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Glycoconjugates in the otolithic membrane of herring larvae: a possible framework for encoding the life history recorder in fishes

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Glycoconjugates in the inner ear of herring *Clupea harengus* larvae, investigated by laser confocal and electron microscopy, were located mainly in the gelatinous layer of the otolithic membranes, forming a collar around the proximal surfaces of the otoliths. The site of secretion was located on the surface of the sensory macula, from which a colonnade of glycoconjugate streamers projected through the subcupular region to connect with the gelatinous layer of each otolith. An electron dense component of the outer gelatinous layers, shown by TEM to be closely associated with the sensory kinocilia, suggested that they provided a basis for the streamers and offered a potential role in directing the path of secretion. It is hypothesized that this highly structured glycoconjugate framework could provide a mechanism for localizing and containing ionic and protein gradients previously detected in this vicinity and which are considered to have a key role in driving the differential growth and mineralization of the otoliths.

Key words: otoliths; otolithic membrane; glycoconjugates; herring; larvae.

INTRODUCTION

The otoliths encapsulate a record of the growth patterns and key events in the life history of individual bony fishes. Calcium carbonate crystals are deposited rhythmically in the protein matrix of the otoliths, producing daily and seasonal growth increments (Panella, 1971; Campana & Neilson, 1980; Gauldie & Nelson, 1990). The spacings between the increments are correlated with the growth of the fish and are influenced by food availability, temperature and key life history events such as migration, settlement, metamorphosis and spawning. Also the shapes of the otoliths, in particular the sagittae, are sufficiently unique to allow identification of individual species (Platt & Popper, 1981; Nolf, 1985; Wilson, 1985). Remarkably, the growth and shaping of the otoliths take place without direct contact with a secretory epithelium. Secretory cells and ionocytes, thought to be responsible for the formation of the protein matrix and calcium carbonate deposition, have been located in the saccular epithelium particularly in and around the macula (Davis *et al.*, 1997; Mayer-Gostan *et al.*, 1997; Pisam *et al.*, 1998). How the secretory products are directed onto the otoliths has not been

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resolved, although local gradients of ions have been measured in the proximity of the otoliths (Payan *et al.*, 1999). Between the otolith and the macula is the otolithic membrane, which may have a possible role in the ontogeny of otoliths in fish larvae (Dunkelberger *et al.*, 1980; Zhang & Runham, 1989) and of otoconia in embryos of higher vertebrates (Endo *et al.*, 1991). Of particular interest is the outer gelatinous layer, the primary function of which is assumed to be the transfer of mechanical energy from the movement of the otolith to the sensory cells of the macula via their cilia (Dunkelberger *et al.*, 1980). The gelatinous layer is porous and where the pores make contact with the sulcus acusticus of the otolith, pits and protrusions are formed on the surface of the otolith, which implicates it in the pattern of mineral deposition. This also raises the intriguing possibility that it may serve as a template for the growth and development of the otolith. The chemical composition of the otolithic membrane is not fully known (Silver *et al.*, 1998), but studies of the otolithic organs and cupulae of birds and mammals have revealed the presence of glycoconjugates (Endo *et al.*, 1991; Sugiyama *et al.*, 1991; Yamashita *et al.*, 1992; Suzuki *et al.*, 1997). In this study the secretion of glycoconjugates and the ontogeny of otoliths in larval herring *Clupea harengus* L. has been investigated, *in vivo*, using a fluorescent lectin marker (concanavalin A) and laser confocal microscopy. A comparison of the distribution of glycoconjugates and the ultrastructure of the otolithic membrane was also made.

