DEVELOPMENTAL DYNAMICS 227:422–430, 2003

PATTERNS

Three-Dimensional Morphology of Inner Ear Development in *Xenopus laevis*

Michele Miller Bever, Ying Y. Jean, and Donna M. Fekete*

The three-dimensional morphology of the membranous labyrinth of *Xenopus laevis* is presented from embryonic through late tadpole development (stages 28 to 52, inclusive). This was accomplished by paint-filling the endolymphatic spaces of *Xenopus* ears at a series of stages, beginning with the embryonic otic vesicle and ending with the complex ear of the late tadpole. At stage 52, the inner ear has expanded approximately 23-fold in its anterior/posterior dimension compared with stage 28 and it is a miniature of the adult form. The paint-filling technique illustrates the dramatic changes required to convert a simple ear vesicle into the elaborate form of the adult, including semicircular canal formation and genesis of vestibular and auditory organs, and it can serve as a basis for phenotype identification in experimentally or genetically manipulated ears. *Developmental Dynamics 227:422–430, 2003.*

Key words: frog; otic vesicle; semicircular canal; basilar papilla; amphibian papilla; saccule; utricle; lagena

Received 24 January 2003; Accepted 20 March 2003

INTRODUCTION

The amphibian ear shares with other vertebrates conserved anatomy of the pars superior, similar mechanisms of auditory and vestibular physiology, and comparable auditory information flow and processing in the brain (Fay and Popper, 1999; Lewis and Narins, 1999). The African clawed frog, *Xenopus,* in particular, is well documented with respect to general development (Nieuwkoop and Faber, 1994) as well as ear development (Paterson, 1948; Nieuwkoop and Faber, 1994) and is commonly the amphibian genus of choice for molecular and genetic techniques (Vize et al., 1991; Amaya and Kroll, 1999; Bronchain et al., 1999; Beck and Slack, 2001; Klein et al., 2002). *Xenopus tropicalis* has been promoted recently because of several advantages over *Xenopus laevis* for transgenic studies (Beck and Slack, 2001; Hirsch et al., 2002). Although this atlas presents morphogenesis of the *Xenopus laevis* inner ear, preliminary work by Serrano (2001) compared the postmetamorphic ears of these two closely related species and demonstrated that the sensory organs are similar in size and organization.

Wever (1985) described the anatomy and functional sensitivity of the ears of adult anura, including the primitive family Pipidae to which *Xenopus* belongs (see also review by Elepfandt, 1996). *Xenopus* continues an aquatic mode of life as an adult, and its ear structure reflects some modifications for efficient aquatic

ABBREVIATIONS: A, anterior; **aa,** anterior ampula; **ab,** abdominal cavity; **ac,** anterior semicircular canal; **acp,** anterior canal pouch; **acr,** anterior crista; **ap,** amphibian papilla; **apr,** amphibian papilla recess; **bp,** basilar papilla; **bpr,** basilar papilla recess; **cc,** common crus; **cm,** contact membrane; **D,** dorsal; **e,** eye; **ed,** endolymphatic duct; **es,** endolymphatic sac; **ha,** horizontal ampulla; **hc,** horizontal semicircular canal; **hcp,** horizontal canal pouch; **hcr,** horizontal crista; **l,** lagena; **lm,** lagenar macula; **M,** medial; **pa,** posterior ampulla; **pc,** posterior semicircular canal; **pcp,** posterior canal pouch; **pcr,** posterior crista; **pi,** pars inferior; **ps,** pars superior; **s,** saccule; **sm,** saccular macula; **u,** utricle; **um,** utricular macula; **VIIIn,** branches of the eighth nerve.

Department of Biological Sciences, Purdue University, West Lafayette, Indiana
Grant sponsor: NIH; Grant number: F32 DC00413; Grant sponsor: March of Dimes Birth Defects Foundation; Grant number: FY00-434.
***Correspondence to: Donna M. Fekete, 915 West State Street, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907. E-mail: dfekete@purdue.edu**

DOI 10.1002/dvdy.10316

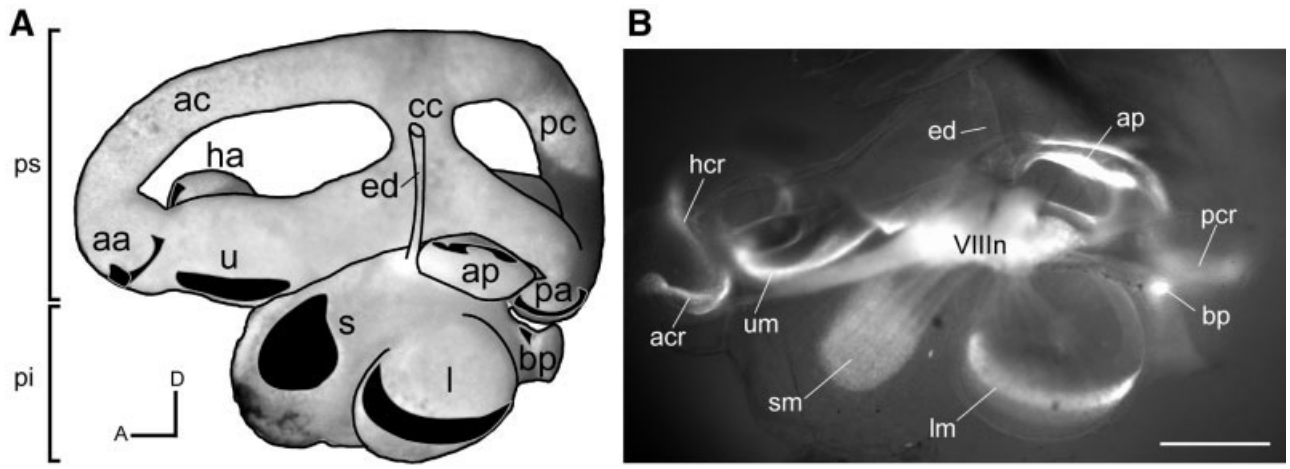

Fig. 1. Structure of the inner ear in *Xenopus laevis*. **A:** Semi-diagrammatic medial view of a right inner ear based on a stage 52 paint-filled ear, with the location of the sensory organs indicated in black. The three semicircular canals and the utricle comprise the pars superior, whereas the pars inferior consists of the saccule, lagena, amphibian papilla, and basilar papilla. The endolymphatic duct is shown joining the pars inferior, but the extensive dorsal endolymphatic sac is not depicted. **B:** Whole-mounted medial view of a stage 60 right inner ear with the sensory epithelia labeled using an anti–hair cell antibody. Branches of the eighth nerve are still attached to the ear and also exhibit fluorescence. For Abbreviations, see list. Scale bar = 0.5 mm in B.

