

Flødevigen rapportser. 1, 1984. ISSN 0333-2594
The Propagation of Cod *Gadus morhua* L.

EMBRYOGENESIS AND GROWTH OF OTOLITHS IN THE COD
(*Gadus morhua* L.)

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ABSTRACT

Dale, T., 1984. Embryogenesis and growth of otoliths in the cod (*Gadus morhua* L.) In: E. Dahl, D.S. Danielssen, E. Moksness and P. Solemdal (Editors), The Propagation of Cod *Gadus morhua* L., Flødevigen rapportser., 1, 1984: 231-250.

The embryogenesis and growth of the otoliths in the cod (*Gadus morhua* L.) was studied by light microscopy and transmission electron microscopy. The first indication of otolith formation was the appearance of electron-dense granules in the anterior and posterior parts of the otocysts at about the 30-somite stage of the embryos. The granules, which consisted of a condensed, flocculent material, were located close to the tips of the developing kinocilia on the sensory cells and accumulated into a cluster, forming the core of the embryonic otoliths. A less condensed organic matrix was deposited around the otolith core, first segmentally, then in continuous layers. The organic matrix consisted of filamentous material, and isolated filaments could be observed in the endolymph around the developing otoliths. The kinocilia remained in contact with the developing otoliths for some days through canals in the deposited matrix, and as they increased in length, they displaced the otoliths upward. Later, the distal part of the cilia disintegrated, but the canals persisted.

In the lapilli (utricle otoliths) of newly hatched larvae reared under a 12L-12D photoperiod, 1-2 faint pre-hatch growth rings could occasionally be observed, but the first distinct growth ring was formed during the first 24 h after hatching and was separated from the central part of the otolith by a prominent boundary layer. Consecutive growth rings seemed to form every 24 h, but the rings were difficult to separate and could not be used for exact age determination. No distinct growth rings formed in the lapilli of larvae reared in constant darkness, and the otolith matrix

was little condensed, making these otoliths somewhat bigger and rounder than "normal" otoliths of the same age. No regular zone formation occurred in the lapilli of larvae reared in constant light. The distribution of precipitated calcium pyroantimonate in the otoliths suggested that the calcification of the otoliths started at hatching.

Light microscopy of transverse sections through the otocysts of larvae showed that the lapillus was located over a horizontal sensory epithelium, whereas the posterior saccular otolith, the sagitta, was located near the medial wall of the otocyst, beside a vertical sensory epithelium. The lapillus was somewhat bigger than the sagitta at the early larval stage. Occasionally, a third body, which probably represented the developing third otolith, the asteriscus, could be observed in the dorsal part of the otocyst at the late embryonic stage.

INTRODUCTION

The structure and composition of teleost otoliths is well understood (Hickling, 1931; Dannevig, 1956; Carlstrøm and Engstrøm, 1963; Morris and Kittleman, 1967; Degens et al., 1969; Liew, 1974; Blacker, 1975; Rannou and Thiriot-Quivreux, 1975; Dale, 1976; Bingel, 1980; Dunkelberger et al. 1980; Radtke, 1984). However, very little is known about the embryogenesis of teleost otoliths. So far, only a few light microscopical observations have been published (Nishio, 1926; Mugiya, 1968; McKern et al., 1974; Radtke and Waiwood, 1980), but nothing at the ultrastructural level, which is necessary to reveal the details in otolith formation.

After Pannella (1971, 1974) discovered the presence of daily growth rings in teleost otoliths, otolith research has been focussed on ring formation, in the hope of getting a reliable method for age determination, especially for the larval stages (see Bergstad, 1984). However, the basic mechanism behind ring formation is still unknown, although there is some evidence that the photoperiod or the activity pattern may be involved (Taubert and Coble, 1977; Pannella, 1980; Tanaka et al., 1981; Neilson and Geen, 1982; Radtke and Dean, 1982).

In the present paper, the formation and growth of the otoliths in the cod (*Gadus morhua* L.) is described at the ultrastructural level. The following problems are elucidated: How, and at what embryonic stage do the otoliths begin to form? How are the otoliths growing, and when is the first growth ring formed? What factors determine the frequency of the growth rings, and when does the calcification of the otoliths start?

