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Morphogenesis of otoliths during larval development in brook lamprey, *Lampetra planeri*

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Abstract
Otolith morphogenesis of the brook lamprey, *Lampetra planeri*, was analysed from larval to adult stages. The brook lamprey remains juvenile for about 4 years, facilitating analysis of otoliths maturation that permits to identify relevant evolutionary traits in this primitive species and to compare our results with more evoluted species of vertebrate taxa. We combined histochemical, immunohistochemical, scanning electron microscopy, elemental analysis and X-ray diffraction of lamprey otoliths to establish possible relationships between otolithic mass, individual crystals, the otolithic organic substance that binds individual otoconia together and the inorganic elements that mineralize the lamprey otoliths. Histochemical analysis of the otoliths suggests that mineralization occurs gradually, beginning near the apex of the secretory epithelium. Then, the otoconia increase in size by deposition of layers of a dense crystalline substance. Immunohistochemical reactivity of calcium binding proteins indicates that calmodulin, calbindin, S-100 and parvalbumin are parts of the uncalcified organic mass that holds otoconia together. Imaging of the immunoreactivity of each protein by Confocal Laser Scanning Microscopy in ammocoete at the first year of the larval stage shows weak reaction products which, however, gradually increase in intensity, with peak value in ammocoete at the fourth year of the larval stage.

Keywords: *Brook lamprey, larval stages, otoliths, calcium binding proteins, histochemistry, immunohistochemistry*

Introduction

The primitive vertebrate *Lampetra planeri* (Block, 1784) analysed in this study is a non-parasitic freshwater lamprey which lives in soft bottoms of brooks and rivers. The number of larval stages in this species spread in central and western Europe, varies between 4 and 6 according to geographical position, and about half a year for the adult stage (Hardisty 1986). Our specimens belong to a population collected in the river Sele, Italy. According to the examinations of combined data reported in two recent contributions, Bianco et al. (2004) and Bianco (2006), four larval stages were identified and the ranges are reported in Table I.

The brook lamprey spends its larval life in the soft bottom of brooks and rivers, the larval growth period ends at metamorphosis. Sexual maturity is reached in the first spring following metamorphosis, when the adult, which does not feed, spawns and dies after a few months (Hardisty 1951). Lamprey larvae, called ammocoetes, are excellent monitors for changes in the environment that are detrimental to their survival. The long juvenile period of *Lampetra* allows the detailed analysis of otolith development (Lychakov 1995), which in evolutively newer species, occurs very rapidly, compressed in short critical periods of embryonic development, thereby making analysis difficult (Riley et al. 1997). The placement of lampreys among primitive vertebrates concerns also the organization of the inner ear (Retzius 1881; de Burlet & Versteegh 1930; Lowenstein et al. 1968; Thornhill 1972; Hoshino & Kodama 1976; Popper 1981; Popper & Hoxter 1981, 1987; Popper & Platt 1983; Avallone

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Table I. Range of total length in ammocoetes of *L. planeri* at four larval stages and adult collected between March–June (2003) and February–April (2006) in rivers Sele of Avellino Province and rivers of Cilento and Vallo di Diano National Park.

Stage	*Ammocoetes* First year	*Ammocoetes* Second year	*Ammocoetes* Third year	*Ammocoetes* Fourth year	Adult
Specimen (no.)	36	66	88	65	8
Total length (mm)	40–80	70–118	90–135	125–164	89–158

et al. 2005), in particular that of their sensory epithelia. The typical arrangement of linear and angular acceleration detectors that is found in mammals and birds consists of three semicircular canals and two maculae: the saccule and utricle. In lampreys, instead, there are only two semicircular canals and a single elongated epithelium or *macula communis*, which is covered by a mass of calcific otoliths (Tret'yakov 1915; Carlström 1963; Fermin et al. 1998).

Despite the primitive organization of the lamprey otoliths, most of the components of the gravity-sensing structures overlaying the hair cells are identical to those of newer species with respect to the effectiveness of gravity detection in their native environment. The greatest difference between the otolith of lampreys and of more recent species is the mode of cation accumulation, which is in turn dictated by refinement of the endolymphatic fluid, its molecular composition, and/or ionic concentration (Peterson et al. 1978; Hara et al. 1989; Wroblewski 1993; Lychakov & Lavrova 1994; Payan et al. 1997). While the nature of the extracellular receptors to which calcium binding proteins (CBPs) attach remains elusive, there is consensus among many investigators that CBPs are important components of sensory and non-sensory structures (Braun 1990; Celio 1990; Kevetter & Leonard 2002), vestibular organs (Yamagishi et al. 1993; Fermin & Martin 1995; Baird et al. 1997; Fermin et al. 1997; Balsamo et al. 2000; Abbate et al. 2002), and inner ear in general (Winsky et al. 1989; Kerschbaum & Hermann 1993; Balsamo et al. 2000; Nakazawa 2001). All CBPs are homologous proteins sharing high sequence identity (Braun 1990; Moncrief et al. 1990; Kerschbaum & Hermann 1993; Yamagishi et al. 1993; Zimmer et al. 1995; Kawasaki et al. 1998), and cross-react with polyclonal antisera produced from different species.

The non-cellular otolithic mass is composed of proteins and an acidic mucosubstance, containing sulphate and carboxy groups (Marmo 1982), glycoproteins (Fermin et al. 1990, 1995) and starches (Kuijpers & Manni 1986). All these molecules are made by nonsensory cells of the end organs (Tachibana & Morioka 1992; Davis et al. 1997; Borelli et al. 2001; Thalmann et al. 2001). Various isoforms of the same family or differing types of CBP react with cellular and non-cellular components of the otoliths in older and younger species such as cichlid fish (Presson 1994), other teleosts (Abbate et al. 2002), amphibians (Hackney et al. 1993; Kerschbaum & Hermann 1993; Baird et al. 1997; Steyger et al. 1997; Heller et al. 2002), lizard (Piscopo et al. 2004), rodents (Chard et al. 1993; Dememes et al. 1993; Raymond et al. 1993; Yamagishi et al. 1993; Nakazawa et al. 1995; Karita et al. 1999; Furness et al. 2002), birds (Fermin & Igarashi 1985; Heil & Scheich 1986; Hiel et al. 2002), other mammals (Pack & Slepecky 1995; Imamura & Adams 1996; Coppens et al. 2000, 2001, 2003), primates (Usami et al. 1995), and humans (Yamashita et al. 1995).

This study was undertaken to evaluate the otolith morphogenesis of lamprey, *L. planeri*, from larval to adult stages. We combined histochemical, immunohistochemical, scanning electron microscopy observations, elemental analysis and X-ray diffraction of lamprey otoliths to determine the relationships that may exist between otolithic mass, individual crystals, the otolithic organic substance that binds individual otoconia together and the inorganic elements that mineralize the lamprey otoliths.

To this aim, in this study we mapped the distribution of the CBPs: calmodulin (CaM), calbindin D28K (CaB), S-100 and parvalbumin (PA) in the non-cellular structures of the end organs of lampreys and determined the expression of these CBPs during the first four years of the ammocoete larval stage and in the adult. In the otolithic membrane of lampreys, only single amorphous otoliths are found (Tret'yakov 1915; Carlström 1963; Volk 1986; Lychakov 1988, 1995; Fermin et al 1998). We performed studies by SEM on otolith morphogenesis of *L. planeri* during the first four years of the ammocoete larval stage and in the adult. Moreover, using histochemical methods, we studied the early appearance of glycoproteins, glycosaminoglycans and calcium. We also established the areas and the stages in which the otolith matrix precursors appear, glycoproteins and glycosaminoglycans, and the possible changes in their secretion during larval development.