sound conduction, especially in the external ear. But, like its terrestrial relatives, the *Xenopus* middle ear is an air-filled cavity containing a columella whose medial end forms a footplate at the oval window and its inner ear has the same general structure as other anuran ears. Paterson (1948) illustrated the development of *Xenopus* inner ears and the associated perilymphatic spaces using drawings of histologic sections and diagrammatic reconstructions. The fully developed inner ear has two major divisions (Fig. 1): the pars superior, consisting of a utricle and three semicircular canals (anterior, posterior, and horizontal); and the pars inferior, consisting of saccule, lagena, basilar papillar recess, and amphibian papillar recess. The extensive endolymphatic sac is connected to the ear by means of an endolymphatic duct that arises from the medial wall of the ear, near the junction of the pars inferior and pars superior. As with other vertebrates, the sensory epithelia of the semicircular canals and utricle (the cristae and utricular macula, respectively, Fig. 1A,B) serve as sensors for equilibrium. In *Xenopus,* the lagenar macula is a band of sensory epithelium located on the ventromedial wall of the lagena (Fig. 1A,B). In anura, the lagenar maculae may provide acoustic information in the form of vibration sensitivity, in addition to gravitational information, at least in the more derived forms (Lewis and Narins, 1999). The saccular macula is a tear-drop shaped patch of sensory epithelium located on the medial wall of the saccule (Fig. 1A,B). At least in terrestrial anura, the saccular maculae serve an acoustic function and appear to respond best to excitation frequencies below 100 Hz, such as substrate-derived seismic signals (Lewis and Narins, 1999). The principal acoustic detectors in frogs, the amphibian papilla and the basilar papilla, are found on the roofs of two recesses of the pars inferior (Fig. 1A,B). In *Xenopus,* these acoustic sensory surfaces provide a hearing range of 200–3,900 Hz, with the amphibian papilla most sensitive to frequencies below 1,000 Hz and the basilar papilla most sensitive to frequencies above 1,000 Hz (Wever, 1985; Elepfandt, 1996). The basilar papilla in anurans is often tuned to a component of the animal's mating call, whereas the amphibian papilla is a "general-purpose acoustic sensor" (Lewis and Narins, 1999) that is functionally and structurally analogous in many ways to the hearing organs of other vertebrates (Fay and Popper, 1999; Lewis and Narins, 1999).

The amphibian is a frequent model for investigations of the ear, including evolutionary or comparative studies (Fritzsch and Wake, 1988; Fritzsch, 1990; Reiss, 1997), anatomic or histologic studies (Diaz et al., 1995; Gao et al., 1998; Gale et al., 2000), or studies that focus on questions of development or regeneration (Wiederhold et al., 1995; Gallagher et al., 1996; Lopez-Anaya et al., 1997; Bever and Fekete, 1999; Horowitz et al., 2001; Kil and Collazo, 2001; Gale et al., 2002). The purpose of this project was to provide an atlas that would illustrate the morphogenetic events of the developing normal inner ear and that could serve as a basis for phenotype identification in experimentally manipulated ears. We present images that demonstrate the three-dimensional features of normal inner ear development in *Xenopus laevis.*

RESULTS AND DISCUSSION

In *Xenopus laevis,* the inner ear begins as a placode in the stage 23 embryo. This placode invaginates and forms a closed vesicle that is completely separated from the overlying epidermis by stage 28 (Nieuwkoop and Faber, 1994). Figure 2 shows the appearance of methyl salicylate-cleared specimens at two stages of development and the location of the developing inner ear with respect to other body struc-

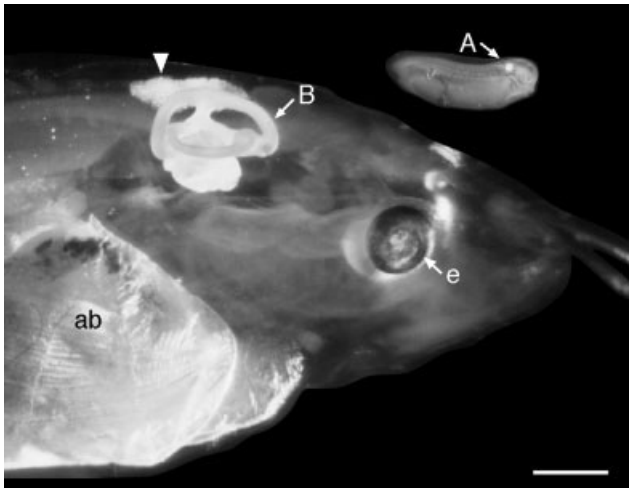

Fig. 2. Paint-filled inner ears are shown in intact, fixed *Xenopus laevis* specimens at stage 28 (**A**) and stage 52 (**B**) to demonstrate the location and relative size of the developing ear. Images are lateral views. Inner ears were filled with paint to visualize the inner surface of the membranous labyrinth. The endolymphatic sac is visible in the stage 52 specimen (arrowhead), even though it did not fill with paint (see text). Total length of stage 52 specimen is 25 mm from tip of snout to tip of tail. Scale bar = 1.0 mm.

tures. With the paint-fill method, diluted paint replaces the fluid that fills the membranous labyrinth, ultimately leaving an "impression" of the inner surface contours of the labyrinth but not labeling the sensory organs directly. In the stage 28 embryo, the otocysts have just pinched off from the overlying epithelium. Each consists of a simple sac approximately 75 μm in anteroposterior (AP) and 110 μm in dorsoventral (DV) diameter. It is located approximately 125 μm posterior and dorsal to the eye (Fig. 2A). By comparison, the ear of a stage 52 tadpole (Fig. 2B), located approximately 1.5 mm posterior and dorsal to the eye, is 1.76 mm in its AP dimension (~23-fold increase) and 1.35 mm in its DV dimension (~12-fold increase) and gross morphogenesis of its component parts is complete. All animals used in this study were staged based on the external criteria described by Nieuwkoop and Faber (1994). Specimen length was not used as an indicator of stage because of its high variability within and between batches of tadpoles raised in captivity. However, to facilitate comparisons with our work, we include length measurements (average ± SD) for specimens used in this study (see Experimental Procedures section).