MATERIAL AND METHODS

Ripe gametes were stripped from coastal cod, *Gadus morhua* L. The eggs were artificially fertilized and incubated in buckets of stagnant sea water at +5°C. One group of eggs was reared under a 12L-12D photoperiod, a second group (24L) was reared in constant light and a third group (24D) was reared in constant darkness. The light source was a 40W daylight fluorescent tube placed 1 m above the eggs. Dead eggs, which sank to the bottom, were removed and the sea water was changed if it became turbid. After one week, samples of eggs were fixed at 24 h intervals at +5°C by immersion in a mixture of 2.5% glutaraldehyde and 2% formaldehyde in Ringer's solution containing 0.1M sucrose and buffered at pH 7.4 with 0.1N sodiumcacodylate/HCl. After an aldehyde fixation of minimum 2 h, the embryos were isolated by removing the chorion and yolk sac with watchmakers forceps under a binocular microscope. The eggs hatched after about 20 days at +5°C, and thereafter, entire larvae were fixed in the same way as the eggs. The larvae were not fed and samples of larvae were taken until the yolk sac was absorbed. This occurred about two weeks after hatching. After the aldehyde fixation, the specimens were rinsed in the fixative vehicle and postfixed for 1 h at +5°C in 1% OsO₄ in the same kind of fixative vehicle as used for the aldehyde fixation. After rinsing, the specimens were dehydrated in acetone and embedded in EPON. For photomicrography, 2 µm thick sections were

stained in toluidine blue and mounted in immersion oil. Some eggs and larvae were photographed *in vivo* by placing them in a spectrophotometric cuvette with sea water and photograph them through a microscope with its optical axis oriented horizontally. The eggs and larvae were kept in position between the wall of the cuvette and a coverslip, and the larvae were immobilized by anaesthesia with MS-222.

For transmission electron microscopy, ultrathin sections were contrasted at room temperature with 2% aqueous uranyl acetate for 1 h and with lead citrate for 5 minutes and examined with a transmission electron microscope at 80 kV. In order to detect calcium in the otoliths, some larvae were prepared according to the potassium-pyroantimonate method (Simson and Spicer, 1975).

RESULTS

Light microscopy

Thick-walled, ellipsoidal otocysts had formed at about the 30-somite stage of the embryo, about one week after fertilization of eggs incubated at +5°C (Fig. 1). Minute, dark dots could be identified close to the epithelial surface in the anterior and posterior parts of the otocysts, although they are hardly visible on the light micrograph (Fig. 1). As the lumen of the otocysts increased, the otocysts became more elongated and the otocyst wall became thinner, except at the locations of the sensory epithelia, which formed thickenings in the otocyst wall under the developing otoliths (Figs. 5, 6). The dark dots in the anterior and posterior parts of the otocysts gradually increased in size, and at hatching, two small lobular otoliths were visible in each otocyst (Figs. 2, 3). The anterior utricular otolith (lapillus) was somewhat bigger than the posterior saccular one (sagitta) and was located more ventro-laterally in the otocyst (Figs. 2, 3). Occasionally, a third dot, probably representing the develop-

ing asteriscus, could be observed in the dorsal part of the otocyst (Fig. 4). Cross sections through the otocysts showed that the anterior otolith was located over a horizontal sensory epithelium, whereas the sensory epithelium belonging to the posterior otolith was oriented in the vertical plane (Figs. 5, 6).

Transmission electron microscopy

The first sign of otolith formation was the appearance of electron-dense granules about 1 μm in diameter in the anterior and posterior parts of the otocysts, at about the 30-somite stage of the embryos (Figs. 7, 8). The granules were located close to the tips of the kinociliary buds on the differentiating sensory cells and consisted of condensed, flocculent material (Figs. 8, 9, 10). Loose flocculent material was present between the granules and the tips of the developing kinocilia, which occasionally contained rows of small vesicle-like structures along their periphery (Fig. 10). The space between the differentiating sensory epithelium and the developing otolith contained membranous material in the form of large vesicles which often contained smaller vesicles (Figs. 8, 9, 11). About 48 h after their appearance, the electron-dense granules had formed clusters consisting of densely packed, multi-faceted bodies (Fig. 9). Some of the bodies had a translucent core and showed a zonation of 3-4 alternating light and dark layers (Fig. 9).

