7. OTOLITH MICROSTRUCTURE IN TROPICAL FISH

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1 INTRODUCTION

This chapter focuses on the fine scale microstructure of otoliths, the details of its formation and the biological information stored within the structures. The examination of otolith microstructure can reveal many different aspects of any individual fish's life events, beyond the information stored as daily incremental deposit of the structures and their respective accretion rates. In tropical environments, and specifically in reef areas, the most singular structure within the otolith is the settlement mark corresponding for some species to the change of habitat between the pelagic and demersal environments (e.g., Thorrold & Milicich 1990, Wilson & McCormick 1997, 1999, McCormick et al. 2002). Such specific marks can be relevant for managers if they want information on the presence of a settlement phase for a species or the duration of the pelagic stage. Nevertheless, the treatment of such information often implies the analysis of the daily increments (see Sponaugle, Chapter 4, this volume). Another type of mark that can be found within the otolith structure is related to reproduction (e.g., Massou et al. 2004b) even though only few studies could demonstrate a direct correspondence between reproduction and otolith checks in experimental or natural conditions. This kind of mark can be particularly informative for resource managers as the knowledge of reproduction is one of the key pieces of information for fishery management. The duration of the reproductive period can be extended, particularly in the tropics, meaning that it is often difficult to evaluate the average birth date for cohorts in populations. Otolith reproductive marks, if they exist, are relevant for calculation of birth dates and also to evaluate the number of individual reproductive events and the link between reproductive output and larval supply within specific populations (McIlwain 2002, Sponaugle & Pinkard 2004). Specific variation in the environment can induce a specific mark in the otolith structure which could be useful afterwards as a reference mark to compare individual life history trait responses (Sponaugle & Pinkard 2004).

In the first part of this chapter we provide a detailed description of the microstructures and the processes and factors (endogenous and exogenous) involved in their formation. This is followed by a listing of the potential applications of structure examination, from a patterns related to life-history traits (metamorphosis, settlement, reproduction, migration, etc.) to tropical ecology and fisheries and an explanation of the advantages and disadvantages of the use of otolith microstructures. We then provide a description of the main experimental procedures for otolith manipulation, followed by discussion of the technical aspects of preparing otoliths for examination and analysis.

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This chapter mainly links with two other chapters of this book – Sponaugle, Chapter 4 and Thorrold and Swearer, Chapter 8 – but also with others, as the knowledge of structure is the basis for interpretation of all kinds of marks laid down and conserved in otoliths throughout a fish's life. The reader should refer directly to Chapters 3 (Fowler) and 4 (Sponaugle) for discussion of ageing fish using primary increments in otoliths.

2 Formation of otolith microstructures

2.1 DESCRIPTION OF MICROSTRUCTURES

2.1.1 Basic constituents and primary increments

Otoliths are mostly composed of calcium carbonate ($CaCO_3$) precipitated in a protein matrix. The $CaCO_3$ is crystallized mainly in the mineral form of aragonite (Carlström 1963) due to the action of an organic matrix in which acidic amino acids predominate (Degens et al. 1969). The aragonite forms acicular (needle shaped) microcrystals about 0.3 µm long grouped in radial prisms of variable thickness, running from the otolith centre to the external surface (Figure 1). The microcrystals show discontinuities at regular intervals in which the organic matter predominates, although some microcrystals may run across several discontinuities (Morales-Nin 1987) (Figure 1a, c).

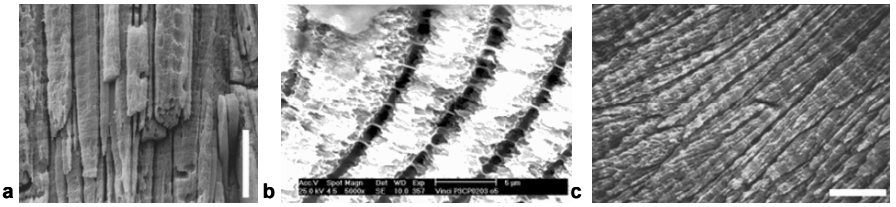

Figure 1. Inorganic constituents of fish otoliths (aragonite crystals within the incremental units) under scanning electron microscopy (SEM) and after acid etching. (**a**) Inorganic constituents within the incremental unit of the otolith of *Stellifer rastrifer*. Scale bar = 7.5 µm (photo B. Morales-Nin). (**b**) Otolith of *Vinciguerria nimbaria*. The radial crystals of aragonite are visible inside the L-zones (carbonate rich zones, light coloured) as well as the bridges formed by water-insoluble proteins inside the D-zones (protein rich zones, *dark coloured*). Scale bar = 5 µm (photo J. Panfili). (**c**) Radial prismatic crystals with primary increments visible on the background (*Lutjanus kasmira*). Scale bar = 10 µm (photo B. Morales-Nin).

The total proportion of the organic matrix is low, between 0.1 and 10% in weight (Borelli et al. 2001), and may vary with otolith growth. For instance, 1 year old *Dicentrarchus labrax* and *Merluccius capensis* had otoliths up to 10 times richer in protein than in older fishes, and there was also a change in the relative proportion of the constituent amino acids with age (Morales-Nin 1986a,c). This organic matter is laid down in the form of a loose reticulum that interpenetrates the crystals and that predominates in the discontinuities, forming thick fibres and layers (Morales-Nin 1987).

The organic matrix is composed of water-soluble proteins (WSP) and water-insoluble proteins (WIP). Both fractions are rich in acidic amino acids. Some of the WSP fractions have calcium-binding capacity and have an important role in mineralisation of the otolith (Asano & Mugiya 1993). These contain a glycoprotein that probably regulates the accretion rate by acting as a calcification inhibitor (Wright et al. 1991). The WIP fraction consists of a single collagen-like protein, termed Otolin-1 (Murayama et al. 2002). The role of WIP is presumably structural (Campana 1999) and might be related to shape control (Figure 2). WSP represent 1.1% by weight of the otolith in *Oreochromis niloticus* whereas total protein accounts for 2.3% (Asano & Mugiya 1993). WSP are composed of a few fractions, including a protein-polysaccharide complex with a moderate calcium-binding capacity and other proteins of low molecular weight that have an intense capacity for calcium binding (Sasagawa & Mugiya 1996).