Stages 28–40

This atlas begins with the otic vesicle of a stage 28 embryo, when the otocyst is a simple fluid-filled sac (Figs. 3A, 4). In the young hatchling (stage 33/34), the vesicle becomes pear-shaped and narrows to a point dorsomedially at the origin of the nascent endolymphatic duct (Figs. 3B, 4). By stage 40, the ear is more heart-shaped in the lateral view and is dominated by the pars superior (Figs. 3C, 4). The ventral pole of the vesicle is the pars inferior. Extending above the pars superior is the tip of the endolymphatic duct. We know from sectioned specimens that the endolymphatic duct is present at this stage as a narrow tube arising from the medial wall near the junction of the pars superior and pars inferior (Paterson, 1948; Bever and Fekete, 1999). The duct and its sac often did not fill with paint, but the duct can be viewed at this stage and in several of the representative older specimens (see below).

Stages 43–46

Although stage 43 and stage 44 hatchlings can be distinguished readily based on external criteria, the morphology and size of the paint-filled inner ear remains similar (Fig. 3D,E). The pars inferior is still a small ventral sac (Fig. 3D,E, lateral and ventral views, and Fig. 4). In the pars superior, however, three pouches expanding in the lateral, anterior, and posterior directions presage the horizontal, anterior, and posterior canals, respectively (Fig. 3D,E, lateral views, and Fig. 4). Haddon and Lewis (1991) showed that canal formation occurs when protrusions originating from the otocyst wall grow centrally and fuse. This process is initiated first for the horizontal canal at stage 43 (Paterson, 1948; Haddon and Lewis, 1991). The crista of the horizontal canal is already evident as an indentation into the paint-filled fluid labyrinth by stage 43 (Fig. 3D, lateral view, and Fig. 4). At stage 44, the dimples representing the anterior and posterior cristae are also discernable with careful oblique lighting and appropriate specimen orientation (not shown).

At stage 46, a constriction is evident where the anterior and posterior canals meet dorsally to join the common crus (arrowheads in dorsal and medial views, Fig. 3F). In addition, the contour of the utricle is visible in the medial view and the anterior crista is more easily distinguished (Fig. 3F, lateral view, and Fig. 4). In the pars inferior, the saccule is now a prominent pouch.

Stages 47–52

The formation of the semicircular canals by fusion of the axial protrusions is completed for all three canal pouches by stage 47 (Fig. 3G,H, lateral and medial views). The process of canal formation is roughly similar in other vertebrates, with pillars (zebrafish) or plates (birds and mammals) joining centrally to demarcate the canals (Knowlton, 1967; Waterman and Bell, 1984; Martin and Swanson, 1993; Haddon and Lewis, 1996). In the early stage 47 paint-filled specimens, the canals are distinct from the utricle but still tightly apposed to its surface. In subsequent stages, the canals gradually arch away from the utricle (Fig. 3I–L) and are separated from it by interposing mesenchyme. An ampulla at the end of each canal increases in size and becomes more bulbous (e.g., compare horizontal ampulla in stage 47 with stage 52 in Fig. 3G,L,

lateral and dorsal views). In addition, the ampullae and the cristae housed within them gradually shift in orientation with respect to the three-dimensional axes of the ear. For example, the crista of the horizontal canal, which at stage 46 is a medial projection into the lumen of the ampulla (Fig. 3F, lateral view), projects more directly posterior into the stage 52 horizontal ampulla (Fig. 3L, lateral, dorsal, and ventral views). Similarly, a pivot of the anterior and posterior ampullae leaves these cristae directed dorsally into their respective ampullae (Fig. 3L, ventral view).

The elaboration of the pars inferior begins around stage 47 (Paterson, 1948; Nieuwkoop and Faber, 1994) and involves the formation of three diverticula from the saccule. Although the differentiation of the sensory epithelia is apparent in histologic sections at earlier stages (Paterson, 1948; Bever, unpublished observations), in paint-filled ears, the outpocketings that house the sensory epithelia are not clearly perceptible until stage 47. The recess for the amphibian papilla is the most evident of the diverticula early in stage 47. It appears as a medial evagination at the junction of the utricle and saccule, just posterior to the endolymphatic duct (Fig. 3G, medial view; see also Fig. 5B). Also during stage 47, the basilar papilla recess evaginates from the posteromedial wall of the dorsal saccule (Fig. 3H, medial view). Later, the location of the contact membrane that separates this recess from the adjacent perilymphatic space is apparent as the flattened posteromedial wall of the recess (Fig. 3I–L). The lagena is a larger medial diverticulum that remains in broad communication with the saccule and is not clearly demarcated in paint-filled specimens until stages 49–50 (Fig. 3J,K).

The endolymphatic duct is visible as a thin tube originating anterior to the amphibian papilla recess and coursing along the medial wall of the common crus (Fig. 3H,I, medial views). At the end of the duct, the endolymphatic sac expands dorsomedially into a highly lobulated structure that is apparent even when unfilled with paint (Figs. 2A, 5A,B) because of the accumulation of calcareous material. We refer readers to the publication of Paterson (1948) for an extensive treatment of the morphogenesis and elaboration of the endolymphatic sac apparatus in *Xenopus*.

Otoconia and Sensory Organs

In paint-filled ears of late stage tadpoles, the locations of most of the sensory epithelia can be identified by indentations (e.g., the cristae, described above) or rough-surfaced areas (e.g., the maculae with their overlying otoconia). A single (saccular) otoconial mass is visible by stage 46 in methyl salicylate-cleared but unfilled specimens (not shown). During stage 47, the otoconia of the utricle and endolymphatic sac emerge, and the lagenar otoconia develop as an extension of the saccular mass to produce the arrangement seen in the stage 49 ear in Figure 5A. Some specimens had otoconia within the endolymphatic duct, extending between the saccular otoconia and the endolymphatic sac otoconia (Fig. 5A). The otoconia sometimes extended the length of the duct but more frequently were scattered within it. Whether this finding is a normal phenomenon or an artifact of processing remains to be determined, although it has been suggested that the endolymphatic sac may generate otoconia that are transported to the vestibule for use by the otolithic organs (Imoto et al., 1983).

