherodactylus* but the footplate and operculum lie at an angle to each other such that their inner surfaces form a bowl-like concavity. The stapes footplate is relatively small; as in *Rana*, it sends a prominent cartilaginous flange under the large operculum. The inferior saccule/periotic cistern region had evidently collapsed to a greater or lesser extent in all four ears examined because it had pulled away from surrounding structures, but its original shape could be determined as the region enclosed between otic capsule, stapes and operculum. The other parts of the inner ear escaped distortion in at least one specimen.

Given the size of the chamber in which it is contained, the inferior saccule must be relatively much smaller than that of *Rana* or *Xenopus*, while the semicircular canals are much wider relative to their

length (Fig. 2). A narrow diverticulum lined with a tegmentum vasculosum extends from the saccular cavity just dorsolateral to the basilar recess (Figs. 6, 7A, B, 8A, C).

The periotic canal (Fig. 6) is relatively longer and more convoluted than in *Rana*. Because the superior saccule is little inflated in *Eleutherodactylus*, the canal, where it emerges from the periotic cistern, is initially not in such close contact with the saccular cavity. However, after turning caudally a diverticulum of the central part of the periotic canal extends downwards and comes into intimate apposition with the superior saccular cavity (Figs. 6, 7B). As in *Rana*, the periotic canal then separates from the otic labyrinth and runs across the lateral semicircular canal en route to the amphibian recess. The contact membrane of the saccule (Figs. 6, 8B) is located just rostral to the contact membrane of the amphibian recess (Figs. 6, 7A, 8A). The contact membrane of the saccule has 26-41% (n=3 ears) of the area of the contact membrane of the amphibian recess. The contact membrane of the basilar recess is around 5-15% (n=4 ears) of the area of the amphibian recess contact membrane.

The *recessus partis basilaris* of the periotic labyrinth (Fig. 6) originates from the periotic sac, which extends out of the otic capsule and into the brain-case. In the whole-head sections which were made from one *Eleutherodactylus* specimen, the periotic sac appears to extend underneath the brain to meet and freely communicate with its contralateral counterpart (Fig. 9). A second fluid space just below this periotic space extends between right and left round windows. The two fluid spaces are separated by a membrane which may be meningeal in origin. It was unclear whether this membrane had simply separated from the basicranial bones due to shrinkage, or whether it really does separate two fluid compartments *in vivo*. The membrane was everted into both the periotic sac and the *recessus partis basilaris* in all four ears examined, perhaps due to shrinkage of the periotic system.

Xenopus laevis

Reconstructions of the inner ear of *Xenopus* are shown in Figs. 2 and 10, and photomicrographs of sections of particular interest are presented as Figs. 8D and 11.

In the sectioned female *Xenopus* specimen, the anterior semicircular canal was damaged, there was a bubble in the saccular region and the sections did not include the lateral passage or stapes. In the male specimen, the utricular and lagenar cavities had collapsed, as judged from a comparison of shapes between the two specimens and the fact that these structures had pulled away from the otic capsule walls. The periotic cistern had pulled away from the otic capsule wall in both specimens. Despite these shrinkage artefacts, the essential features of the inner ear remained intact in at least one of the two specimens, permitting the following description.

The saccular chamber is relatively large and shifted dorsally compared to that of *Rana* (Fig. 2); it is not divided into superior and inferior compartments. The anterior and especially the lateral semicircular canals are elongated rostro-caudally; the posterior canal is shorter. The amphibian recess projects as a diverticulum from the caudomedial end of the saccule, and ventral to this extends a second diverticulum which divides into the basilar recess laterally and the prominent lagenar recess medially (Fig. 10). No special subcavity of the saccular chamber containing a tegmentum vasculosum could be found.

The periotic cistern completely enwraps the saccule (Fig. 10) and is interposed between this and the basilar recess, giving rise to a ‘tympanal area’ rostrolateral to the basilar recess (Figs. 8D, 11; see Discussion). The relatively short, sickle-shaped periotic canal runs very close to the dorsal part of the periotic cistern, but the two remain separate (Fig. 2). Although the periotic canal is also close to the dorsal wall of the saccular cavity, the two are not in such close apposition as in *Rana* and *Eleutherodactylus* and there is no distinct contact membrane of the saccule. The periotic canal forms a small contact membrane with the amphibian recess before turning caudally and expanding into

the periotic sac, which forms a second contact membrane directly with the basilar recess (Figs. 10, 11). The periotic sac then extends out of the otic cavity.

No trace of an operculum was identified in *Xenopus*. The stapes footplate caps the end of a tubular passage projecting laterally from the otic capsule (Figs. 2, 11). The contents of this passage had picked up a pale blue stain in the histological sections, suggesting that a precipitate had formed there. The periotic cistern was not similarly stained and was clearly separated from the stapes footplate by whatever was in this lateral passage. In gross dissection of frogs of both sexes, the lateral passage was found to be filled with a clear, colourless fluid. A very thin membrane was seen at the medial end of the passage, separating its contents from the periotic cistern. The lateral end was sealed by the tough membrane of the oval window.

Although the reconstructions made from the male and female *Xenopus* specimens were generally very similar, the female's inner ear apparatus, particularly the saccular chamber, was more elongated rostro-caudally. The contact membrane of the amphibian recess was just over twice the area of the contact membrane of the basilar recess in the female, whereas in the male the contact membrane of the basilar recess was 1.5 times the area of that of the amphibian recess.

Discussion

Wever's diagram

Wever's (1973) schematic section through the ear of a leopard frog (Fig. 1A) has been widely reproduced in the literature. Although presented as a "frontal section", Wever did not claim that his diagram was based on a single, histological section and he may have amalgamated several slides in its construction. In order to address this possibility, MicroView software was used to reorient a stack of registered *Rana* section photomicrographs and section it in a new plane, thus revealing a 'virtual section' through the inner ear. The orientation was chosen such that the 'virtual section' (Fig. 12A)

was as close to Wever's illustration as possible, the main criteria being that the section should show both amphibian and basilar recesses as well as the lateral chamber of the inner ear. Falling somewhere between frontal and transverse planes, its orientation is best described as oblique (Fig. 12C).

Assuming that our original section photomicrographs were well-aligned, which by comparison with CT scan data appeared to be the case, our 'virtual section' shows Wever's schematic figure to be anatomically inaccurate in several respects. The orientation of the stapes footplate in our 'virtual section' differs substantially, revealing the process of the stapes *pars media* which articulates with the otic capsule (marked with an asterisk in Fig. 12B). It is easy to visualise the stapes footplate rocking about this process, as has been shown to be the case in ranid frogs (Jørgensen and Kanneworff, 1998; Mason and Narins, 2002; Werner, 2003), rather than acting as a piston as Wever's diagram might imply. Our 'virtual section' also passes through all three semicircular canals, but only touches the periphery of the operculum. It does not include the contact membrane of the saccule.

Wever's figure therefore appears not to represent a single, real section through the ear, but it is useful in diagrammatically illustrating the likely pathways for acoustic energy flow from the stapes to the amphibian and basilar papillae, and thence to the round window. Acoustic energy is also thought to be able to pass from periotic cistern to round window via the periotic canal, bypassing the otic labyrinth and auditory papillae entirely (Purgue and Narins, 2000a, b). That portion of the periotic canal which ascends from the periotic cistern is visible in both our 'virtual section' (Fig. 12B) and Wever's diagram (Fig. 1B), but Wever (1973, 1985) made surprisingly little mention of the canal in his otherwise detailed descriptions of frog inner ears.

Of the other schematic illustrations of the frog inner ear which exist in the literature, that of Frishkopf & Goldstein (1963) may be the best-known. More obviously diagrammatic than Wever's

illustration, this older representation shows the periotic and semicircular canals, and it represents the extrastapes more accurately.

The ears of *Eleutherodactylus* and *Xenopus*

The inner ear of *Eleutherodactylus limbatus* was found generally to resemble that of *Rana*, but there were some pronounced differences in terms of the relative sizes and shapes of the various structures. These differences were not highlighted by Wever (1985), who examined three other *Eleutherodactylus* species. The very small saccule and the relatively short, wide semicircular canals (Fig. 2) closely resemble reconstructions of the inner ear in 'stage 8' *Rana temporaria* tadpoles (30 mm long, just before emergence of hindlimbs) made by Birkmann (1940). *E. limbatus* has direct development which omits a tadpole stage, but our frogs had been vocalizing in life and were therefore believed to be reproductively mature. The inner ear of this species may therefore be paedomorphic.

The inner ear of *Xenopus laevis* has been described, among others, by Paterson (1949, 1960), Wever (1985) and Bever et al. (2003). Our reconstructions of *Xenopus* ears largely agree with their descriptions. The saccular cavity contains a dense otoconial mass (very obvious in the CT scans) which, relative to the rest of the inner ear, is much larger and more dorsally positioned than its equivalent in *Rana* and *Eleutherodactylus*. The size and position of the saccular cavity in *Xenopus* gives its inner ear a striking morphological similarity to that of the fish *Gobius niger*, as illustrated by Retzius (1881). The possible functional convergence between these two aquatic species remains to be explored.

The association of the basilar papilla with the lagenar recess in urodeles, caecilians and amniotes has been said to be the "single most influential piece of evidence supporting a [basilar papilla] homology among all terrestrial vertebrates", but the separate opening of the basilar recess into the saccule in frogs was seen as a complication to this theory (Smotherman and Narins, 2004). We have found that

the basilar and lagenar recesses are in fact conjoined in *Xenopus* (Fig. 10), suggesting that this represents the primitive condition for all lissamphibians and perhaps tetrapods in general.

At the caudal end of the basilar recess, periotic contact occurs via the periotic sac directly in *Xenopus*, rather than via a *recessus partis basilaris*. Rostrally, the recess is separated from the periotic cistern by a thin "tympanal area", discussed later. These features have been previously described by Paterson (1949, 1960).

In *Xenopus*, the periotic cistern is separated from the stapes footplate by the fluid contained within a tubular extension of the otic capsule (Fig. 11). A shorter separation between stapes and cistern is shown in Wever's (1985) diagrams of the ear of this frog, but Wever found a much longer, fluid-filled "lateral passage" in the related species *Pipa pipa*. In both our histological slides of *Xenopus* and Wever's slides of *Pipa* it looked like a precipitate had formed within this lateral passage, but not in the periotic labyrinth. Paterson (1960) found only a short lateral passage in her immature specimen of *Pipa* which she refers to as a "fossa fenestrae ovalis", filled with "delicate connective tissue"; she did not describe anything similar in *Xenopus*. Perhaps the separation between footplate and periotic labyrinth increases in pipids as the skull grows, such that it is less obvious in younger specimens. The lateral chamber of *Rana* differs from the lateral passage of *Xenopus* because the ranid lateral chamber has a narrower connection with the main otic capsule, it bends sharply rostrally to reach the stapes footplate and it is filled with a diverticulum of the periotic cistern. The ranid lateral chamber is also in contact with the operculum, an element lacking in *Xenopus*.

The otic labyrinth in the female *Xenopus* was around 1.5 times the linear dimensions of that of the male. The saccular cavity was more elongated in the female and there was a difference in the relative sizes of the contact membranes of the amphibian and basilar recesses, noted earlier. Further investigation of a larger number of specimens is needed in order to establish whether these inner ear differences represent sexual dimorphism, which has been observed in the middle ear of this species (Mason et al., 2009).

The blind branch of the periotic canal

The discrete "blind branch" of the periotic canal which Purgue & Narins (2000b) found in the bullfrog *Rana catesbeiana* could not be identified in the *Rana* or *Eleutherodactylus* specimens examined here. However, the membranous wall of the periotic canal as it curves around the superior saccule was particularly thin in *Rana pipiens*, and in some slides it appeared to be ruptured. An apparent short diverticulum found in the female *Xenopus* specimen only (Fig. 10) might also have been the result of periotic canal rupture. Purgue & Narins injected silicone into the periotic labyrinth of their frogs to make casts: it is possible that they experienced a similar problem.

The contact membranes

Harrison (1902) referred to three 'tympanal areas' in the frog inner ear where otic and periotic labyrinths are in particularly close apposition. One of these is the extensive, membranous division between periotic cistern and saccule, while the other two 'tympanal areas' are now more generally known as the 'contact membranes' of the amphibian and basilar recesses. To these three may be added the contact membrane of the saccule and an additional 'tympanal area' in *Xenopus*, discussed later.

Sound energy from stapedial vibrations is widely presumed to enter the otic labyrinth through the first 'tympanal area' between periotic cistern and saccule (the membrane surrounding the inferior saccular chamber, labelled IS in Fig. 4, forms part of this). The division between the periotic and otic systems remains very thin where the periotic canal wraps around the anterior wall of the superior saccule; a special, ventral diverticulum of the canal makes additional contact with the superior saccule in *Eleutherodactylus* (Figs. 6, 7B).

In both *Rana* and *Eleutherodactylus*, the periotic canal separates from the saccule but returns to meet the otic labyrinth at three contact membranes within the otherwise thickened limbic tissue at the posterior end of the otic capsule. The contact membranes of the amphibian and basilar recesses represent pathways through which sound energy can travel from the otic labyrinth back into the periotic system, via the auditory epithelia of the amphibian and basilar papillae (Purgue and Narins, 2000a, b). Although not considered by Purgue & Narins, Wever (1973) showed in his diagram a third contact membrane located between the two papillae, marked only with a tiny arrow (Fig. 1A). Wever wrote that "this thin area acts as a bypass and allows some fraction of the fluid motion to go directly into the perilymphatic duct [= periotic canal] without being detected". This membrane between superior saccule and periotic canal was described in *Rana catesbeiana* by Lewis (1976), who referred to it as the "contact membrane of the saccule". Lewis & Narins (1999) state that it is found in "the more derived anurans", referring this statement to Lewis (1984); Lewis & Narins may have meant Lewis' 1976 publication.

The contact membrane of the saccule was identified in this study in both *Rana* (Figs. 3, 5C) and *Eleutherodactylus* (Figs. 6, 8B), located close to the contact membrane of the amphibian recess. Although it appears to be thicker than the other two contact membranes, it might indeed represent a second route by which sound energy could bypass the amphibian and basilar papillae, additional to the periotic canal route described by Purgue & Narins (2000a, b). The frequency-dependent impedance of such a bypass, and hence its functional significance, remains to be determined. The so-called 'round window' within the metotic fissure, which may not be homologous with the round window of other tetrapods (Henson, 1974), represents a point of pressure release for all these routes (Wever, 1985).

As well as the usual contact membrane at the posterior end of the basilar recess, *Xenopus* has an additional 'tympanal area' between the rostrolateral wall of this recess and a posterior extension of the periotic cistern (Paterson, 1949, 1960). Of the frogs studied here only *Xenopus* has this

'tympanal area' (Figs. 8D, 11) because its basilar and lagenar recesses both arise from the same caudoventral diverticulum of the saccular cavity (Fig. 10; see earlier), and part of the periotic cistern has come to occupy the space between this diverticulum and the saccule proper. Elsewhere, the membranous walls of the basilar recess are enclosed within thick limbic tissue (Fig. 8D). In principle, acoustic energy might flow from periotic cistern through this 'tympanal area' directly into the basilar recess, exiting at the posterior end of the recess via the contact membrane formed here with the periotic sac. This short and direct pathway through the basilar recess was represented diagrammatically by Wever (1985).

The tegmentum vasculosum

'Tegmentum vasculosum', literally meaning 'vascular covering', is a term most often used to describe the well-vascularized, thickened wall of the cochlear duct in birds and crocodilians. Separating the scala media from the scala vestibuli, this archosaur tegmentum vasculosum is believed to combine the roles of the stria vascularis and Reissner's membrane in mammals (Baird, 1974; Lewis et al., 1985; Hossler et al., 2002). The same term has long been used in the German anatomical literature to describe the thickened layer of epithelial cells found in the superior saccule of certain frogs (e.g. Deiters, 1862; Hasse, 1868; Kuhn, 1880; Retzius, 1881; Gaupp, 1904; Birkmann, 1940; Hagmann and Giebel, 1978). Retzius (1881) described a tegmentum vasculosum in *Bufo*, *Hyla* and *Pelobates* but found it to be very poorly-developed in *Alytes*; it is not found in *Ascaphus* or *Liopelma* (Wagner, 1934), nor in pipids including *Xenopus* (Paterson, 1960; this study). Although lacking in some 'archaeobatrachians', the tegmentum vasculosum has apparently been identified in all neobatrachian ears in which it has been sought. It is not found in urodeles (Birkmann, 1940).

Retzius (1881) produced several illustrations of the otic labyrinth of *Rana esculenta*, which were redrawn and modified by Gaupp (1904). Retzius and Gaupp both labelled the whole of the superior saccular wall as the tegmentum vasculosum, as did Birkmann (1940) in his reconstructions of the otic labyrinth of *Rana temporaria*. Several illustrations from Gaupp and Birkmann were redrawn by

Wever (1985) in *The Amphibian Ear*, but in each case the region originally labelled as tegmentum vasculosum was relabelled as part of the saccule. In the present study, the tegmentum vasculosum was readily identifiable in *Rana* and *Eleutherodactylus* as a thickened epithelium lining an otherwise unoccupied diverticulum of the saccular chamber. However, the extent of its vascularization could not be ascertained and, as Gaupp (1904) noted, its rostral borders are indistinct in *Rana*. A tegmentum vasculosum was not found in *Xenopus*.

Wever (1985) made only one, brief mention of the anuran tegmentum vasculosum in his book (p.78), in which he commented on the mistake of "early anatomists" in assigning to it a sensory function. Nevertheless, the unusual epithelium suggests a functional distinction from the rest of the superior saccular chamber. Hagmann & Giebel (1978), working on *Rana temporaria*, confirmed that this region is richly vascularized and found high levels of metabolic enzymatic activity. This supports the contention that the tegmentum vasculosum in frogs is responsible for the secretion of endolymph, like the tegmentum of archosaurs and the stria vascularis of mammals. Subsequent studies of endolymph secretion in frogs, however, have focused on the dark cells located in the utricle and semicircular canal ampullae (Burnham and Stirling, 1984; Bernard et al., 1986); the anuran tegmentum vasculosum has fallen into obscurity.

The apparent interaural fluid connection in *Eleutherodactylus*

Of the frogs examined, sections through the whole head were only available for one specimen of *Eleutherodactylus*. Although our interpretation may have been affected by shrinkage, these sections appeared to show the left and right periotic sacs converging to form a fluid space immediately beneath the brain (Fig. 9). Wever (1978) showed that vibrations applied to the operculum of one ear in a salamander can excite the contralateral ear: his proposed mechanism involved a similar intracranial pathway below the brain, but he believed that the two periotic sacs in his species communicated only indirectly, via the cerebrospinal fluid. Harrison (1902) challenged the notion of

earlier authors that amphibians possess a connection between the periotic system and a "subdural space".