MATERIALS AND METHODS

Larvae were reared from gonads from Buchan herring stocks, which were flown to Bergen, Norway, where the eggs were fertilized artificially (Tytler & Ireland, 2000) and incubated at 10·1° C in a natural photoperiod regime (60°24′ N; 5°18′ E). The larvae were reared at 10·3° C and fed natural plankton from 4 days posthatch (Folkvord *et al.*, 2000). Between days 10 and 11 posthatch, one batch of larvae was processed for electron microscopy (Tytler & Ireland, 1995). A second batch was prepared for injection of concanavalin A (Con A) fluorescein conjugate (Molecular Probes) which binds to α-mannopyranosyl and α-glucopyranosyl residues of glycoconjugates. Using the method of Tytler *et al.* (1996), 15 nl of isotonic saline solution of Con A (0·2 mg ml^{-1}) were injected into the otocysts of six larvae anaesthetized by immersion in a solution of MS222 (Sandoz) in sea water. The injections were made between 0900 and 1100 hours local time. The larvae were allowed to recover in isotonic sea water (11 psu) for 24 h. Each larva was then re-anaesthetized, placed on a glass slide and compressed by withdrawing excess anaesthetic solution from under the cover slip, sufficient to immobilize it and shorten the working distance for microscopy. The otolithic membrane was then viewed by a Bio-Rad MRC1024 UV laser confocal microscope, equipped with a Krypton/Argon laser (488 nm) and a 522/35-emission filter, under a $\times 40$ objective lens. Digitized images were captured and processed using Bio-Rad Confocal Assistant 4·02. The experiments were conducted in accordance with the Norwegian Animal Welfare Act.

RESULTS

Two spheroid otoliths, the sagitta and lapillus, are present in the otocyst of 11 day posthatch herring larvae [Fig. 1(a),(b)]. The sagitta is placed, posterior and ventral to the lapillus and in a more medial position. The main concentration of the glycoconjugates in the otolithic membrane of the sagitta, revealed by the intensity and distribution of fluorescence of Con A, was found in a layer,

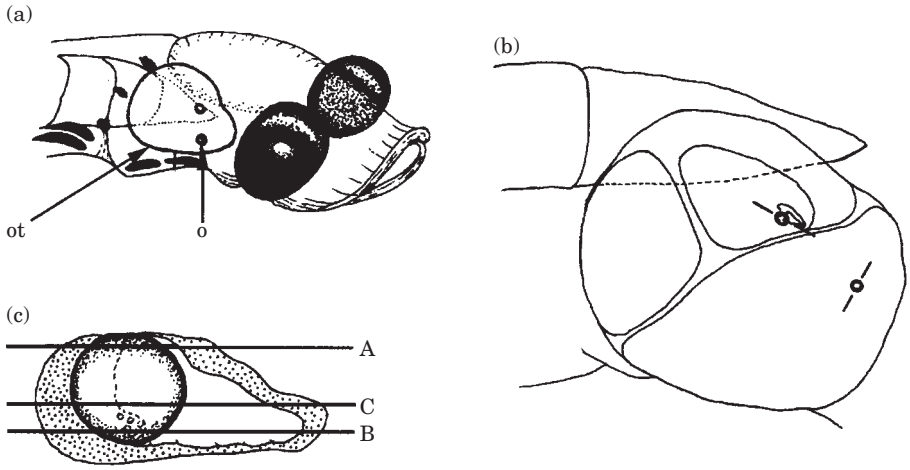

FIG. 1. (a) The head of a herring larva (12 days posthatch) showing the location of the otocyst (ot) and otolith (o). (b) Dorsolateral view of the otocyst of an 11 day herring larva, showing the location of the sagitta and lapillus in relation to the notochord. The lines represent the long axes of the otolithic membranes. (c) A plan view of the sagitta in relation to the gelatinous layer and the macula (stippled), as revealed by Con A fluorescence. The lines indicate the sections A, B and C in Fig. 4, which are part of a series used to construct this drawing. Bar = 25 μm.

proximal to the otolith and corresponding to the location of the gelatinous layer (Fig. 2). The layer was 6–7 μm thick, about half that of the underlying subcupular meshwork (15 μm). The otolithic membrane forms a sub-equatorial collar around the otoliths, proximal to the macula. It was also seen to extend anteriorly, beyond its attachment to the sagitta, in the long axis of the larva [Fig. 1(c)]. This extension of the sagittal otolithic membrane is even evident in newly hatched herring larvae (Fig. 2). By contrast, at this stage, the glycoconjugates were found to be restricted to the rim of the lapillus forming a fluorescent cup with the greatest concentrations in the ventral sectors at right angles to the axis of the sagitta (Fig. 3).