Because the presence of otoconia often disturbs paint flow within the ear, these areas can be recognized in many specimens, especially with foreknowledge of their final location and appearance (Fig. 1B). A note of caution, however: the quality and density of the paint-loading and the integrity of the otoconial masses will affect how readily and accurately one can distinguish the locations of sensory epithelia. The utricular macula is apparent in the ventral view as early as stage 47 (Fig. 3G) as an ovoid rough spot on the utricular floor just posterior and medial to the anterior ampulla. By stage 48, the saccular macula is visible as a teardrop-shaped impression on the medial wall of the saccule (Fig. 3I–L, medial views). The lagenar sensory epithelium is evident in stages 49–52 as a band that extends from anterodorsal to posteroventral along the medial wall of the lagenar diverticulum (Fig. 3J–L, medial views). The amphibian and basilar papillae are not otolithic organs, but their papillar structures can form impressions in the paint. The tiny basilar papilla (Fig. 3L, medial view) is located on the saccular side of a transverse ridge that appears in paint-filled specimens as an indentation on the roof of the cylindrical basilar papilla recess (Fig. 3I–L) and is discernable in some paint-filled specimens by stage 48. The amphibian papilla, located on the roof of the AP recess (Fig. 1B), is difficult to discern in paint-fills, unless the recess is viewed from a dorsomedial perspective with appropriate oblique lighting. In stage 48 and older specimens, it is visible as a gentle depression (Fig. 5B).

Metamorphosis

Our paint-fill analysis stops before metamorphosis because the components of the inner ear are formed by late tadpole stage 50 (Paterson, 1960; Nieuwkoop and Faber, 1994). The perilymphatic (periotic) system, which we have not addressed here, is also completed before metamorphosis (Paterson, 1948; Nieuwkoop and Faber, 1994). However, fine-tuning of the inner ear continues through and past metamorphosis with, for example, the formation of the contact membrane in the AP recess and elongation of the AP sensory epithelium (Smirnov, 1993), increases in the numbers of hair cells and VIIIth nerve axons (Fritzsch et al., 1988; Diaz et al., 1995; Lopez-Anaya et al., 1997), and continued expansion and lobulation of the endolymphatic sac (Paterson, 1948). Additional critical events in the development of the adult ear occur during metamorphosis, such as the formation of the middle ear from its anlage beginning at stage 56 and the appearance of the tympanic membrane anlage at stage 63 (Nieuwkoop and Faber, 1994).

Comparison with Other Vertebrates

There is a paint-fill–based atlas of inner ear development for one com-

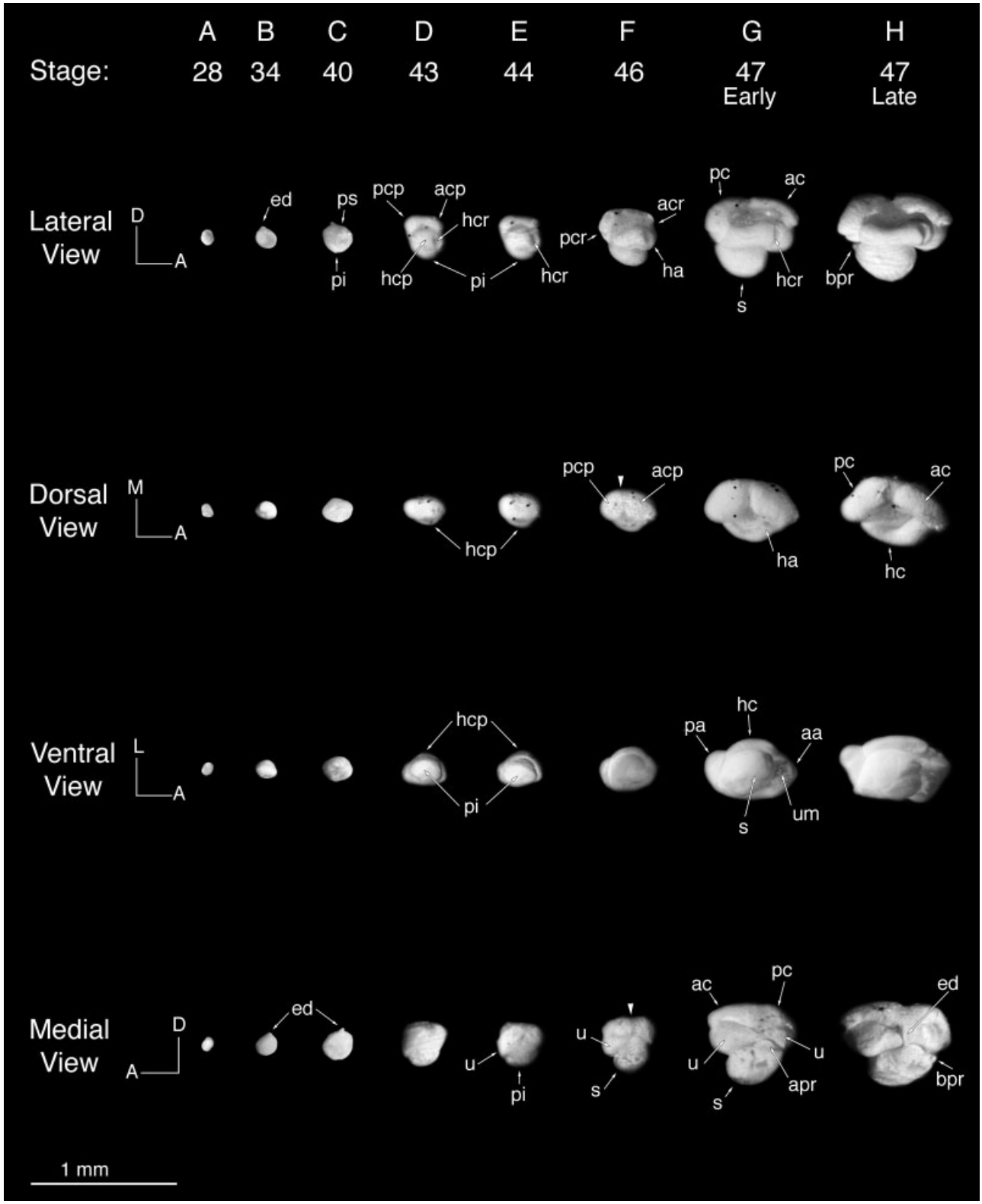

Fig. 3. Atlas of the developing *Xenopus laevis* inner ear. The endolymph-filled spaces of the inner ear were injected with a solution of alkyd paint and imaged to present three-dimensional views of the inner surface of the ear as it develops from stage 28 to stage 52 (staging based on Nieuwkoop and Faber, 1994). All specimens are shown at the same magnification. For Abbreviations, see list. Scale bar = 1 mm.

