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ANNUAL CHECK FORMATION IN OTOLITHS AS A STARTING POINT FOR AGEING BY MEANS OF COMPUTER ANALYSIS

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Abstract

This paper is an attempt to summarize the basic principles behind commonly used ageing routines of otoliths. Age determination is based on the interpretation of optically distinct zones. However, there is still no theoretical basis for allocating a time scale to the visible ring pattern. The reliability of the decision-making process in ageing can be improved when subjective interpretations are eliminated and when the chemistry beyond contrast enhancing techniques is fully understood. An analytic approach of age determination in terms of calcium mineralization and physiology is therefore in this study the first step in the development of an automatic ageing system.

1. Introduction

Growth and mortality represent two population processes that are essential to the assessment and management of fisheries. Estimation procedures for rates of change in weights and numbers depends heavily on knowledge of the age structure of the populations and ageing of fish can be considered the back bone of fish stock assessment. Interests in ageing methods go back many years and calcareous structures were first used for ageing pike in 1759 (HEDERSTRÖM, 1759). Scales, otoliths, opercular bones, fin rays, spines, cleithra and vertebrae are commonly used in modern times. The basic principle is that the age of fish can be deduced from the ring features identified on these structures, when a time scale can be allocated to the visible pattern. This latter assumption can often still be questioned and even these days there appears to be no general theoretical basis for the age reading of hard tissues. Sometimes, age reading is considered more an art then a scientific activity. Even though, the annual nature of the ring features is widely accepted and it is believed that, whenever proper techniques can be developed, a large amount information on the life history of the individual fish is accessible.

Ageing of fish continues to be a labour intensive routine activity in fisheries research. There is a clear need for less time consuming and more objective ways to produce age compositions and it has been suggested that the use of image analysis systems could improve the efficiency and reliability of age determination (e.g. FAWELL, 1974). As a first step, an understanding of the structural characteristics, chemical composition and formation of otoliths would make it feasible to determine a suitable enhancement-technique.

Not all studies relating to age reading can be summarized in a short paper. Therefore a selection of topics in otolith reading has been made. One of the major advance in ageing of fish, the discovery of daily growth zones (PANNELLA, 1971), is briefly discussed. The current trend in this topic has recently been well reviewed, but the implications for the determination of yearly events in otoliths and the process of ageing are not yet fully understood (BEAMISH AND MCFARLANE, 1987). Whereas daily growth increments appear to form as a consequence of diurnal rhythm in the mobilization of calcium (MUGIYA, 1987) and neuropeptide production (GAULDIE AND NELSON, 1988), annual check rings of otoliths are supposed to have a more complicated origin. Some basic chemical and mineralogical aspects of otolith mineralization mechanisms will be discussed and related to currently used ageing techniques.

2. Otolith microstructure

In teleost fishes, the sagittae are the largest of the three otoliths and apparently the most easy to use in ageing. They are composed of calcium carbonate crystals deposited on an organic matrix (CARLSTROM, 1963). The mineral part is aragonite and the organic part is called otolin (DEGENS *et al.*, 1969). In comparison with skeletal bones there are some basic differences. The organic compound of bone are mucoproteins with a different amino acid composition than otolin. Most importantly, osteogenesis in bone is either cellular or acellular and osteoblasts or scleroblasts are involved, while no such cells have been reported in otolith mineralization.

Otoliths are surrounded by a fluid medium in the labyrinth. The only connective tissue is the sensory epithelium of the macula acustica. Sensory hairs of this nervous-end material project in a membrane that cover the surface of the otolith. This membrane is probably permeable to material diffusing from the endolymph fluid towards the otolith. An ultrastructural study of the otolithic membrane (DUNKELBERGER *et al.*, 1980) revealed two distinct zones, a structured gelatinous layer and a non-structured subcupular meshwork. The gelatinous layer extends from the otolith surface to the tips of the sensory hairs only over the sensory region. The subcupular meshwork, not limited to the sensory region, consists of a loose network of fibrous material. This fibrous material was relatively more abundant over areas of the non-sensory region. At the periphery of the otolith, a continuation of fibrous material into the otolith was found (DUNKELBERGER *et al.*, 1980). The full significance of this otolithic membrane in regulating the calcium deposition and/or organic matrices within the otolith has not yet been established.