Materials and methods

The evaluation of lamprey otoliths was carried out on five specimens for each of four ammocoete larval stages and on five adult *L. planeri*. Specimens were collected from basins of rivers Sele and Bussento in Campania region, National Park of Cilento and Vallo di Diano, southern Italy, on February and March 2005. Animals were deeply anaesthetized with Ms 222, sacrificed, and the cartilaginous otic capsule exposed.

Light microscopy

The cartilaginous otic capsules were fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) pH 7.4 for 3 h at 4°C. After several washes in the same buffer, the membranous labyrinths were dissected, dehydrated, and paraffin embedded. Sections (4 μm) were prepared and stained with periodic acid–Schiff (PAS), Alcian blue–Neutral red or von-Kossa solutions (Luna 1968). Observations were carried out with a Zeiss Axioskop microscope,

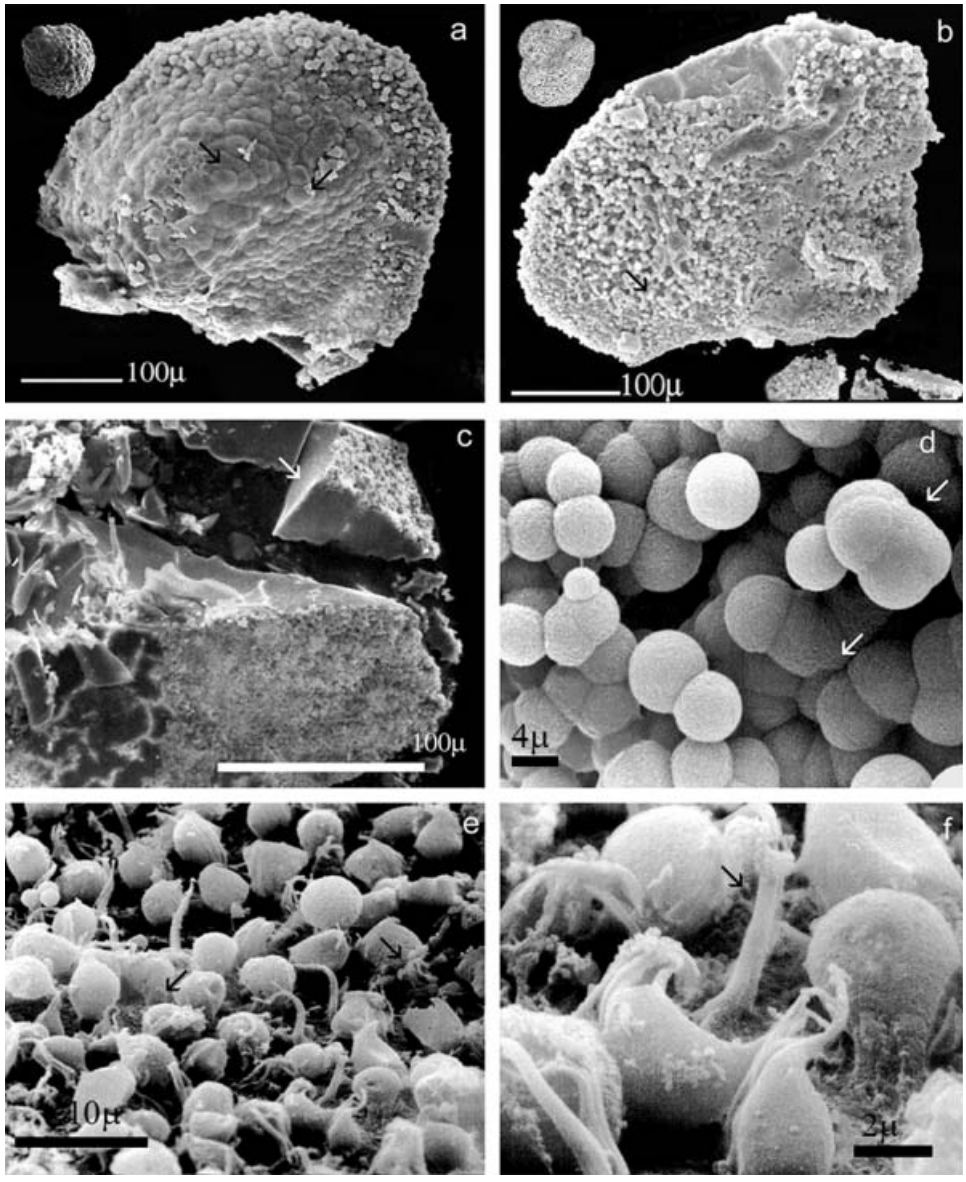
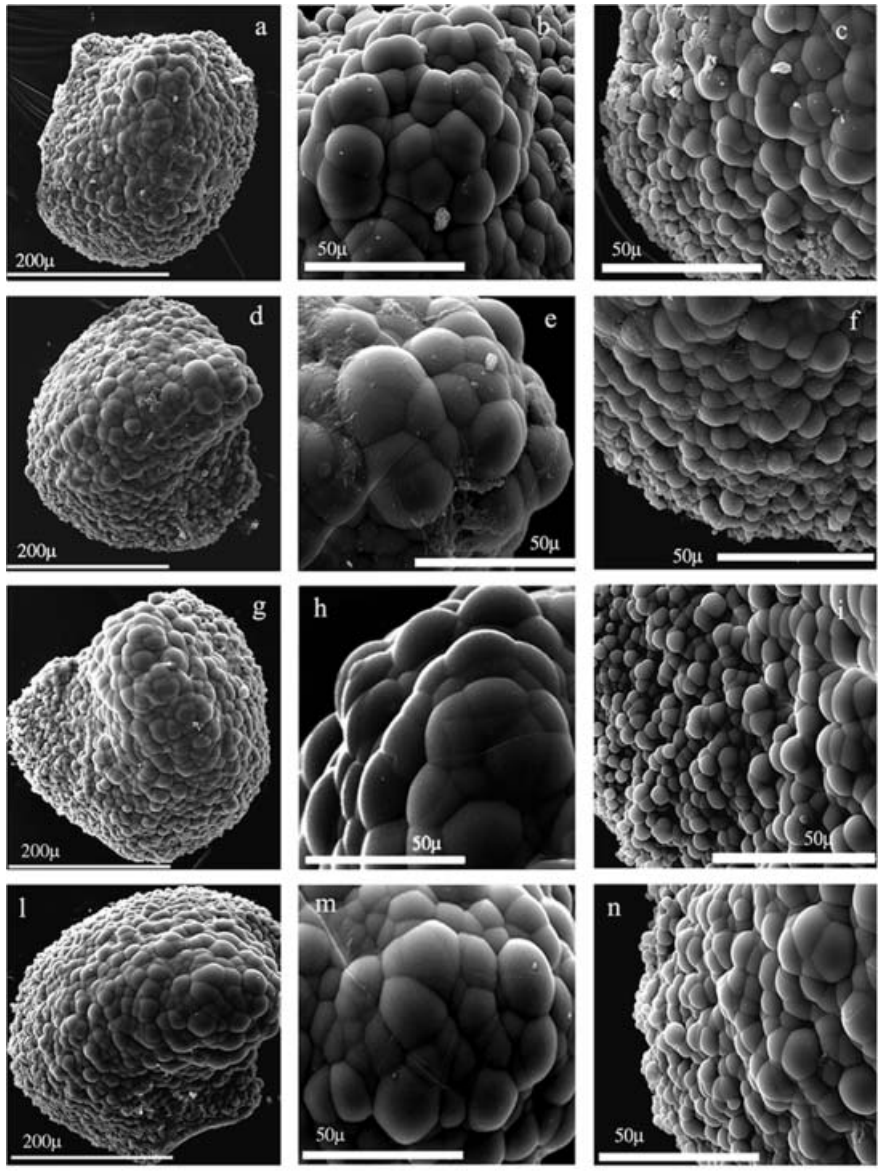


Figure 1. SEM: otoliths in adult stage of *L. planeri*. Two otoliths, a larger one measuring 400–450 μm in diameter and a smaller one measuring 90–100 μm are present. The otoliths are conically shaped. The largest otoconia were arranged on the top of the otolith (arrows) (**a**); the smallest otoconia (arrow) were located on the side of the otolith facing the sensory epithelium (**b**); the otolith appears to be an aggregation of numerous otoconia forming a homogeneous stone (arrow) (**c**); many large spherical otoconia were present on the sensory epithelium and some appear to be fused (arrows) (**d**); potential precursors at the spherical otoconia are granules found near the microvilli of the supporting cells (arrows) (**e**) and not over the hair cells bundles (arrow) (**f**).