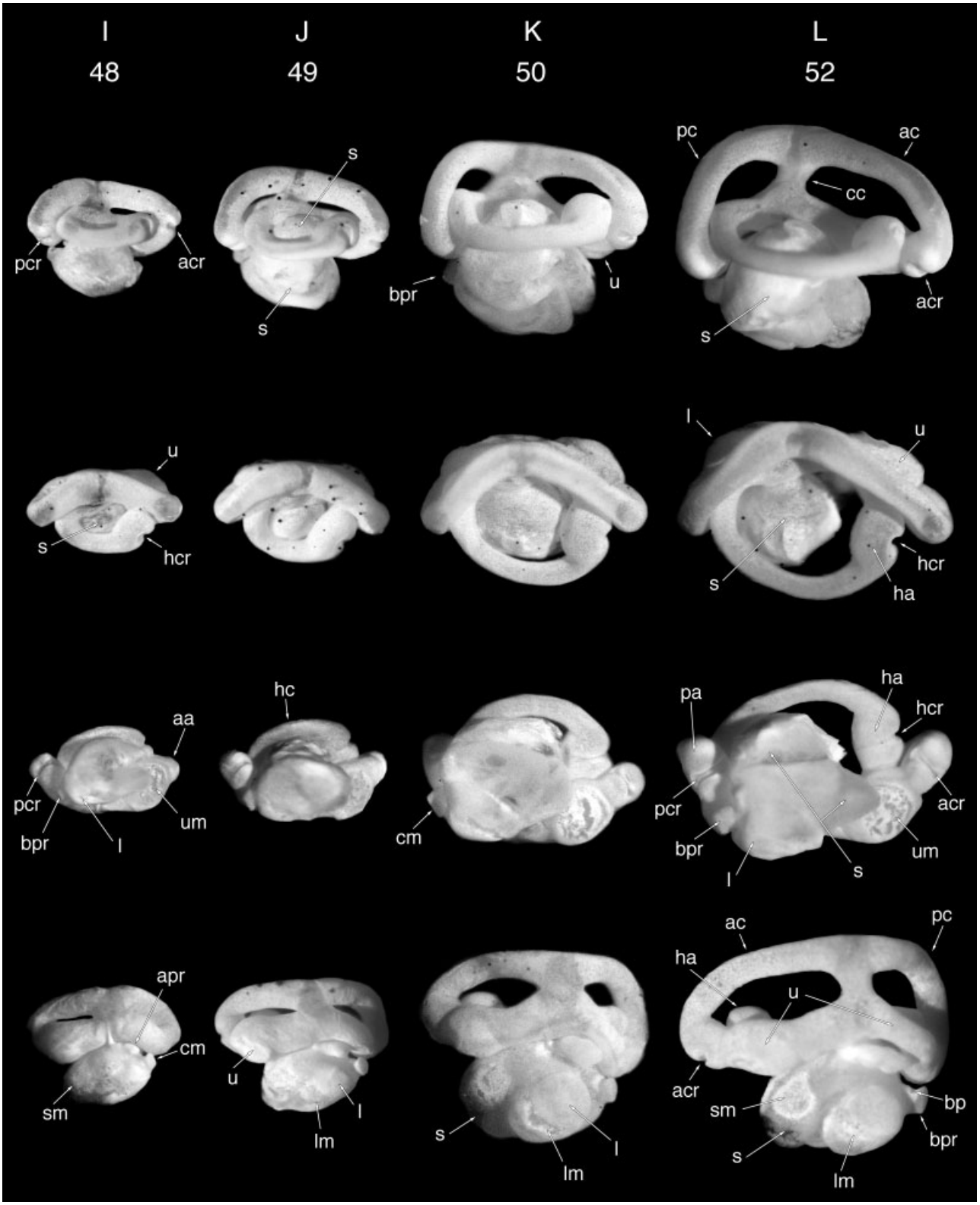

Fig. 3. (Continued.)

monly studied species in each of four vertebrate classes: zebrafish (Bever and Fekete, 2002), chicken (Bissonnette and Fekete, 1996), mouse (Morsli et al., 1998), and now *Xenopus*. A comparison across classes illustrates the morphologic similarities of the ears, especially in the pars superior, as well as the

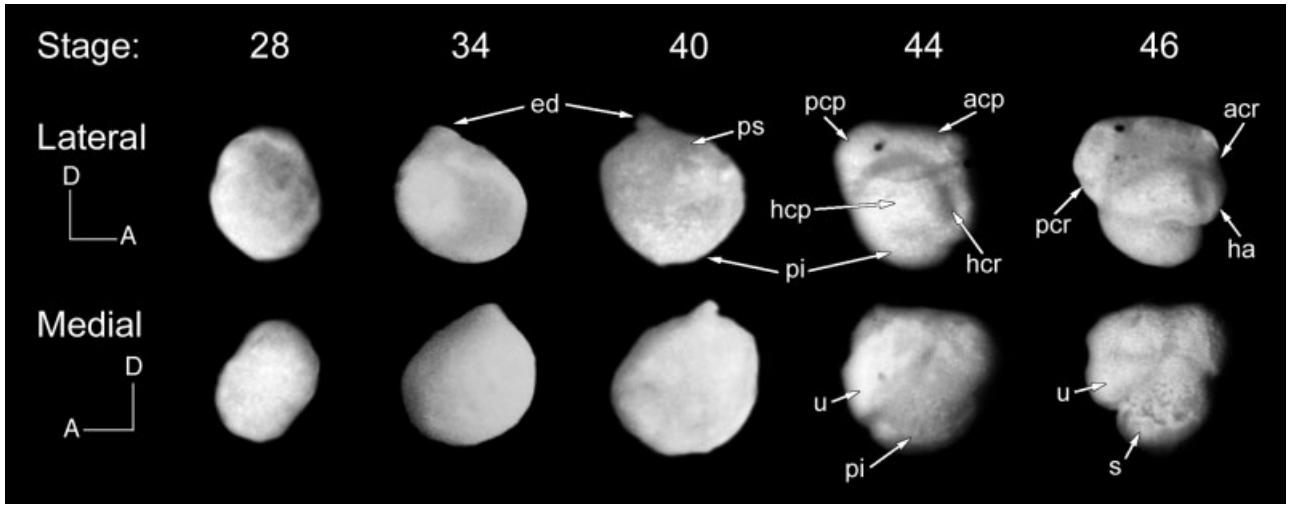

Fig. 4. Selected images enlarged from Figure 3. Lateral and medial aspects of ears at early stages reveal the emerging morphologic features of the developing otocyst. Ear images from stages 28–44 were approximately normalized to the stage 46 images; refer to Figure 3 for accurate size comparisons. For Abbreviations, see list.