2.1. The organic matrices

In almost all cases of biomineralization, proteinous macromolecules are found in solution and many of them have one particular characteristic: they are highly acidic (LOWENSTAM AND WEINER, 1989). These proteins (otolin, in case of otoliths) assemble to a so called organic matrix, a three-dimensional framework and they are rich in highly oxygenated aspartic and glutamic acid and low in aromatic and basic amino acids. This fibrous protein is uniform in composition and unlikely to be affected by environmental or phylogenetic events at the molecular level (DEGENS *et al.*, 1969).

Organic material is found between individual calcified layers, between individual crystals in each layer, as well as within subunits of the crystals (respectively termed as interlamellar, intercrystalline and intracrystalline; DUNKELBERGER *et al.*, 1980). In the latter type it has not been resolved whether each individual subunit is an independent crystal or, very unlikely, just a portion of a single crystal. The tightly packed fibers of the intercrystalline and interlamellar matrices were similar to the observed fibers in the subcupular component of the surrounding otolithic membrane.

Over a wide variety of fish species the amino acid composition of otolin is rather uniform. The total organic content in different species varies within a range between 0.2 and 10 % of the total otolith weight (DEGENS *et al*, 1969).

2.2. The mineral form of calcium carbonate

Structures of calcium carbonate deposited in biomineralization are mostly calcite or aragonite. Calcite is the most stable form at low temperature and low pressure, and aragonite (and vaterite) transform to calcite in aquatic environments under normal conditions. Electron diffraction studies of teleost otoliths revealed that they were crystalline in the form of aragonite (DEGENS et al.,1969; GAULDIE AND NELSON, 1988). For instance, otoliths of plaice have been described as polycrystalline aragonite (CARLSTROM, 1963). The great stability of biological aragonite is assumed to be caused by the highly stable lattice of aragonite formed in the biological system, although the exact reasons remain to be studied (KITANO *et al.*, 1980).

Aragonitic crystals in otoliths are orientated with their crystallographic *c*-axis perpendicular to their projected surfaces. Electron micrographs showed twinning of the individual crystals. This twinning often occurs in biological mineralization and in the presence of foreign ions. It is supposed to be an efficient mechanism for rapid crystal growth (GAULDIE AND NELSON, 1988).

Nevertheless some otoliths do contain more than one mineral form of calcium carbonate (GAULDIE AND NELSON, 1988). In a few studies, the sagittae were vateritic, or partially aragonitic and partially vateritic (CAMPANA, 1983b). Also calcite is known to occur as a thin layer on the distal surface of the otolith (GAULDIE, 1990).

Differences between geologically and biologically formed aragonitic minerals.

The electron diffraction patterns of otolithic fragments and geological aragonite have been used to recognize the crystal structures. Growth in the three-dimensional plane of mineral aragonite is always observed to expose the (010) face only whereas the aragonitic biominerals also exposed the (001) face (MANN *et al.*, 1983). In biologically formed aragonite impurity with several inorganic elements in trace quantities occurred while the geological sample of aragonite was pure $CaCO3$.

The ratio of strontium to calcium concentrations in otolith aragonite has been found to be correlated negatively with temperature, but in a different manner compare to geologically formed aragonite. Therefore, assuming a constant level of Sr/Ca in the environment, the amount of strontium incorporated into otolith aragonite could be governed by physiological processes (RADTKE, 1984).

The outstanding difference is that mineral crystals have sharp edges and are different in size while the single crystals from biological sources have rounded edges and a fairly uniform diameter of about 1000 Å. The observed differences in the faces together with the assumption of the basic building blocks of these 1000 Å single crystals indicate that biological and geological minerals differ in the way in which they are formed. MANN *et al.* (1983) suggested that the matrix-mediated precipitation of otoliths is sufficiently different from the mode of mineral formation to explain these observed differences in morphology.