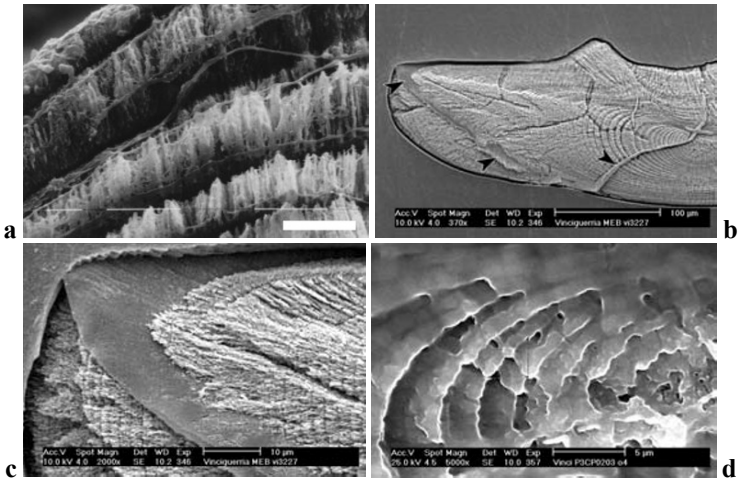

Figure 2. Organic constituents of otoliths seen under SEM, after acid etching. (**a**) Framework of proteins in the microincremental unit of an otolith of *Dicentrarchus labrax*. Scale bar = 10 µm (photo B. Morales-Nin). (**b**) Framework of organic material (*black arrows*) inside the otolith of *Vinciguerria nimbaria*. Scale bar = 100 µm. (**c**) Detail of the image in (**b**) on the ventral edge showing a protein plate crossing the aragonite crystals. Scale bar = 10 µm. (**d**) Water-insoluble proteins in the core area of the otolith of *Vinciguerria nimbaria*. Each protein plate constitutes an element of the primary increment. Scale bar = 5 µm (photos J. Panfili).

Otoliths grow by successive deposition of increments, the so-called primary increments, which constitute bi-partite structures with each composed of one L-zone and one D-zone (Figure 3). The L-zone is a band rich in calcium carbonate crystals, translucent to light and appearing raised in scanning electron microscopy (SEM), with a width varying between 0.4 and 10 µm. The D-zone is a band rich in organic material, opaque to light and appearing as a groove in SEM, with a width smaller than 1 µm (Panfili et al. 2002). The total width of a primary increment ranges from less than 1–12 µm (Pannella 1974) and is thus mainly constituted by the L-zone. The width of the

increments depends on the growth rate and the metabolism of the fish (but see Sponaugle, Chapter 4).

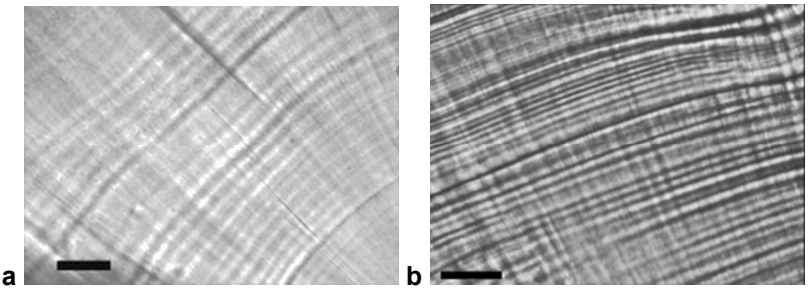

Figure 3. D-zones (protein rich, *dark coloured*) and L-zones (carbonate rich, *light coloured*) of primary increments in the otoliths. (**a**) *Coryphaena hippurus* (scale bar = 10 µm, photo J. Tomás), (**b**) *Ethmalosa fimbriata* (scale bar = 20 µm, photo K. Diop). Note the variations of the L-zone compared to the D-zone in both species.

2.1.2 Initial growth structures

Otolith formation begins with the aggregation of free-floating protein core particles, which are directed to the developing sensory maculae through the action of ciliated cells that line the otosac cavity (Riley et al. 1997). These pre-otoliths are rapidly mineralised forming the primordia or the core, which aggregate to form an optically opaque spot (Figure 4a). X-ray maps have shown that these are formed of non-calcium material, as seen in the otoliths of the tropical tilapia *Oreochromis niloticus* (Zhang & Runham 1989) or the tropical *Anguilla* spp. (Arai et al. 2001), as well as other species. The first primary increment surrounding this aggregation delineates an area, generally lentil-shaped, called the core, which includes mineral crystals at this stage (Figure 4).

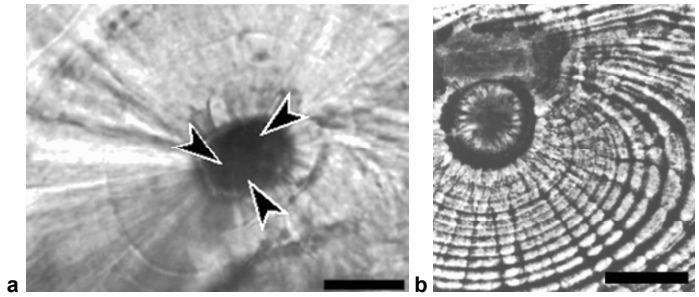

Figure 4. Examples of otolith cores. Scale bar = 10 µm. (**a**) Core area of a *Sarotherodon melanotheron* otolith viewed with a compound microscope. The *arrows* indicate the primordial granules constituting the core (photo K. Diop). (**b**) Central area of a *Vinciguerria nimbaria* otolith viewed under SEM after acid etching. The core, surrounded by a deep depression, is formed by an aggregation of multiple granules (photo L. Marec).

This first increment – also named the first ring, band or check – is generally thought to be formed at hatching and appears like a discontinuity in the otolith (Zhang & Runham 1992b) but this first ring may not have the same physiological basis in all species (Wright et al. 2002b). For example, in the armoured catfish *Hoplosternum littorale*, a few primary increments seem to be laid down prior to the hatching ring (Ponton et al. 2001). In some species, such as tropical clupeids (*Stolothrissa tanganicae* and *Limnothrissa miodon*), otolith growth may initiate increment deposition at yolk sac absorption up to 2–8 days after hatching (Kimura 1995). The biological significance of this first mark is probably species specific and may correspond to several life history events such as yolk-sac absorption, hatching or first feeding (Figure 5) (Wright et al. 2002b). After this, the incremental growth proceeds in a way that has been described as an "onion model" (Figures 4b and 5), with concentric layers of material deposited over the surface of the otolith.