Such a fluid system extending between the two ears via the cranial cavity might be functionally significant in (1) communicating vibrations from the cerebrospinal fluid to the inner ears, (2) acoustically coupling the two ears, which might affect sound localization, or (3) providing increased possibilities for pressure release from the inner ear, affecting sensitivity. Further work is clearly needed to confirm the presence of an interaural fluid connection in *Eleutherodactylus* and other frogs, so as to assess whether this condition is widespread among anurans.

Conclusion

The anuran inner ear is a complex, three-dimensional structure consisting of the intertwined canals of the periotic and otic labyrinths. Although his representation appears not to be anatomically accurate, Wever's (1973) diagram of the leopard frog inner ear does an admirable job of clearly illustrating some of the possible routes of acoustic energy flow from stapes to round window. Its main shortcoming in this respect is that it does not label the periotic canal, which represents another potential route. Although some anatomical differences were identified, the inner ear of *Eleutherodactylus* is broadly similar to that of *Rana*, so Wever's diagram is clearly representative of a wider range of neobatrachian frogs. The illustration is less useful in describing the ear of *Xenopus*; to what extent this reflects the 'archaeobatrachian' status of *Xenopus* or its aquatic habits remains to be determined.

The tegmentum vasculosum and the contact membrane of the saccule, found here in *Rana* and *Eleutherodactylus* but not *Xenopus*, have been neglected in the recent literature and deserve further attention from auditory physiologists. The intriguing possibility of a fluid pathway extending between right and left ears also demands investigation.

Acknowledgements

The authors wish to thank Emanuel Mora for his help and support with this project. Dave Simpson kindly provided the *Xenopus* specimens. The CT scan of *Xenopus* was made by Alan Heaver of the University of Cambridge Department of Engineering, with thanks going also to Norman Fleck for the use of his equipment. The authors are very grateful to Dolores Bozovic, Alan D. Grinnell, Tammy Hoang, Victoria Sandoval and Felix E. Schweizer for facilitating the *Rana* CT scan, which was made by Ting-Ling Chang at the UCLA School of Dentistry, Division of Advanced Prosthodontics. Stephan Kamrad helped with translations. The research of JMS and PvD was supported by the Heinsius Houbolt Foundation and is part of the research program *Healthy Ageing and Communication* of the Department of Otorhinolaryngology at the University Medical Center Groningen. Finally, the authors wish to thank the reviewers and editors of the manuscript for their very helpful comments.

Conflicts of interest

The authors declare that they have no conflicts of interest.

References

Baird IL (1974) Anatomical features of the inner ear in submammalian vertebrates. In: Handbook of Sensory Physiology, volume V/1: Auditory System (Keidel WD, Neff WD, eds), pp 159-212. Berlin: Springer-Verlag.

Bernard C, Ferrary E, Sterkers O (1986) Production of endolymph in the semicircular canal of the frog *Rana esculenta*. Journal of Physiology 371:17-28.

Bever MM, Jean YY, Fekete DM (2003) Three-dimensional morphology of inner ear development in *Xenopus laevis*. Developmental Dynamics 227:422–430.

Birkmann K (1940) Morphologisch-anatomische Untersuchungen zur Entwicklung des häutigen Labyrinthes der Amphibien. Zeitschrift für Anatomie und Entwicklungsgeschichte 110:443-488.

Blood DC, Studdert VP (1999) Saunders Comprehensive Veterinary Dictionary, 2nd Edition. Edinburgh: W.B. Saunders.

Burnham JA, Stirling CE (1984) Quantitative localization of Na-K pump site in frog inner ear dark cells. Hearing Research 13:261-268.

Deiters O (1862) Ueber das innere Gehörorgan der Amphibien. Archiv für Anatomie, Physiologie und Wissenschaftliche Medicin 1862:262-275, 277-310.

Dubois A (2007) Naming taxa from cladograms: a cautionary tale. Molecular Phylogenetics and Evolution 42:317-330.

Duellman WE, Trueb L (1986) Biology of Amphibians. Baltimore: The Johns Hopkins University Press.

Fay RR, Popper AN (1985) The octavolateralis system. In: Functional Vertebrate Morphology (Hildebrand M, Bramble DM, Liem KF, Wake DB, eds), pp 291-316. London: Belknap Press.

Frishkopf LS, Goldstein MH (1963) Responses to acoustic stimuli from single units in the eighth nerve of the bullfrog. Journal of the Acoustical Society of America 35:1219-1228.

Gaupp E (1904) A. Ecker's und R. Wiedersheim's Anatomie des Frosches, part 3, 2nd Edition. Braunschweig: Druck und Verlag von Friedrich Vieweg und Sohn.

Gridi-Papp M, Narins PM (2010) Seismic detection and communication in amphibians. In: The Use of Vibrations in Communication: Properties, Mechanisms and Function across Taxa (O'Connell-Rodwell CE, ed), pp 69-83. Kerala: Research Signpost.

Hagmann B, Giebel W (1978) Enzymhistochemische Untersuchungen am Innenohr des Frosches (*Rana temporaria*). Archives of Oto-Rhino-Laryngology 220:89-103.

Harrison HS (1902) On the perilymphatic spaces of the amphibian ear. Internationale Monatsschrift für Anatomie und Physiologie 19:221-261.

Hasse C (1868) Das Gehörorgan der Frösche, Reprinted from Zeitschrift für wissenschaftliche Zoologie, Bd. 18 Edition. Leipzig: Verlag von Wilhelm Engelmann.

Henson OW (1974) Comparative anatomy of the middle ear. In: Handbook of Sensory Physiology, volume V/1: Auditory System (Keidel WD, Neff WD, eds), pp 39-110. Berlin: Springer-Verlag.

Hillis DM (2007) Constraints in naming parts of the Tree of Life. Molecular Phylogenetics and Evolution 42:331-338.

Hoegg S, Vences M, Brinkmann H, Meyer A (2004) Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. Molecular Biology and Evolution 21:1188-1200.

Hossler FE, Olson KR, Musil G, McKamey MI (2002) Ultrastructure and blood supply of the tegmentum vasculosum in the cochlea of the duckling. Hearing Research 164:155-165.

Jørgensen MB, Kanneworff M (1998) Middle ear transmission in the grass frog, *Rana temporaria*. Journal of Comparative Physiology A 182:59-64.

Kuhn (1880) Ueber das häutige Labyrinth der Amphibien. Archiv für Mikroskopische Anatomie 17:479-550.

Lewis ER (1976) Surface morphology of the bullfrog amphibian papilla. Brain, Behavior and Evolution 13:196-215.

Lewis ER (1984) On the frog amphibian papilla. Scanning Electron Microscopy 1984 (IV):1899-1913.

Lewis ER, Narins PM (1999) The acoustic periphery of amphibians: anatomy and physiology. In: Comparative Hearing: Fish and Amphibians (Fay RR, Popper AN, eds), pp 101-154. New York: Springer.

Lewis ER, Leverenz EL, Bialek WS (1985) The Vertebrate Inner Ear. Boca Raton: CRC Press, Inc.

Lombard RE (1977) Comparative morphology of the inner ear in salamanders (*Caudata*: *Amphibia*). Basel: S. Karger.

Mason MJ, Narins PM (2002) Vibrometric studies of the middle ear of the bullfrog *Rana catesbeiana* I. The extrastapes. Journal of Experimental Biology 205:3153-3165.
Mason MJ, Wang M, Narins PM (2009) Structure and function of the middle ear apparatus of the aquatic frog, *Xenopus laevis*. Proceedings of the Institute of Acoustics 31:13-21.
Paterson NF (1949) The development of the inner ear of *Xenopus laevis*. Proceedings of the Zoological Society of London 119:269–291.
Paterson NF (1960) The inner ear of some members of the Pipidae (Amphibia). Proceedings of the Zoological Society of London 134:509-546.
Pauly GB, Hillis DM, Cannatella DC (2009) Taxonomic freedom and the role of official lists of species names. Herpetologica 65:115–128.
Purgue AP, Narins PM (2000a) A model for energy flow in the inner ear of the bullfrog (*Rana catesbeiana*). Journal of Comparative Physiology A 186: 489-495.
Purgue AP, Narins PM (2000b) Mechanics of the inner ear of the bullfrog (*Rana catesbeiana*): the contact membranes and the periotic canal. Journal of Comparative Physiology A 186: 481-488.
Retzius G (1881) Das Gehörorgan der Wirbelthiere. Morphologisch-Histologische Studien. I. Das Gehörorgan der Fische und Amphibien. Stockholm: Samson & Wallin.
Simmons DD, Meenderink SWF, Vassilakis PN (2007) Anatomy, physiology, and function of auditory end-organs in the frog inner ear. In: Hearing and Sound Communication in Amphibians (Narins PM, Feng AS, Fay RR, Popper AN, eds), pp 184-220. New York: Springer.
Smotherman M, Narins P (2004) Evolution of the amphibian ear. In: Evolution of the Vertebrate Auditory System (Manley GA, Popper AN, Fay RR, eds), pp 164-199. New York: Springer.
Thévenaz P, Ruttimann UE, Unser M (1998) A pyramid approach to subpixel registration based on intensity. IEEE Transactions on Image Processing 7:27-41.
van Dijk P, Mason MJ, Schoffelen RLM, Narins PM, Meenderink SWF (2011) Mechanics of the frog ear. Hearing Research 273:46-58.
Wagner DS (1934) The structure of the inner ear in relation to the reduction of the middle ear in the Liopelmidae (Noble). Anatomischer Anzeiger 79:20-36.
Werner YL (2003) Mechanical leverage in the middle ear of the American bullfrog, *Rana catesbeiana*. Hearing Research 175:54-65.
Wever EG (1973) The ear and hearing in the frog, *Rana pipiens*. Journal of Morphology 141:461-477.
Wever EG (1978) Sound transmission in the salamander ear. Proceedings of the National Academy of Sciences of the USA 75:529-530.
Wever EG (1985) The Amphibian Ear. Princeton: Princeton University Press.

Figure Captions

Fig. 1

A: Ernest Glen Wever's schematic diagram of the peripheral auditory apparatus of a ranid frog. The original caption is included. From: Wever, E.G. (1973) The ear and hearing in the frog, *Rana pipiens*. *Journal of Morphology* 141(4): 461-477. Copyright © 1973 Wiley-Liss, Inc. B: A representation of the same diagram with the colour-coding used elsewhere in this paper, to facilitate comparison between figures. Where Wever's nomenclature differs from that used in the present paper, structures have been relabelled. Additionally, based on the interpretations of the present study, new labels have been introduced for structures that were not explicitly identified in Wever's original diagram, including the contact membranes which separate otic and periotic labyrinths.

Colour code: white = otic labyrinth (endolymph); green = periotic labyrinth (perilymph); red = sensory epithelium; dark grey = limbic tissue; cream = bone; yellow = cartilage.

Fig. 2

WinSurf reconstructions of the left inner ear structures of (left) *Xenopus laevis*, male specimen, (middle) *Rana pipiens*, (right) *Eleutherodactylus limbatus*. Lateral views are shown in the top row, dorsal views in the bottom row. *Eleutherodactylus* reconstructions are 2.5× enlarged relative to the others. In the *Eleutherodactylus* sections used for these reconstructions, the periotic cistern in the region marked with an asterisk, which lies lateral to the very small saccular cavity, had collapsed. Its approximate shape has been restored here by comparison with the contralateral ear and the extent of the space available for it within the otic capsule. Colour code: white = otic labyrinth (endolymph); green = periotic labyrinth (perilymph); red = sensory epithelium; semitranslucent grey = internal walls of the otic capsule.

Key to this and subsequent figures: AR = amphibian recess (endolymph); AS = anterior semicircular canal; B = brain; BR = basilar recess (endolymph); CA = contact membrane of amphibian recess; CB = contact membrane of basilar recess; CC = *crus commune* (confluence of anterior and posterior semicircular canals); CS = contact membrane of saccule; IS = inferior saccular chamber; LC = lateral chamber; LP = lateral passage; LR = lagenar recess (endolymph); LS = lateral semicircular canal; LT = limbic tissue; O = operculum; PC = periotic canal; POC = periotic cistern; POS = periotic sac; PS = posterior semicircular canal; PT = periotic tissue; PU = posterior utricular cavity; RPB = *recessus partis basilaris* (perilymph); RW = round window; S = saccule; SPI = stapes *pars interna*; SPM = stapes *pars media*; SS = superior saccular chamber; TV = tegmentum vasculosum, or the saccular diverticulum within which this epithelial lining is found; VIII = branch of eighth cranial nerve.

Fig. 3

WinSurf reconstructions of left inner ear structures of *Rana pipiens*, seen from an approximately posterior view. The reconstruction on the left shows the otic labyrinth (white), sensory epithelia (red) and contact membranes separating endo- and perilymph (purple). Part of the posterior semicircular canal has been removed to reveal the diverticula of the superior saccule. The reconstruction on the right shows the same, with the periotic labyrinth added in (semitranslucent green). Scale bar 2.5 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 4

Composite photomicrographs of two approximately transverse sections through the posterior part of the inner ear of *Rana pipiens*. Section B is in a plane 240 µm anterior to Section A. Relative to the centre of each photomicrograph, dorsal is upwards and slightly away from the viewer, lateral is to

the left. Scale bar applies to both A and B and represents 1 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 5

Photomicrographs of sections through the inner ear of *Rana pipiens*. A: Basilar recess (BR; endolymph), *recessus partis basilaris* (RPB; perilymph) and contact membrane of basilar recess (CB) separating the two. B: Amphibian recess (AR) and its contact membrane (CA), expanded from Fig. 4A. C: Periotic canal (PC) and contact membrane of the saccule (CS), expanded from Fig. 4B. D: Tegmentum vasculosum (TV), expanded from Fig. 4A. All scale bars 200 µm. See Fig. 2 caption for full list of abbreviations.

Fig. 6

WinSurf reconstructions of left inner ear structures of *Eleutherodactylus limbatus*, seen from an approximately lateral view. Part of the lateral semicircular canal has been removed to reveal the contact membrane of the amphibian recess (CA) and the contact membrane of the saccule (CS). The reconstruction on the left shows the otic labyrinth and associated structures only; the reconstruction on the right includes the dorsal and posterior parts (only) of the periotic labyrinth. The asterisk indicates the ventral diverticulum of the central part of the periotic canal, which is closely apposed to the superior saccular chamber (SS). Scale bar 0.5 mm. Colour code: white = otic labyrinth (endolymph); semitranslucent green = periotic labyrinth (perilymph); red = sensory epithelium; purple = contact membrane separating endo- and perilymph. See Fig. 2 caption for full list of abbreviations.

Fig. 7

Composite photomicrographs of two sections through the inner ear of *Eleutherodactylus limbatus*. Section A is in an approximately transverse plane, through the posterior part of the inner ear. Relative to the centre of the photomicrograph, dorsal is upwards and slightly away from the viewer, lateral is to the left. Section B is in an oblique plane (between transverse and sagittal), taken from the ear on the contralateral side to Section A and laterally inverted to aid comparison. Relative to the centre of the photomicrograph, dorsal is upwards, lateral is to the left and away from the viewer. Note in particular the membrane marked with an asterisk, located between the ventral diverticulum of the central part of the periotic canal (PC) and the superior saccular chamber (SS). The membranous labyrinth has pulled away from the otic capsule wall on the right hand side. Scale bar applies to both A and B and represents 200 µm. See Fig. 2 caption for full list of abbreviations.

Fig. 8

Photomicrographs of sections through the inner ears of *Eleutherodactylus limbatus* and *Xenopus laevis*. A: Tegmentum vasculosum (TV), amphibian recess (AR) and its contact membrane (CA) in *E. limbatus*, scale bar 100 µm. B: Periotic canal (PC) and the contact membrane of the saccule (CS) in *E. limbatus*, scale bar 100 µm. C: Tegmentum vasculosum of *E. limbatus*, scale bar 50 µm. D: Basilar recess (BR) of *X. laevis* (female specimen), scale bar 200 µm. The asterisk indicates the additional 'tympanal area' identified by Paterson (1949, 1960), located just rostrolateral to the basilar recess (see text). See Fig. 2 caption for full list of abbreviations.

Fig. 9

Photomicrograph of a transverse section through the head of *Eleutherodactylus limbatus*, at the level of the posterior half of the otic capsule. The three fluid compartments collectively denoted '1'

appear to be separate in this section, but inspection of other sections in the same series suggests that they are actually part of one continous fluid system extending between the periotic labyrinths of each ear and passing underneath the brain (B). The left-hand arrow marked '1' points to the left periotic sac, which can be seen emerging from the left otic capsule. Within the capsule, it is immediately adjacent to the amphibian recess (AR), from which it is separated by a thin contact membrane. Between fluid space 1 and the base of the skull is a second fluid space (2), which is also continuous across the head and extends between the two round windows (RW). Distortion resulting from shrinkage may have changed the relative sizes of fluid spaces 1 and 2. Scale bar 0.5 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 10

WinSurf reconstructions of left inner ear structures of *Xenopus laevis* (female specimen), seen from an approximately caudoventral view. Part of the posterior semicircular canal has been removed to reveal the diverticula of the saccule. The reconstruction on the left shows the otic labyrinth and associated structures only; the reconstruction on the right includes all but the most rostrolateral part of the periotic labyrinth too. The anterior semicircular canal had been obliterated in the slides used to make this reconstruction and the saccular cavity was distorted owing to the presence of a bubble. The presumed original shape of the saccule (S) has been restored here. The asterisk indicates a short, blind branch of the periotic canal (see text). Scale bar 1.5 mm. Colour code: white = otic labyrinth (endolymph); semitranslucent green = periotic labyrinth (perilymph); red = sensory epithelium; purple = contact membrane separating endo- and perilymph. See Fig. 2 caption for full list of abbreviations.