3. Otolith growth

There is little knowledge about the extracellular process how calcium is deposited. In the analysis of the mineralization process attention should be paid for mechanisms of the nucleation of the mineral, the transformation of unstable mineral to a more stable form, the polymorph determination of the calcium carbonate deposition, and the control of crystal growth and crystal orientation. Although the knowledge about these mechanisms is incomplete, the organic matrix is supposed to play a principal role. The matrix hypothesis assumes that the organic material is deposited as a crystal nucleating substrate (WATABE *et al*, 1982). The organic components supply locations for the initial fixation of ions, in which aspartic acid itself appears to be highly charged. This implies an active role in regulating the mineral formation.
DEGENS *et al.* (1969) suppose that oxygen ions of the bicarbonate are bound in the polyhedra structure of otolin. This will result in a more stable confirmation and crystal nucleation is initiated by the arrangement of calcium to form metal ion coordination polyhedra's. Then carbonate groups will be attached to the matrix by hydrogen linkages and the oxygen from the carbonate will exchange with oxygen from the metal ion coordination polyhedra's into a stable structure.
Acidic glycoproteins from molluscs, echinoderms, tooth enamel and bone all adopt the ß-sheet conformation in presence of calcium. The result of this orientation is that the carboxylate groups of the aspartic and glutamic acid residues are perpendicular to the plane of the sheet. This possible mode of calcium binding to the surface of the ß-sheet could be an explanation for the manner in which the proteins function. Furthermore, in vitro experiments showed that the ß-sheet can interact with certain specific crystal faces only (LOWENSTAM AND WEINER, 1989). Since otolin is rich in aspartic and glutamic acids, in theory a similar sort of mechanism may occur.

Ceased growth of aragonite could be explained if nucleation sites on the matrix are blocked, for instance when calcite at those sites interferes with the aragonite growth. However X-ray diagrams did not indicate any traces of calcite (DEGENS *et al.*, 1969). But there are more possible explanations for growth interruptions: the same authors refer to MORRIS AND KITTLEMAN (1967) who suggested the presence of other orthorhombic minerals because they found a significant concentration of sodium in otoliths (calcium substitution by sodium in aragonitic minerals is rather unlikely).

To some extent mineralization must be a dynamic process because otoliths are surrounded by a membrane in a fluid medium. Initiation, inhibition and control of the proposed extracellular mechanisms of otolith mineralization are likely to be regulated by one or several of the following possible principal factors: the organic matrix, hormonal or neurosecretory factors, and the environment (temperature, salinity, element concentrations). However, little is known of the factors governing the crystal modifications.

Organic matrix

The formation of crystal nucleation is possible when concentrations of ions exceed their solubility product. CRENSHAW (1982) proposed the ionotropy theory as a likely mechanism of crystal nucleation. The hydrophilic sites of the soluble part of the matrix (the acidic groups) bind calcium and around this sites a second carbonate enriched layer is formed. Crystal nuclei (seeds) are formed whenever a local increase in pH or a decrease in carbon dioxide occurs. The survival of the crystal seed is determined by ion-transport processes or the local concentration of the calcium-binding matrix.
Organic material probably provides binding sites for ions or removes substances which inhibit mineralization (WILBUR, 1980; MANN *et al.*, 1983). The macromolecules are secreted

into the extracellular space where they assemble into a continuous sheetlike structure. Once calcification has begun, the organic matrix is likely a mold for the mineralization (DEGENS *et al.*, 1969). LOWENSTAM AND WEINER (1989) proposed that the structure of the nucleation site is responsible for the formation of aragonite or calcite, but the composition of the surrounding endolymphe or the otolithic membrane could also be responsible. In this process an interfering physiological control can not be excluded since the stable isotopic composition and additives of mineralized parts are not in equilibrium with the environment.