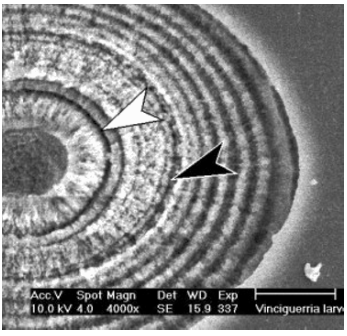

Figure 5. First ring (*white arrow*) and first feeding (*black arrow*) marks interpreted in the central area of an otolith of *Vinciguerria nimbaria* viewed in SEM after acid etching. Scale bar = 5 μm (photo L. Marec & E. Dabas).

2.1.3 Structural discontinuities

Structural discontinuities interrupt the rhythmic growth pattern and can be of different natures. Some are minor, appearing as disruptions in the rhythm. Others are more important and are formed by organic fibres and lamellae, probably due to the cessation of crystal deposition (Figure 6). Once crystal growth is resumed, crystals may have their orientation changed after such a discontinuity. Several possible causes of discontinuities include metamorphosis, settlement, stress or fish reproduction among other changes in life-history characteristics (Victor 1982). Moreover, the discontinuities might determine otolith shape by controlling growth direction and rate by the formation of accessory growth centres, which act as new centres of crystallisation away from the primary core (Figure 7) (Zhang & Runham 1992b). The initiation of their formation is generally concomitant with major developmental, behavioural or ontogenetic changes in the life of the individual. In some species accessory growth centres have been described on the outer surface of adult otoliths resulting in bumps around the outside of the otolith (Wright et al. 2002b, Tomás 2006) or have been observed in the *sulcus acusticus* (Lombarte & Morales-Nin 1995).

The sulcal area, formed in close contact to the *macula acustica*, also is characterised by a major proportion of organic matrix (turning darker brown in charred otoliths) and discontinuities and irregularities both at daily and macro-structural levels (Figure 6a).

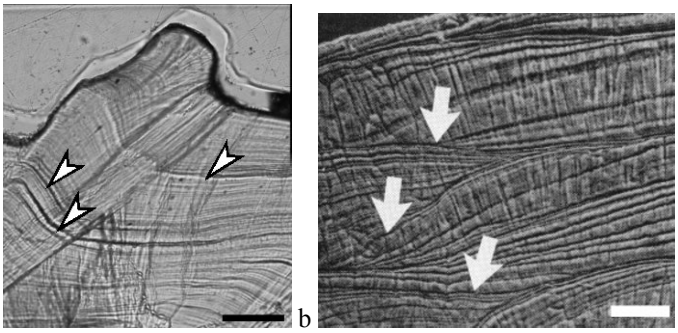

Figure 6. Checks (*arrows*) inside the otolith microstructure. (**a**) Area along the sulcus on the ventral face of an otolith (thin transverse section) of *Oreochromis niloticus* viewed with compound microscopy. Scale bar = 100 µm (photo K. Diop & A. Malam Massou). (**b**) Checks (*arrows*) in the structure of the otolith of *Calamus brachysomus*. Scale bar = 30 µm (photo Morales-Nin 1986b).

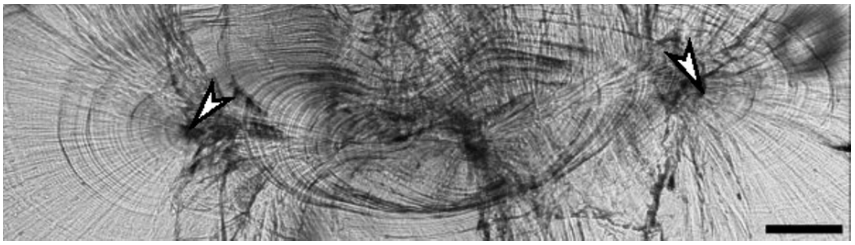

Figure 7. Example of accessory growth centres (arrows) in the central area of the otolith of *Sarotherodon melanotheron*. Thin transverse section observed under compound microscopy. Scale bar = 100 µm (photo K. Diop).

2.1.4 Rhythmical growth patterns

Rhythmical patterns in the deposition of microstructures in the otoliths of tropical fish are the basis of age estimation. Micro-incremental patterns in the otoliths of tropical species vary from sub-daily to daily, lunar and seasonal scales. Daily alternating deposition of calcium and organic matrix results in the formation of daily primary increments predominated by aragonite crystals (L-zone) and a discontinuous organic matrix unit (D-zone) (Watabe et al. 1982, Zhang 1992). This daily rhythm in the formation of the alternating zones is controlled by a circadian variation in plasma chemistry, since during the dark period there is a parallel decline in otolith calcification and total and free plasma calcium concentrations (Mugiya 1987b, Wright et al. 1992). Significant diurnal fluctuations have been recorded in calcium and protein concentrations in the plasma and endolymph of turbot (Edeyer et al. 2000). Incremental

growth is general in all ecosystems and the daily nature of the process has been validated for a number of species in the tropics (Sponaugle, Chapter 4). The otolith growth process is a continuous precipitation of aragonite in the endolymph, with incorporation into the otolith structure modulated by neuroproteins secreted at the macula controlling the rate of aragonite crystal growth and possibly crystal size. The effect of this modulation results in a narrow, mineral deficient increment (D-zone) and a wider, mineral-dense increment (L-zone) but also may result in sub-daily rhythmical patterns superimposed on the daily increments (Gauldie & Nelson 1988). Sub-daily increments have been suggested by Pannella (1980) and Campana and Neilson (1985) and described in the otoliths of several species such as *Lepomis sp.* (Taubert & Coble 1977), *Fundulus heteroclitus* (Radtke & Dean 1982) and *Oreochromis niloticus* (Zhang & Runham 1992b, Panfili & Tomás 2001). Note that distinguishing daily and sub-daily increments may be difficult (Taubert & Coble 1977, Sponaugle, Chapter 4).

Lunar patterns in the otolith microstructure have been identified in the juvenile stages of several species inhabiting shallow waters under the influence of tidal rhythms in tropical (Rahman & Cox 2006) and temperate environments (Campana 1984, Geffen & Nash 1995). The influence of lunar rhythms in the recruitment of coral reef species has been sufficiently emphasised (Sponaugle & Pinkard 2004) and the concomitant effect of recruitment and lunar timing may act in phase to alter the otolith microstructural pattern. Nevertheless, lunar rhythms also have been identified and described in the otoliths of pelagic fish inhabiting open oceanic waters, such as *Vinciguerria nimbaria* (Linkowski 1996) or the myctophid *Myctophum asperum* (Hayashi et al. 2001), for which otolith increment widths were narrower around the full moon compared to the new moon.