Fig. 11

Photomicrograph of an oblique section through the inner ear of *Xenopus laevis* (male specimen). Note that the periotic cistern (POC), the contents of which have picked up only a small amount of stain, is separated from the stapes footplate (SPI and SPM) by the lateral passage (LP), which is filled with diffuse material staining pale blue. The contact membrane of the basilar recess is the thin membrane between the basilar recess (BR) and the periotic sac (POS); the membrane marked with an asterisk between the basilar recess and the periotic cistern (POC) is Paterson's (1949, 1960) additional 'tympanal area' (see text). Relative to the centre of the picture, dorsal is upwards and away from the viewer, lateral is to the left and towards the viewer. Scale bar 1 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 12

Reconstructions created from a stack of serial section images of the ear of *Rana pipiens*, digitally resectioned in a plane as close as possible to Wever's 1973 illustration (Fig. 1). See text for details. The extrastapes, tympanic membrane, periotic sac and round window were not within the original sections and are consequently not shown in these reconstructions. A: 'Virtual section'; the faint, diagonal striations indicate the planes of the original section photomicrographs from which this was reconstructed. B: Expanded, diagrammatic illustration of the same. Out of the plane of this particular section, the periotic canal is in communication with the periotic fluid which abuts the contact membranes of the amphibian and basilar recesses; these regions are all components of the periotic labyrinth and are hence shaded in green. The asterisk indicates a process of the stapes *pars media* which articulates with the otic capsule. C: WinSurf reconstructions of the right inner ear of *R. pipiens* from (left) lateral, (middle) posterior and (right) dorsal views, showing the position of the 'virtual section' as a grey plane. The stapes and operculum are included in these reconstructions but the internal walls of the otic capsule are not. Both scale bars 2 mm. Colour code: white = otic labyrinth

(endolymph); green = periotic labyrinth (perilymph); dark grey = limbic tissue; lighter grey = looser periotic tissue; cream = bone; yellow = cartilage.

The frog inner ear: picture perfect?

Matthew J. Mason[1]

Johannes M. Segenhout[2]

Ariadna Cobo-Cuan[3]

Patricia M. Quiñones[4]

Pim van Dijk[2,5]

[1] University of Cambridge

Department of Physiology, Development & Neuroscience

Downing Street

Cambridge CB2 3EG

U.K.

Tel. +44 1223 333829

Fax +44 1223 333840

mjm68@cam.ac.uk

[2] University of Groningen

University Medical Center Groningen

Department of Otorhinolaryngology, Head & Neck Surgery

P.O. Box 30.001

9700 RB Groningen

The Netherlands

jmsegenhout@hotmail.com

p.van.dijk@umcg.nl

[3] Department of Animal and Human Biology

Havana University

Street 25 No. 455

Havana CP 10400

Cuba

cobo_cuan@fbio.uh.cu

[4] Department of Physics and Astronomy

1-129 Knudsen Hall, 475 Portola Plaza

University of California Los Angeles

Los Angeles, CA 90095

U.S.A.

yukiq@ucla.edu

[5]University of Groningen

Graduate School of Medical Sciences, Research School of Behavioral and Cognitive Neurosciences

P.O. Box 196

9700 AD Groningen

The Netherlands

p.van.dijk@umcg.nl

Abstract

Many recent accounts of the frog peripheral auditory system have reproduced Wever's (1973) schematic cross-section of the ear of a leopard frog. We sought to investigate to what extent this diagram is an accurate and representative depiction of the anuran inner ear, using three-dimensional reconstructions made from serial sections of *Rana pipiens*, *Eleutherodactylus limbatus* and *Xenopus laevis*.

In *Rana*, three discrete contact membranes were found to separate the posterior otic (= endolymphatic) labyrinth from the periotic (= perilymphatic) system: those of the amphibian and basilar recesses and the contact membrane of the saccule. The amphibian 'tegmentum vasculosum' was distinguishable as a thickened epithelial lining within a posterior recess of the superior saccular chamber. These features were also identified in *Eleutherodactylus*, but in this tiny frog the relative proportions of the semicircular canals and saccule resemble those of ranid tadpoles. There appeared to be a complete fluid pathway between the right and left periotic labyrinths in this species, crossing the cranial cavity. *Xenopus* lacks a tegmentum vasculosum and a contact membrane of the saccule; the *Xenopus* ear is further distinguished by a lateral passage separating stapes from periotic cistern and a more direct connection between periotic cistern and basilar recess. The basilar and lagenar recesses are conjoined in this species.

Wever's diagram of the inner ear of *Rana* retains its value for diagrammatic purposes but it is not anatomically accurate, nor representative of all frogs. Although Wever identified the contact membrane of the saccule, most recent studies of frog inner ear anatomy have overlooked both this and the amphibian tegmentum vasculosum. These structures deserve further attention.

Keywords

Inner ear; frog; amphibian papilla; basilar papilla; tegmentum vasculosum; contact membrane

Introduction

The inner ear structures of ranid frogs have been the subject of detailed anatomical accounts dating back over 150 years, many of which were written in German (see e.g. Deiters, 1862; Hasse, 1868; Retzius, 1881; Gaupp, 1904). Among the best known of the English-language descriptions are those of Ernest Glen Wever. The first figure from Wever's 1973 paper in *Journal of Morphology* (reproduced here as Fig. 1A) shows "A schematic representation of the ear and labyrinth of *Rana pipiens*, in frontal section". An almost identical diagram appeared in two of Wever's later publications including his 1985 book *The Amphibian Ear*, which remains the most comprehensive account of the subject. It has been reproduced, sometimes in a modified form, in ~~high-profile~~ review papers (e.g. Fay and Popper, 1985; Lewis and Narins, 1999; Simmons et al., 2007; Gridi-Papp and Narins, 2010), a leading text-book on amphibian biology (Duellman and Trueb, 1986) and several other articles. As such, it must be the world's most widely-consulted scientific illustration of an amphibian peripheral auditory system. However, as explained below, there is some uncertainty as to the species depicted, the orientation and the accuracy of this important diagram.

The species examined

The taxonomy of the North American ranid frogs is currently in a state of flux (Dubois, 2007; Hillis, 2007; Pauly et al., 2009). Some authors place leopard frogs in the genus *Lithobates*, but following Hillis' (2007) recommendation, we retain the genus *Rana* for North American ranids.

Wever (1973) stated that his illustration shows the ear of *Rana pipiens*, but in his 1985 book he distinguished between this species (the northern leopard frog) and the very similar "*Rana utricularia sphenocephala*", now *R. sphenocephala utricularia* (the southern leopard frog). Wever (1985) did not make it clear which of these species is represented in the figure of interest to us here, which appears in slightly modified form as his Fig. 3-17. However, another illustration also reproduced from his 1973 paper (Fig. 3-79 in Wever, 1985) is ~~clearly~~ labelled as *R. sphenocephala*. Wever's 1973 paper

may therefore describe the ear of the southern leopard frog, but it is likely that the ears of these two species are practically identical. The articles that have reproduced Wever's illustration have often implied that a generalized anuran morphology is represented ~~(Table 1)~~.

The orientation of Wever's illustration

Within his 1973 paper, Wever stated that he sectioned his specimens in "a horizontal plane (dorsal to ventral)...frontally (anterior to posterior) or laterally (right to medial, then continued from medial to left)". This description is somewhat confusing, and perhaps for this reason Wever later redefined the three planes. In Wever's (1985) figure 3-34, a frontal plane is clearly shown dividing the head into dorsal and ventral components. Such a plane would pass through most of the teeth on both left and right maxillae and may be considered horizontal. A sagittal plane vertically divides the head into left and right components. A transverse plane is a vertical plane perpendicular to the frontal and sagittal planes, which divides the head into anterior and posterior components. These definitions agree with the standard veterinary anatomical nomenclature (Blood and Studdert, 1999) and are those used in the present study. In our interpretation, "horizontal" (Wever, 1973) is actually frontal, "lateral" (Wever, 1973) is actually sagittal and "frontal" (Wever, 1973) is actually transverse.

The present study focuses on the first figure from Wever (1973), the caption of which reads "the ear and labyrinth...in frontal section" (Fig. 1A). Was this in fact a transverse section, according to standard nomenclature?

The accuracy of Wever's illustration

When preparing a previous article (van Dijk et al., 2011), two of the current authors had reason to question the accuracy of Wever's 1973 diagram. Wever shows the extrastapes (= extracolumella) as a very short extension of the bony stapes shaft (stapes *pars media*), connecting it to the centre of the tympanic membrane (Fig. 1). It would be natural to assume that this apparatus must operate as a stiff piston, an inflection of the tympanic membrane driving the stapes directly into the inner ear. In reality, the extrastapes is much longer than this and has an angled articulation with the *pars*

media: the stapes/extrastapes system works as a flexible, first-order lever (Jørgensen and Kanneworff, 1998; Mason and Narins, 2002; Werner, 2003).

Turning to the inner ear, Wever shows three pathways for "fluid flow" to pass between the stapes on the right of his diagram to the periotic (= perilymphatic) sac on the left, one via the amphibian papilla, a second via the basilar papilla and a third between the two papillae, each being indicated by arrows in Fig. 1A. The endolymph within the otic (= endolymphatic) labyrinth in frogs is separated from the perilymph within the periotic labyrinth by so-called 'contact membranes' (Fig. 1B), so fluid cannot actually flow between the two systems and the three arrows should instead be taken to indicate three pathways of acoustic energy flow. In their more recent account of energy flow pathways through the ear of the bullfrog (*Rana catesbeiana*), Purgue & Narins (2000a, b) considered the routes passing through the amphibian and basilar recesses but made no mention of Wever's middle pathway. Purgue & Narins regarded the periotic canal as an alternative route for low-frequency energy flow which bypasses the otic system entirely, but this is not labelled in Wever's diagram and there is scant reference to it in his written descriptions. Four potential pathways for sound energy flow between stapes and periotic sac ~~in frogs~~ have therefore been described in frogs, but are all four consistently present?

In this study, histological sections were made from the inner ears of three species of frogs, including leopard frogs (these were believed to be *Rana pipiens* rather than *R. sphenocephala*, but this could not be confirmed beyond doubt by the suppliers). Photomicrographs and three-dimensional reconstructions were used to assess the accuracy of Wever's accounts and other recent descriptions of ranid inner ear morphology.

While leopard frogs are in the family Ranidae, within the Ranoidea clade of the Neobatrachia, *Eleutherodactylus limbatus* (Eleutherodactylidae) is placed within the other major neobatrachian clade, the Hyloidea (Hoegg et al., 2004). *Xenopus laevis*, family Pipidae, is an aquatic 'archaeobatrachian', the 'Archaeobatrachia' being a paraphyletic assemblage of frogs which

diverged before the Neobatrachia (Hoegg et al., 2004). In order to assess whether Wever's diagram ~~the ranid ear~~ is representative of a more diverse range of frogs, the leopard frog ear was compared here with the ears of *E. limbatus*, one of the world's smallest frogs, and with those of *X. laevis*.

Materials and methods

Twelve frogs from three different species were used in this study. Three leopard frogs believed to be *Rana pipiens* (40-50 g body mass) were obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, U.S.A.) via Exoterra Schaudi GmbH, Holzheim, Germany. They were housed at the University of Groningen laboratory animal facilities. The frogs were euthanized using the double pith procedure and then decapitated. The lower jaw was removed and the remaining part of the head was divided sagittally. ~~This resulted in two preparations, each containing one complete ear.~~ Skin was removed and small holes were made in various places in the skull, away from the structures of interest, to improve fluid impregnation. The ears were fixed by immersion in a 10% neutral buffered formalin solution (pH 7.4) for at least 24 hours at 4°C. The fresh corpse of a male *Rana pipiens*, originating from Nasco (Fort Atkinson, WI, U.S.A.), was used for micro-computed tomography (micro-CT) scanning, as described below.

Two *Eleutherodactylus limbatus* specimens (each around 0.2 g body mass) were captured at Las Terrazas, Artemisa province, Cuba. They were euthanized by double pithing and decapitation, the palatal skin was removed and their heads were preserved in 10% formalin and sent to Groningen for further processing. The head of one specimen was halved prior to sectioning, while the other was sectioned whole.

Six *Xenopus laevis* specimens (males 55-60 g, females 120-220 g, all gonadectomized body masses) were obtained as fresh corpses from a breeding colony in the Wellcome Trust/Cancer Research U.K. Gurdon Institute, Cambridge, U.K. They had been euthanized via tricaine overdose followed by

cooling, as part of another study. The otic capsules of one male and one female specimen were cut out and placed in 4% buffered formaldehyde solution within two hours of euthanasia. They were then sent to the University of Groningen where they were processed as the *Rana* specimens. A micro-CT scan was made of the head of another male specimen at the University of Cambridge, and the head was then dissected under light microscopy. The remaining three specimens, two females and a male, were also dissected.

Animal care and euthanasia procedures conformed to local and national regulations and were approved by the appropriate institutional Animal Care and Use Committees.

Histological procedures

After fixation the *Rana* and *Xenopus* specimens were rinsed in distilled water, refreshed several times. All subsequent steps were performed on a rolling bank to keep the specimens moving in the experimental solutions. Decalcification took place in a 10% EDTA solution (Sigma, ED5SS, pH 7.34) at a temperature of 50°C in a microwave oven (T/T MEGA microwave histo-processor, Milestone), in four sessions of twelve hours. After decalcification the specimens were rinsed again in distilled water and dehydrated in a graded, seven-step ethanol series (30%, 50%, 70%, 90%, 96%, 100%, 100%) where each step took one hour and solutions were refreshed three times. If necessary, specimens were stored overnight in 70% ethanol. Next, specimens were placed in a 100% ethanol/ hydroxypropyl methacrylate (HPMA) solution (50:50) for 4-8 hours and then put in pure HPMA solution for 24-48 hours. The specimens were then embedded in pure HPMA solution with addition of a plasticizer (around 25:1). The HPMA solution contained 45 ml HPMA, 5 ml ethylene glycol monobutyl ether, 0.5 g benzoyl peroxide, 1.25 ml glycerol and 0.25 ml ethylene glycol dimethacrylate. The plasticizer consisted of 1 ml n,n-dimethylaniline and 10 ml polyethylene glycol 400.

The *Eleutherodactylus* sections were prepared using a faster procedure owing to time constraints. The decalcification was performed in only two steps of 7 and 12 hours and the ethanol dehydration

series was also slightly altered (30% for 30 minutes, three times; 70% for 30 minutes, three times; 90% for 10 minutes, three times; 96% for 10 minutes, three times and 100% for 15 minutes, twice). The specimens were placed in the ethanol/HPMA solution for two sessions of 1 hour, then overnight.

After polymerization, transverse sections of 4 µm thickness were cut using a motorized microtome (HM350S, Microm, Heidelberg, Germany). In some cases, the otoconial mass of the saccule, if identified during the sectioning procedure, was removed from the embedded specimen using a fine needle so as to avoid damaging the microtome. A subset of sections was stained with toluidine blue 1% (10 min) and contrast-stained with basic fuchsin (15-20 s).

3-D reconstruction from serial sections

Digital photographs of *Rana* and *Xenopus* sections were made with an Olympus Camedia C-5050 digital camera and stored as tiff files. Digital photographs of the smaller *Eleutherodactylus* specimens were made using a Leica DM RXA microscope fitted with a Colorview 1 MP camera (Soft Imaging System), working with AnalySiS software (Olympus). Individual files, in some cases reduced in size by cropping and/or conversion to greyscale, were then loaded into ImageJ 1.45s (W. Rasband, 2011, National Institutes of Health) and autoregistered using the StackReg plug-in (P. Thévenaz, 2011, Biomedical Imaging Group, Swiss Federal Institute of Technology, Lausanne; see Thévenaz et al., 1998). StackReg uses a recursive procedure based on rigid-body translation and rotation to align each consecutive section. WinSurf 4.0 (S. Lozanoff & D. Moody, 2001) was then used to construct three-dimensional images, following visual identification of relevant structures. Where wall thickness was significant, the internal rather than external walls of the otic and periotic labyrinths were traced and modelled. The choice of interval between sections used to make the final reconstruction depended upon the size of the structure being reconstructed and the level of detail required. In the production of Fig. 12, MicroView 2.1.2 (GE Healthcare, 2006) was used to reorient the registered image stacks.

One potential problem with 3D reconstruction from serial sections is systematic misalignment of the sections, resulting in a distorted (skewed or twisted) representation, and it can also be difficult to determine orientation. *Eleutherodactylus* was small enough that a whole head could be sectioned and reconstructions from right and left ears compared. In the case of *Rana* and *Xenopus*, the reconstructions from serial sections were compared with micro-CT reconstructions of the whole skull and the ear regions within it (see below). Although soft tissue could not be visialized in our CT scans, hard-tissue structures including otic capsule walls and stapes shaft provided sufficient landmarks for comparison with the serial section reconstructions.

Photomicrographs and reconstructions were laterally inverted where necessary, to facilitate comparison.

Micro-CT reconstructions

Micro-CT images were obtained of the head of one male *Xenopus* specimen at the University of Cambridge. The posterior part of the head was skinned and tissues between the mandibles were removed. The head was then wrapped in cellophane to reduce the rate of drying, and the head was scanned using a Metris X-Tek HMX 160 micro-CT scanner operating at 50 kV and 50 µA with no prefilter. The stepping rotational angle was 0.5 degrees. The software used in the processing of the scan data included iXS Integrated X-ray System Control version 4.1.29 (X-Tek Systems Ltd., 2002), NGI CT Control version 1.5.4 (X-Tek Systems Ltd., 2005) and CT-Pro 2.0 (Metris, 2008). At UCLA, a micro-CT scan was made of the head of one male *Rana pipiens* specimen, immersed in a buffered salt solution within a sample holder. A desktop micro-CT machine was used (MicroCT 40; Scanco Medical, Bassersdorf, Switzerland), operating at 55 kV and 145 µA with a 0.5 mm Al prefilter. The stepping rotational angle was 0.36 degrees. The image was processed using Scanco proprietary software. For both animals, the voxels in the scan images were of 30 µm side length.

VGStudio Max 2.0.1 (Volume Graphics GmbH, 2008), MicroView 2.1.2 and WinSurf 4.0 were used to construct 3D images from the CT data obtained. The CT reconstructions were used to verify that the

reconstructions made from serial sections of *Rana* and *Xenopus* were not distorted, and to determine their orientation relative to the skull.

Results

WinSurf reconstructions of the inner ears of the three anuran species are presented for comparison in Fig. 2. There was no evidence of systematic distortion of the reconstructions made from serial sections, as determined by comparison between different ears and/or comparison with micro-CT reconstructions. Histological artefacts inevitably affected the reconstructions, however, as described below.

Rana pipiens

Reconstructions of the inner ear of *Rana* are shown in Figs. 2 and 3, and photomicrographs of sections of particular interest are presented as Figs. 4 and 5.