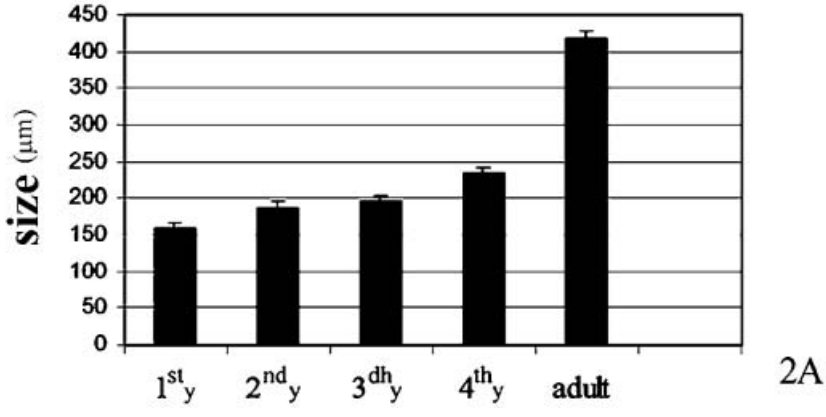


Figure 2. SEM: progressive increase in size of the larger otolith: **a**–**c**, in ammocoete at the first year of the larval stage; **d**–**f**, in ammocoete at the second year of the larval stage; **g**–**i**, in ammocoete at the third year of the larval stage; **l**–**n**, in ammocoete at the fourth year of the larval stage. **2A** shows the progressive increase in size of large otolith from the first year to the adult stage. Values represent means ± SE.

using the KS300 software to acquire microscope images and ProPalette 8000 for digital colour film recorder.

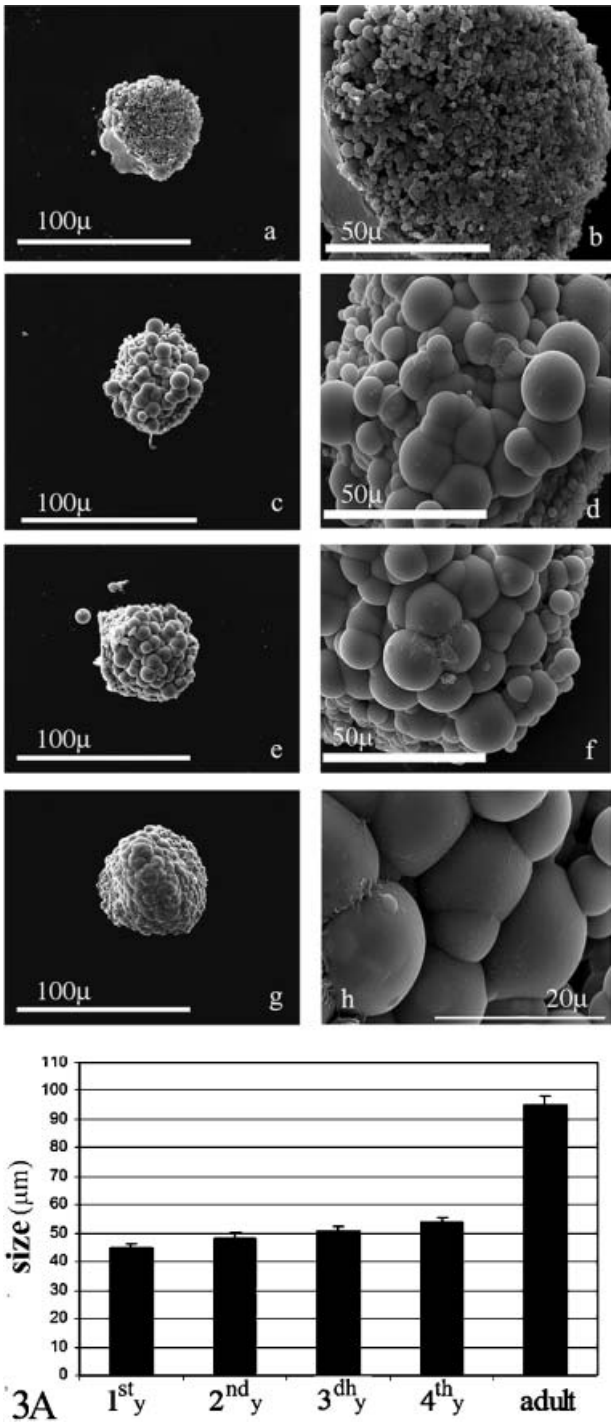


Figure 3. SEM: progressive increase in size of the smaller otolith: **a,b**, in ammocoete at the first year of the larval stage; **c,d**, in ammocoete at the second year of the larval stage; **e,f**, in ammocoete at the third year of the larval stage; **g,h**, in ammocoete at the fourth year of the larval stage. **3A** shows the progressive increase in size of small otolith from the first year to the adult stage. Values represent means ± SE.

X-ray diffraction

Unfixed otoliths were attached to the tips of glass fibres using a collodion wire holder. Diffraction patterns (Glauert 1972) were recorded with Ni-filtered Cu-Kα radiation (1.5418 Å, 40 kV and 30 mA) using a Debye–Scherrer camera with a diameter of 114.6 mm and evaluated for changes.

Confocal laser scanning microscopy (CLSM)

The cartilaginous otic capsules were fixed in 100% methanol at 4°C for 2 h after which the otoliths were dissected. For the identification of calcium binding proteins in the otolithic organic mass, the otoliths were treated with 0.25% Triton X-100 and 0.1% Tween 20 in 0.1 M PBS, pH 7.4 for 1 h. After several washes in PBS, the otoliths were incubated overnight at 4°C, with primary antibodies against CaB, rabbit anti-calbindin-D28K (KD-15) 1:500, (Sigma, St. Louis, MO, USA) and against CaM, mouse monoclonal anti-calmodulin (clone 6D4), 1:1000 (Sigma). After transfer in PBS (6 washes × 10 min), the otoliths were incubated in the same buffer for 2 h at room temperature in a moist, dark chamber in the presence of FITC-labelled sheep anti-rabbit IgG (1:10) to reveal antibodies against CaB, and in the presence of rodhamine-labelled sheep anti-mouse IgG (1:10), to reveal anti CaM mAb. After washing again several times in PBS, the otoliths were mounted on slides using Diazabicyclo-octane (DABCO) (Sigma). The same procedure was followed using primary antibodies against S-100, rabbit anti-S-100 (1:80; Chemicon), and against PA, using mouse monoclonal anti-Parvalbumin (clone PARV-19) (1:2,000; Sigma).

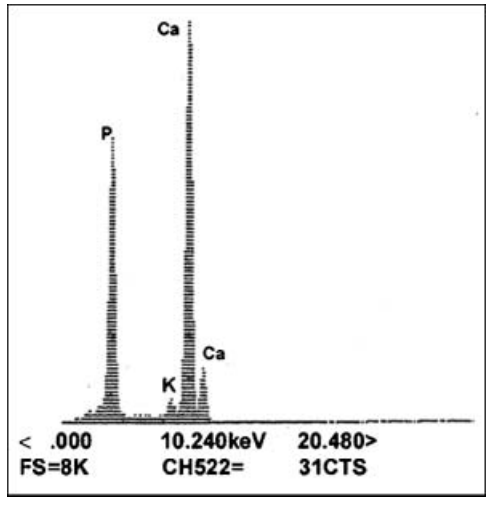


Figure 4. SEM elemental microanalysis of large otoliths. Peaks corresponding to Ca, K, and P, are present suggesting that these elements are components of the Lamprey otolith.