The micro-architecture of the subcupular meshwork of the sagitta was characterized by thin parallel columns of fluorescence arising from a regular array of ' blebs ' on the surface of the macula and terminating in the gelatinous layer. The columns were generally orientated at right angles to the plane of gelatinous layer (Figs 2 and 4). Similar fluorescent columns were found in the subcupular layer of the lapillus, but their distribution was more regular than for the sagitta (Fig. 2).

In sagittal confocal sections [Fig. 4(c)] the glycoconjugate layer on the surface of the sagitta, proximal to the macula, was markedly thinner with a regular pattern of ' blebs ' associated with the termination of the fluorescent columns in this region. Some low-level fluorescence was detected on the surface of the otolith, particularly on this medial surface, but the intensity was much less than in the gelatinous layer. On the surface of the macula the ' blebs ', taken to be the sources of secretion, were most conspicuous dorsally, under the anterior extension of the otolithic membrane and ventrally under the otolith [Fig. 4(a),(b)]. The fluorescent columns were also more conspicuous in these regions. The distances between the fluorescing columns, ranging from 3·4 to 5·4 μm, were

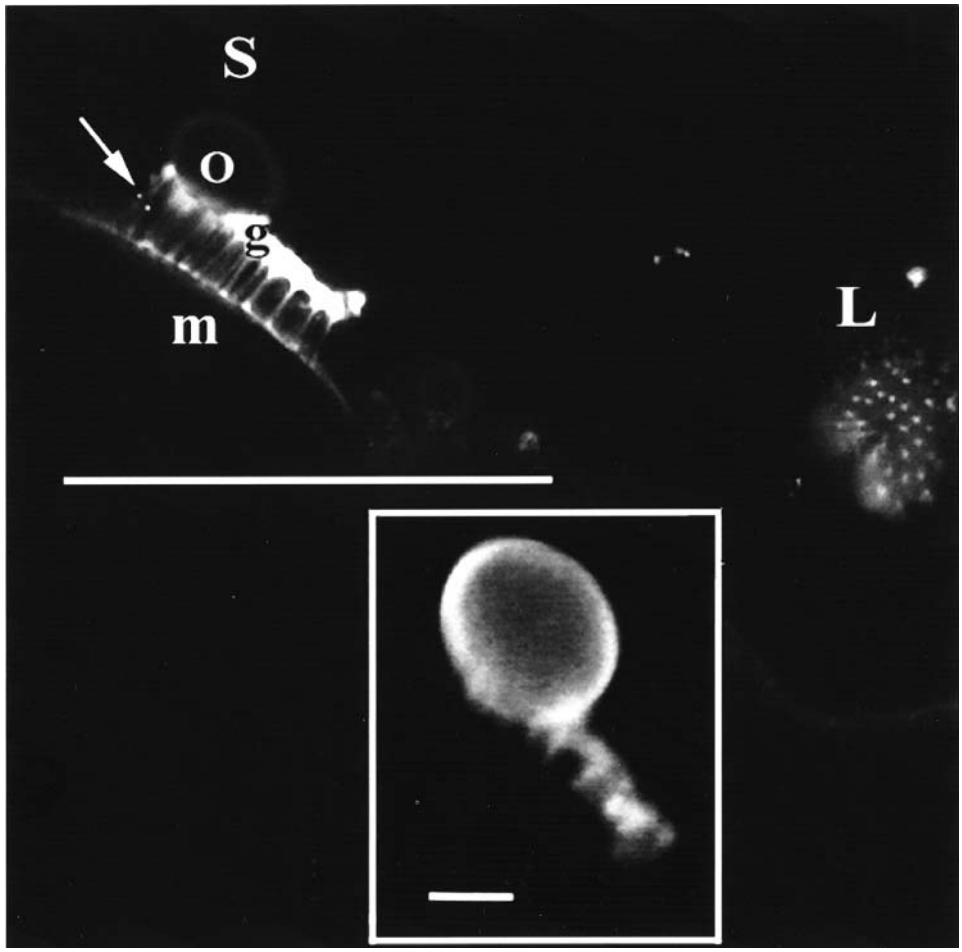

FIG. 2. A confocal section through the sagitta (S) of a herring larva at 11 days posthatch, showing the otolith (o), the gelatinous layer (g), overlying the subcupular layer and the macula (m). The arrow indicates fluorescent beads associated with the fluorescent columns. To the right is a section through the subcupular layer of the lapillus (L). Bar = 100 μm. The insert shows the distribution of Con A in the otolithic membrane and on the sagitta of a 44 somite yolk sac herring larva. Bar = 10 μm.