Considering first the otic labyrinth, the saccule is partially divided by a central constriction into inferior and superior compartments (Fig. 3). The **inferior saccule** is an ovoid chamber, flattened rostromedially. The saccular macula (sensory epithelium) is at the centre of the flattened surface. The **superior saccule** has an expanded dorsal chamber and four relatively small, posterior diverticula:

1) The prominent **amphibian recess** (Figs. 3, 4A, B, 5B) extends medially from the dorsomedial part of the superior saccule before turning caudally. The sensory epithelium on its dorsal wall is known as the **amphibian papilla**, although this term is sometimes used to refer to the whole chamber and its contents.
2) The **lagenar recess** (Figs. 3, 4A) extends medially from the caudoventral part of the superior saccule, below the amphibian recess. Its sensory epithelium covers its medial wall.

3) A third small diverticulum, the only one to lack a sensory end-organ, extends caudally from the dorsolateral part of the superior saccule (Fig. 3). The thick epithelium forming the internal lining of this diverticulum is known as the **tegmentum vasculosum** (Figs. 4A, 5D). This lining extends rostrally into the posterior part of the superior saccular chamber.

4) The **basilar recess** (Figs. 3, 4A, 5A) is located between the cavity of the tegmentum vasculosum and the lagenar recess. Its sensory epithelium (**basilar papilla**) lies on its medial wall.

Rostral to the amphibian recess, the superior saccule communicates via a constricted region, the utriculo-saccular foramen, with the elongated **utricular chamber**. The sensory epithelium of the utricle is on the ventral wall of the free, rostral portion of this chamber, which then divides to form the ampullae of the anterior and lateral **semicircular canals** (Fig. 2). From the caudal end of the utricular chamber arise the other end of the lateral semicircular canal and the ***crus commune***, a short, vertical segment representing the convergence of the anterior and posterior semicircular canals (Fig. 3). The ampulla of the posterior semicircular canal is located just underneath the caudal-most part of the lateral semicircular canal; the two are not in contact.

We turn now to the periotic system, which may be divided (after Lombard, 1977) into periotic tissue and the periotic labyrinth proper. **Periotic tissue** is the connective tissue found separating both otic and periotic labyrinths from the walls of the otic capsule. In places, it takes the form of condensed and cartilage-like 'limbic tissue' (Wever, 1973). Limbic tissue forms a thin layer around the membranes of the semi-circular canals and utriculus, but it is much thicker around the amphibian and basilar recesses (Figs. 5A, B). The lagenar recess and part of the tegmentum vasculosum are also supported by limbic tissue. Elsewhere, the periotic tissue consists of little more than a diffuse collection of fibres within a fluid space. The semicircular canals, within their thin shells of limbic tissue, are separated from the otic capsule walls by such a fluid space, as is much of the superior saccule (Figs. 4A, B).

The other component of the periotic system, the **periotic labyrinth**, is a membranous sac of complex shape containing apparently acellular fluid. Its three main subdivisions are the periotic cistern, the periotic canal and the periotic sac. The capacious **periotic cistern** (Figs. 3, 4A, B) almost completely surrounds the inferior saccule, extending around it on the medial side as far dorsally as the utricular chamber. A diverticulum of the lateral part of the periotic cistern extends through a narrow, oval-shaped foramen in the wall of the otic capsule and turns sharply rostrally to expand into a **lateral chamber** (Figs. 2-4). The cartilaginous **operculum** lies immediately over the foramen (Figs. 4A, B), while the **stapes footplate** is rostral to this. The footplate comprises the expanded medial part of the bony *pars media* and, around its periphery, the U-shaped, cartilaginous *pars interna*. The operculum and stapes footplate interlock: a flange of the *pars interna* extends a short distance medial to the operculum, while the rostrolateral corner of the operculum fills the gap between the *pars interna* and a ventral process of the *pars media* which articulates with the otic capsule.

The **periotic canal** is a long, narrow tube which ascends dorsally from the lateral part of the periotic cistern and wraps closely around the anterior aspect of the superior saccule (Figs. 2, 3). There is a thin, shared membrane between periotic and otic labyrinths throughout this course. The periotic canal then parts from the saccule near the *crus commune*, turns sharply caudolaterally and bends down around the lateral semicircular canal to meet the superior saccule again between the amphibian recess and the diverticulum of the tegmentum vasculosum. The oval region of apposition found here between otic and periotic labyrinths is the **contact membrane of the saccule** (Figs. 3, 5C), identified in all three specimens of *Rana pipiens* just lateral to the contact membrane of the amphibian recess. The contact membrane of the saccule has 33-76% (n=3 ears) of the area of the contact membrane of the amphibian recess and it is more than twice as thick, but it still represents a relatively thin window between otic and periotic labyrinths, in a region where much of the otic system is surrounded by thick limbic tissue.

The periotic canal then turns ventromedially to form an elongated, curved contact membrane with the lateral wall of the amphibian recess (Fig. 5B). Leaving the otic capsule, the canal runs for a short distance parallel to the ***recessus partis basilaris***, a blind-ending periotic diverticulum heading rostrally towards the basilar recess (Figs. 3, 5A). There is a small contact membrane between the apposed tips of the *recessus partis basilaris* and the basilar recess (Fig. 5A), which is 6-14% (n=3 ears) of the area of the contact membrane of the amphibian recess. The sections of the three *Rana* specimens stopped at this point, so the relationship between the *recessus partis basilaris* and the rest of the periotic system could not be examined. From the literature (see e.g. Lewis and Narins, 1999), the *recessus partis basilaris* and the periotic canal are expected to communicate with each other via the **periotic sac**, a caudal expansion of the periotic canal which projects out of the otic capsule.

Eleutherodactylus limbatus

Reconstructions of the inner ear of *Eleutherodactylus* are shown in Figs. 2 and 6, and photomicrographs of sections of particular interest are presented as Figs. 7-9.

There is no distinct lateral chamber in *Eleutherodactylus* but the footplate and operculum lie at an angle to each other such that their inner surfaces form a bowl-like concavity. The stapes footplate is relatively small; as in *Rana*, it sends a prominent cartilaginous flange under the large operculum. The inferior saccule/periotic cistern region had evidently collapsed to a greater or lesser extent in all four ears examined because it had pulled away from surrounding structures, but its original shape could be determined as the region enclosed between otic capsule, stapes and operculum. The other parts of the inner ear escaped distortion in at least one specimen.

Given the size of the chamber in which it is contained, the inferior saccule must be relatively much smaller than that of *Rana* or *Xenopus*, while the semicircular canals are much wider relative to their

length (Fig. 2). A narrow diverticulum lined with a tegmentum vasculosum extends from the saccular cavity just dorsolateral to the basilar recess (Figs. 6, 7A, B, 8A, C).

The periotic canal (Fig. 6) is relatively longer and more convoluted than in *Rana*. Because the superior saccule is little inflated in *Eleutherodactylus*, the ~~periotic~~ canal, where it emerges from the periotic cistern, is initially not in such close contact with the saccular cavity. However, after turning caudally a diverticulum of the central part of the periotic canal extends downwards and comes into intimate apposition with the superior saccular cavity (Figs. 6, 7B). As in *Rana*, the periotic canal then separates from the otic labyrinth and runs across the lateral semicircular canal en route to the amphibian recess. The contact membrane of the saccule (Figs. 6, 8B) is located just rostral to the contact membrane of the amphibian recess (Figs. 6, 7A, 8A). The contact membrane of the saccule has 26-41% (n=3 ears) of the area of the contact membrane of the amphibian recess. The contact membrane of the basilar recess is around 5-15% (n=4 ears) of the area of the amphibian recess contact membrane.

The *recessus partis basilaris* of the periotic labyrinth (Fig. 6) originates from the periotic sac, which extends out of the otic capsule and into the brain-case. In the whole-head sections which were made from one *Eleutherodactylus* specimen, the periotic sac appears to extend underneath the brain to meet and freely communicate with its contralateral counterpart (Fig. 9). A second fluid space just below this periotic space extends between right and left round windows. The two fluid spaces are separated by a membrane which may be meningeal in origin. It was unclear whether this membrane had simply separated from the basicranial bones due to shrinkage, or whether it really does separate two fluid compartments *in vivo*. The membrane was everted into both the periotic sac and the *recessus partis basilaris* in all four ears examined, perhaps due to shrinkage of the periotic system.

Xenopus laevis

Reconstructions of the inner ear of *Xenopus* are shown in Figs. 2 and 10, and photomicrographs of sections of particular interest are presented as Figs. 8D and 11.

In the sectioned female *Xenopus* specimen, the anterior semicircular canal was damaged, there was a bubble in the saccular region and the sections did not include the lateral passage or stapes. In the male specimen, the utricular and lagenar cavities had collapsed, as judged from a comparison of shapes between the two specimens and the fact that these structures had pulled away from the otic capsule walls. The periotic cistern had pulled away from the otic capsule wall in both specimens. Despite these shrinkage artefacts, the essential features of the inner ear remained intact in at least one of the two specimens, permitting the following description.

The saccular chamber is relatively large and shifted dorsally compared to that of *Rana* (Fig. 2); it is not divided into superior and inferior compartments. The anterior and especially the lateral semicircular canals are elongated rostro-caudally; the posterior canal is shorter. The amphibian recess projects as a diverticulum from the caudomedial end of the saccule, and ventral to this extends a second diverticulum which divides into the basilar recess laterally and the prominent lagenar recess medially (Fig. 10). No special subcavity of the saccular chamber containing a tegmentum vasculosum could be found.

The periotic cistern completely enwraps the saccule (Fig. 10) and is interposed between this and the basilar recess, giving rise to a ‘tympanal area’ rostrolateral to the basilar recess (Figs. 8D, 11; see Discussion). The relatively short, sickle-shaped periotic canal runs very close to the dorsal part of the periotic cistern, but the two remain separate (Fig. 2). Although the periotic canal is also close to the dorsal wall of the saccular cavity, the two are not in such close apposition as in *Rana* and *Eleutherodactylus* and there is no distinct contact membrane of the saccule. The periotic canal forms a small contact membrane with the amphibian recess before turning caudally and expanding into

the periotic sac, which forms a second contact membrane directly with the basilar recess (Figs. 10, 11). The periotic sac then extends out of the otic cavity.

No trace of an operculum was identified in *Xenopus*. The stapes footplate caps the end of a tubular passage projecting laterally from the otic capsule (Figs. 2, 11). The contents of this passage had picked up a pale blue stain in the histological sections, suggesting that a precipitate had formed there. The periotic cistern was not similarly stained and was clearly separated from the stapes footplate by whatever was in this lateral passage. In gross dissection of frogs of both sexes, the lateral passage was found to be filled with a clear, colourless fluid. A very thin membrane was seen at the medial end of the passage, separating its contents from the periotic cistern. The lateral end was sealed by the tough membrane of the oval window.

Although the reconstructions made from the male and female *Xenopus* specimens were generally very similar, the female's inner ear apparatus, particularly the saccular chamber, was more elongated rostro-caudally. The contact membrane of the amphibian recess was just over twice the area of the contact membrane of the basilar recess in the female, whereas in the male the contact membrane of the basilar recess was 1.5 times the area of that of the amphibian recess.

Discussion

Wever's diagram

Wever's (1973) schematic section through the ear of a leopard frog (Fig. 1A) has been widely reproduced in the literature. Although presented as a "frontal section", Wever did not claim that his diagram was based on a single, histological section and he may have amalgamated several slides in its construction. In order to address this possibility, MicroView software was used to reorient a stack of registered *Rana* section photomicrographs and section it in a new plane, thus revealing a 'virtual section' through the inner ear. The orientation was chosen such that the 'virtual section' (Fig. 12A)

was as close to Wever's illustration as possible, the main criteria being that the section should show both amphibian and basilar recesses as well as the lateral chamber of the inner ear. Falling somewhere between frontal and transverse planes, its orientation is best described as oblique (Fig. 12C).

Assuming that our original section photomicrographs were well-aligned, which by comparison with CT scan data appeared to be the case, our 'virtual section' shows Wever's schematic figure to be anatomically inaccurate in several respects. The orientation of the stapes footplate in our 'virtual section' differs substantially, revealing the process of the stapes *pars media* which articulates with the otic capsule (marked with an asterisk in Fig. 12B). It is easy to visualise the stapes footplate rocking about this process, as has been shown to be the case in ranid frogs (Jørgensen and Kanneworff, 1998; Mason and Narins, 2002; Werner, 2003), rather than acting as a piston as Wever's diagram might imply. Our 'virtual section' also passes through all three semicircular canals, but only touches the periphery of the operculum. It does not include the contact membrane of the saccule.

Wever's figure therefore appears not to represent a single, real section through the ear, but it is useful in diagrammatically illustrating the likely pathways for acoustic energy flow from the stapes to the amphibian and basilar papillae, and thence to the round window. Acoustic energy is also thought to be able to pass from periotic cistern to round window via the periotic canal, bypassing the otic labyrinth and auditory papillae entirely (Purgue and Narins, 2000a, b). That portion of the periotic canal which ascends from the periotic cistern is visible in both our 'virtual section' (Fig. 12B) and Wever's diagram (Fig. 1B), but Wever (1973, 1985) made surprisingly little mention of the canal in his otherwise detailed descriptions of frog inner ears.

Of the other schematic illustrations of the frog inner ear which exist in the literature, that of Frishkopf & Goldstein (1963) may be the best-known. More obviously diagrammatic than Wever's

illustration, this older representation shows the periotic and semicircular canals, and it represents the extrastapes more accurately.

The ears of *Eleutherodactylus* and *Xenopus*

The inner ear of *Eleutherodactylus limbatus* was found generally to resemble that of *Rana*, but there were some pronounced differences in terms of the relative sizes and shapes of the various structures. These differences were not highlighted by Wever (1985), who examined three other *Eleutherodactylus* species. The very small saccule and the relatively short, wide semicircular canals (Fig. 2) closely resemble reconstructions of the inner ear in 'stage 8' *Rana temporaria* tadpoles (30 mm long, just before emergence of hindlimbs) made by Birkmann (1940). *E. limbatus* has direct development which omits a tadpole stage, but our frogs had been vocalizing in life and were therefore believed to be reproductively mature. The inner ear of this species may therefore be paedomorphic.

The inner ear of *Xenopus laevis* has been described, among others, by Paterson (1949, 1960), Wever (1985) and Bever et al. (2003). Our reconstructions of *Xenopus* ears largely agree with their descriptions. The saccular cavity contains a dense otoconial mass (very obvious in the CT scans) which, relative to the rest of the inner ear, is much larger and more dorsally positioned than its equivalent in *Rana* and *Eleutherodactylus*. The size and position of the saccular cavity in *Xenopus* gives its inner ear a striking morphological similarity to that of the fish *Gobius niger*, as illustrated by Retzius (1881). The possible functional convergence between these two aquatic species remains to be explored.

The association of the basilar papilla with the lagenar recess in urodeles, caecilians and amniotes has been said to be the "single most influential piece of evidence supporting a [basilar papilla] homology among all terrestrial vertebrates", but the separate opening of the basilar recess into the saccule in frogs was seen as a complication to this theory (Smotherman and Narins, 2004). We have found that

the basilar and lagenar recesses are in fact conjoined in *Xenopus* (Fig. 10), suggesting that this represents the primitive condition for all lissamphibians and perhaps tetrapods in general.

At the caudal end of the basilar recess, periotic contact occurs via the periotic sac directly in *Xenopus*, rather than via a *recessus partis basilaris*. Rostrally, the recess is separated from the periotic cistern by a thin "tympanal area", discussed later. These features have been previously described by Paterson (1949, 1960).

In *Xenopus*, the periotic cistern is separated from the stapes footplate by the fluid contained within a tubular extension of the otic capsule (Fig. 11). A shorter separation between stapes and cistern is shown in Wever's (1985) diagrams of the ear of this frog, but Wever found a much longer, fluid-filled "lateral passage" in the related species *Pipa pipa*. In both our histological slides of *Xenopus* and Wever's slides of *Pipa* it looked like a precipitate had formed within this lateral passage, but not in the periotic labyrinth. Paterson (1960) found only a short lateral passage in her immature specimen of *Pipa* which she refers to as a "fossa fenestrae ovalis", filled with "delicate connective tissue"; she did not describe anything similar in *Xenopus*. Perhaps the separation between footplate and periotic labyrinth increases in pipids as the skull grows, such that it is less obvious in younger specimens. The lateral chamber of *Rana* differs from the lateral passage of *Xenopus* because the ranid lateral chamber has a narrower connection with the main otic capsule, it bends sharply rostrally to reach the stapes footplate and it is filled with a diverticulum of the periotic cistern. The ranid lateral chamber is also in contact with the operculum, an element lacking in *Xenopus*.

The otic labyrinth in the female *Xenopus* was around 1.5 times the linear dimensions of that of the male. The saccular cavity was more elongated in the female and there was a difference in the relative sizes of the contact membranes of the amphibian and basilar recesses, noted earlier. Further investigation of a larger number of specimens is needed in order to establish whether these inner ear differences represent sexual dimorphism, which has been observed in the middle ear of this species (Mason et al., 2009).

~~Some other aspects of the inner ear of the three frog species studied that are worthy of particular attention are discussed in more detail in the sections to follow.~~

The blind branch of the periotic canal

The discrete "blind branch" of the periotic canal which Purgue & Narins (2000b) found in the bullfrog *Rana catesbeiana* could not be identified in the *Rana* or *Eleutherodactylus* specimens examined here. However, the membranous wall of the periotic canal as it curves around the superior saccule was particularly thin in *Rana pipiens*, and in some slides it appeared to be ruptured. An apparent short diverticulum found in the female *Xenopus* specimen only (Fig. 10) might also have been the result of periotic canal rupture. Purgue & Narins injected silicone into the periotic labyrinth of their frogs to make casts: it is possible that they experienced a similar problem.

The contact membranes

Harrison (1902) referred to three 'tympanal areas' in the frog inner ear where otic and periotic labyrinths are in particularly close apposition. One of these is the extensive, membranous division between periotic cistern and saccule, while the other two 'tympanal areas' are now more generally known as the 'contact membranes' of the amphibian and basilar recesses. To these three may be added the contact membrane of the saccule and an additional 'tympanal area' in *Xenopus*, discussed later.

Sound energy from stapedial vibrations is widely presumed to enter the otic labyrinth through the first 'tympanal area' between periotic cistern and saccule (the membrane surrounding the inferior saccular chamber, labelled IS in Fig. 4, forms part of this). The division between the periotic and otic systems remains very thin where the periotic canal wraps around the anterior wall of the superior

saccule; a special, ventral diverticulum of the canal makes additional contact with the superior saccule in *Eleutherodactylus* (Figs. 6, 7B).