Despite the widespread assumption of a lack of seasonal environmental variations in tropical environments, tropical regions have marked hydrological seasons, mainly annual, that interact with temperature variations (Morales-Nin & Panfili 2005). These seasonal variations may leave a print in the otolith of tropical fish in the form of opaque and translucent zones which display differences at the microstructure level (Fowler 1995). The seasonal environmental conditions modulate the relative width of primary increments to the point that zones formed at low temperature and high temperature (macrostructures) can be distinguished at low magnification (Morales-Nin & Ralston 1990). Opaque zones are constituted by thick daily microincrements whereas translucent zones consist of thin microincrements, as observed in many coral reef fish (Fowler 1995 and references therein). Finally, the primary increments become narrower as the fish grows older and growth tends to reach an asymptote, leading to possible interruptions in the rhythm of deposition (Ralston & Miyamoto 1983, Hoedt 1992).

2.2 REGULATION OF INCREMENT FORMATION

Otoliths are located within an anatomically closed sac and cells constituting the sac are responsible for synthesising otolith proteins and secreting calcium into the saccular endolymph. The extracellular otolith biomineralisation is complex and requires an orchestrated process bringing proteinaceous and ionic components together in time and space, as proposed in recent models for these processes based on studies of zebrafish (Hughes et al. 2006). Otolith biocalcification is related to endolymph composition

(Romanek & Gauldie 1996), which is heterogeneous inside the otosac probably due to the presence of several types of ionocytes in the membrane surrounding the otolith (Mayer-Gostan et al. 1997). The spatial heterogeneity may be necessary for otolith mineralisation. Payan et al. (1999) proposed that $CaCO_3$ deposition is initiated in the proximal side of the otolith resulting in an heterogeneity of growth along different sides. Increment formation is regulated by both endogenous and exogenous factors.

2.2.1 Endogenous influences on microincrement periodicity

The daily formation of microstructures in the otoliths of fish is under the regulation of an endogenous circadian rhythm. Experimental manipulation of exogenous factors (photoperiod and feeding) showed that these alone did not explain the daily formation of microstructures in *Tilapia mossambica* (Taubert & Coble 1977). Nonetheless, little is known about the endogenous regulation of otolith formation in fish. Early studies ruled out the influence of estradiol (a hormone causing hypercalcemia) upon calcium deposition or resorption in the otolith of the goldfish *Carassius auratus* (Mugiya 1977, 1978). It has been suggested since that otolith growth could be controlled by neuroprotein secretion (Gauldie & Nelson 1988), yet evidence of hormonal regulation of otolith growth has so far only been demonstrated in the otoliths of *Carassius auratus* (Shinobu & Mugiya 1995). It was shown recently in the tropical freshwater green swordtail (*Xiphophorus helleri*) that otolith growth and, more precisely, calcium uptake is neurally regulated, since calcium incorporation into the otolith ceased in fishes that had the vestibular nerve sectioned (Anken et al. 2002).

Changes associated with the development of the embryo also result in noticeable changes in the microstructure of the otolith. Ontogenetic changes usually take the form of: (1) changes in the width of microstructures, as in *Vinciguerria nimbaria* (Figure 5) (Tomás & Panfili 2000), myctophids (Gjøsaeter 1987, Gartner Jr. 1991) or the tropical species *Ambassis vachelli* (Molony 1996); and (2) structural changes associated with accessory primordia and the prismatic growth of the otolith (Figure 7, Zhang & Runham 1992b, Lagardère et al. 1995, Panfili & Tomás 2001). In some cases, the same ontogenetic changes, such as settlement in reef fish, may encompass different changes in the otolith structure at the microincremental level depending on the family. For example, settlement is associated with wider increments in *Scarus rivulatus* and narrower increments in *Ctenochaetus binotatus* (Lou 1993). Ontogenetic trends also may result in the cessation or absence of otolith formation. For example, clupeids only deposit their first microincrement a few days after hatching, possibly coinciding with yolk sac resorption (Kimura 1995).

The sequence of developmental stages (hatching, flexion, metamorphosis, among others) leads to structural changes in the otolith. For example, the formation of the sulcus and increase in otolith concavity (Lagardère et al. 1995) and the appearance of accessory primordia (as new sites of crystal nucleation) redirect the otolith accretion in juvenile (Figure 7) (Panfili & Tomás 2001) and adult (Zhang & Runham 1992b) tilapia *Oreochromis niloticus*. The formation of checks often is associated with metamorphosis (Gjøsaeter 1987). Even reproduction has been shown to leave a mark in the otoliths of certain species (Pannella 1980, Massou et al. 2004b). Therefore, ontogenetic patterns should be identified prior to interpreting variations in microincrement structure potentially caused by exogenous factors.

2.2.2 Exogenous influences on microincrement periodicity

Exogenous influences on microincrement periodicity can only be identified and assessed once the ontogenetic (endogenous) patterns of variation of microstructure are known (Molony 1996). This Section lists the major exogenous factors that affect otolith microstructure periodicity in tropical fish species (photoperiod, temperature, food availability, salinity and dissolved oxygen, stress and rearing). The application of using the resulting marks or patterns will be reviewed in the following section on analysis of otolith microstructure.

Otolith microstructure is strongly influenced by alternating light and dark cycles of day and night. Increments were deposited daily in fish reared under a 24 h light-dark periodicity, while microincrements deviated from daily deposition under other light cycles in *Tilapia mossambica* (Taubert & Coble 1977) and the temperate fishes *Fundulus heteroclitus* (Radtke & Dean 1982), *Oncorhynchus mykiss* (Mugiya 1987a) and *Salmo salar* (Wright et al. 1991). Deposition of the aragonite fraction takes place during the light phase for tropical species (Brothers 1981, Ré 1984, Jenkins & Davis 1990, Zhang & Runham 1992c, Tanabe et al. 2003), declining at dusk and resuming at dawn. Nonetheless, other authors have shown the opposite in rainbow trout *Salmo gairdneri* and turbot *Psetta maxima* (Mugiya 1984, Edeyer et al. 2000). It is not clear whether these controversial findings are related to climatic zones, species or seasons. Hence, the timing of formation of the D- and L-zones should be verified for each species where it is important for a particular study. The mechanisms by which photoperiod influences the daily formation of microstructures have yet to be elucidated (Boeuf & Le Bail 1999) and may be related to internal rhythms or other environmental variations related to diel cycle.