similar to those between the kinocilia of the sensory hair cells revealed by electron microscopy. Also a comparison of phase contrast and confocal images of the same section shows close matching of the fluorescent columns and the cilia in the otolithic membrane of the lapillus (Fig. 5). Electron-dense particles were also observed in electron micrographs of the otolithic membrane, particularly in the subequatorial regions of the otolith, with a ' bleb ' on the medial surface, corresponding to the distribution of glycoconjugates seen in confocal sections (Fig. 6). These particles were seen to be closely associated with the kinocilia of the hair cells. In the subcupular meshwork numerous, apparently empty, vesicles were observed, suggesting vigorous secretory activity in this region. Fluorescent particles, which may correspond to these, are seen associated with the peripheral posterior columns (Fig. 2) and within the subcupular zone (Fig. 4), but not in the profusion seen in electron microscopy.

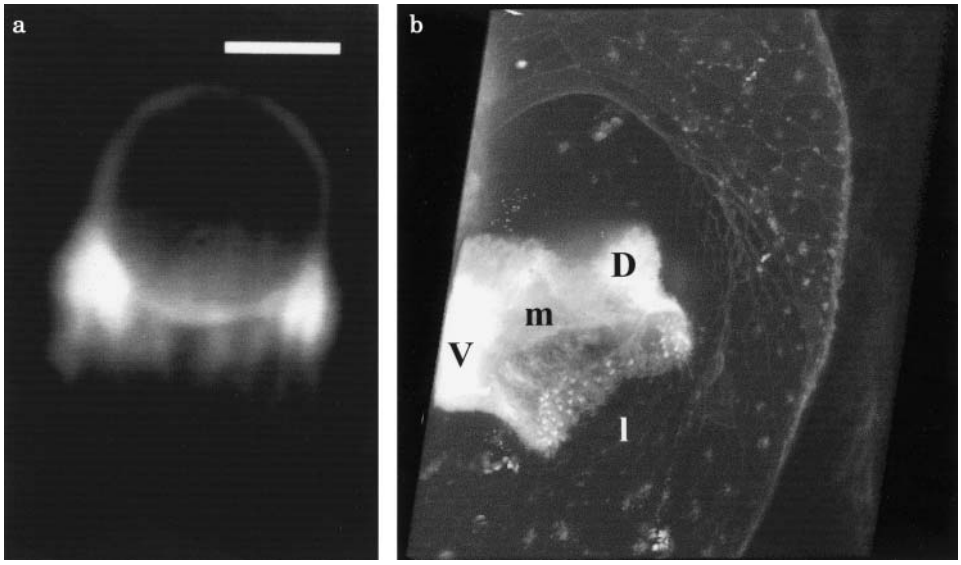

FIG. 3. (a) The lapillus of a 55 somite late yolk sac larva, showing two subequatorial patches of Con A fluorescence. Bar = 10 μm. (b) A three dimensional projection of a series of 18 confocal sections taken at 1·8 μm through the otolithic membrane (m) of an 11 day posthatch larva, showing the concentrations of glycoconjugate secretion in the dorsum (D) and ventrum (V) of the otolithic membrane (m). The location of the lapillus (l) is indicated.