In both *Rana* and *Eleutherodactylus*, the periotic canal separates from the saccule but returns to meet the otic labyrinth at three contact membranes within the otherwise thickened limbic tissue at the posterior end of the otic capsule. The contact membranes of the amphibian and basilar recesses represent pathways through which sound energy can travel from the otic labyrinth back into the periotic system, via the auditory epithelia of the amphibian and basilar papillae (Purgue and Narins, 2000a, b). Although not considered by Purgue & Narins, Wever (1973) showed in his diagram a third contact membrane located between the two papillae, marked only with a tiny arrow (Fig. 1A). Wever wrote that "this thin area acts as a bypass and allows some fraction of the fluid motion to go directly into the perilymphatic duct [= periotic canal] without being detected". This membrane between superior saccule and periotic canal was described in *Rana catesbeiana* by Lewis (1976), who referred to it as the "contact membrane of the saccule". Lewis & Narins (1999) state that it is found in "the more derived anurans", referring this statement to Lewis (1984); Lewis & Narins may have meant Lewis' 1976 publication.

The contact membrane of the saccule was identified in this study in both *Rana* (Figs. 3, 5C) and *Eleutherodactylus* (Figs. 6, 8B), located close to the contact membrane of the amphibian recess. Although it appears to be thicker than the other two contact membranes, it might indeed represent a second route by which sound energy could bypass the amphibian and basilar papillae, additional to the periotic canal route described by Purgue & Narins (2000a, b). The frequency-dependent impedance of such a bypass, and hence its functional significance, remains to be determined. The so-called 'round window' within the metotic fissure, which may not be homologous with the round window of other tetrapods (Henson, 1974), represents a point of pressure release for all these routes (Wever, 1985).

As well as the usual contact membrane at the posterior end of the basilar recess, *Xenopus* has an additional 'tympanal area' between the rostrolateral wall of this recess and a posterior extension of the periotic cistern (Paterson, 1949, 1960). Of the frogs studied here only *Xenopus* has this 'tympanal area' (Figs. 8D, 11) because its basilar and lagenar recesses both arise from the same caudoventral diverticulum of the saccular cavity (Fig. 10; see earlier), and part of the periotic cistern has come to occupy the space between this diverticulum and the saccule proper. Elsewhere, the membranous walls of the basilar recess are enclosed within thick limbic tissue (Fig. 8D). In principle, acoustic energy might flow from periotic cistern through this 'tympanal area' directly into the basilar recess, exiting at the posterior end of the recess via the contact membrane formed here with the periotic sac. This short and direct pathway through the basilar recess was represented diagrammatically by Wever (1985).

The tegmentum vasculosum

'Tegmentum vasculosum', literally meaning 'vascular covering', is a term most often used to describe the well-vascularized, thickened wall of the cochlear duct in birds and crocodilians. Separating the scala media from the scala vestibuli, this archosaur tegmentum vasculosum is believed to combine the roles of the stria vascularis and Reissner's membrane in mammals (Baird, 1974; Lewis et al., 1985; Hossler et al., 2002). The same term has long been used in the German anatomical literature to describe the thickened layer of epithelial cells found in the superior saccule of certain frogs (e.g. Deiters, 1862; Hasse, 1868; Kuhn, 1880; Retzius, 1881; Gaupp, 1904; Birkmann, 1940; Hagmann and Giebel, 1978). Retzius (1881) described a tegmentum vasculosum in *Bufo*, *Hyla* and *Pelobates* but found it to be very poorly-developed in *Alytes*; it is not found in *Ascaphus* or *Liopelma* (Wagner, 1934), nor in pipids including *Xenopus* (Paterson, 1960; this study). Although lacking in some 'archaeobatrachians', the tegmentum vasculosum has apparently been identified in all neobatrachian ears in which it has been sought. It is not found in urodeles (Birkmann, 1940).

Retzius (1881) produced several illustrations of the otic labyrinth of *Rana esculenta*, which were redrawn and modified by Gaupp (1904). Retzius and Gaupp both labelled the whole of the superior saccular wall as the tegmentum vasculosum, as did Birkmann (1940) in his reconstructions of the otic labyrinth of *Rana temporaria*. Several illustrations from Gaupp and Birkmann were redrawn by Wever (1985) in *The Amphibian Ear*, but in each case the region originally labelled as tegmentum vasculosum was relabelled as part of the saccule. In the present study, the tegmentum vasculosum was readily identifiable in *Rana* and *Eleutherodactylus* as a thickened epithelium lining an otherwise unoccupied diverticulum of the saccular chamber. However, the extent of its vascularization could not be ascertained and, as Gaupp (1904) noted, its rostral borders are indistinct in *Rana*. A tegmentum vasculosum was not found in *Xenopus*.

Wever (1985) made only one, brief mention of the anuran tegmentum vasculosum in his book (p.78), in which he commented on the mistake of "early anatomists" in assigning to it a sensory function. Nevertheless, the unusual epithelium suggests a functional distinction from the rest of the superior saccular chamber. Hagmann & Giebel (1978), working on *Rana temporaria*, confirmed that this region is richly vascularized and found high levels of metabolic enzymatic activity. This supports the contention that the tegmentum vasculosum in frogs is responsible for the secretion of endolymph, like the tegmentum of archosaurs and the stria vascularis of mammals. Subsequent studies of endolymph secretion in frogs, however, have focused on the dark cells located in the utricle and semicircular canal ampullae (Burnham and Stirling, 1984; Bernard et al., 1986); the anuran tegmentum vasculosum has fallen into obscurity.

The apparent interaural fluid connection in *Eleutherodactylus*

Of the frogs examined, sections through the whole head were only available for one specimen of *Eleutherodactylus*. Although our interpretation may have been affected by shrinkage, these sections appeared to show the left and right periotic sacs converging to form a fluid space immediately

beneath the brain (Fig. 9). Wever (1978) showed that vibrations applied to the operculum of one ear in a salamander can excite the contralateral ear: his proposed mechanism involved a similar intracranial pathway below the brain, but he believed that the two periotic sacs in his species communicated only indirectly, via the cerebrospinal fluid. Harrison (1902) challenged the notion of earlier authors that amphibians possess a connection between the periotic system and a "subdural space".

Such a fluid system extending between the two ears via the cranial cavity might be functionally significant in (1) communicating vibrations from the cerebrospinal fluid to the inner ears, (2) acoustically coupling the two ears, which might affect sound localization, or (3) providing increased possibilities for pressure release from the inner ear, affecting sensitivity. Further work is clearly needed to confirm the presence of an interaural fluid connection in *Eleutherodactylus* and other frogs, so as to assess whether this condition is widespread among anurans.

Conclusion

The anuran inner ear is a complex, three-dimensional structure consisting of the intertwined canals of the periotic and otic labyrinths. Although his representation appears not to be anatomically accurate, Wever's (1973) diagram of the leopard frog inner ear does an admirable job of clearly illustrating some of the possible routes of acoustic energy flow from stapes to round window. Its main shortcoming in this respect is that it does not label the periotic canal, which represents another potential route. Although some anatomical differences were identified, the inner ear of *Eleutherodactylus* is broadly similar to that of *Rana*, so Wever's diagram is clearly representative of a wider range of neobatrachian frogs. The illustration is less useful in describing the ear of *Xenopus*; to what extent this reflects the 'archaeobatrachian' status of *Xenopus* or its aquatic habits remains to be determined.

The tegmentum vasculosum and the contact membrane of the saccule, found here in *Rana* and *Eleutherodactylus* but not *Xenopus*, have been neglected in the recent literature and deserve further attention from auditory physiologists. The intriguing possibility of a fluid pathway extending between right and left ears also demands investigation.

Acknowledgements

The authors wish to thank Emanuel Mora for his help and support with this project. Dave Simpson kindly provided the *Xenopus* specimens. The CT scan of *Xenopus* was made by Alan Heaver of the University of Cambridge Department of Engineering, with thanks going also to Norman Fleck for the use of his equipment. The authors are very grateful to Dolores Bozovic, Alan D. Grinnell, Tammy Hoang, Victoria Sandoval and Felix E. Schweizer for facilitating the *Rana* CT scan, which was made by Ting-Ling Chang at the UCLA School of Dentistry, Division of Advanced Prosthodontics. Stephan Kamrad helped with translations. The research of JMS and PvD was supported by the Heinsius Houbolt Foundation and is part of the research program *Healthy Ageing and Communication* of the Department of Otorhinolaryngology at the University Medical Center Groningen. Finally, the authors wish to thank the reviewers and editors of the manuscript for their very helpful comments.

Conflicts of interest

The authors declare that they have no conflicts of interest.

References

Baird IL (1974) Anatomical features of the inner ear in submammalian vertebrates. In: Handbook of Sensory Physiology, volume V/1: Auditory System (Keidel WD, Neff WD, eds), pp 159-212. Berlin: Springer-Verlag.

Bernard C, Ferrary E, Sterkers O (1986) Production of endolymph in the semicircular canal of the frog *Rana esculenta*. Journal of Physiology 371:17-28.

Bever MM, Jean YY, Fekete DM (2003) Three-dimensional morphology of inner ear development in *Xenopus laevis*. Developmental Dynamics 227:422–430.

Birkmann K (1940) Morphologisch-anatomische Untersuchungen zur Entwicklung des häutigen Labyrinthes der Amphibien. Zeitschrift für Anatomie und Entwicklungsgeschichte 110:443-488.

Blood DC, Studdert VP (1999) Saunders Comprehensive Veterinary Dictionary, 2nd Edition. Edinburgh: W.B. Saunders.

Burnham JA, Stirling CE (1984) Quantitative localization of Na-K pump site in frog inner ear dark cells. Hearing Research 13:261-268.

Deiters O (1862) Ueber das innere Gehörorgan der Amphibien. Archiv für Anatomie, Physiologie und Wissenschaftliche Medicin 1862:262-275, 277-310.

Dubois A (2007) Naming taxa from cladograms: a cautionary tale. Molecular Phylogenetics and Evolution 42:317-330.

Duellman WE, Trueb L (1986) Biology of Amphibians. Baltimore: The Johns Hopkins University Press.

Fay RR, Popper AN (1985) The octavolateralis system. In: Functional Vertebrate Morphology (Hildebrand M, Bramble DM, Liem KF, Wake DB, eds), pp 291-316. London: Belknap Press.

Frishkopf LS, Goldstein MH (1963) Responses to acoustic stimuli from single units in the eighth nerve of the bullfrog. Journal of the Acoustical Society of America 35:1219-1228.

Gaupp E (1904) A. Ecker's und R. Wiedersheim's Anatomie des Frosches, part 3, 2nd Edition. Braunschweig: Druck und Verlag von Friedrich Vieweg und Sohn.

Gridi-Papp M, Narins PM (2010) Seismic detection and communication in amphibians. In: The Use of Vibrations in Communication: Properties, Mechanisms and Function across Taxa (O'Connell-Rodwell CE, ed), pp 69-83. Kerala: Research Signpost.

Hagmann B, Giebel W (1978) Enzymhistochemische Untersuchungen am Innenohr des Frosches (*Rana temporaria*). Archives of Oto-Rhino-Laryngology 220:89-103.

Harrison HS (1902) On the perilymphatic spaces of the amphibian ear. Internationale Monatsschrift für Anatomie und Physiologie 19:221-261.

Hasse C (1868) Das Gehörorgan der Frösche, Reprinted from Zeitschrift für wissenschaftliche Zoologie, Bd. 18 Edition. Leipzig: Verlag von Wilhelm Engelmann.

Henson OW (1974) Comparative anatomy of the middle ear. In: Handbook of Sensory Physiology, volume V/1: Auditory System (Keidel WD, Neff WD, eds), pp 39-110. Berlin: Springer-Verlag.

Hillis DM (2007) Constraints in naming parts of the Tree of Life. Molecular Phylogenetics and Evolution 42:331-338.

Hoegg S, Vences M, Brinkmann H, Meyer A (2004) Phylogeny and comparative substitution rates of frogs inferred from sequences of three nuclear genes. Molecular Biology and Evolution 21:1188-1200.

Hossler FE, Olson KR, Musil G, McKamey MI (2002) Ultrastructure and blood supply of the tegmentum vasculosum in the cochlea of the duckling. Hearing Research 164:155-165.

Jørgensen MB, Kanneworff M (1998) Middle ear transmission in the grass frog, *Rana temporaria*. Journal of Comparative Physiology A 182:59-64.

Kuhn (1880) Ueber das häutige Labyrinth der Amphibien. Archiv für Mikroskopische Anatomie 17:479-550.

Lewis ER (1976) Surface morphology of the bullfrog amphibian papilla. Brain, Behavior and Evolution 13:196-215.

Lewis ER (1984) On the frog amphibian papilla. Scanning Electron Microscopy 1984 (IV):1899-1913.

Lewis ER, Narins PM (1999) The acoustic periphery of amphibians: anatomy and physiology. In: Comparative Hearing: Fish and Amphibians (Fay RR, Popper AN, eds), pp 101-154. New York: Springer.

Lewis ER, Leverenz EL, Bialek WS (1985) The Vertebrate Inner Ear. Boca Raton: CRC Press, Inc.

Lombard RE (1977) Comparative morphology of the inner ear in salamanders (*Caudata*: *Amphibia*). Basel: S. Karger.

Mason MJ, Narins PM (2002) Vibrometric studies of the middle ear of the bullfrog *Rana catesbeiana* I. The extrastapes. Journal of Experimental Biology 205:3153-3165.

Mason MJ, Wang M, Narins PM (2009) Structure and function of the middle ear apparatus of the aquatic frog, *Xenopus laevis*. Proceedings of the Institute of Acoustics 31:13-21.

Paterson NF (1949) The development of the inner ear of *Xenopus laevis*. Proceedings of the Zoological Society of London 119:269–291.

Paterson NF (1960) The inner ear of some members of the Pipidae (Amphibia). Proceedings of the Zoological Society of London 134:509-546.

Pauly GB, Hillis DM, Cannatella DC (2009) Taxonomic freedom and the role of official lists of species names. Herpetologica 65:115–128.

Purgue AP, Narins PM (2000a) A model for energy flow in the inner ear of the bullfrog (*Rana catesbeiana*). Journal of Comparative Physiology A 186: 489-495.

Purgue AP, Narins PM (2000b) Mechanics of the inner ear of the bullfrog (*Rana catesbeiana*): the contact membranes and the periotic canal. Journal of Comparative Physiology A 186: 481-488.

Retzius G (1881) Das Gehörorgan der Wirbelthiere. Morphologisch-Histologische Studien. I. Das Gehörorgan der Fische und Amphibien. Stockholm: Samson & Wallin.

Simmons DD, Meenderink SWF, Vassilakis PN (2007) Anatomy, physiology, and function of auditory end-organs in the frog inner ear. In: Hearing and Sound Communication in Amphibians (Narins PM, Feng AS, Fay RR, Popper AN, eds), pp 184-220. New York: Springer.

Smotherman M, Narins P (2004) Evolution of the amphibian ear. In: Evolution of the Vertebrate Auditory System (Manley GA, Popper AN, Fay RR, eds), pp 164-199. New York: Springer.

Thévenaz P, Ruttimann UE, Unser M (1998) A pyramid approach to subpixel registration based on intensity. IEEE Transactions on Image Processing 7:27-41.

van Dijk P, Mason MJ, Schoffelen RLM, Narins PM, Meenderink SWF (2011) Mechanics of the frog ear. Hearing Research 273:46-58.

Wagner DS (1934) The structure of the inner ear in relation to the reduction of the middle ear in the Liopelmidae (Noble). Anatomischer Anzeiger 79:20-36.

Werner YL (2003) Mechanical leverage in the middle ear of the American bullfrog, *Rana catesbeiana*. Hearing Research 175:54-65.

Wever EG (1973) The ear and hearing in the frog, *Rana pipiens*. Journal of Morphology 141:461-477.

Wever EG (1978) Sound transmission in the salamander ear. Proceedings of the National Academy of Sciences of the USA 75:529-530.

Wever EG (1985) The Amphibian Ear. Princeton: Princeton University Press.

Figure Captions

Fig. 1

A: Ernest Glen Wever's schematic diagram of the peripheral auditory apparatus of a ranid frog. The original caption is included. From: Wever, E.G. (1973) The ear and hearing in the frog, *Rana pipiens*. *Journal of Morphology* 141(4): 461-477. Copyright © 1973 Wiley-Liss, Inc. B: A representation of the same diagram with the ~~same~~ colour-coding ~~as~~ used elsewhere in this paper, to facilitate comparison between figures. Where Wever's nomenclature differs from that used in the present paper, structures have been relabelled. Additionally, based on the interpretations of the present study, new labels have been introduced for structures that were not explicitly identified in Wever's original diagram, including the contact membranes which separate otic and periotic labyrinths.

Colour code: white = otic labyrinth (endolymph); green = periotic labyrinth (perilymph); red = sensory epithelium; dark grey = limbic tissue; cream = bone; yellow = cartilage.

Fig. 2

WinSurf reconstructions of the left inner ear structures of (left) *Xenopus laevis*, male specimen, (middle) *Rana pipiens*, (right) *Eleutherodactylus limbatus*. Lateral views are shown in the top row, dorsal views in the bottom row. *Eleutherodactylus* reconstructions are 2.5× enlarged relative to the others. In the *Eleutherodactylus* sections used for these reconstructions, the periotic cistern in the region marked with an asterisk, which lies lateral to the very small saccular cavity, had collapsed. Its approximate shape has been restored here by comparison with the contralateral ear and the extent of the space available for it within the otic capsule. Colour code: white = otic labyrinth (endolymph); green = periotic labyrinth (perilymph); red = sensory epithelium; semitranslucent grey = internal walls of the otic capsule.

Key to this and subsequent figures: AR = amphibian recess (endolymph); AS = anterior semicircular canal; B = brain; BR = basilar recess (endolymph); CA = contact membrane of amphibian recess; CB = contact membrane of basilar recess; CC = *crus commune* (confluence of anterior and posterior semicircular canals); CS = contact membrane of saccule; IS = inferior saccular chamber; LC = lateral chamber; LP = lateral passage; LR = lagenar recess (endolymph); LS = lateral semicircular canal; LT = limbic tissue; O = operculum; PC = periotic canal; POC = periotic cistern; POS = periotic sac; PS = posterior semicircular canal; PT = periotic tissue; PU = posterior utricular cavity; RPB = *recessus partis basilaris* (perilymph); RW = round window; S = saccule; SPI = stapes *pars interna*; SPM = stapes *pars media*; SS = superior saccular chamber; TV = tegmentum vasculosum, or the saccular diverticulum within which this epithelial lining is found; VIII = branch of eighth cranial nerve.