One of the various explanations for reduced growth of crystals is tanning of the organic layer at its surface (WILBUR, 1976). A newly secreted layer of the organic material then again initiates a new layer of crystals and this alternation continues. In this supposed mechanism, crystal thickness depends upon the secretion frequency of inhibiting organic material. The width of the crystal layer changes when the secretion of organic material does not proceed at a constant rate. The growth of the crystal layer is interrupted by organic material and as a result a bipartite structure can be seen (WILBUR, 1980). The study of DUNKELBERGER *et al.* (1980) showed that aragonitic crystals in otoliths of the mummichog (*Fundulus heteroclitus*) appeared to be relatively short and limited in length by the intercrystalline matrix. Furthermore, a relatively thick interlamellar matrix limits each individual growth layer. But again there is very little knowledge about the structures in organic matrices that are responsible for crystal growth inhibition. In mollusc shells it is obvious that two distinct organic structures are present in connection with the initiation and growth of calcium carbonate crystals. The 'sheetlike' interlamellar matrix is composed of glycine and alanine and limits or ceases crystal growth. The 'envelope' structure which intimately surrounds the surface of growing crystals, is thought to be involved in the acceleration of crystal growth (BEVELANDER AND NAKAHARA, 1980).

A deficiency of ions to the mineral site, for instance through trace quantities of interacting molecules, is probably a more common and main reason why crystals stop growing. Besides lack of ions, the mechanical properties of the crystal can be changed by these interactions. An interaction during crystal formation retards growth on these faces causing them to grow more slowly. Although there is no documentation available, LOWENSTAM AND WEINER (1989) suspect that these type of effects are widely utilized by organisms for controlling biomineralization.

Internal regulation

The otolith grows in the endolymphatic sac, a fluid medium, and the only connective tissue is the macula acustica. Thus calcium carbonate deposition sites on otoliths are isolated from the environment and the calcium concentration in this plasma is low in comparison with sea-water (SIMKISS, 1974). This is in agreement with the observation that the regulation mechanism of cation plasma levels in fish is quite effective (NORDLIE AND WHITTIER, 1983). By studying the composition of the endolymph fluid during mineralization, one might be able to unravel the chemical driving force for the calcium carbonate mineralization rate and type.

MUGIYA (1974) found that injected labeled calcium is transported through the blood capillaries and secreted into the otolith fluid by some macular cells in at least 3 hours. These experiments have revealed that a large quantity of calcium deposition in rainbow trout takes place on the medial surface near the macula cells except for the sulcus acusticus (Figure 1). The author suggests that both organic and inorganic constituents of teleost otoliths are secreted, at least in part, by a cellular activity in the macular region.

MUGIYA *et al.* (1981) determined that there is a strong correlation between the plasma calcium level of fish blood and the calcium deposition rate on the otolith. They assume that this is caused by the diel rhythm in branchial uptake of calcium from the environment. Experiments with coho salmon, exposed to experimental stress conditions, support the hypothesis. Stress resulted in microstructural check formation on otoliths and interfered with a lowered calcium uptake from the water (CAMPANA, 1983a).

The calcium physiology of otolith growth appears to be much different from bones and scales. Comparative aspects of the calcium dynamics in different calcified structures have been described by ICHII AND MUGIYA (1983). Experiments with coho salmon, in water with radioactive labeled calcium, showed that the retention of radioactivity in otoliths was exceptionally large compared to bone and scales. Even when the fish were put in non-radioactive water after the exposure, the radioactivity of otoliths increased by 312% during the first 14 days. This result supports the belief that once deposited calcium is hardly withdrawn from otoliths after initial mineralization and contradicts the suggestion of PANNELLA (1980) that resorption produces checks reflecting growth discontinuities.

The daily deposition of calcium carbonate upon the organic matrix ceases near dawn (PANNELLA, 1980). An endogenous circadian rhythm entrained to the photoperiod may be directly responsible for the reduced branchial uptake of calcium (MUGIYA *et al.*, 1981; TANAKA *et al.*, 1981). Moreover, GAULDIE AND NELSON (1988) concluded that there are strong physiological arguments for daily deposition of micro-increments, in the anti-sulcul section, tied to diel rhythms of the brain of the fishes. The authors hypothesize that neuroproteins are transported down the pallial axon from the central ganglia and secreted at the nervous end-organ (the macula region).