Temperature also acts as a primary forcing factor for microstructure formation and appearance in otoliths of fish. The temperature effect on increment periodicity has mostly been studied in temperate species but a few examples are available from tropical environments. A decrease in water temperature from 25ºC to 10ºC resulted in the cessation of microstructure deposition and the formation of an annulus in the green sunfish, *Lepomis cyanellus* (Taubert & Coble 1977), and a 2–4ºC variation at low temperatures (from 8ºC to 6 and 4ºC) resulted in the absence of microincrement deposition in the otoliths of the European lake whitefish *Coregonus lavaretus* (Klink & Eckmann 1992). Examples of temperature manipulation effects upon otolith microincrement periodicity in tropical species are scarce. Zhang and Runham (1992a) studied the effect of food ration and temperature (18ºC and 26ºC imposed on 23ºC acclimated fish) on the otolith microstructure of tilapia *Oreochromis niloticus*. Results showed that increment deposition persisted in the otoliths of fish kept at low temperatures but increments were less easily discernible. The effects of temperature, reflected in the periodicity of microincrement formation and the width of microstructures, may last for more than one day (Gutiérrez & Morales-Nin 1986). So long as the temperature is high enough to sustain growth (Campana & Neilson 1985), however, a daily temperature fluctuation is not needed for increment formation. For example, daily microstructures were produced in the otoliths of *Tilapia mossambica* held at constant temperature (Taubert & Coble 1977). Microincrements in the otoliths of the Chinese sucker *Myxocyprinus asiaticus*, however, were less discernible in the otoliths of fish reared under constant temperature as opposed to fish that experienced

temperature variations between day and night (Song et al. 2008b). The lack of contrast between increments resulted in errors in age estimation for fish reared under constant temperature, at least when the lapillus was used.

Food intake and food deprivation also have an influence on microstructure deposition and increment width and periodicity appear to be sensitive indicators of recent feeding history in fish larvae and juveniles (McCormick & Molony 1992, Molony 1996, Massou et al. 2002). Daily microincrement widths in adult fish decline enough to be not discernible as fish grow older (Hoedt 1992), thus possibly not recording changes caused by food deprivation. Moreover, older fish may have accumulated more reserves and thus be in better condition to cope with food deprivation (Molony & Choat 1990). Hence, the effects of food intake upon otolith microstructure are of main application in the studies of larvae and juveniles (but see comments on adult studies below). In tropical marine waters, which are thought to constitute nutrient poor waters where food can be patchy, it may be of great interest to know whether food deprivation results in changes at the level of otolith microstructure. It has been shown that the feeding regime may lead to the formation of sub-daily or supra-daily microstructures if young fish are fed more or less than once a day (Pannella 1980, Neilson & Geen 1985), although multiple daily feeds did not result in the formation of multiple microstructures in the otoliths of *Chanos chanos* larvae (Tzeng & Yu 1992). Ultimately, food deprivation may result in smaller increment widths but not in the cessation of microincrement deposition (Massou et al. 2002) if the fish has enough body energy reserves (Campana & Neilson 1985). Starvation during 33 days was recorded in the otolith microstructure of the tropical species *Ambassis vachelli* in the form of narrower increment widths, although daily deposition persisted (Molony & Choat 1990). Interestingly, the trends in microincrement widths caused by food deprivation in this species were detectable only after a few days.

The effect of food deprivation on the microstructure of otoliths of adult fish has been studied for the tropical species *Ambassis vachelli* (Molony & Sheaves 1998). Fish exposed to starvation (for a length of time at least 12% of the maximum age of the species) had narrower increments than fed fish. Moreover, increment widths returned to similar widths of fed fish after a period of recovery feeding. Compared to other variables such as lipid content, microincrement widths showed a more conservative pattern of variation indicating that microstructure changes are modulated (probably due to the buffering effect of the endolymph chemistry tending to preserve the functionality of an important organ for hearing) and microstructure will never provide an absolute measurement of the extent of food deprivation. Incidentally, the feeding time during the day does not have any influence on otolith structure. Changes in daily feeding time did not alter the formation of the crystalline part of the microstructure in *Tilapia nilotica* otoliths (Tanaka et al. 1981).

Salinity or dissolved oxygen concentration are also exogenous factors potentially affecting microincrement periodicity but they have scarcely been studied. It is known that salinity induces changes in otolith composition at the microstructure level (Thorrold & Swearer, Chapter 8), yet little is known on how salinity can affect microstructure deposition. Salinity alone does not seem to affect otolith structure but the combination of low food rations and high salinities resulted in the deposition of less than one increment per day in the otoliths of silver perch *Bairdiella chrysoura,* suggesting that

salinity could have a physiological effect at low rations (Hales Jr. & Hurley 1991). Oxygen depletion in the water has also been hypothesised to cause disruption of microstructure deposition in *Tilapia sp*. (Pannella 1980) and even resorption of the otolith edge in *Carassius auratus* (Mugiya & Uchimura 1989).

Stress leads to changes in the structure of the otolith in the form of "checks" (sensu Kalish et al. 1995). Stress-induced marks indicate the cessation of otolith growth and appear under transmitted light microscopy as opaque, regular, thin marks (Pannella 1980). It has been suggested that cessation of otolith growth under stress conditions results from disruption in calcium metabolism, although stress is not believed to cause the resorption of otolith calcium (Campana 1983). Stress marks were identified in the otoliths of tilapia *Oreochromis niloticus* associated with fish tagging (Panfili & Tomás 2001) and in juveniles of a subtropical flatfish species, the fringed flounder, *Etropus crossotus* (Reichert et al. 2000), caused by handling the fish to carry out measurements.