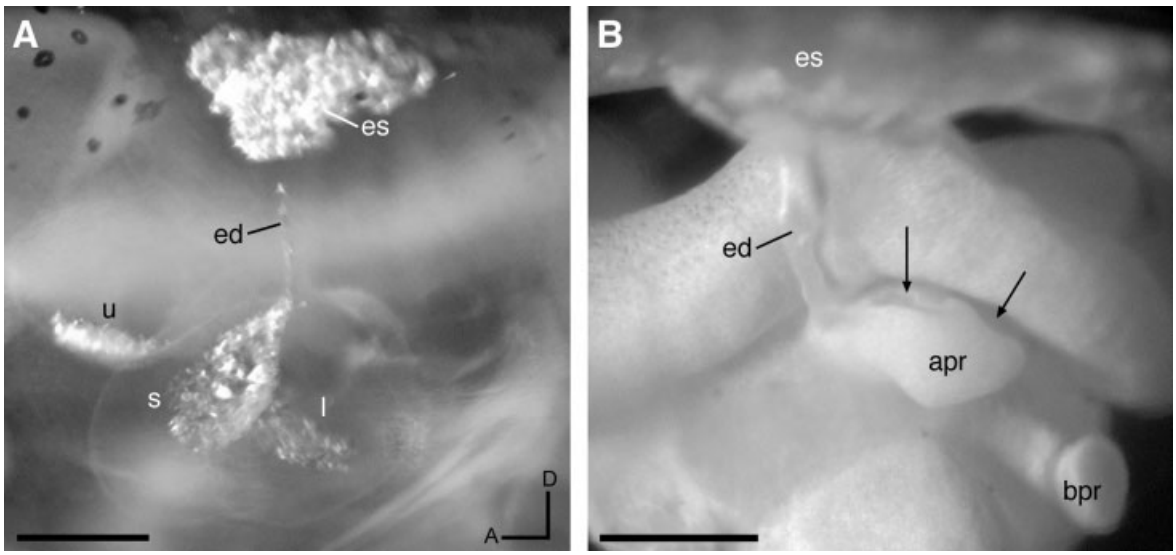

Fig. 5. Anatomic details of the *Xenopus laevis* inner ear. **A:** Otoconia in a stage 49 left inner ear, lateral perspective, in a methyl salicylate-cleared specimen that was not injected with paint. **B:** The location of the amphibian papilla (arrows) on the roof of its recess and the position of the endolymphatic duct are demonstrated in this dorsomedial view of a stage 51 paint-filled right inner ear. The endolymphatic sac is out of focus in the dorsal foreground and partially obscures the anterior and posterior semicircular canals. Axis serves both images. For Abbreviations, see list. Scale bars = 250 μm in A,B.

unique properties of each ear "type," most notably highlighted by morphologic differences in the auditory organs of each class and chronotypic variations in the ear development process. By using the staging provided by Nieuwkoop and Faber (1994), *Xenopus* requires approximately 15 days to transform an otocyst to an inner ear. In contrast, the chicken completes this process in 6 days, the mouse in 8 days, and the zebrafish in approximately 20 days. Notably, the ectotherms are significantly slowed in their completion of ear morphogenesis compared with the endotherms. While the *Xenopus* ear at hatching stage is merely a simple vesicle with a small dorsal endolymphatic protrusion, the hatchling zebrafish already has a completed set of semicircular canals. In mouse and chicken, the semicircular canals are completed within the first half of the ear development period, whereas in *Xenopus,* the canals are completed within the first third of the ear development period. The appearance of the diverticulae that house the hearing organs also varies across species. In mouse and chicken, the outgrowth of the pars inferior that presages the cochlear duct occurs before formation of the semicircular canals and before differentiation of any auditory hair cells (Sher, 1971; Cotanche, 1987). In zebrafish, an overt diverticulum for the hearing organ (saccule) of the pars inferior forms over a week after the first differentiated hair cells appear (Haddon and Lewis, 1996).

And, for *Xenopus* pars inferior, the recesses for the amphibian and basilar papillae appear at stage 47, at the same time that canal genesis is being completed and during a period in which, at least in the bullfrog (Boatright-Horowitz and Simmons, 1997), tadpole auditory sense is already functional.

EXPERIMENTAL PROCEDURES

To initiate breeding, adult male and female albino *Xenopus laevis* frogs were injected with human chorionic gonadotropin (HCG). Males were injected into the dorsal lymph sac with 300 international units (IU) of HCG, followed 4 hr later with a secondary dose (100 IU). Females received a 150 IU of HCG and a 600 IU secondary dose 4 hr later. After the secondary dose, males and females were placed together in breeding tanks. The embryos were collected approximately 12–14 hr later and maintained in aerated well water at 22°C. Beginning on the fifth day, tadpoles were fed *Xenopus* Tadpole Food (Carolina Biological).

Specimens were staged according to the external characteristics described by Nieuwkoop and Faber (1994, N/F), fixed in Bodian's fixative (71.2% ethanol, 5% glacial acetic acid, 5% formalin, 18.8% water), dehydrated through a graded alcohol series and cleared in several changes of methyl salicylate. After processing for paint-filling, specimen lengths (tip of snout to tip of tail, Fig. 6) were measured by using an analog caliper. On the whole, specimen lengths for each stage were shorter than that reported by N/F, possibly owing to slightly cooler water temperature and/or more specimens per unit volume, in addition to shrinkage based on fixation and dehydration. We compared length measurements in live vs. fixed and dehydrated specimens at stage 48 to determine that the processed specimens are approximately 10% shorter (data not shown). Developmental categories used to describe specimens are defined as follows (McDiarmid and Altig, 1999): embryo, N/F stages 1–32; hatchling, N/F stages 33–44; tadpole, N/F stages 45–58; metamorph, N/F stages 59–66.