As a result of the seasonal change in width of daily micro-increments the optical pattern of translucent and opaque zones in otoliths could be formed (RADTKE *et al.*, 1985). This deduction provides a physiological mechanism for the argument that opaque and translucent zones are optical markers of seasonal changes in the growth rate of otoliths. GAULDIE (1990) stated: "Thus the information in the anti-sulcul section that is used to estimate age of fish is on a firm scientific footing which would allow one to confidently assay the validity of the patterns of opaque and hyaline (translucent) zonation in the anti-sulcul section of the otolith".

Environment

The internal water en salt balance, and thus the calcification rate, of fish seem to be directly or indirectly regulated by environmental variables such as temperature, pH, salinity and ionic ratios. As PANNELLA (1976) stated, the control by temperature, photoperiod, tides, salinity and probably food intake on the microstructural mineral deposition is evident. The influence of these factors is seen in the size and frequency changes of the mineralized increments. Both the calcium mobilization and the protein synthetic rate could be responding to these growth altering factors.

The growth rate of body mass is affected by variations in growth hormones. In turn, hormone levels are related to food consumption. Also, a relationship between calcium deposition and feeding activity seems to exist. For instance, when *Tilapia* sp., reared in experimental ponds, were fed twice a day instead of once, the number of incremental zones increased (PANNELLA, 1980). In contradiction to these results, CAMPANA (1983b) found no relation between the growth increments and the feeding frequency of young-of-the-years. Even starvation of trout and flounder for periods over 32 and 26 days, respectively, had no effect on the deposition of daily growth increments on their otoliths.

4. Age determination

One of the visible periodicities in otoliths is the alternation of opaque and translucent zones, commonly interpreted to determine the age of fish (WILLIAMS AND BEDFORD, 1974). In temperate regions, intensive growth is reflected by the formation of the opaque spring summer zone. Probably due to a much reduced growth rate in winter, the translucent zone is being formed (IMMERMAN, 1908). Although originally narrow translucent zones were interpreted as 'false' or 'check' rings (JENSEN, 1965), in this paper the term 'check' is referring to translucent zones regardless of their width, corresponding to the annually, or otherwise, occurring decreases in somatic growth. The reason for this change is given by GAULDIE (1990). Since JENSEN (1965) introduced a standard terminology to describe otolith marks, the interpretation of which features could be equivalent to annual marks or not has been changed. GAULDIE (1988) stated that on the crystal level there are no criteria to differentiate between annual check rings and additional check rings. If this statement is correct, it would follow that contrast-enhancing techniques which are specific for annual checks are bound to fail.

Beside the description of false and annual rings, there is a more confusing disagreement on the terms hyaline and opaque, especially in relation with the zone with richest in organic matter. According to DANNEVIG (1956), MOLANDER (1947) and IRIE (1960), the organic material is present only in the opaque zones, while the hyaline zones consist entirely of aragonite. However, the burning technique of MØLLER CHRISTENSEN (1964) applied by BLACKER (1974) showed that the optical dark parts (after burning) correspond to the parts of the hyaline zones, suggesting that this zone has the higher concentration of organic compounds. This phenomenon made the term hyaline to become ambiguous because it is being used to describe both an optical feature as well as a structural feature. Therefore, in this paper a terminological distinction is made in the otolith features depending on the aspect that is being considered according to Table 1.

Table 1. Used descriptions and allocated components of otoliths.

general interpretation	optical feature	after burning	organic material	inorganic material
check zone	translucent	dark	protein-rich	short crystals
growth zone	opaque	light	protein-poor	long crystals

The ring pattern is often hard to define objectively and a variety of recipes is used to enhance the contrast in ring features. It is generally believed that a large amount of information on the individual life-history could become accessible if proper techniques were employed. However, in the literature only theoretical mechanisms which could explain the bipartite nature of otoliths have been proposed so far. At a microstructural level, the overall conclusion can be drawn that daily ring features are a result of the rhythm in neuropeptide regulation and the mobilisation of calcium. These two processes are governed within the organism but undoubtedly are also influenced by environmental factors (e.g. photoperiod, temperature). Since calcium metabolism of fish is poorly understood and protein synthesis is not easy to measure, it is hard to believe that any attempt to point out one principal factor that determines otolith growth is realistic.