Rearing also has been shown to affect otolith structure at the microincrement level. Being a combination of artificial environmental conditions, it may be the actual rearing per se (stress associated with high densities, manipulation of fish, etc.) that affects the microincrement formation but it may also be the relatively homogenous environment provided in most rearing mesocosms. The stress of confinement has been shown to affect the microstructure of tilapia *Oreochromis niloticus* in the form of checks and reduced increment deposition (Massou et al. 2004a). Otolith structure is also affected by fish confinement, as shown by comparing otoliths from laboratory and field caught individuals (Strelcheck et al. 2003). Experimental mesocosms are also unnatural environments where exogenous factors such as photoperiod, temperature and feeding are held constant as independent variables. Various studies have reported that microincrements are more easily discernible in wild fish than in reared fish, in both marine (Al Husaini et al. 2001) and freshwater fish (Vilizzi 1998), to the point that errors in counting microstructures were smaller in field-caught larvae than in reared larvae of *Pagrus major* (Tsuji & Aoyama 1982). The absence of differences in water temperature between night and day led to less discernible increments in the otoliths of reared Chinese sucker compared to fish that experienced diel temperature variations closer to their natural environment (Song et al. 2008a).

3 Analysis of otolith microstructure in tropical fisheries and ecological studies

Otolith microstructure presents a broad spectrum of characteristics that are of immediate application in ecology and fishery management. The first choice to make is which otolith of the three pairs available (sagitta, lapillus, asteriscus) is to be used. The second choice is about the method of analysis, which is highly specific because of the need for a precise examination of the internal structure, requiring time-consuming preparations and sophisticated techniques (compound microscopy, SEM, microprobes, etc.).

3.1 SAGITTA, LAPILLUS OR ASTERISCUS?

All otolith types cannot be employed indiscriminately when studying the structure of the first increments because their formation is not simultaneous, the sagitta and lapillus

being formed first (Thorrold 1989). The earliest formed increments in tropical species often are not clearly discernible, depending on the otolith. For example, the asteriscus, with an ambiguous core, renders difficult the identification of the first increment in Cyprinidae (Morioka 2002, Morioka & Matsumoto 2003). The primary increments are often not clearly discernible in smaller lapilli during the first growth phase in the tropical eastern rainbowfish, *Melanotaenia splendida splendida* (Humphrey et al. 2003), probably due to the compression of the growth in smaller otoliths. Some crystallographic and composition differences exist between otoliths, since asterisci are made of the glassy appearing vaterite and sagittae and lapilli are preferentially precipitated in the form of aragonite (Chesney et al. 1998, Campana 1999).

The sagitta is the most commonly used of the three otolith pairs for structural and ultrastructural examination (Morales-Nin & Panfili 2002). This is probably due to the larger size of this otolith compared to lapillus and asteriscus and, therefore, to the larger relative size of its primary increments. There is an exception in the order Ostariophysi, taxa very common in the tropics, in which the asteriscus is bigger than the other two otolith types. Nevertheless, the lapillus has also been preferred in some studies in the tropics of juvenile pelagic species (Morales-Nin et al. 1999, Morioka 2002), juvenile reef species (Allman & Grimes 2002, Zapata & Herron 2002, Nemeth 2005) and even for adult reef fish (e.g., Lou 1993), mainly for ageing purposes and specifically to measure the extent of the planktonic larval duration. Different studies have chosen different otoliths indicating that each new study should carefully select which otolith to use prior to large scale observation of the otolith microstructure (Sponaugle, Chapter 4). Lapilli can be small enough to allow the observation of their internal structure with little or no preparation (Morales-Nin et al. 1999, Morioka 2002, Sponaugle, Chapter 4). Adult fish have thicker otoliths and they are usually sectioned to reveal the primary increments (e.g., in Cichlidae, Zhang & Runham 1992b). In this case (Figure 8), the sagitta is preferred because its increments are wider, whereas the increments of the lapillus are less discernible and the asteriscus structures are more complex (Figure 8). If the comparison between the three otoliths is impossible, the use of the sagitta is recommended because it contains all information from the earliest growth stages.

3.2 PATTERNS RELATED TO LIFE HISTORY EVENTS

The structure and ultrastructure of otoliths are particularly useful to reveal patterns related to life history events such as ontogenetic or environmental changes experienced by individual fish. Investigations often attempt to relate marks with specific events like transition between ontogenetic stages (e.g., metamorphosis, settlement), migration, reproduction or different stresses. The evidence presented below results from experimental rearing in some cases and correlative observations in others. In as much as possible, this Section aims at presenting sound results that invite applying microstructure observation to identifying and analysing metamorphosis, migration, settlement or reproduction in tropical fish species. Otoliths are most powerful, however, when used as a tool combining microstructures, microchemistry and individual growth trajectories to elucidate individual fish life histories.