The next stage in otolith formation was the deposition of filamentous material around the clusters of multi-faceted bodies, which constituted the core of the embryonic otoliths. The deposition started at the top of the clusters (Fig. 9), forming cup-shaped segments separated by narrow furrows (Figs. 11, 12). The deposited filamentous material was less condensed than the flocculent material of the otolith core and seemed to consist of globular subunits (Fig. 12). Isolated filaments were in contact with the surface of the

developing otolith and could also be observed free-floating in the endolymph around the otolith (Fig. 12).

About 48 h after the deposition of filamentous material had started, the otolith cores were embedded in a matrix of filamentous material, except at the locations of the kinocilia, which remained in contact with the otolith through canals in the deposited matrix (Fig. 13). As the kinocilia increased in length, they pushed the otolith upward, as could be seen by the increasing distance between the otolith core and the epithelial surface (Figs. 8, 13). Later, the distal part of the kinocilia, located in the ciliary canals, disintegrated as the otolith increased in diameter, but the canals persisted (Figs. 14, 18).

The deposited filamentous matrix was more condensed adjacent to the otolith core than more peripherally, where it was arranged in concentric, undulating bundles, but without forming growth rings before hatching. However, 1-2 faint pre-hatch growth rings could occasionally be observed (Fig. 14).

In the lapillus, the first distinct growth ring formed during the first 24 h after hatching and can accordingly be called the "hatching ring" (Fig. 15). Consecutive growth rings formed every 24 h. Thus five growth rings could be identified in the lapillus five days after hatching. The growth rings were separated by either sharp discontinuity lines or more diffuse boundary zones (Figs. 16, 17). The thickness of the growth rings varied both between different rings and within the same ring (Fig. 16). The growth rings followed the outline of the otoliths, which were deeply invaginated at the entrances of the ciliary canals (Fig. 16).

A fine, radial texture and a narrow zone of minute, electron-dense granules were visible in the peripheral growth rings of some lapilli five days after hatching (Fig. 17). The deposited matrix was more condensed on the peripheral side of the discontinuity lines than on the central side (Fig. 17).

Ten days after hatching, 10 growth rings could be interpreted in the lapillus, although some of the rings were rather questionable (Figs. 18, 19). Ciliary canals were still present, but the underside of the otolith was less invaginated than it was five days after hatching (Fig. 18).

In lapilli of larvae reared in constant darkness, no growth rings formed, except for a few thin discontinuity lines (Fig. 20). The otolith matrix was also little condensed, making these otoliths somewhat bigger and rounder than "normal" otoliths of the same age (cf. Figs. 16, 20).

In lapilli of larvae reared in constant light, a few distinct and some diffuse growth rings occurred. The surface of the otolith appeared to have collapsed due to shrinkage (Fig. 21).

Precipitation of calcium by means of potassium-pyroantimonate, showed that calcium was lacking in the central part of the lapillus, which corresponds to the pre-hatch area, whereas heavy deposits were present in the post-hatch area (Fig. 22). The calcium deposits seemed to follow the same zonation as that of the incremental layers of the organic matrix.

DISCUSSION

The fact that the granules, which aggregated to form the core of the embryonic otoliths, appeared in close proximity to the tips of the developing kinocilia on the differentiating sensory cells, suggests that the material forming the granules was secreted by the kinocilia. This hypothesis is supported by the presence of flocculent material between the granules and the tips of the kinocilia and of small vesicles along the peripheral tubuli in some of the kinocilia (Fig. 10). Thus the granule material may be produced by the differentiating sensory cells and transported along the ciliary microtubuli to be secreted at the tips of the kinocilia where it condenses to form the granules. This could

happen in the initial stage of cilia formation, when the kinocilia form short buds with a short distance of transportation for the granule material. The ciliary vesicles on Fig. 10, however, may well be fixation artefacts. The large vesicles between the sensory epithelium and the developing otolith are probably also fixation artefacts caused by osmotic disruption of cell membranes, as can be seen on some of the ciliary shafts (Fig. 13).

Tanaka et al. (1981), using SEM on ground and etched sagittae of *Tilapia nilotica*, found a central aggregate of small crystallized spherules, which corresponds to the core of the cod otoliths. In the cod, however, the granules constituting the core are more densely packed than in *Tilapia* forming faceted, crystalline bodies rather than isolated spherules (Fig. 9).