The macrostructural ring pattern is supposed to be a result of the change in width of daily increments and the length of crystals would then be the major difference between translucent and opaque zones. On the other hand, the success of the burning techniques is explained by the differences in protein content between the two zones. It is of course possible that both suggestions are true. Because the protein covers the crystal faces and/or smaller crystals reflect less light, the dark zone is optically translucent (Table 1).

Structural differences between the zones are often enhanced by treatments such as acid etching, staining or burning of otoliths. The most common and widespread method is still MØLLER CHRISTENSEN's burning technique (1964). A main disadvantage of this method is that the chemistry beyond the technique is not fully understood. Whenever, as stated before, the protein content and the crystal length in the two zones are both distinct, it is probable that burning not only affects the organic layers but the crystal configuration too. Aragonite is not the most stable form of calcium carbonate (crystallized at low temperature and low pressure) and heating will probably change the mineral form to calcite (MANN *et al.*, 1983). The degradation rate of minerals is not only temperature dependent but also influenced by the size of the crystals. Smaller crystals will degradate at a lower temperature. In practice the burning technique has another disadvantage. Great care has to be taken not to crumble burned otoliths and it is difficult to achieve standardization of the burning process.

Recently, attempts have been made to dye fish otoliths in a simple way which is suitable for mass processing. RICHTER AND DERMOTT (1990) succeeds in staining otoliths of several economic species. The appearance of the ring features in treated otoliths resembled the burning technique. However, striking was the result that collagen (related to otolin) specific stains were not the most suitable ones. The authors found that, in general, the darker the stain, the better the contrast enhancement. This does not exclude the possibility that the rougher zones are more easily stained, because after sectioning the zones consisting of smaller crystals appear to have a relatively rough surface.

Considering these two contrast enhancing techniques, only one conclusion can be drawn. It is not certain that they act only as a chemical reagent and are physically neutral. Only if it is clear what kind of mechanism is involved, the interpretation of the contrast-enhanced structures can be deduced in a straightforward manner.

5. Prospects

The original aim of this study was to achieve some insights in the formation of translucent and opaque zones before starting to develop an automatic ageing method by means of image analysis. In principle, a video camera fitted to a stereomicroscope and combined with a computer can be used in ageing (FAWELL, 1974, MCGOWAN et al., 1987). It is expected that image analysis can reduce the amount of subjective interpretation in the ageing process. The setting of a threshold level for automatic identification of annual checks would serve as a fixed kind of error in judgement that could be relatively easily detectable in due course compared to subjective thresholds by expert readers.
One of the possible advance that can be expected from an image analysis system is that, besides number of growth zones and width of zones, non-conventional data can be retrieved such as shape factors and transformation coefficients (e.g. fourier, DE PONTUAL AND PROUZET, 1988). Sophisticated image processing computer products are now within reach and can be applied for many biological topics (e.g. egg and larvae recognition and counting, zooplankton determination, sorting of fish, as well as pattern recognition and counting in otoliths). Digitized information from the camera on a video monitor can be displayed at a resolution of, at least, 512 times 512 pixels each with the full 8 bits of information. This means that 256 intensities (grey levels) are available for image processing, which is far beyond the detail that can be derived by direct visual reading. It is important to distinguish full automatic or semi-automatic devices. In the latter an operator moves a cursor on the video image and thus selects the coordinates of the cursor and the corresponding grey level values of the original image that are stored to the computer for further processing. The information

on growth zones is thereby user controlled and in this case validation of automatic counting is just as critical as it is in conventional methods. The potentional for automatic reading can be developed by incorporating numerical techniques. One of them, the graphic display of grey levels along a specified counting path in a histogram, has already been applied in different ageing studies (MCGOWAN *et al.*, 1987; GANDELIN AND LAVAL, 1987).