Fig. 3

WinSurf reconstructions of left inner ear structures of *Rana pipiens*, seen from an approximately posterior view. The reconstruction on the left shows the otic labyrinth (white), sensory epithelia (red) and contact membranes separating endo- and perilymph (purple). Part of the posterior semicircular canal has been removed to reveal the diverticula of the superior saccule. The reconstruction on the right shows the same, with the periotic labyrinth added in (semitranslucent green). Scale bar 2.5 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 4

Composite photomicrographs of two approximately transverse sections through the posterior part of the inner ear of *Rana pipiens*. Section B is in a plane 240 µm anterior to Section A. Relative to the centre of each photomicrograph, dorsal is upwards and slightly away from the viewer, lateral is to

the left. Scale bar applies to both A and B and represents 1 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 5

Photomicrographs of sections through the inner ear of *Rana pipiens*. A: Basilar recess (BR; endolymph), *recessus partis basilaris* (RPB; perilymph) and contact membrane of basilar recess (CB) separating the two. B: Amphibian recess (AR) and its contact membrane (CA), expanded from Fig. 4A. C: Periotic canal (PC) and contact membrane of the saccule (CS), expanded from Fig. 4B. D: Tegmentum vasculosum (TV), expanded from Fig. 4A. All scale bars 200 µm. See Fig. 2 caption for full list of abbreviations.

Fig. 6

WinSurf reconstructions of left inner ear structures of *Eleutherodactylus limbatus*, seen from an approximately lateral view. Part of the lateral semicircular canal has been removed to reveal the contact membrane of the amphibian recess (CA) and the contact membrane of the saccule (CS). The reconstruction on the left shows the otic labyrinth and associated structures only; the reconstruction on the right includes the dorsal and posterior parts (only) of the periotic labyrinth. The asterisk indicates the ventral diverticulum of the central part of the periotic canal, which is closely apposed to the superior saccular chamber (SS). Scale bar 0.5 mm. Colour code: white = otic labyrinth (endolymph); semitranslucent green = periotic labyrinth (perilymph); red = sensory epithelium; purple = contact membrane separating endo- and perilymph. See Fig. 2 caption for full list of abbreviations.

Fig. 7

Composite photomicrographs of two sections through the inner ear of *Eleutherodactylus limbatus*. Section A is in an approximately transverse plane, through the posterior part of the inner ear. Relative to the centre of the photomicrograph, dorsal is upwards and slightly away from the viewer, lateral is to the left. Section B is in an oblique plane (between transverse and sagittal), taken from the ear on the contralateral side to Section A and laterally inverted to aid comparison. Relative to the centre of the photomicrograph, dorsal is upwards, lateral is to the left and away from the viewer. Note in particular the membrane marked with an asterisk, located between the ventral diverticulum of the central part of the periotic canal (PC) and the superior saccular chamber (SS). The membranous labyrinth has pulled away from the otic capsule wall on the right hand side. Scale bar applies to both A and B and represents 200 µm. See Fig. 2 caption for full list of abbreviations.

Fig. 8

Photomicrographs of sections through the inner ears of *Eleutherodactylus limbatus* and *Xenopus laevis*. A: Tegmentum vasculosum (TV), amphibian recess (AR) and its contact membrane (CA) in *E. limbatus*, scale bar 100 µm. B: Periotic canal (PC) and the contact membrane of the saccule (CS) in *E. limbatus*, scale bar 100 µm. C: Tegmentum vasculosum of *E. limbatus*, scale bar 50 µm. D: ~~Section through the~~ Basilar recess (BR) of *X. laevis* (female specimen), scale bar 200 µm. The asterisk indicates the additional 'tympanal area' identified by Paterson (1949, 1960), located just rostrolateral to the basilar recess (see text). See Fig. 2 caption for full list of abbreviations.

Fig. 9

Photomicrograph of a transverse section through the head of *Eleutherodactylus limbatus*, at the level of the posterior half of the otic capsule. The three fluid compartments collectively denoted '1'

appear to be separate in this section, but inspection of other sections in the same series suggests that they are actually part of one continous fluid system extending between the periotic labyrinths of each ear and passing underneath the brain (B). The left-hand arrow marked '1' points to the left periotic sac, which can be seen emerging from the left otic capsule. Within the capsule, it is immediately adjacent to the amphibian recess (AR), from which it is separated by a thin contact membrane. Between fluid space 1 and the base of the skull is a second fluid space (2), which is also continuous across the head and extends between the two round windows (RW). Distortion resulting from shrinkage may have changed the relative sizes of fluid spaces 1 and 2. Scale bar 0.5 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 10

WinSurf reconstructions of left inner ear structures of *Xenopus laevis* (female specimen), seen from an approximately caudoventral view. Part of the posterior semicircular canal has been removed to reveal the diverticula of the saccule. The reconstruction on the left shows the otic labyrinth and associated structures only; the reconstruction on the right includes all but the most rostrolateral part of the periotic labyrinth too. The anterior semicircular canal had been obliterated in the slides used to make this reconstruction and the saccular cavity was distorted owing to the presence of a bubble. The presumed original shape of the saccule (S) has been restored here. The asterisk indicates a short, blind branch of the periotic canal (see text). Scale bar 1.5 mm. Colour code: white = otic labyrinth (endolymph); semitranslucent green = periotic labyrinth (perilymph); red = sensory epithelium; purple = contact membrane separating endo- and perilymph. See Fig. 2 caption for full list of abbreviations.

Fig. 11

Photomicrograph of an oblique section through the inner ear of *Xenopus laevis* (male specimen). Note that the periotic cistern (POC), the contents of which have picked up only a small amount of stain, is separated from the stapes footplate (SPI and SPM) by the lateral passage (LP), which is filled with diffuse material staining pale blue. The contact membrane of the basilar recess is the thin membrane between the basilar recess (BR) and the periotic sac (POS); the membrane marked with an asterisk between the basilar recess and the periotic cistern (POC) is Paterson's (1949, 1960) additional 'tympanal area' (see text). Relative to the centre of the picture, dorsal is upwards and away from the viewer, lateral is to the left and towards the viewer. Scale bar 1 mm. See Fig. 2 caption for full list of abbreviations.

Fig. 12

Reconstructions created from a stack of serial section images of the ear of *Rana pipiens*, digitally resectioned in a plane as close as possible to Wever's 1973 illustration (Fig. 1). See text for details. The extrastapes, tympanic membrane, periotic sac and round window were not within the original sections and are consequently not shown in these reconstructions. A: 'Virtual section'; the faint, diagonal striations indicate the planes of the original section photomicrographs from which this was reconstructed. B: Expanded, diagrammatic illustration of the same. Out of the plane of this particular section, the periotic canal is in communication with the periotic fluid which abuts the contact membranes of the amphibian and basilar recesses; these regions are all components of the periotic labyrinth and are hence shaded in green. The asterisk indicates a process of the stapes *pars media* which articulates with the otic capsule. C: WinSurf reconstructions of the right inner ear of *R. pipiens* from (left) lateral, (middle) posterior and (right) dorsal views, showing the position of the 'virtual section' as a grey plane. The stapes and operculum are included in these reconstructions but the internal walls of the otic capsule are not. Both scale bars 2 mm. Colour code: white = otic labyrinth

(endolymph); green = periotic labyrinth (perilymph); dark grey = limbic tissue; lighter grey = looser periotic tissue; cream = bone; yellow = cartilage.

Figure 1
Click here to download high resolution image

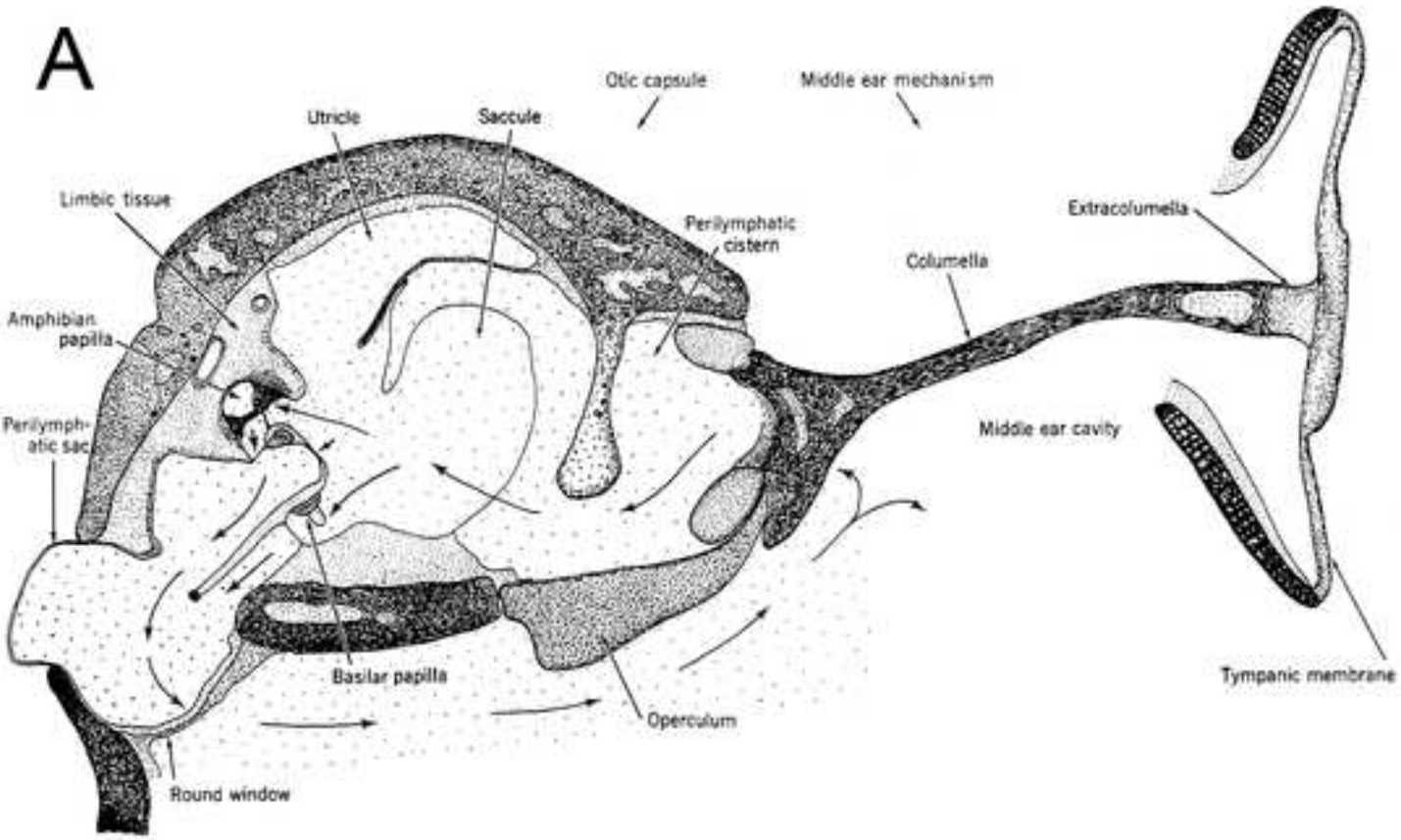


Fig. 1 A schematic representation of the ear and labyrinth of *Rana pipiens*, in frontal section. The large arrows indicate the paths of fluid flow for an inward thrust of the columella. Scale × 20.