After transfer in PBS (6 washes × 10 min), the otoliths were incubated in the presence of FITC-labelled sheep anti-rabbit IgG (1:10), to reveal antibodies against S-100 and in presence of rodhamine-labelled sheep anti-mouse IgG (1:10), to reveal anti-PA mAb. All antibodies were diluted in PBS. All histochemical incubations were done simultaneously to allow a comparison of the staining intensity. Preimmune sera were used as negative controls. Finally, fluorescence observations were carried out using a confocal microscope (Leica TCSNT) with laser argon-krypton 7.5 mW multilines. Focal series of horizontal planes of sections were simultaneously monitored for FITC using the 488-nm and 568-nm laser line, a FITC band-pass 530/30, and a long-pass filter 590 for TRITC. The Leica TCSNT confocal microscope was equipped with an AOTF filter. This filter minimizes cross talk during simultaneous detection of FITC/TRITC labels.

Scanning electron microscopy (SEM)

The cartilaginous otic capsules were fixed in 2.5% glutaraldehyde in PBS for 3 h at 4°C. After micro dissection of the membranous labyrinth, the specimens were transferred in PBS and post-fixed in 1% OsO_4 in the same buffer for 1 h at 4°C. After several washes in PBS, the specimens were dehydrated in a step gradient of Freon in ethanol to final 100% Freon, then critical point dried. Specimens were mounted on aluminium stubs, coated with gold for about 30 s at a distance of 15 cm from the source at 40 kV and 2–3 mA. For SEM microanalysis, the otoliths were mounted on carbon stubs without gold coating. The observations were carried out with a Cambridge Stereoscan 250 MK III microscope fitted with an elemental microanalysis system.

Statistical analysis

Ten images of left and right otoliths alike for each stage of development were used for quantitative analysis. Statistical analysis was performed with a single factorial analysis of variance (ANOVA). A P value of less than 0.01 was considered to be significant. Values represent means ± SE.

Results

SEM observations showed that all animals examined (ammocoetes at either larval or in adult stages) had

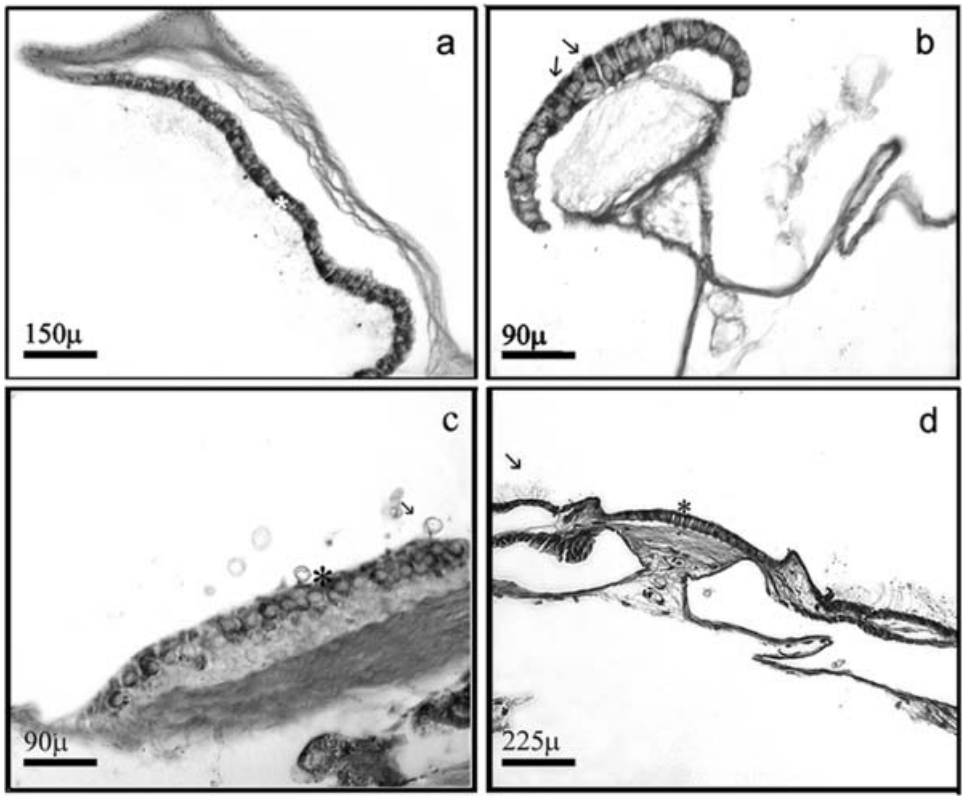


Figure 5. PAS staining shows an accumulation of glycoproteins in ammocoete at the second year of the larval stage. **a**, all ciliated chamber cells are positive (asterisk); **b**, *crista ampullaris*: positive signal in the cytoplasm of sensory cells, (arrows); **c**, *macula communis*: sensory cell cytoplasm (asterisk) and secreted vesicles (arrow) are positive; **d**, *crista ampullaris* (asterisk) and ciliated chamber (arrows).

two otoliths, conical-shaped, one larger otolith with maximum chord of the base measuring 400–450 µm and one smaller with maximum chord of the base measuring otolith 90–100 µm, in adult stage (Figure 1a, b). During the four larval stages the sizes of both otoliths increase (Figures 2, 3). Figures 2a and 3a show the progressive increase in size of otolith in ammocoete from the first year to the adult stage. The analysis indicates that a significant increase in size of otolith exists from the progressive four larval stages to the adult. Values represent means ± SE. Both otoliths were conical-shaped (Figure 1a) and appeared to be formed by fusion of a large number of spherical otoconia (Figure 1a, b, d). Each otolith had a homogeneous appearance and no boundaries were detected between the fused otoconia (Figure 1c). Fused otoconia differed in size such that the largest otoconia were arranged on the top of the otoliths (Figures 1a, 2a–n) whereas the smallest otoconia were located on the side of the otolith facing the sensory epithelium (Figure 1b). Moreover, SEM observations revealed the presence of numerous, probably secretory, vesicles on the sensory epithelium surface (Figure 1e, f), apparently in association with the supporting microvilli rather than the stereocilia of hair cells.

The elemental microanalysis at the SEM of otoliths of all larval stages examined showed peaks