To start with, video images should be of the highest possible quality. Although image enhancement by mathematic transformations can lead to spectacular results, the desired information needs to be present in the original image. Even though a new technology may give the impression of greater objectivity, there is always the risk that arbitrary decisions, such as contrast enhancing preparation of otoliths, have resulted in bias. Therefore physical changes in the ring structures should be excluded whenever only chemical reactions are interpreted and vice versa. The most suitable enhancement technique for mass processing is not (yet) available. In case of staining, some decisive experiments with chemically neutral dyes, for instance indian ink, need to be done. When working with automatic ageing systems, this will be more important than trying to understand the possible clues behind the formation of yearly checks and a-periodic checks since the structural argument fails until now. The latter kind of checks should become detectable in a consequent manner by using automatic ageing routines.

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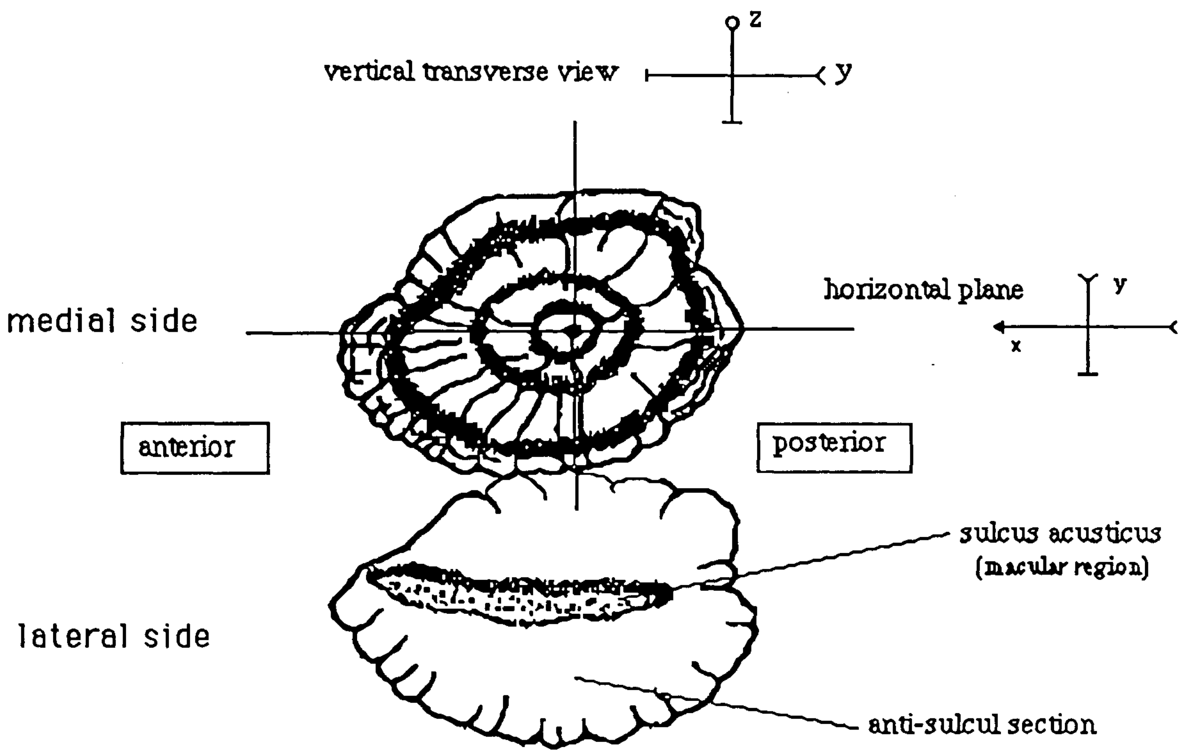

Figure 1. The sagittal view of the medial (concave) and lateral surface of an otolith. The two axes in the upper picture represents the often used transverse and horizontal sectioning through the nucleus.