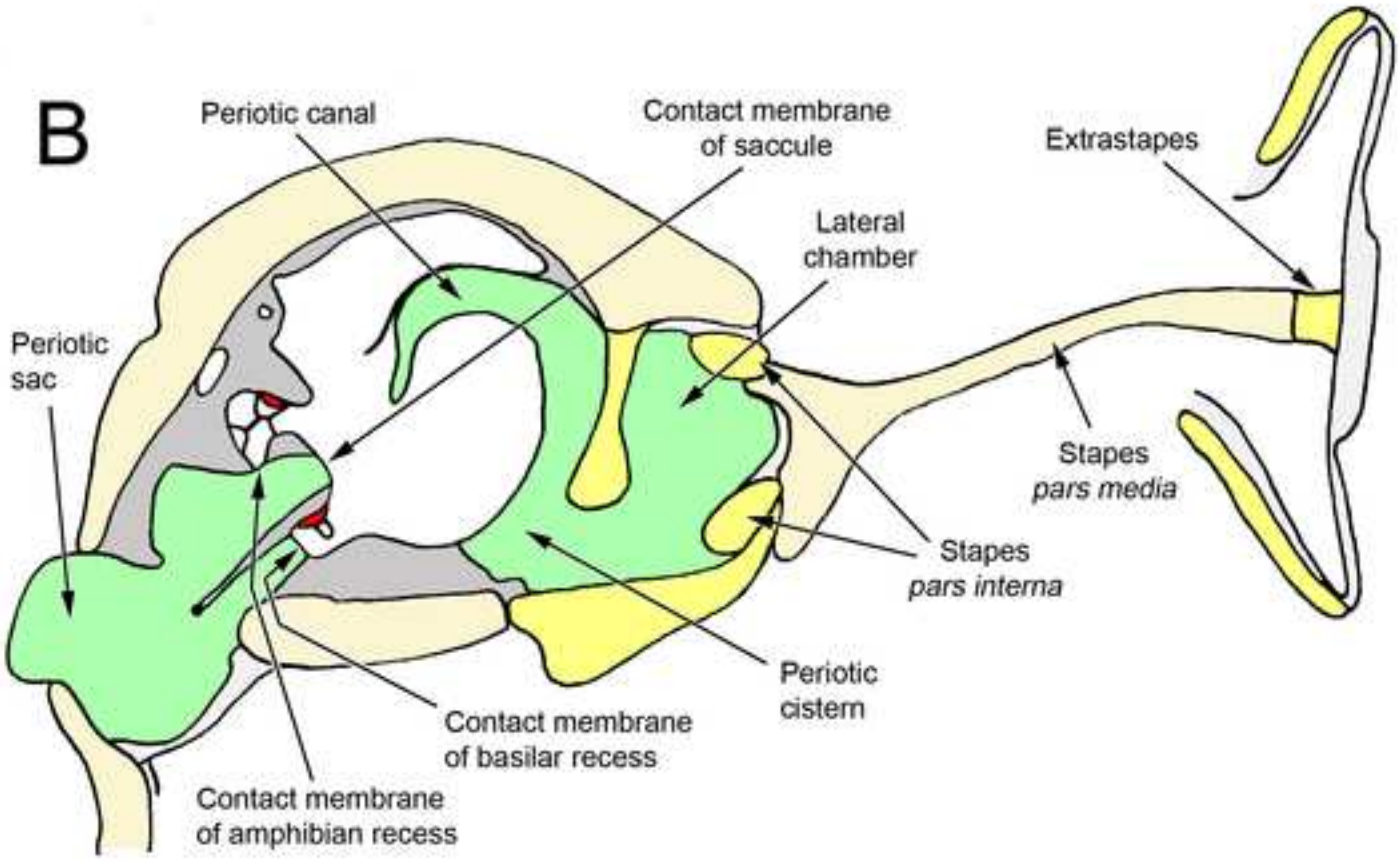


Figure 2
Click here to download high resolution image

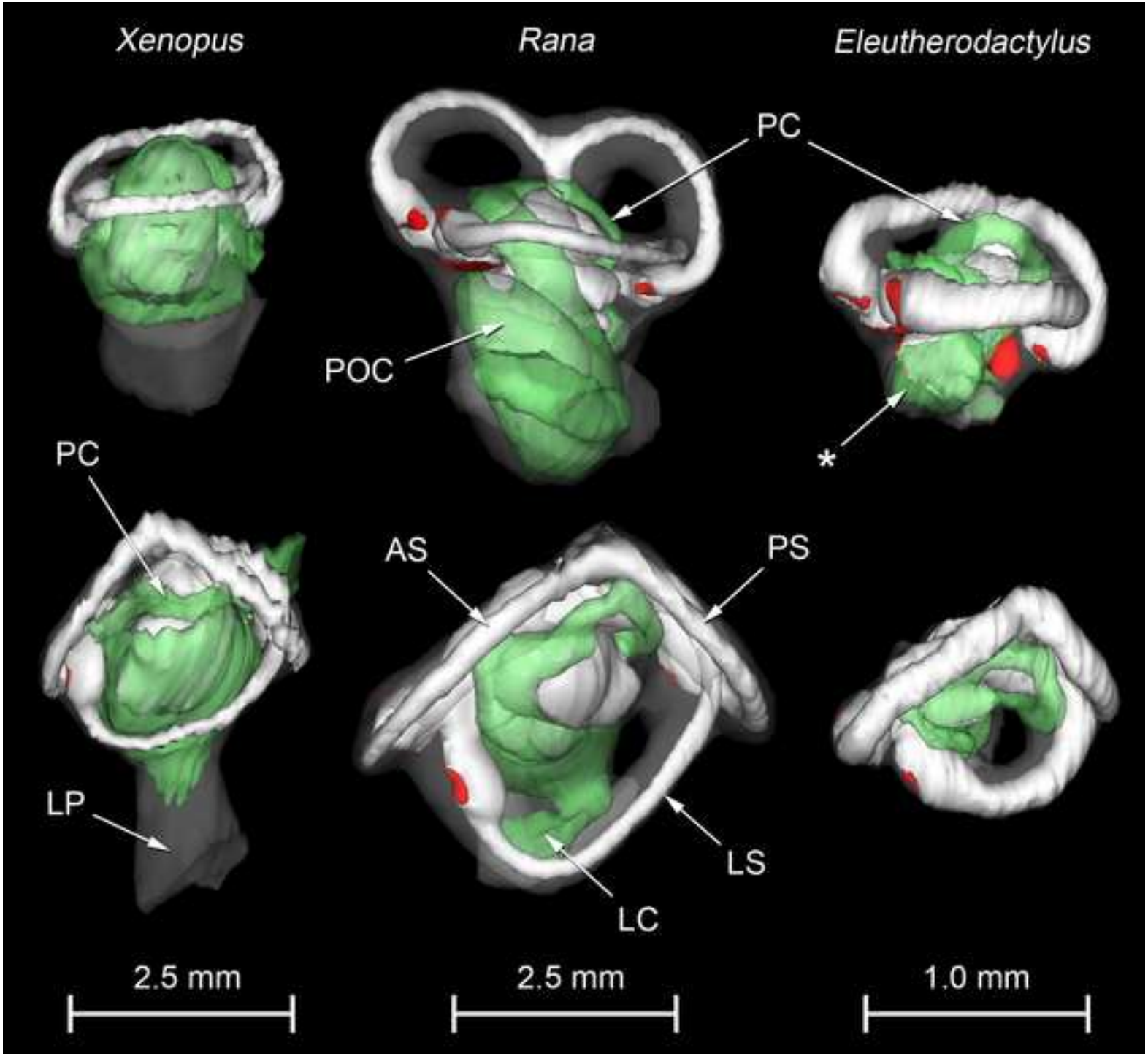


Figure 3
Click here to download high resolution image

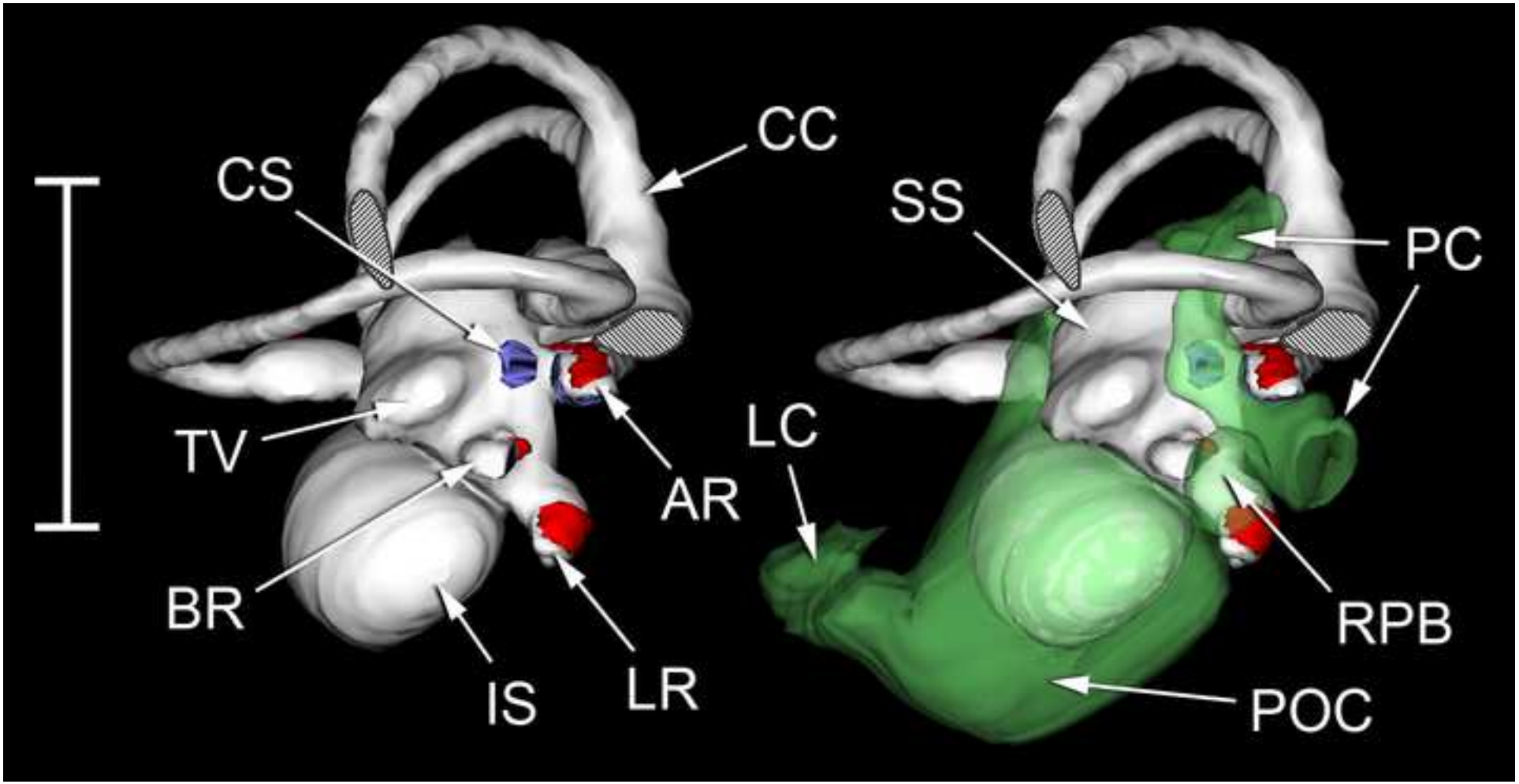


Figure 4
Click here to download high resolution image

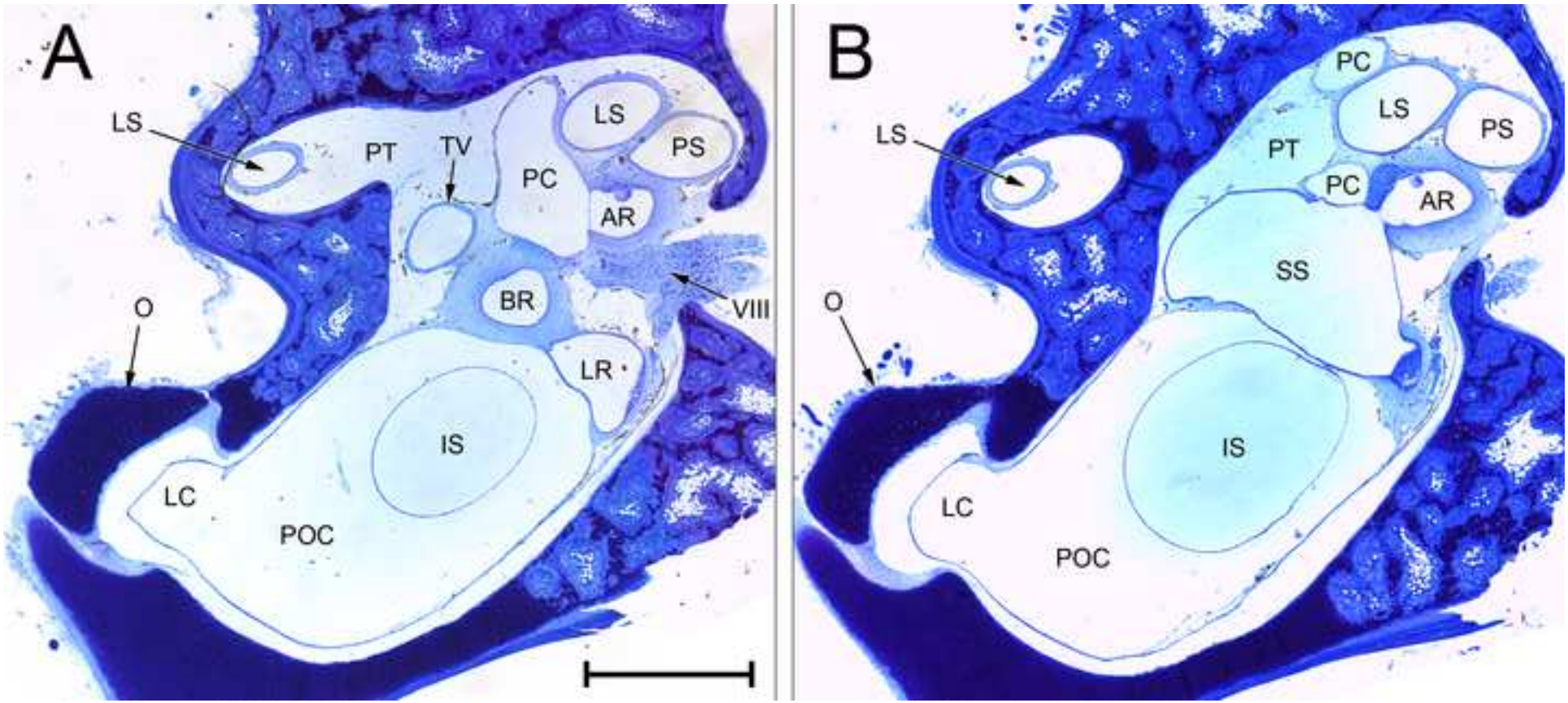


Figure 5
Click here to download high resolution image

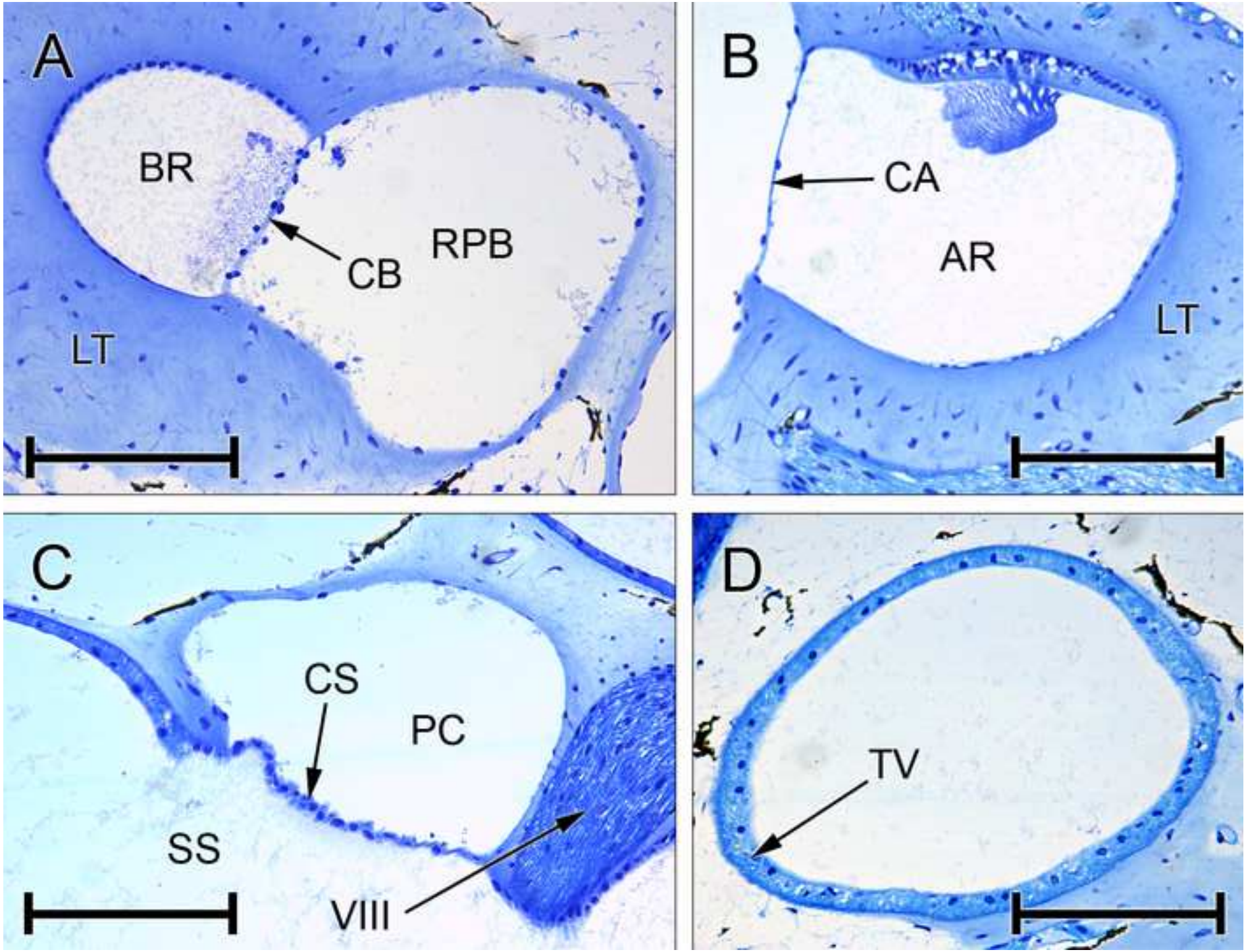


Figure 6
Click here to download high resolution image

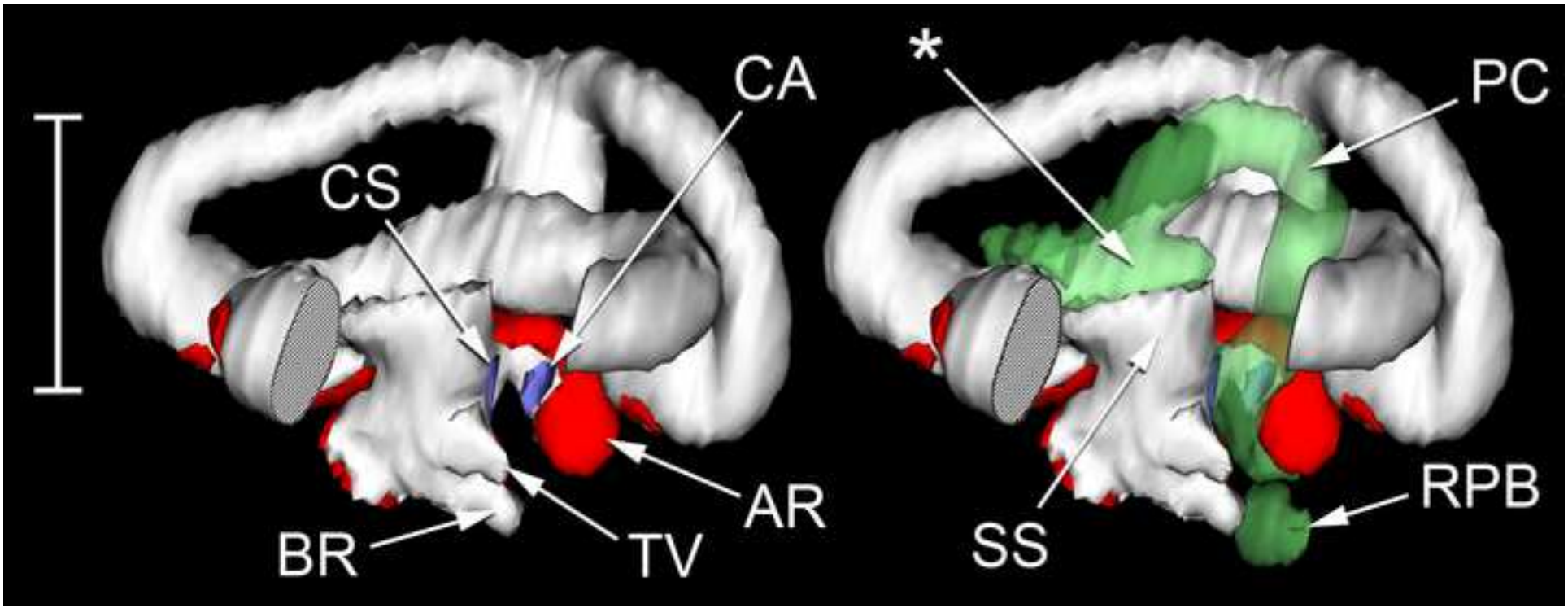


Figure 7
Click here to download high resolution image

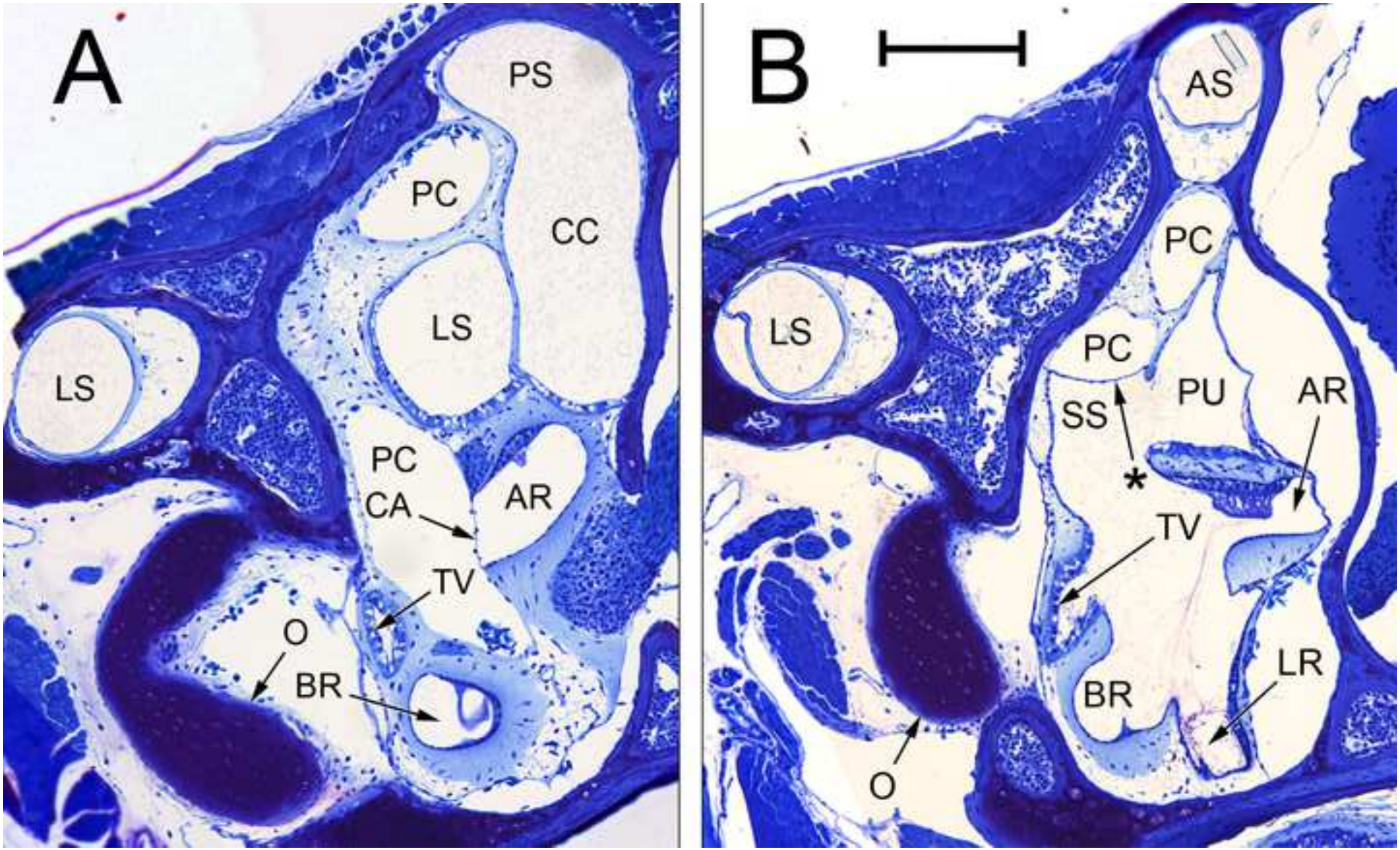