The filamentous material deposited around the otolith core constitutes the organic matrix of the otolith. As shown by Degens et al. (1969), the organic part of teleost otoliths consists of a special protein called otolin. This protein is probably synthesized and secreted by the supporting cells of the sensory epithelium, because these cells are densely packed with rough endoplasmic reticulum, indicating a high rate of protein synthesis (Dale, unpublished data).

Salamat et al. (1980) claim that the filamentous material of the otoconia in the fetal rat was secreted by vesicles extruded from the epithelium beneath the otoconia. In the cod otocyst, however, the vesicles between the sensory epithelium and the developing otolith do not contain filamentous material (Figs. 9, 11). However, the presence of free-floating filamentous material in the endolymph around the developing otolith suggests that the filaments are formed by precursors secreted by the supporting cells of the sensory epithelium (Fig. 12).

The formation of growth rings in the otoliths is probably caused by differential production and/or deposition of growth material, as reflected by differential density of the deposited material in the otoliths. The lack of growth rings in

the initial stages of otolith formation suggests that the deposition of growth material takes place at a steady rate up to one or two days before hatching, when the first differentiation in the deposition can be seen as sharp discontinuity lines or layers of increased density of the organic matrix (Figs. 14, 15). The sharp discontinuity lines may represent a paucity in the deposition of growth material, whereas the more diffuse dividing layers may represent a temporary increase in the density of the growth material.

The highest density of the organic matrix occurs when the deposition is resumed after a resting phase (Fig. 17). This corresponds to the growth phase after the light period has started in *Tilapia* otoliths exposed to a 12L-12D photoperiod (Tanaka et al., 1981). This layer also corresponds to the interlamellar organic matrix in *Fundulus* otoliths (Dunkelberger et al. 1980). It is also in accordance with the observation of Tanaka et al. (1981), that the discontinuous layers in *Tilapia* otoliths contain more organic material than the incremental layers. These electron-dense layers most probably also correspond to the dark rings in larval cod otoliths observed by light microscopy (Radtke and Waiwood, 1980; Gjøsaeter and Tilseth, 1982; Bergstad, 1984).

Taubert and Coble (1976) and Tanaka et al. (1981) have suggested that otolith growth is controlled by an endogenous rhythm synchronized with the environmental photoperiod. On the other hand, Neilson and Geen (1982) found that the formation of growth rings was dependent on the feeding rhythm, but independent on the photoperiod. Further, Pannella (1980) found that the activity pattern was an important factor in the formation of growth rings in teleost otoliths.

This suggests that the activity rhythm, and not the photoperiod, is the decisive factor in the formation of growth rings in teleost otoliths. However, the photoperiod may control the activity rhythm which in turn induces the formation of growth rings in the otoliths. The mechanism may be explained as follows: During activity, the otoliths are more "excited", causing the otolith growth material to be

more densely packed than during inactivity, when the otoliths are "quiet" and there is no turbulence in the endolymph. During inactivity, the otoliths can therefore grow undisturbed at a steady rate, without forming growth rings and with a uniform, little condensed matrix. This can be seen in otoliths of larvae reared in constant darkness, when the larvae are rather inactive, as indicated by lowered energy consumption (Solberg and Tilseth, 1984). The loosely packed matrix also make these otoliths bigger than "normal" otoliths of corresponding age (cf. Figs. 16, 20).

Periods of high activity may also explain the formation of the prominent "hatching ring". Thus the heavy struggle of the larvae to get rid of the chorion at hatching may cause an extra dense packing of the otolith growth material, resulting in the formation of a condensed "hatching zone" (Figs 15, 16). Activity periods may also explain the formation of "pre-hatch" rings (Fig. 14). Thus jerks of the embryos could be observed several days before hatching after the trunk musculature was developed and the embryos were sensitive to light.