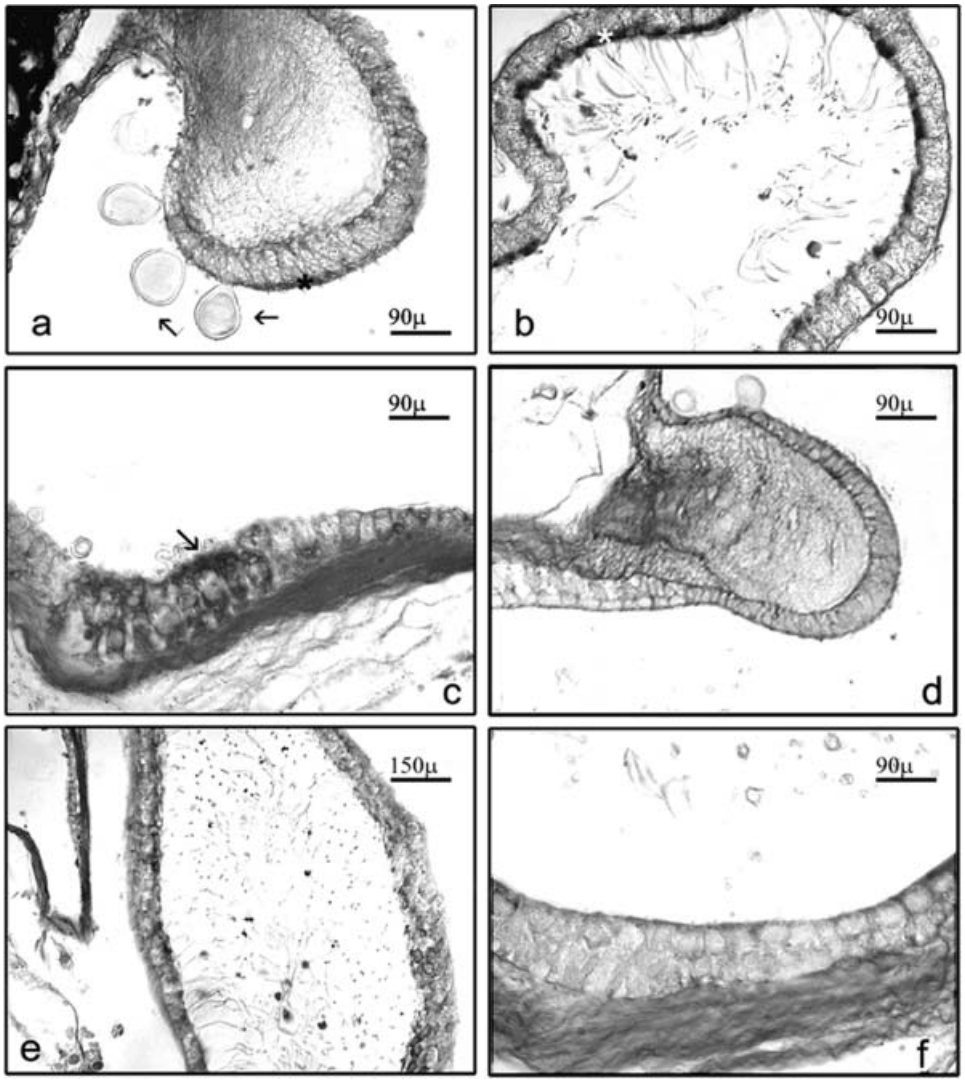


Figure 6. A comparison between the PAS reaction in ammocoete at the third year of the larval stage (**a**–**c**) and at the fourth year of the larval stage (**d**–**f**), showing that PAS reaction is progressively reduced. **a**, *crista ampullaris*: positive signal is present in the cytoplasm of sensory cells (asterisk) and secreted vesicles (arrows); **b**, ciliated chamber: many cells are positive (asterisk); **c**, *macula communis*: expression is present in the cytoplasm of some sensory cells (arrow); **d**, crista ampullaris: PAS reaction is very reduced.; **e**, ciliated chamber: PAS reaction is reduced.; **f**, *macula communis*: PAS reaction is almost absent.

corresponding to Ca, K, and P (Figure 4). But the peaks were absent in the small otoliths in ammocoete at the first year of the larval stage.

The otoliths examined by X-ray diffraction were formed by carbonate–fluorapatite, in agreement with the JCPDS–ICDD (Joint Committee on Powder Diffraction Standards–International Centre for Diffraction Data) database for inorganic structures. Their three strongest diffraction peaks were 2.69 (strong), 2.79 (medium/strong), and 2.24 (medium).

PAS revealed the presence of glycoproteins in the cytoplasm of all cells in the ciliated chamber (Figure 5a, d), in the cytoplasm of sensory cells at the *crista ampullaris* level (Figure 5b, d), and in both the sensory cell cytoplasm and secreted vesicles of *macula communis* (Figure 5c) mainly in the early stages of larval development. In late ammocoete larval stages, the PAS reaction was progressively reduced (Figure 6).

By Alcian blue–Neutral red stain, glycosaminoglycans were clearly present in all the examined samples, mainly in the material over the sensory epithelium, both of the *macula communis* and *crista ampullaris* (Figure 7a). The von Kossa method showed calcium in otoconia beginning in the second larval stage (Figure 7b–d).

CLSM observations showed a weak presence of CaB, CaM, S-100, and PA at the first larval stage around the otoconia (Figure 8a, g, h). In the successive larval stages, these proteins progressively increased and, in the ammocoete at the fourth larval stage, were present in the fibrous ground substance that surrounded and held together the otoconia (Figure 8b, c, d, i, l, m). In the adult stage these CBPs were always present in the fibrous ground substance that surrounded and held together the otoconia but their presence was again weak (Figure 8e, f, n). Conversely, in all larval stages and in the adult these CBPs were not detected in the central core of spherical otoconia.

Incubation with preimmune sera resulted in complete lack of immunoreactivity.

Discussion

According to Lychakov (1995), during evolution, the appearance of otolithic mass is associated with the need of vertebrate to have a constant growing

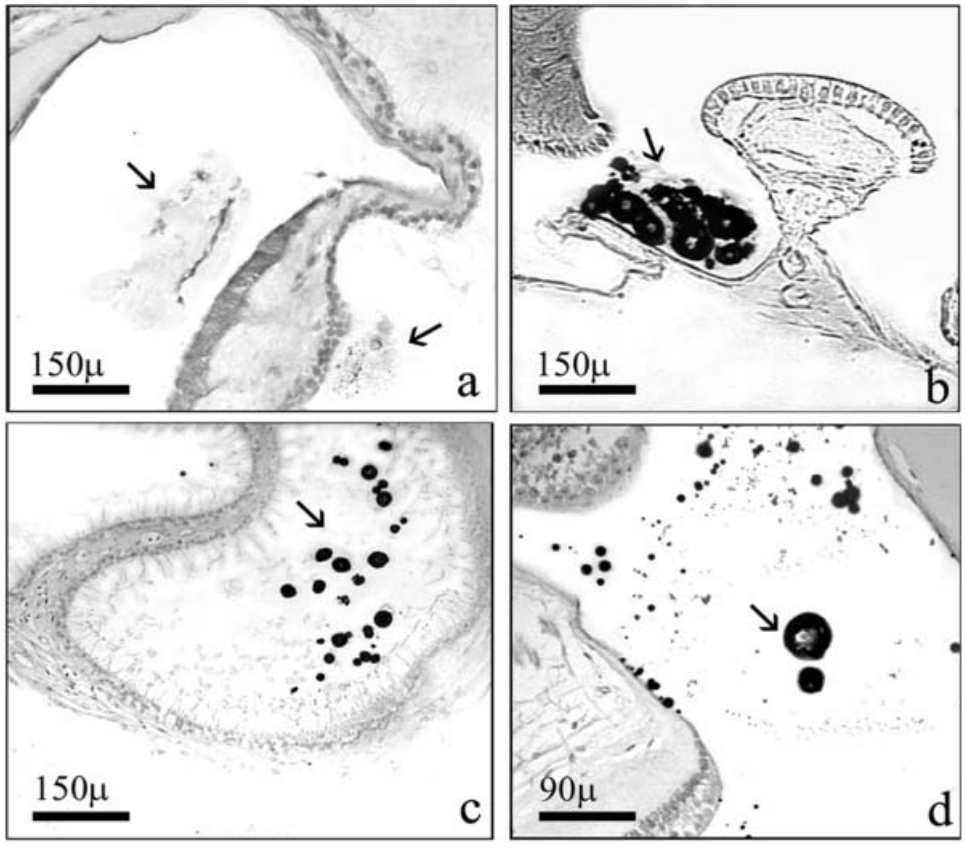


Figure 7. **a**, Alcian blue–Neutral red in ammocoete at the second year of the larval stage, *macula communis*: glycosaminoglycans are present on the sensory epithelium (arrow); **b**–**d**, von Kossa method reveals calcium in otoconia (arrows) of ammocoete at the second year of the larval stage (**b,c**), and in ammocoete at the third year of the larval stage (**d**).

and a load mass to stimulate the hair cells of the macular epithelia. In lampreys, only amorphous otoliths, arising from the fusion of spherical otoconia are found in the macula of adults (Tret'yakov 1915; Carlström 1963; Volk 1986; Fermin et al. 1998). This arrangement resembles the incorporation of calcium positive granules studied in avian otoconia by Fermin and Igarashi (1985, 1986). In birds, accretion occurs at the single crystal level, whereas in older species accumulation occurs onto a single large otolith. Earlier investigations on lamprey otolith morphology and development have shown that spherules seen at the apex of the epithelia are not artefacts of fixation (Lychakov 1995).