Three-dimensional projections of confocal serial sections [Figs 1(c) and 7] show a plan view of the sagitta, in which the gelatinous layer is seen to taper anteriorly and to have a bulge at its widest post, ventral to the otolith.

DISCUSSION

The glycoconjugates of the otolithic membranes in herring larvae are similar to that found in the cupular and gelatinous membranes of adult fishes and higher vertebrates, consisting of glycoproteins (Endo *et al.*, 1991; Munyer & Schulte, 1991; Sugiyama *et al.*, 1991; Yamashita *et al.*, 1992; Cohen-Salmon *et al.*, 1997; Davis *et al.*, 1997; Suzuki *et al.*, 1997). The protein of the saccular (sagitta) otolithic membrane of the bluegill sunfish *Lepomis macrochirus* Rafinesque has been identified as a 95 kDa glycoprotein (Davis *et al.*, 1997). The main location of glycoconjugates is in the outer gelatinous layer. The source of the secretion of proteins and glycoproteins in particular has been identified as the supporting cells of the sensory macula in various species (Endo *et al.*, 1991; Davis *et al.*, 1997; Pisam *et al.*, 1998). The products appear to be directed up the kinocilia towards the gelatinous layer, in much the same way as the cupula is formed in neuromast organs. The supporting evidence for this study is that electron dense material of the gelatinous layer of the otolithic membrane is associated with the kinocilia and that the spacing between the fluorescent columns is similar to that between kinocilia seen in light and electron micrographs. In his histological study of the sacculae of *Salmo salar* L. and *Gasterosteus aculeatus* L. Wright (1990) identified PAS positive globules associated with kinocilia. Therefore it is proposed that the fluorescent columns seen in this study are kinocilia coated with

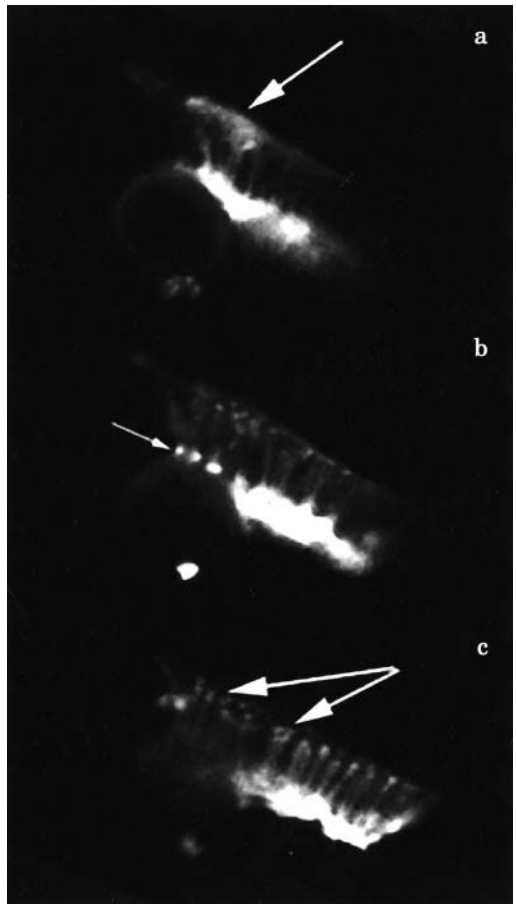

FIG. 4. Confocal sections taken through the sagitta of an 11 day posthatch herring larva and its otolithic membrane, showing the localization of the sites of glycoconjugates on the surface of the macula (⟹) and fluorescent beads on the proximal surface of the otolith (⇨). A, B & C are dorsal, ventral and sagittal sections respectively [see Fig. 1(c)].