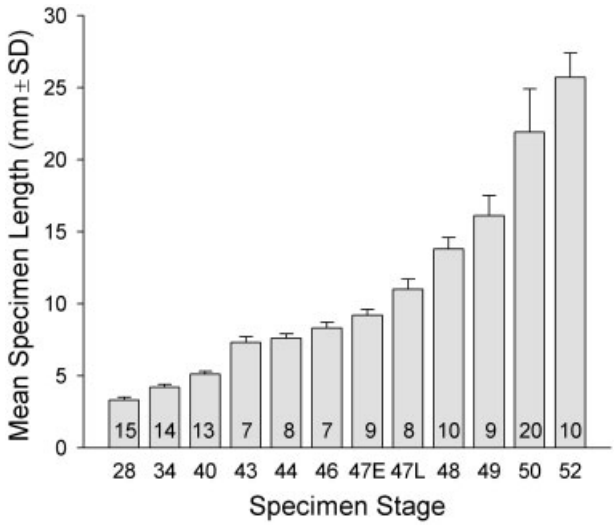

Fig. 6. Mean length (tip of snout to tip of tail) of fixed, cleared *Xenopus laevis* specimens. Staging was based on Nieuwkoop and Faber, 1994 (see Experimental Procedures section), and sample size for each stage is given on the bar. E, early; L, late.

The paint-fill technique (Martin and Swanson, 1993; Bissonnette and Fekete, 1996; Morsli et al., 1998; Bever and Fekete, 2002) serves as a tool for rapid determination of otic membranous labyrinth structure. To visualize inner ear morphogenesis in *Xenopus*, the membranous labyrinth was injected with 1% alkyd enamel paint (Benjamin Moore & Co.; Base 1 235 1A) diluted in methyl salicylate. This procedure was accomplished by using a pulled glass micropipette (with tip diameter 7-17 μm, depending on the size of the ear) coupled to a positive-displacement micro injector (Stolting). During injection, minutien pins (Fine Science Tools) were used to secure embryos or tadpoles in grooves cut into a resin-filled dish (Sylgard 182 Encapsulating Resin Kit, Dow Corning Corporation). Ears were injected from a dorsolateral approach. After canal genesis began, the anterior canal was targeted for injection.

Images of the paint-filled ears illuminated by an obliquely applied gooseneck fiber optic light source (KL1500, Schott) were obtained by using a Wild Heerbrugg M5A microscope, OM-4T Olympus camera, and Kodak 160 T film. Images from these color slides were scanned (Polaroid SprintScan 35) into Adobe Photoshop (versions 5.0.2 and 7.0). Some dissection of overlying tissue was necessary for unobstructed medial and ventral views, and images from some specimens were reversed so that all ears are presented as though they are right ears in the final figures (excepting Fig. 5A). In order that readers might readily compare ears from different views and various stages, ears for the atlas presentation in Figure 3 were digitally excised from the scanned images and pasted onto a black background to produce a composite figure. This process eliminated the variation in background from one specimen image to the next and removed the occasional paint leak from the edge of an ear. Likewise, both specimens in Figure 2 were pasted onto a black background to facilitate comparison.

For whole-mount antibody staining, inner ears from 4% paraformaldehyde-fixed specimens were dissected free of surrounding otic capsule and the lateral epithelium of the saccule was opened to enhance reagent access to the inner surface. Ears were treated with 50 μg/ml subtilisin to assist in removal of the otoliths (Gale et al., 2000), washed 35 min with phosphate-buffered saline+0.1% Tween (PBST), and incubated in blocking solution (3% bovine serum albumin, 0.4% Triton X-100 in PBS) overnight at 4°C. HCS-1 primary antibody (courtesy of Jeff Corwin; Finley et al., 1997) was applied at a dilution of 1:250 in 0.1 M PBS, 5% normal goat serum, and

0.1% Triton X-100 and incubated on a shaker for 2 days at 4°C. Ears were washed 4 × 15 min in PBST then incubated in fluorescein-conjugated horse anti-mouse immunoglobulin G (1:200) on a shaker for 2 days at 4°C. After washing at least 4 × 15 min in PBS (pH 7.8), ears were viewed with a Leica stereofluorescence microscope (MZ FLIII). Images were acquired by using a SPOT RT color digital camera (Diagnostic Instruments, Inc.).

ACKNOWLEDGMENTS

We thank Drs. Joseph W. Vanable and Kenneth R. Robinson for access to their *Xenopus* colonies and Fekete lab members for careful reading of the manuscript.

REFERENCES

Amaya E, Kroll KL. 1999. A method for generating transgenic frog embryos. In: Sharpe PT, Mason I, editors. Methods in molecular biology. Vol. 97. Molecular embryology: methods and protocols. Totowa, NJ: Humana Press. p 393-414.

Beck CW, Slack JM. 2001. An amphibian with ambition: a new role for *Xenopus* in the 21st century. Genome Biol 2: REVIEWS1029.

Bever MM, Fekete DM. 1999. Ventromedial focus of cell death is absent during development of *Xenopus* and zebrafish inner ears. J Neurocytol 28:781-793.

Bever MM, Fekete DM. 2002. Atlas of the developing inner ear in zebrafish. Dev Dyn 223:536-543.

Bissonnette JP, Fekete DM. 1996. Standard atlas of the gross anatomy of the developing inner ear of the chicken. J Comp Neurol 368:620-630.

Boatright-Horowitz SS, Simmons AM. 1997. Transient "deafness" accompanies auditory development during metamorphosis from tadpole to frog. Proc Natl Acad Sci U S A 94:14877-14882.

Bronchain OJ, Hartley KO, Amaya E. 1999. A gene trap approach in *Xenopus*. Curr Biol 9:1195-1198.

Cotanche DA. 1987. Development of hair cell stereocilia in the avian cochlea. Hear Res 28:35-44.

Diaz ME, Varela-Ramirez A, Serrano EE. 1995. Quantity, bundle types, and distribution of hair cells in the sacculus of *Xenopus laevis* during development. Hear Res 91:33-42.