In this study we find that the otoliths, examined by X-ray diffraction, are formed by carbonate–fluor-apatite. Considering phylogenetic position of the lamprey, it appears likely that their otoliths may be

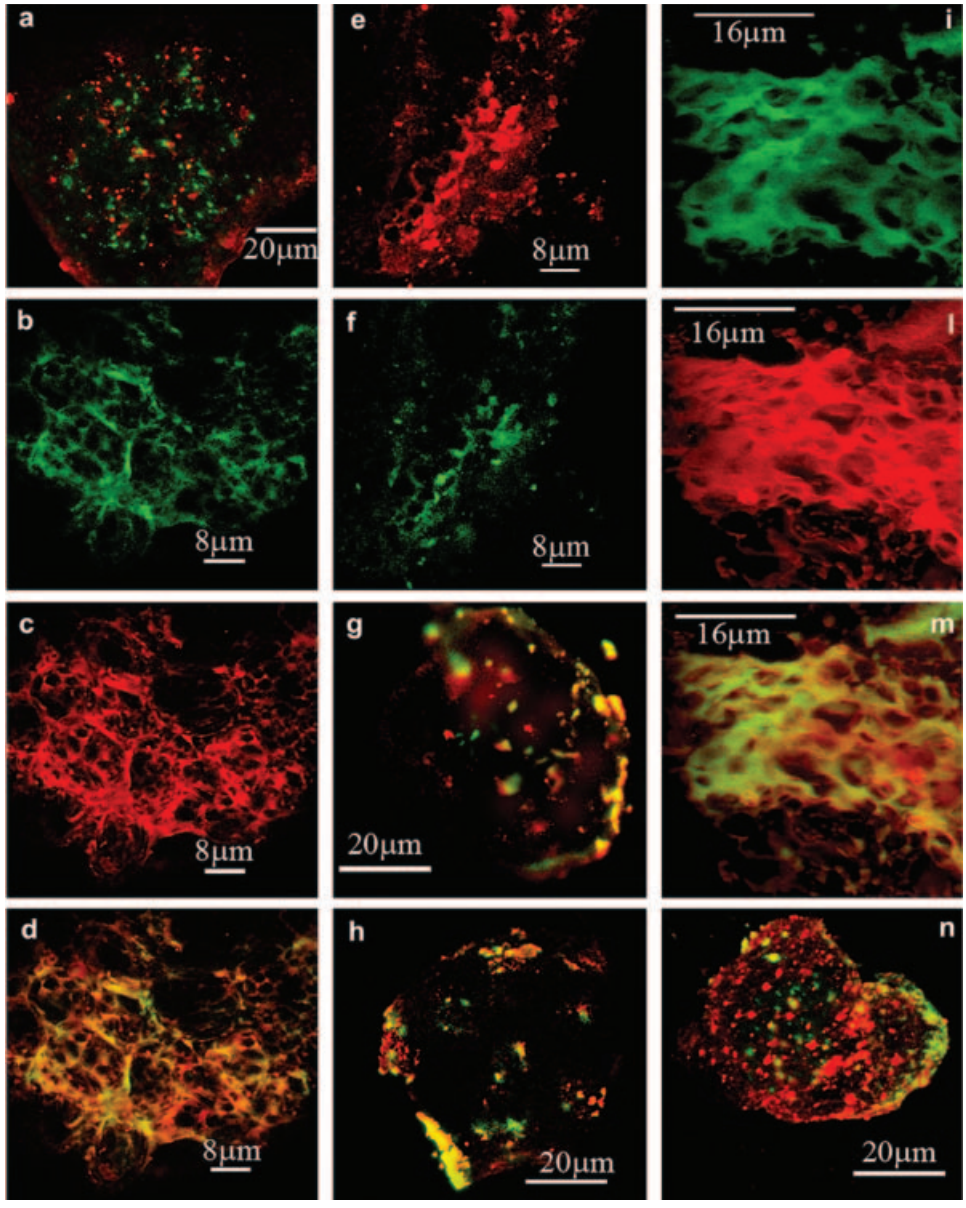


Figure 8. CLSM, CaB (green) and CaM (red) localization (**a**–**f**). **a**, in ammocoete at the first year of the larval stage both proteins are weakly present around the otoconia; **b**, CaB, **c**, CaM, **d**, CaB and CaM colocalization (yellow) in ammocoete at the fourth year of the larval stage. These proteins are present in the fibrous ground substance that surrounds and holds together the otoconia. In the adult (**e, f**), the fluorescence intensity for these proteins is weak. S-100 (green) and PA (red) localization (**g**–**n**). **g**, in ammocoete at the first year of the larval stage and **h**, in ammocoete at the second year of the larval stage, both proteins are weakly present around the otoconia; **i**, S-100, **l**, PA, **m**, S-100 and PA colocalization (yellow) in ammocoete at the fourth year of the larval stage. These proteins are present in the fibrous ground substance that surrounds and holds together the otoconia; **n**, adult stage, the presence of these proteins is again weak.

the nontetrapod ancestral otoliths. The different chemical composition between otoliths of lamprey and fishes (calcium carbonate) may be due to the need of continuously adding more weight to the otolithic mass and permitted otoliths to remain unfused as well.

Our histochemical data, together with SEM and microanalysis suggest that the round vesicles on the sensory epithelium surface may be an immature organic matrix. Therefore, it is possible that the otoliths arise near the apex of the sensory cells as small organic masses, followed by precocious mineralization. Indeed, microanalysis data show peaks corresponding to Ca in ammocoete even at the second larval stage. Otoconia mineralization in the large precedes that in the small otoliths. Our microanalysis data, in fact, show that the peaks corresponding to Ca are absent in the small otolith of ammocoete at the second larval stage. Then, in agreement with the von Kossa stain, otoliths appear to increase in size by both repeated deposition of layers of a dense crystalline substance and the fusion of otoconia. Moreover, we propose also that the lamprey otoliths increase in size by binding of newly formed spherical otoconia to the base of the otolith. The contribution of glycoproteins to the formation of the organic matrix that initiates otolith formation and holds otoconia together has been demonstrated in different species and reviewed (Fermin et al. 1998). The data presented here suggest that the organic matrix of this very old fish contains varying amounts of glycoproteins at different times during development. This indicates the existence of a differential accumulation of glycomolecules at critical stages of otolith mineralization, a point that has been made in earlier otolith analysis of mammalian and non-mammalian species.

CLSM data show the localization of CaM, CaB, S-100, and PA proteins in a fibrous ground substance that surrounds and holds together the otoconia. These proteins were not detected in the otoconia central core. According to Usami et al. (1995), a Ca^{2+} transport mechanism in the otoconial membrane would be required to form otoconia. As shown in the confocal images of the otolith in the early larval stages, the CBPs used in our studies and that have already been shown by others to be part of the otoliths of birds and mammals, are differently expressed and only in the organic matrix that surrounds and holds together the otoconia. The presence of these four calcium-binding proteins, CaM, CaB, S-100 and PA, in the otolith indicates that they participate in the formation and mineralization of otoconia. Moreover, the unequal distribution of these proteins in the otoconia during larval development and in the adult might be due to a progressive increase in size of the otoliths spanning the four years of the ammocoete larval stage. Since the adult lamprey settles on the substrate, reproduces, stops feeding and generally becomes inactive to let itself die, we suppose that otoconia turnover is similarly reduced like the other physiological functions. In the lamprey, as in other more recent species, CBPs are expressed more abundantly earlier in the formation of otoconia and otolith than later in development and when animals reach adulthood. Thus, CBP contribution to inner ear maturation started very early in the evolutionary ladder, as suggested by their presence in the organic matrix of this old fish and newer teleosts as well as higher vertebrates (Rhoten et al. 1986; Drescher et al. 1989; Foster et al. 1993; Baird et al. 1997; Abbate et al. 2002; Heller et al. 2002; Piscopo et al. 2003, 2004).