the glycoconjugate secretion. Glycoconjugates are often electrically charged molecules and electrochemical gradients are known to occur along sensory hair cells, particularly when stimulated. Possibly these gradients, created by the activity of the larva, could provide the driving force for the movement of the glycoconjugates towards the gelatinous layer and the otolith. Although low level Con A fluorescence was often seen on the surface of the otoliths it is not clear whether or not these glycoconjugates are incorporated into the matrix of the otoliths. Two types of proteins have been identified in the matrix of otoliths. Degens *et al.* (1969) described a high-molecular weight fibrous protein, which resembles keratin and forms the matrix of the otolith. More recently water-soluble PAS positive glycoproteins, with a capacity for calcium binding, have been identified in the otoliths of *S. salar* (Wright, 1991) and *Oreochromis niloticus* L. (Sasagawa & Mugiya, 1996). It may be significant that electron dense material, which may be calcium carbonate, is found in association with the kinocilia. This is where the fluorescent marker for glycoconjugates has also been

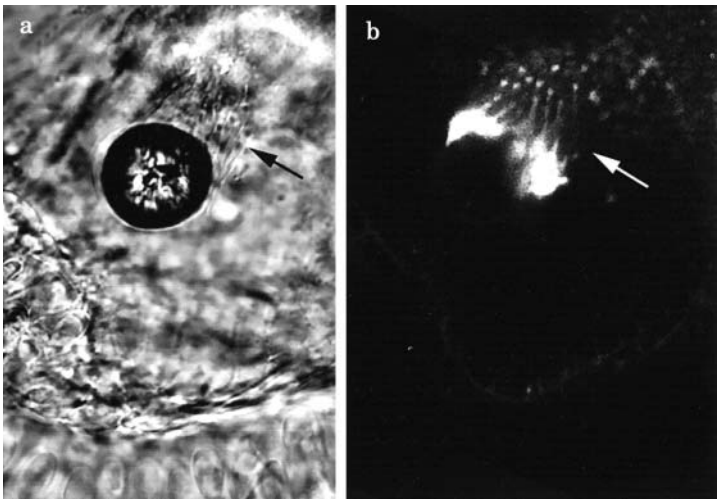

FIG. 5. Coincident phase contrast (a) and confocal (b) sections through the lapillus, showing the relative locations of the kinocilia (arrowed) and Con A fluorescence.

located in this study. Clearly analysis of the chemical composition of the electron dense material is required to resolve this question.

The subcupular meshwork part of the otolithic membrane contains a regular array of strands effectively producing a sponge-like structure. From earlier ultrastructural studies of *O. niloticus* it has been shown that there is an even finer meshwork of filaments within the subcupular layer, with a pore size of *c*. 150 nm (Zhang & Runham, 1989). This structural arrangement is ideally suited for the localization and containment of the gradients of ions and proteins in the endolymph found about the otoliths of rainbow trout *Oncorhynchus mykiss* (Walbaum) and the turbot *Scophthalmus maximus* (L.) (Payan *et al*., 1999). The growth of otoliths has been described as a pattern of incremental deposition of the calcified layers (Dunkelberger *et al*., 1980), which were thickest on the medial (proximal to the macula) and peripheral surfaces of the otoliths. In other words the primary growth zone is where the gelatinous layer makes contact with the otolith. It may be significant that large ionocytes and granular (secretory) cells have been found in the corresponding underlying areas in the maculae of rainbow trout and turbot (Mayer-Gostan *et al*., 1997; Pisam *et al*., 1998). The products of these cells are thought to be incorporated in the otolith and that the driving forces for the differential growth and mineralization of the otolith are the ionic and protein gradients created about the otolith (Mayer-Gostan *et al*., 1997; Pisam *et al*., 1998; Payan *et al*., 1999).