Elepfandt A. 1996. Underwater acoustics and hearing in the clawed frog, *Xenopus*. In: Tinsley RC, Kobel HR, editors. The biology of *Xenopus*. Oxford: Clarendon Press. p 177-193.

Fay RR, Popper AN. 1999. Hearing in fishes and amphibians: an introduction. In: Fay RR, Popper AN, editors. Comparative hearing: fish and amphibians. New York: Springer. p 1-14.

Finley JE, Xia B, Corwin JT. 1997. Monoclonal antibodies raised as markers for supporting cells and hair cells. Assoc Res Otolaryngol Abstr 20:134.

Fritzsch B. 1990. The evolution of metamorphosis in amphibians. J Neurobiol 21:1011-1021.

Fritzsch B, Wake MH. 1988. The inner ear of gymnophione amphibians and its nerve supply: a comparative study of regressive events in a complex sensory system (Amphibia, Gymnophiona). Zoomorphology 108:201-217.

Fritzsch B, Wahnschaffe U, Bartsch U. 1988. Metamorphic changes in the octavolateralis system of amphibians. In: Fritzsch B, Ryan MJ, Wilczynski W, Hetherington TE, Walkowiak W, editors. The evolution of the amphibian auditory system. New York: John Wiley & Sons. p 359-376.

Gale JE, Meyers JR, Corwin JT. 2000. Solitary hair cells are distributed throughout the extramacular epithelium in the bullfrog's saccule. J Assoc Res Otolaryngol 1:172-182.

Gale JE, Meyers JR, Periasamy A, Corwin JT. 2002. Survival of bundleless hair cells and subsequent bundle replacement in the bullfrog's saccule. J Neurobiol 50:81-92.

Gallagher BC, Henry JJ, Grainger RM. 1996. Inductive processes leading to inner ear formation during *Xenopus* development. Dev Biol 175:95-107.

Gao W, Wiederhold ML, Hejl R. 1998. Production of otoconia in the endolymphatic sac in the Japanese red-bellied newt, Cynops pyrrhogaster: light and transmission electron microscopic study. Acta Otolaryngol 118:540-548.

Haddon CM, Lewis JH. 1991. Hyaluronan as a propellant for epithelial movement: the development of semicircular canals in the inner ear of *Xenopus*. Development 112:541-550.

Haddon C, Lewis J. 1996. Early ear development in the embryo of the zebrafish, *Danio rerio*. J Comp Neurol 365:113-128.

Hirsch N, Zimmerman LB, Grainger RM. 2002. *Xenopus*, the next generation: *X. Tropicalis* genetics and genomics. Dev Dyn 225:422-433.

Horowitz SS, Chapman JA, Kaya U, Simmons AM. 2001. Metamorphic development of the bronchial columella of the larval bullfrog (Rana catesbeiana). Hear Res 154:12-25.

Imoto T, Rask-Andersen H, Bagger-Sjoback D. 1983. The role of the endolymphatic sac in statoconial formation and degradation. Acta Otolaryngol 96:227-235.

Kil SH, Collazo A. 2001. Origins of inner ear sensory organs revealed by fate map and time-lapse analyses. Dev Biol 233: 365-379.

Klein SL, Strausberg RL, Wagner L, Pontius J, Clifton SW, Richardson P. 2002. Genetic and genomic tools for *Xenopus* research: The NIH *Xenopus* initiative. Dev Dyn 225:384-391.

Knowlton VY. 1967. Correlation of the development of membranous and bony labyrinths, acoustic ganglia, nerves, and brain centers in the chick embryo. J Morphol 121:179-208.

Lewis ER, Narins PM. 1999. The acoustic periphery of amphibians: anatomy and physiology. In: Fay RR, Popper AN, editors. Comparative hearing: fish and amphibians. New York: Springer. p 101-154.

Lopez-Anaya VL, Lopez-Maldonado D, Serrano EE. 1997. Development of the *Xenopus laevis* VIIIth cranial nerve: increase in number and area of axons of the saccular and papillar branches. J Morphol 234:263-276.

Martin P, Swanson GJ. 1993. Descriptive and experimental analysis of the epithelial remodellings that control semicircular canal formation in the developing mouse inner ear. Dev Biol 159:549-558.

McDiarmid RW, Altig R. 1999. Research: materials and techniques. In: McDiarmid RW, Altig R, editors. Tadpoles: the biology of anuran larvae. Chicago: University of Chicago Press. p 7-23.

Morsli H, Choo D, Ryan A, Johnson R, Wu DK. 1998. Development of the mouse inner ear and origin of its sensory organs. J Neurosci 18:3327-3335.

Nieuwkoop PD, Faber J. 1994. Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. New York: Garland Publishing, Inc.

Paterson NF. 1948. The development of the inner ear of *Xenopus laevis*. Proc Zool Soc Lond 119:269-291.

Paterson NF. 1960. The inner ear of some members of the Pipidae (Amphibia). Proc Zool Soc Lond 134:509-546.

Reiss JO. 1997. Early development of chondrocranium in the tailed frog Ascaphus truei (Amphibia: Anura): implications for anuran palatoquadrate homologies. J Morphol 231:63-100.

Serrano EE. 2001. Confocal laser scanning microscopy of the inner ears of *Xenopus tropicalis* and *Xenopus laevis*. ARO Abstr 24:244.

Sher AE. 1971. The embryonic and postnatal development of the inner ear of the mouse. Acta Otolaryngol 285:1-77.

Smirnov SV. 1993. The anuran amphibian papilla development, with comments on its timing, rate, and influence on adult papilla morphology. Zool Jahrb Anat 123:273-289.

Vize PD, Melton DA, Hemmati-Brivanlou A, Harland RM. 1991. Assays for gene function in developing *Xenopus* embryos. In: Kay BK, Peng HB, editors. Methods in cell biology. San Diego, CA: Academic Press. p 367-387.

Waterman RE, Bell DH. 1984. Epithelial fusion during early semicircular canal formation in the embryonic zebrafish, *Brachydanio rerio*. Anat Rec 210:101-114.

Wever EG. 1985. The amphibian ear. Princeton, NJ: Princeton University Press.

Wiederhold ML, Yamashita M, Larsen KA, Batten JS, Koike H, Asashima M. 1995. Development of the otolith organs and semicircular canals in the Japanese red-bellied newt, Cynops pyrrhogaster. Hear Res 84:41-51.