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References

Abbate F, Catania S, Germana A, Gonzalez T, Diaz-Esnal B, Germana G, Vega JA. 2002. S-100 protein is a selective marker for sensory hair cells of the lateral line system in teleosts. Neuroscience Letters 329:133–136.

Avallone B, Fascio U, Senatore A, Balsamo G, Bianco PG, Marmo F. 2005. The membranous labyrinth during larval development in lamprey (*Lampetra planeri* Bloch, 1784). Hearing Research 201:37–43.

Baird RA, Steyger PS, Schuff NR. 1997. Intracellular distributions and putative functions of calcium-binding proteins in the bullfrog vestibular otolith organs. Hearing Research 103:85–100.

Balsamo G, Avallone B, Del Genio F, Trapani S, Marmo F. 2000. Calcification processes in the chick otoconia and calcium binding proteins: patterns of tetracycline incorporation and calbindin-D28K distribution. Hearing Research 148:1–8.

Bianco PG, Picariello O, Belfiore C. 2004. Carta Ittica della Provincia di Avellino Avellino: Provincia di Avellino, Assessorato Agricoltura, Caccia e Pesca.

Bianco PG. 2006. Progetto di ricerca sul recupero degli agnati nel Parco Nazionale del Cilento e Vallo di Diano: relazione finale. Ente PNCVD. pp 44..

Borelli G, Mayer-Gostan N, De Pontual H, Boeuf G, Payan P. 2001. Biochemical relationships between endolymph and otolith matrix in the trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). Calcified Tissue International 69:356–364.

Braun K. 1990. Calcium-binding proteins in avian and mammalian central nervous system: Localization, development and possible functions. Progress in Histochemistry and Cytochemistry 21:1–64.

Carlström D. 1963. A crystallographic study of vertebrate otoliths. The Biological Bulletin 125:441–463.

Celio MR. 1990. Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35:375–475.

Chard PS, Bleakman D, Christakos S, Fullmer CS, Miller RJ. 1993. Calcium buffering properties of calbindin D28k and parvalbumin in rat sensory neurones. Journal of Physiology 472:341–357.

Coppens AG, Kiss R, Heizmann CW, Schafer BW, Poncelet L. 2001. Immunolocalization of the calcium binding S100A1, S100A5 and S100A6 proteins in the dog cochlea during postnatal development. Brain Research 126:191–199.

Coppens AG, Resibois A, Poncelet L. 2000. Immunolocalization of calbindin D28k and calretinin in the dog cochlea during postnatal development. Hearing Research 145:101–110.

Coppens AG, Salmon I, Heizmann CW, Kiss R, Poncelet L. 2003. Postnatal maturation of the dog stria vascularis: an immunohistochemical study. The Anatomical Record A 270:82–92.

Davis JG, Burns FR, Navaratnam D, Lee AM, Ichimiya S, Oberholtzer JC. 1997. Greene MI. Identification of a structural constituent and one possible site of postembryonic formation of a teleost otolithic membrane. Proceedings of the National Academy of Sciences USA 94:707–712.

de Burlet HM, Versteegh C. 1930. Über Bau und Funktion des Petromyzonlabyrinthes. Acta Oto-Laryngologica 13 (Supplement):1–58.

Dememes D, Eybalin M, Renard N. 1993. Cellular distribution of parvalbumin immunoreactivity in the peripheral vestibular system of three rodents. Cell and Tissue Research 274:487–492.

Drescher DG, Khan KM, Arden R. 1989. Protein associated with the sensory cell layer of the rainbow trout saccular macula. Brain Research 485:225–235.

Fermin C, Martin D, Li YT, Li SC. 1995. The glycan keratan sulfate in inner ear crystals. Cellular and Molecular Biology 41:577–591.

Fermin C, Martin D. 1995. Expression of S100 β in sensory and secretory cells of the vertebrate inner ear. Cellular and Molecular Biology 41:213–225.

Fermin CD, Igarashi M. 1985. Development of otoconia in the embryonic chick (*Gallus domesticus*). Acta Anatomica 123:148–152.

Fermin CD, Igarashi M. 1986. Review of statoconia formation in birds and original research in chicks (*Gallus domesticus*). Scanning Electron Microscopy 4:1649–1665.

Fermin CD, Lovett AE, Igarashi M, Dunner K Jr. 1990. Immunohistochemistry and cytochemistry of the inner ear gelatinous membrane and statoconia of the chick (*Gallus domesticus*). Acta Anatomica 138:75–83.

Fermin CD, Lychakov D, Campos A, Hara H, Sondag E, Jones T, Jones S, Taylor M, Meza-Ruiz G, Martin DS. 1998. Otoconia biogenesis, phylogeny, composition and functional attributes. Histology and Histopathology 3:1103–1154.

Fermin CD, Martin D, Hara H. 1997. Color threshold and ratio of S100ß, MAP5, NF68/200, GABA & GAD I. Distribution in inner ear afferents. Cell Vision 4:280–297.

Foster JD, Drescher MJ, Khan KM, Drescher DG. 1993. Immunohistochemical localization of S-100 protein in the saccule of the rainbow trout (*Salmo gairdnerii R.*). Hearing Research 68:180–188.

Furness DN, Karkanevatos A, West B, Hackney CM. 2002. An immunogold investigation of the distribution of calmodulin in the apex of cochlear hair cells. Hearing Research 173:10–20.

Glauert MA. 1972. Practical methods in electron microscopy. Vol. 1. New York: American Elsevier Publishing Co., Inc.

Hackney CM, Fettiplace R, Furness DN. 1993. The functional morphology of stereociliary bundles on turtle cochlear hair cells. Hearing Research 69:163–175.

Hara A, Salt AN, Thalmann R. 1989. Perilymph composition in scala tympani of the cochlea—influence of cerebrospinal fluid. Hearing Research 42:265–271.

Hardisty MW. 1951. Duration of the larval period in the brook lamprey (*Lampetra planeri*). Nature 167:38–39.

Hardisty MW. 1986. *Lampetra planeri* (Bloch, 1784). In: Holčik J, editor. The freshwater fishes of Europe. Vol. 1/I. Petromyzontiformes. Wiesbaden: AULA-Verlag. pp 279–285.

Heil P, Scheich H. 1986. Effects of unilateral and bilateral cochlea removal on 2-deoxyglucose patterns in the chick auditory system. The Journal of Comparative Neurology 252:279–301.

Heller S, Bell AM, Denis CS, Choe Y, Hudspeth AJ. 2002. Parvalbumin 3 is an abundant Ca^{2+} buffer in hair cells. Journal of the Association for Research in Otolaryngology 3:488–498.

Hiel H, Navaratnam DS, Oberholtzer JC, Fuchs PA. 2002. Topological and developmental gradients of calbindin expression in the chick's inner ear. Journal of the Association for Research in Otolaryngology 3:1–15.

Hoshino T, Kodama A. 1976. A scanning electron microscopic study of the crista ampullaris in vertebrates. ANL 3:41–52.

Imamura S, Adams JC. 1996. Immunolocalization of peptide 19 and other calcium-binding proteins in the guinea pig cochlea. Anatomy and Embryology 194:407–418.