Figure 8
Click here to download high resolution image

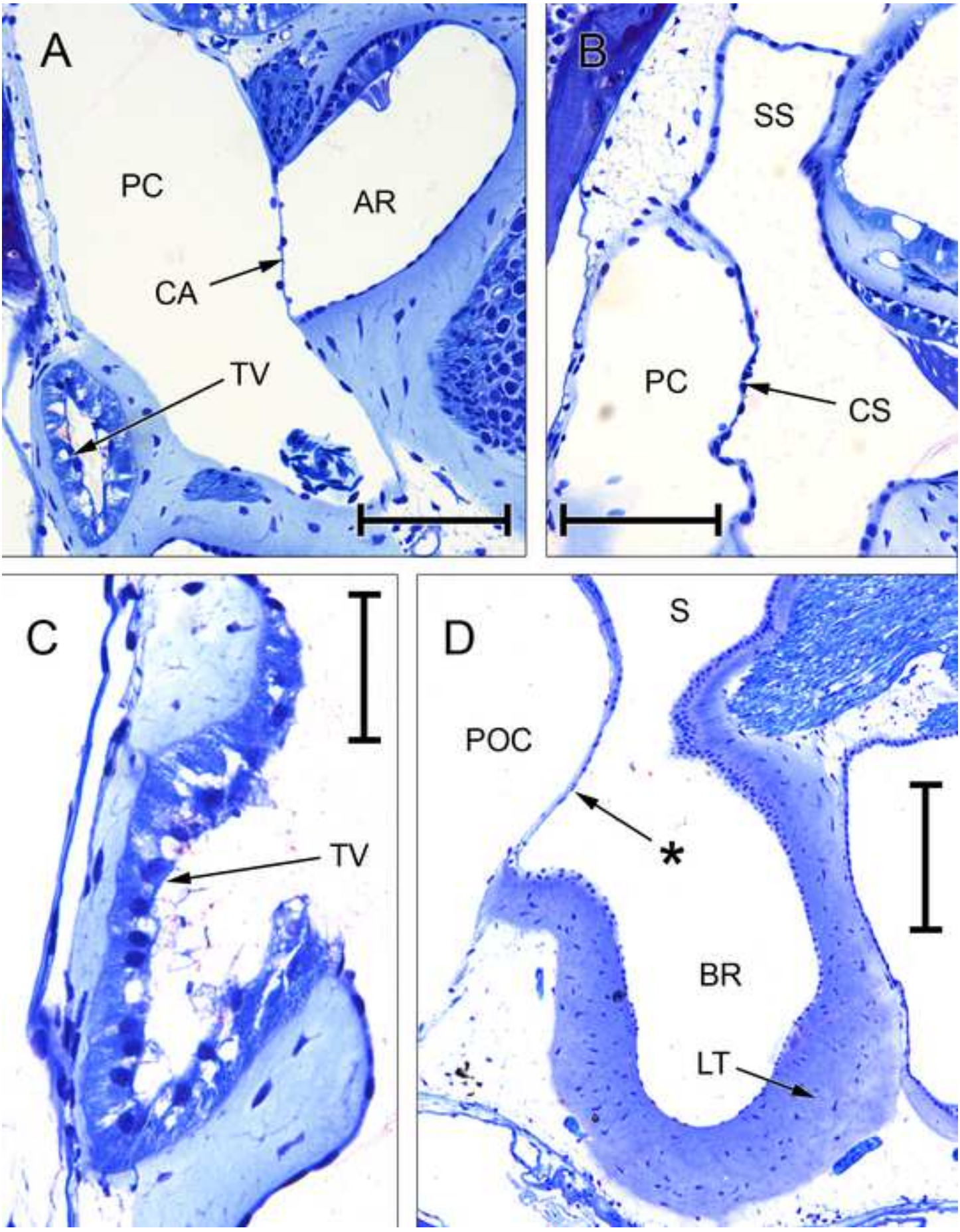


Figure 9
Click here to download high resolution image

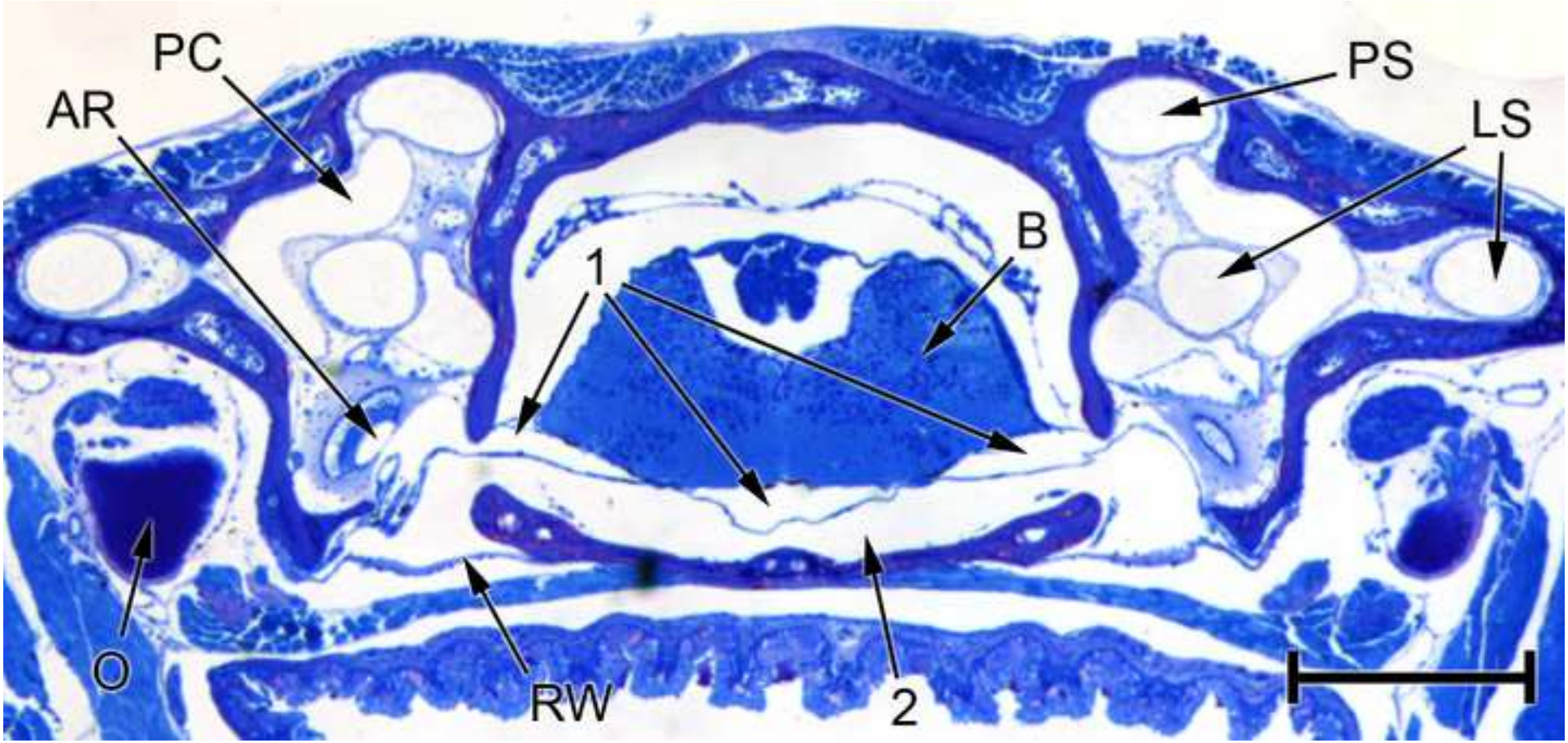


Figure 10
Click here to download high resolution image

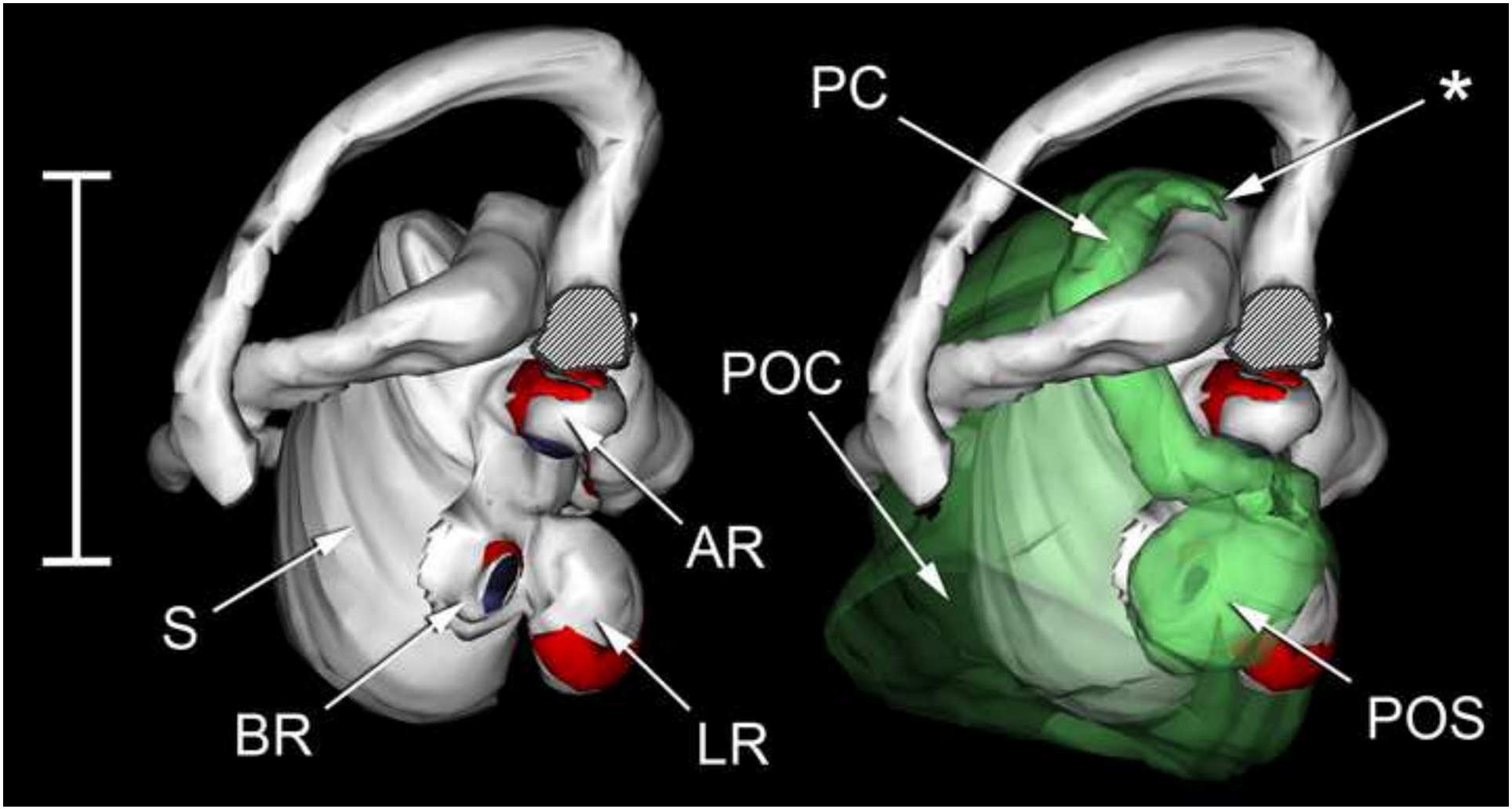


Figure 11
Click here to download high resolution image

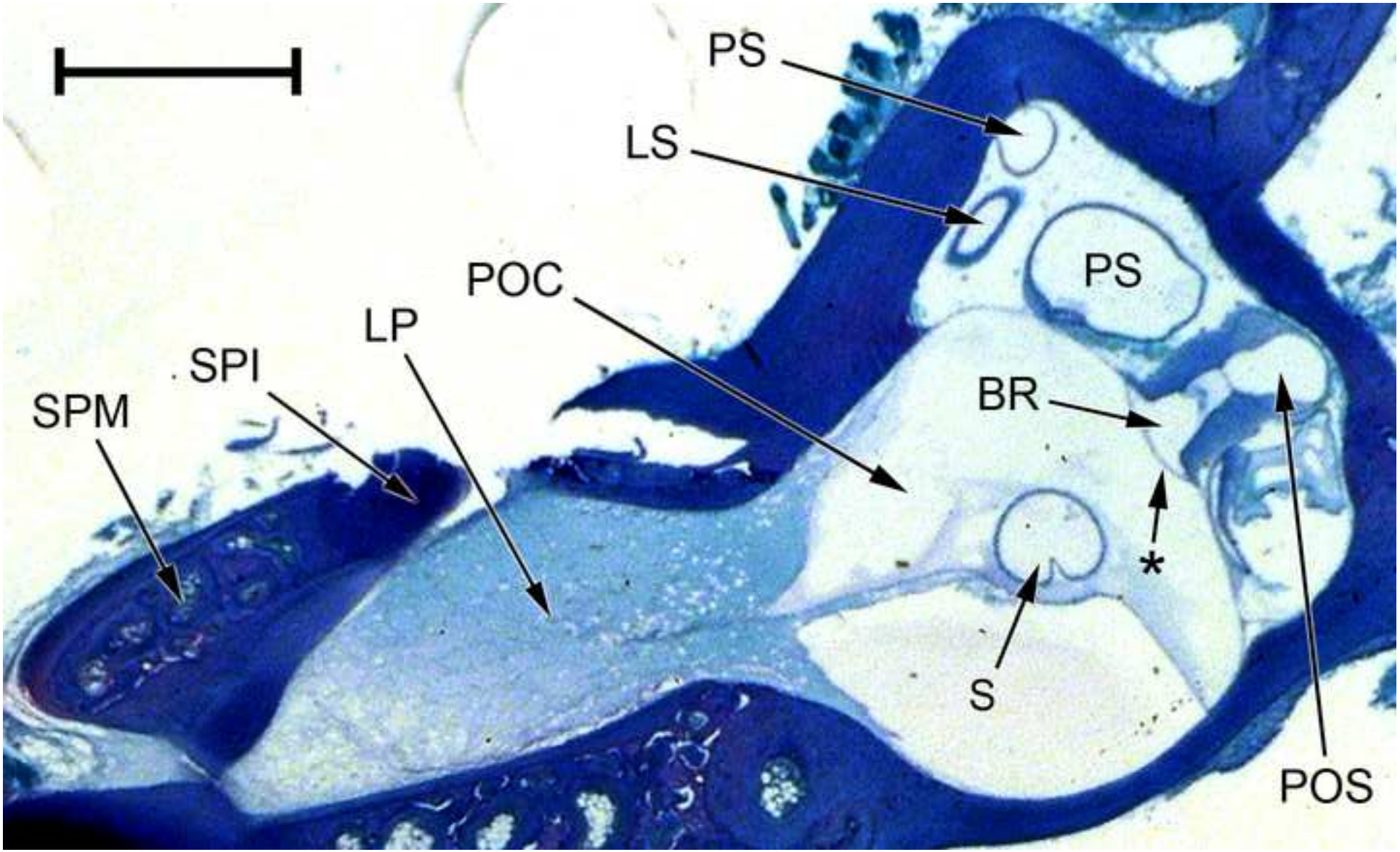
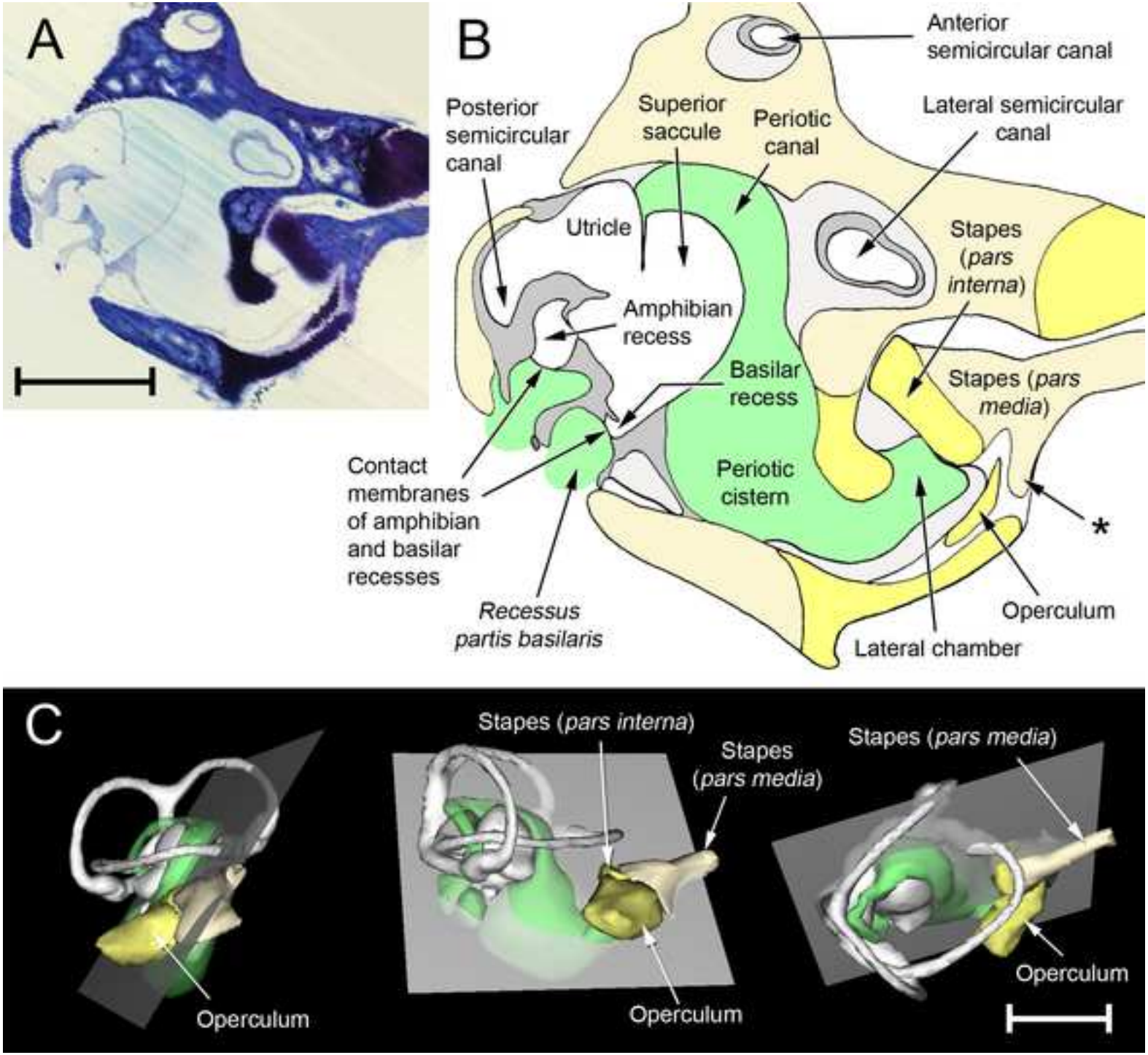


Figure 12
Click here to download high resolution image



Conflicts of interest disclosure statement

The authors declare that they have no conflicts of interest.