The striking resemblance between the plan view of the otolithic membrane and the eventual shape of the sagitta, implies that the otolithic membrane may act as a template for the otolith. Such a suggestion must be tenuous, since the adult form of the sagitta in herring does not appear until *c*. 2–3 months later, at metamorphosis. Also daily growth increments continue to be deposited up to metamorphosis. It is more likely that the shape of the otolithic membrane may have functional significance (Gauldie, 1988), although this proposal does not exclude the former. The primary site for deposition of growth increments in

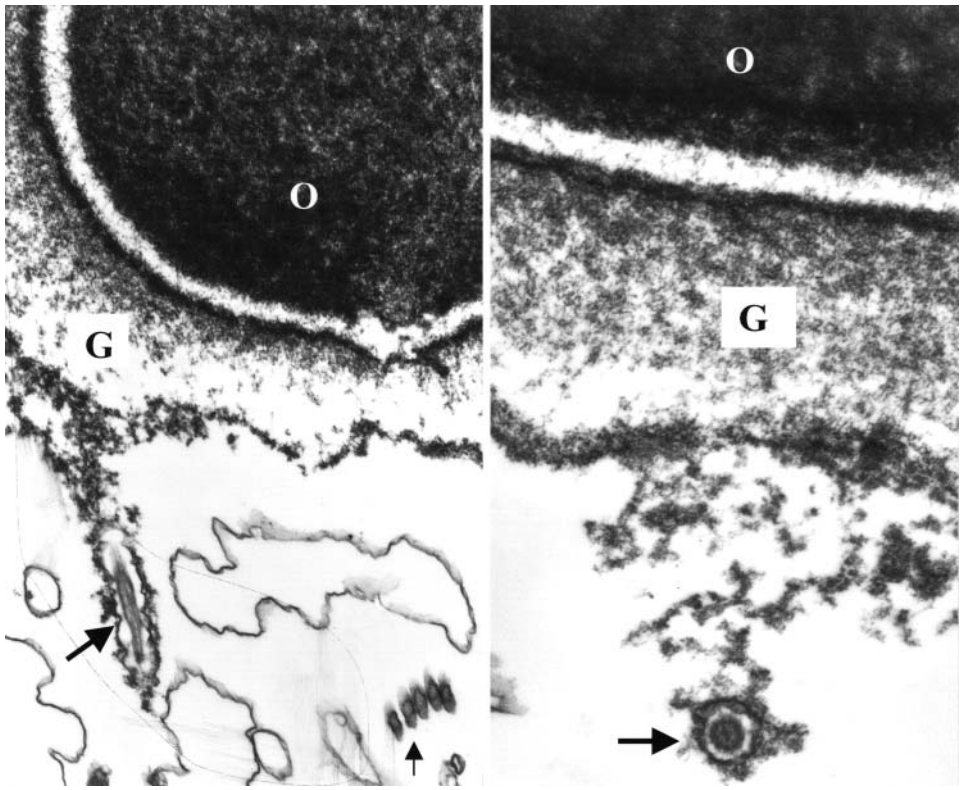

FIG. 6. Electron micrographs of the otolithic membranes of the lapilli of 10 day posthatch herring larvae. The kinoclia (➞) are shown enveloped in the same electron dense material, which is found in the outer layer of the gelatinous layer (G). Stereocilia are indicated by the ➞. O, otolith [(a) × 15 000 and (b) × 20 000].

otoliths is where it makes contact with the otolithic membrane (Dunkelberger *et al.*, 1980). The glycoconjugates of the otolithic membranes of herring larvae were located on the median surface of the sagitta and on the rim of the lapillus. In this context it was interesting that Mugiya (1977) found that the deposition of ^{45}calcium was heavily deposited on the median surface of the sagitta, while the lateral sides of the lapillus were favoured in *Carassius auratus* (L). It remains to be explained how the secretory products are directed through or around the outer gelatinous layer onto the otolith. One explanation is based on the observation that the gelatinous layer is porous and that a regular calcification pattern is associated with the pores (Dunkelberger *et al.*, 1980), implying that these may be conduits for the secretory products. Variations in pH and calcium concentrations are known to have marked effect on the viscosity or hardness of mucous and glycoconjugate secretions. The pH and ionic gradients located within the otolithic membrane (Payan *et al.*, 1997) may have some significance in terms of a possible role for the otolithic membrane in regulating the movement of the glycoconjugates along the kinocilia and thus influencing the periodicity of incorporation of secretory products in the otolith.