Karita K, Nishizaki K, Nomiya S, Masuda Y. 1999. Calbindin and calmodulin localization in the developing vestibular organ of the musk shrew (*Suncus murinus*). Acta Oto-Laryngologica 540 (Supplement):16–21.

Kawasaki H, Nakayama S, Kretsinger RH. 1998. Classification and evolution of EF-hand proteins. Biometals 11:277–295.

Kerschbaum H, Hermann A. 1993. Calcium-binding proteins in the inner ear of the Xenopus laevis (Daudin). Brain Research 617:43–49.

Kevetter GA, Leonard RB. 2002. Decreased expression of calretinin and calbindin in the labyrinth of old gerbils. Brain Research 957:362–365.

Kuijpers W, Manni JJ. 1986. Developmental aspects of glycoprotein secretion and migration in the endolymphatic space. Acta Oto-Laryngologica 429:35–43.

Lowenstein O, Osborne MP, Thornhill RA. 1968. The anatomy and ultrastructure of the labyrinth of the lamprey (*Lampetra fluviatilis* L.). Proceedings of the Royal Society of London B 170:113–134.

Luna LG. 1968. Manual of histologic staining methods of the armed forces institute of pathology. 3rd edn. New York: McGraw-Hill Book Company.

Lychakov D. 1988. Evolution of the otolithic membrane: Structural organization. Journal of Evolutionary Biochemistry and Physiology 24:188–197.

Lychakov D. 1995. Study on structure of the otolith membrane in the lamprey *Lampetra fluviatilis* in the context of otolith and otoconium evolution. Journal of Evolutionary Biochemistry and Physiology 31:90–97.

Lychakov DV, Lavrova EA. 1994. [The content of electrolytes (Na, K, Ca, Mg) in vertebrate otoliths and otoconia]. Zhurnal Evoliutsionnoĭ Biokhimii I Fiziologii 30:99–105.

Marmo F. 1982. Development, structure and composition of the otoliths of vertebrates. Basic and Applied Histochemistry 26:117–130.

Moncrief ND, Kretsinger RH, Goodman M. 1990. Evolution of EF-hand calcium-modulated proteins. I. Relationships based

on amino acid sequences. Journal of Molecular Evolution 30:522–562.

Nakazawa K. 2001. Ultrastructural localization of calmodulin in gerbil cochlea by immunogold electron microscopy. Hearing Research 151:133–140.

Nakazawa K, Spicer SS, Schulte BA. 1995. Ultrastructural localization of Na, K-ATPase in the gerbil cochlea. The Journal of Histochemistry and Cytochemistry 43:981–991.

Pack AK, Slepecky NB. 1995. Cytoskeletal and calcium-binding proteins in the mammalian organ of Corti: Cell type-specific proteins displaying longitudinal and radial gradients. Hearing Research 91:119–135.

Payan P, Kossmann H, Watrin A, Mayer-Gostan N, Boeuf G. 1997. Ionic composition of endolymph in teleosts: Origin and importance of endolymph alkalinity. Journal of Experimental Biology 200:1905–1912.

Peterson SK, Frishkopf LS, Lechene C. 1978. Element composition of inner ear lymphs in cats, lizards, and skates determined by electron probe microanalysis of liquid samples. Journal of Comparative Physiology A 126:1–14.

Piscopo M, Avallone B, D'Angelo L, Fascio U, Balsamo G, Marmo F. 2004. Localization of Calbindin-D28K in the otoconia of lizard *Podarcis sicula*. Hearing Research 189:76–82.

Piscopo M, Balsamo G, Mutone R, Avallone B, Marmo F. 2003. Calbindin D28K is a component of the organic matrix of lizard *Podarcis sicula* otoconia. Hearing Research 178:89–94.

Popper A. 1981. Comparative scanning electron microscopic investigations of the sensory epithelia in the teleost sacculus and lagena. The Journal of Comparative Neurology 200:357–374.

Popper A, Hoxter B. 1981. The fine structure of the sacculus and lagena of a teleost fish. Hearing Research 5:245–263.

Popper A, Hoxter B. 1987. Sensory and nonsensory ciliated cells in the ear of sea lamprey, *Petromyzon marinus*. Brain, Behavior and Evolution 30:43–61.

Popper A, Platt C. 1983. Sensory surface of the saccule and lagena in the ears of ostariophysan fishes. Journal of Morphology 176:121–129.

Presson JC. 1994. Immunocytochemical reactivities of precursor cells and their progeny in the ear of a cichlid fish. Hearing Research 80:1–9.

Raymond J, Dechesne CJ, Desmadryl G, Dememes D. 1993. Different calcium-binding proteins identify subpopulations of vestibular ganglion neurons in the rat. Acta Oto-Laryngologica 503 (Supplement):114–118.

Retzius G. 1881. Das Gehörorgan der Wirbeltiere. Das Gehörorgan der Fische und Amphibien. Vol. 1. S. Stockholmamoson and Wallin.

Rhoten WB, Gona O, Christakos S. 1986. Calcium-binding protein (28,000 Mr calbindin-D28k) in kidneys of the bullfrog *Rana catesbeiana* during metamorphosis. The Anatomical Record 216:127–132.

Riley BB, Zhu C, Janetopoulos C, Aufderheide KJ. 1997. A critical period of ear development controlled by distinct populations of ciliated cells in the zebrafish. Developmental Biology 191:191–201.

Steyger PS, Burton M, Hawkins JR, Schuff NR, Baird RA. 1997. Calbindin and parvalbumin are early markers of non-mitotically regenerating hair cells in the bullfrog vestibular otolith organs. International Journal of Developmental Neuroscience 15:417–432.

Tachibana M, Morioka H. 1992. Glucuronic acid-containing glycosaminoglycans occur in otoconia: cytochemical evidence by hyaluronidase-gold labeling. Hearing Research 62:11–15.

Thalmann R, Ignatova E, Kachar B, Ornitz DM, Thalmann I. 2001. Development and maintenance of otoconia: biochemical considerations. Annals of the New York Academy of Sciences 942:162–178.

Thornhill RA. 1972. The development of the lamprey (*Lampetra fluviatilis*, Linn. 1758). Proceedings of the Royal Society of London B 181:175–198.

Tret'yakov D. 1915. Sense organs of the river lamprey. Zapiski Imperators Novorossisk University (in Russian) 8:1–644.

Usami SI, Shinkawa H, Inoue Y, Kanzaki J, Anniko M. 1995. Calbindin-28K localization in the primate inner ear. Journal Oto-Rhino-Laryngology and its Related Specialties 57:94–99.

Volk E. 1986. Use of calcareous (statoliths) to determine age of sea lamprey amomocoetes *(Petromyzon marinus*). Canadian Journal of Fisheries and Aquatic Sciences 43:718–722.

Winsky L, Harvey JA, McMaster SE, Jacobowitz DM. 1989. A study of proteins in the auditory system of rabbits using 2-dimensional gels—identification of glial fibrillary acidic protein and vitamin D-dependent calcium binding protein. Brain Research 493:136–146.

Wroblewski R. 1993. Intracellular and extracellular elemental composition of the endolymphatic sac studied by X-Ray microanalysis. Scanning Microscopy 7:1221–1232.

Yamagishi M, Ishizuka Y, Fujiwara M, Nakamura H, Igarashi S, Nakano Y, Kuwano R. 1993. Distribution of calcium binding proteins in sensory organs of the ear, nose and throat. Acta Oto-Laryngologica 506 (Supplement):85–89.

Yamashita H, Takahashi M, Bagger-Sjoback D. 1995. Expression of S-100 protein in the human fetal inner ear. European Archives of Oto-Rhino-Laryngology 252:312–315.

Zimmer DB, Cornwall EH, Landar A, Song W. 1995. The S100 protein family: history, function, and expression. Brain Research Bulletin 37:417–429.