According to the above hypothesis, the formation of an irregular number of growth rings in otoliths of larvae reared in constant light (Fig. 21), may reflect an irregular activity rhythm in these larvae. Further, the presence of faint discontinuity lines in some of the otoliths of larvae reared in constant darkness may be due to short activity periods during inspection of the larvae. This is also in accordance with the observation of Radtke and Dean (1982), that a 1-min light stimulus on day 10 resulted in increment formation in *Fundulus* otoliths of larvae reared in constant darkness. However, if light is a necessary factor in the formation of growth rings in teleost otoliths, growth rings should also be lacking or irregular in otoliths from deep-sea fishes living in the aphotic zone, but this does not seem to be the case. Thus Rannou and Thiriou-Quivreux (1975) found the same zonation in otoliths from the deep-sea macrourid *Coryphaenoides*

guentheri as that of otoliths from fishes living in the upper layers. *C. guentheri* may not live in a complete aphotic environment, however.

Temperature and feeding were kept constant and therefore cannot have induced the formation of growth rings in the otoliths. In fact, the larvae were not fed at all, but used their yolk sac energy. This may explain why the growth rings were thinner and more difficult to read after day 5, when most of the yolk sac is utilized and the larvae start to feed on prey under natural conditions (Ellertsen et al., 1980). Also Taubert and Coble (1976) found daily rings in otoliths of fish held at constant temperature, and Tanaka et al. (1981) showed that the formation of growth rings was independent of feeding times. On the other hand, temperature and nutrition influence the growth rate and thereby also the thickness of the growth rings and their content of organic material, like the formation of seasonal zones in otoliths of fish from temperate waters (Pannella, 1974). However, Bergstad (1984) found that there is some indication that the rate of zone formation is related to the individual growth rate of cod larvae between hatching and metamorphosis at day 35-40 after hatching.

Another hypothesis for the formation of growth rings in teleost otoliths is that the photoperiod may regulate the quantity of organic growth material secreted into the otocyst. The fluctuations in available growth material may then be reflected in differential growth of the otoliths, seen as growth rings. In fact, Delahunty and De Vlaming (1980) have shown that the photoperiod alters the level of plasma proteins in *Carassius*. There may thus be a combined effect of photoperiod, activity rhythm and plasma protein fluctuation in the formation of otolith growth rings.

Nakahara and Bevelander (1979) have suggested that the otoconiae of fetal mice are formed as organic "preotoliths" which are calcified after birth. On the other hand, Peacor et al. (1980) claim that the "preotoliths" have a definite crystal structure from the beginning. Although mammalian

otoconiae and teleost otoliths cannot be directly compared, the present study shows that the growth of cod otoliths is initiated by the deposition of a filamentous, organic matrix around a core of densely packed, crystalline granules consisting of organic, flocculent material which seems to be secreted by the kinociliary buds.

As demonstrated by the calcium-pyroantimonate technique (Fig. 22), the mineralization of the otoliths probably starts at hatching, when the otoliths are more exposed to sea water after the chorion is removed. Mugiya (1980) has shown that environmental ^{45}Ca was heavily incorporated into regenerating scales of *Carassius*, and this may also be the case for the otoliths. Thus sea water may be the ultimate calcium source for the otoliths, at least in the initial stages when nutritional calcium is not available. Further, Mugiya et al. (1981) found a diurnal rhythm in the calcium uptake in *Carassius* otoliths. Thus the calcium uptake slowed down or stopped at sunrise and resumed in 3 h. This may be correlated with the formation of the matrix-dominant discontinuity zone after light onset under a 12L-12D photoperiod, as discussed above.

The concentric zones of precipitated calcium (Fig. 22) probably correspond to the incremental zones of calcified interlamellar matrix. The calcification cannot be seen on the ultrathin sections prepared for transmission electron microscopy because the use of standard preparation methods results in demineralization of the sections, mainly because of the acidity of the stains. Thus the stained otolith sections can be considered as decalcified, showing the differential deposition of organic matrix only. This supports the hypothesis that the growth rings are formed primarily by differential deposition of an organic matrix consisting of calcium-binding proteins which in turn are calcified.

ACKNOWLEDGEMENTS

This work was supported by the Norwegian Fishery Research Council (NFFR). The author is also indebted to the Institute of Marine Research, Bergen, for supplying the material, and to Zoological Laboratory, University of Bergen, for working facilities.

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LEGENDS TO FIGURES

Fig. 1. Photomicrograph of embryo *in vivo*, about one week after fertilization. Thick-walled otocysts (O) with anterior and posterior otolith primordia have developed.

Fig. 2. Photomicrograph of the head of a newly hatched larva *in vivo*, dorsal view focussed on the otocysts with otoliths. L=lapillus, S=sagitta.