This work has demonstrated that fluorescent dyes injected into the otocyst can be used to visualize otolith growth *in vivo* in young transparent fish larvae.

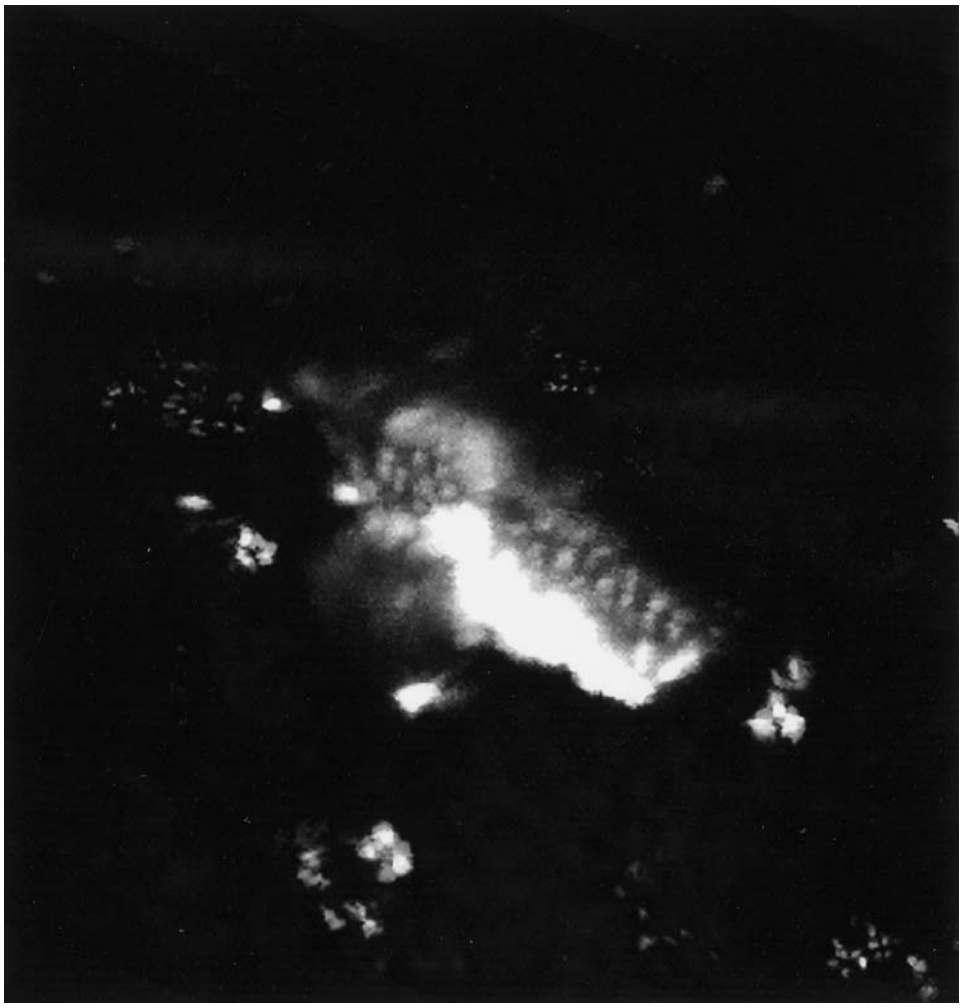

FIG. 7. A three-dimensional projection of a series of confocal sections taken 1·8 μm apart, through the sagitta and otolithic membrane of an 11 day posthatch herring larva. The view is at 60° to the plane of sectioning.

Further studies using this technique and employing specific fluorescent markers for calcium and proteins are needed to investigate the periodicity of growth and deposition of micro-increments within the otoliths.

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