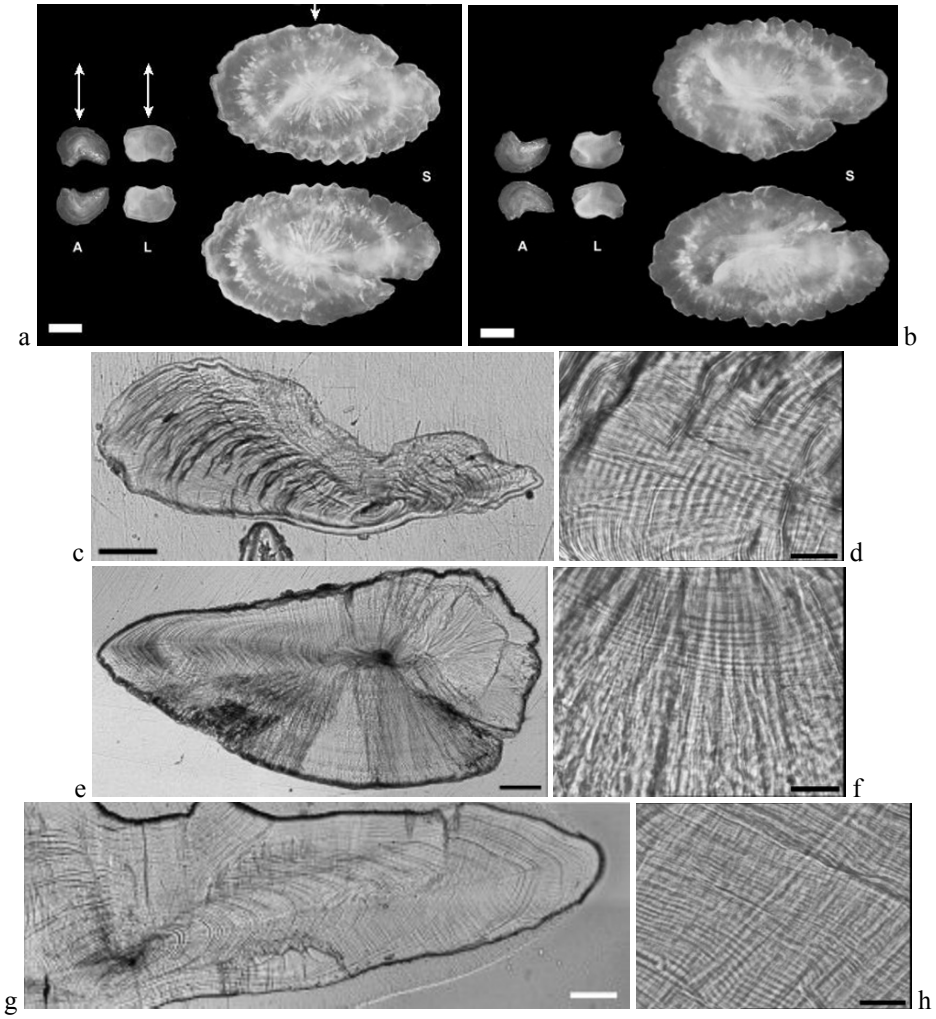

Figure 8. Asteriscus (A), lapillus (L) and sagitta (S) of a *Sarotherodon melanotheron* viewed whole under reflected light against a dark background: (**a**) External faces. (**b**) Internal faces. Thin transverse sections following section plane indicated by arrows in (**a**) of the same asteriscus (**c**, **d**), lapillus (**e**, **f**) and sagitta (**g**, **h**) Scale bars : (**a**, **b**) = 1 mm; (**c**, **e**, **g**) = 100 µm; (**d**, **e**, **f**) = 20 µm (photos K. Diop).

3.2.1 Metamorphosis or environmental changes (migration, settlement)

First feeding can be a critical period in fish from tropical waters as the transitional period between yolk absorption and first feeding is short (Houde 1974). Both hatching and first feeding are frequently recorded in the otolith as distinctive marks (see above). The next major steps for a developing fish can be metamorphosis (i.e., the transition between larval and juvenile life) and then settlement (i.e., final installation in one specific environment), a particularly sensitive stage in tropical areas and especially for

reef species (Leis 1991). These developmental stages and environmental conditions are reflected by changes in otolith microstructure which can be used as reference marks.

The metamorphosis stage varies greatly between species but it is generally a strong transition associated with the end of ossification and for that reason it constitutes a tremendous physiological and morphological stress for the fish. Metamorphosis also can be associated with habitat change (Wilson & McCormick 1997, 1999, McCormick et al. 2002) including changes in environmental conditions and behavioural changes, for example in mesopelagic marine species (Myctophidae, Photichthyidae). Metamorphosis in these species leads to a clear change in otolith pattern (Linkowski 1991, Tomás & Panfili 2000) and also to strong morphological, behavioural (from passive pelagic drifting to active diel vertical migration) and habitat transitions (from surface waters to deep waters). Width of daily increments increases with metamorphosis and a distinct shift in habitat (Jenkins 1987, Wilson & McCormick 1999). Metamorphosis in tropical eels appears with a morphological transition from leptocephali and elver to yellow eel stages accompanying movements from marine to inland habitats and produces a marked check on the otolith (Arai et al. 2000a,b, Sugeha et al. 2001). The otolith check induced by metamorphosis will then constitute a reference mark useful for back-calculating the age at metamorphosis for the different species.

Tropical fish species can be involved in either horizontal or vertical migrations. Horizontal migrations often imply changes of habitat and result in changes of environmental conditions, which can mark the otolith microstructure through a corresponding change in growth rate. This kind of migration can also be linked to metamorphosis (e.g., eel species Arai et al. 2000a) or to settlement (Wilson & McCormick 1997) and it is often difficult to distinguish the effects of ontogeny from those of physiology or behavioural changes. Effects of migration across environmental clines may be more pronounced in terms of microchemical otolith composition. For example, the transition between different salinity conditions affects the Sr/Ca ratio, although in eel species metamorphosis changes are also associated with microchemical modifications (Kotake et al. 2003, Thorrold & Swearer, Chapter 8 of this volume). Daily, seasonal or irregular vertical migrations frequently occur among pelagic species in tropical oceanic waters. These can also be associated with metamorphosis and behavioural changes, as is the case for small mesopelagic species. As for horizontal migrations, associated changes in otolith microstructure are mainly reflected in a change in otolith growth rates.

Fish settlement often corresponds with the transition from a pelagic habitat to a demersal or benthic habitat (Wilson & McCormick 1997), as the larvae of many marine and freshwater species are planktonic and, in tropical reef fish, this is often followed by a relatively sedentary, site-attached mode of life. This transition is reflected in both the increment pattern and growth rates in otoliths (Figure 9b) (Wilson & McCormick 1997, 1999). Settlement checks have been documented for many reef species (e.g., Thorrold & Milicich 1990, Danilowicz 1997b, Suthers 1998, Searcy & Sponaugle 2000, Bergenius et al. 2002) but these checks are not found in all tropical species. They are most common in families living close to reefs, such as Acanthuridae, Chaetodontidae, Labridae, Lethrinidae, Lutjanidae, Mullidae, Pomacentridae, Pseudochromidae and Serranidae (Thorrold & Milicich 1990, Risk 1997, Robertson et al. 1999, Wilson & McCormick 1999, Zapata & Herron 2002, Nemeth 2005). In contrast, non-reef species, such as pelagic or estuarine fish, do not present settlement checks in their otoliths,

probably because the transition between the larval and the juvenile stages does not correspond to major changes of environment. The transition can correspond to changes in the widths of the primary increments within the otolith (Stequert et al. 1996, Panfili & Tomás 2001) but often not to a check.