Fig. 3. Photomicrograph of the head of a newly hatched larva *in vivo*, left lateral view, focussed on the otocyst with otoliths. L=lapillus, S=sagitta.

Fig. 4. Photomicrograph of embryo *in vivo*, just before hatching, left lateral view focussed on the otocyst with otoliths. A third body, probably representing the developing asteriscus (A) is visible in the dorsal part of the otocyst.

Fig. 5. Photomicrograph of a transverse section through the head of a larva and the anterior otolith pair, the lapilli (L). Note that the sensory epithelia (SE) are located ventrally to the otoliths, in the horizontal plane.

Fig. 6. Photomicrograph of a transverse section through the head of a larva and the posterior otolith pair, the sagittae (S). Note that the sensory epithelia (SE) are located medially to the otoliths, in the vertical plane.

Fig. 7. Low power transmission electron micrograph of otocyst shown in Fig. 1, with otolith primordia consisting of clusters (C) of electron-dense granules in the anterior and posterior parts of the otocyst.

Fig. 8. Transmission electron micrograph showing the relationship between the developing kinocilia (kc) and the electron-dense granules (g). se=sensory epithelium.

Fig. 9. Transmission electron micrograph of aggregated electron-dense granules forming a cluster (c). Note the faceting of the granules and the initial deposition of organic matrix (om) on top of the cluster. kc=kinocilium, m="membraneous material".

Fig. 10. Detail from Fig. 9, showing the relationship between the tip of a kinocilium (kc) and an electron-dense granule (g). Note the flocculent material (fm) between the kinocilium and the condensed, granular material. The kinocilium contains a row of small, vesicle-like structures (v).

Fig. 11. Transmission electron micrograph showing initial deposition of organic matrix (om) around the cluster (c) of electron-dense granules. sc=stereocilia, se=sensory epithelium.

Fig. 12. High power transmission electron micrograph of deposited organic matrix (om) with attached organic filaments (f).

Fig. 13. Transmission electron micrograph of a longitudinal section through a developing lapillus about two weeks after fertilization. The cluster of electron-dense granules (c) is embedded in organic matrix (om), except at the location of the kinocilium (kc), which forms a ciliary canal (cc) in the deposited matrix. se= sensory epithelium.

Fig. 14. Transmission electron micrograph of a longitudinal section through a lapillus at hatching. Three faint pre-hatch growth rings are present (1-3). Note part of the ciliary canal (cc). c= cluster of electron-dense granules forming the otolith core.

Fig. 15. Transmission electron micrograph of a longitudinal section through the lapillus one day after hatching. Note the distinct "hatching zone" (hz). c= core of otolith.

Fig. 16. Transmission electron micrograph of a longitudinal section through a lapillus 5 days after hatching. Five growth rings can be identified (1-5). c= core of otolith. hz= hatching zone.

Fig. 17. Detail from Fig. 16, with growth rings numbered 1-5. The organic matrix has a fine, radial texture. Note the sharp discontinuity line (dl) and the peripheral, electron-dense layer (el).

Fig. 18. Transmission electron micrograph of longitudinal section through the lapillus 10 days after hatching. 10 growth layers can be interpreted (see Fig. 19). Note the distinct hatching zone (hz), and the ciliary canal (cc). se= sensory epithelium.

Fig. 19. Detail from Fig. 18, showing the interpretation of the number of growth rings (1-10). Note the distinct hatching zone (hz) and the sharp discontinuity line (dl) 3 days after hatching. cc= ciliary canal.

Fig. 20. Transmission electron micrograph of a longitudinal section through a lapillus of a larva reared in constant darkness. The organic matrix is rather loose and homogeneous, without forming distinct growth rings, except for a few faint discontinuity lines (dl) se= sensory epithelium.

Fig. 21. Transmission electron micrograph of a longitudinal section through a lapillus of a larva reared in constant light. A few distinct, and some diffuse growth rings have formed. Note the folded surface of the otolith.

Fig. 22. Transmission electron micrograph of a longitudinal section through a lapillus of a larva prepared by the potassium pyroantimonate technique. The calcium is precipitated as electron-dense calcium pyroantimonate deposits. Note that there is no deposition in the central part of the otolith.