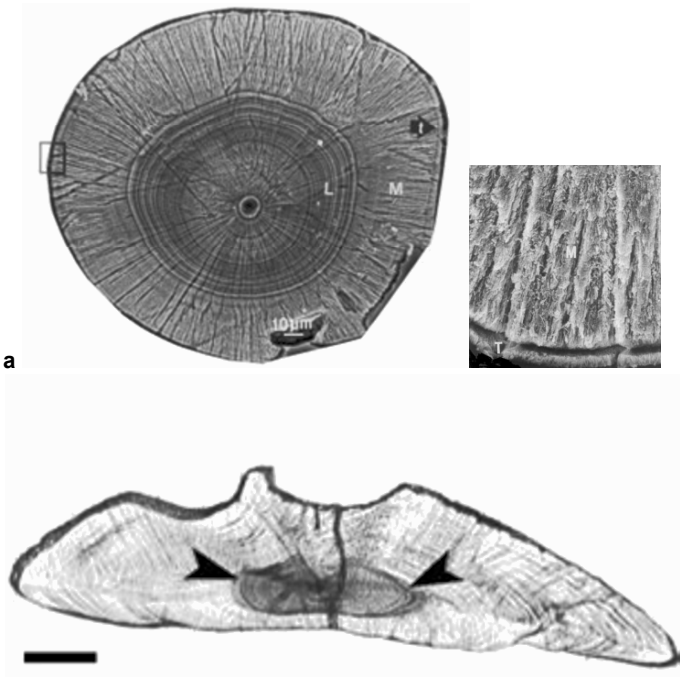

Figure 9. (**a**) Example of metamorphosis zonation on the otolith of *Anguilla marmorata* from Tahiti (French Polynesia). Otolith section viewed in SEM after acid etching. The *right image* shows a detail of the area framed at the *left margin* of the *left image*. L, leptochephalus stage; M, metamorphosis; T and t, transition mark. Scale bar = 10 µm (photos R. Lecomte-Finiger). (**b**) Example of settlement mark (*black arrows*) on the thin transverse section, observed in compound microscopy, of an otolith of *Chaetodon speculum* from the New Caledonian lagoon. Scale bar = 200 µm (photo E. Morize & L. Marec).

Otolith settlement marks in reef species can be grouped into categories based on changes in increment width and optical qualities of the mark (Wilson & McCormick 1999). The most common type corresponds to an "abrupt" settlement mark characterised by a rapid decrease in increment width, whereas other types present more gradual decreases in increment width during the settlement period. Evidence suggests that the increment profile over early development and the increment transitions associated with the settlement event are taxon-specific and may enable late-larval stage fish to be identified to species level (Wilson & McCormick 1999). This microstructural feature is mainly used for the back-calculation of settlement patterns and estimation of larval planktonic durations (Wilson & McCormick 1997). The settlement mark is used for age estimation prior to (e.g., Colin et al. 1997) or after settlement (e.g., Robertson

et al. 1999, Rooker et al. 2004, Nemeth 2005), as well as for calculating growth rates during these two periods (Allman & Grimes 2002) or a combination of all types of analyses (Thorrold & Milicich 1990, Suthers 1998, Robertson et al. 1999, Rooker et al. 1999, Searcy & Sponaugle 2000). The planktonic larval duration of each species is then calculated from the otolith (Raventos & Macpherson 2001). Studies on recruitment of fish in the tropics often rely on the measurements of settlement marks. Growth rates of pelagic larvae which have been argued to be one of the principal determinants of recruitment success have also been estimated from otoliths (Bergenius et al. 2002). The identification of settlement location is also possible using the differences in individual growth rates (Danilowicz 1997a) and the spawning dates of arriving recruits can be estimated from back-calculation (Danilowicz 1997b). Body lengths at settlement are also directly back-calculated from otolith settlement checks (Radtke et al. 2001, Vigliola & Meekan 2002, Chapter 6 in this volume).

3.2.2 Reproduction and maturity

Reproduction can slow somatic growth by redirecting energy resources to the reproductive process and the production of gametes instead of whole body metabolism (Roff 1983). As such, reproduction could have an effect on the otolith growth via a slower body growth during this stage. “Spawning checks” or marks on the otolith which coincide with spawning were first described by Williams and Bedford (1974). Nevertheless, experimental studies establishing a precise relationship between reproduction and otolith features are particularly difficult to conduct because spawning should be strictly controlled to establish the exact location of its effect on the otolith. These observations are even more difficult to obtain in the field because the control of spawning or individual maturity is not possible. Massou et al. (2004b) have clearly shown for the first time an effect of reproduction on both somatic and otolith growth in females of the freshwater tilapia *Oreochromis niloticus*. In this study, the otolith had an irregular series of checks during the reproductive period (Figure 10). The responses varied among individuals, however, and there was not a common signal of reproduction on the otolith structure for all fish. The effect could be related to the peculiar behaviour of this mouth-brooding tilapia because of reduction on food intake or oxygen availability. This appearance of checks in the otolith could also be compared with the scale resorption that occurs during the spawning season in salmonids (Persson 1997). Such marks in the otoliths probably could be found in other mouth brooders in marine environments, like apogonids for example, but so far have not been documented. The information given by reproductive checks could allow managers to know the number of reproductive events per individual or within populations but more studies are needed on the characterisation of these checks, at both structural and composition levels.

There are a number of examples of correlative evidence describing checks in otoliths concurrent with change in sex (Walker & McCormick 2004). This study was the first to clearly demonstrate a strong temporal link between sex change from female to male and accelerated growth in an annual species. To our knowledge, however, there is no demonstration in the literature of a direct effect of reproductive activity on otolith microstructures of individuals caught in the field. Research in this direction could be a goal for management because knowledge of reproduction is key for population management.

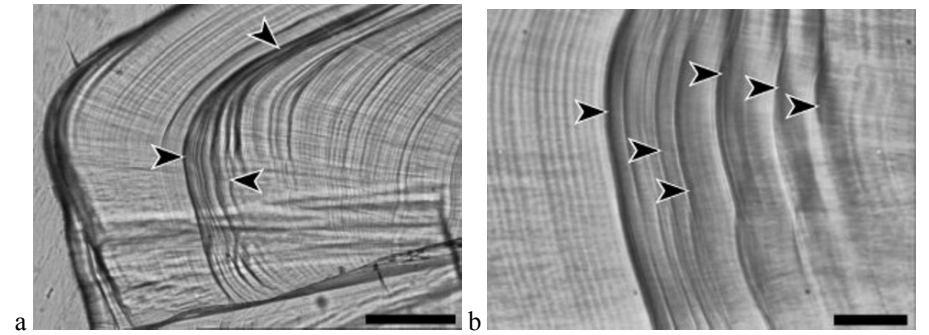

Figure 10. Checks (*arrows*) in the otoliths of *Oreochromis niloticus* females induced by reproduction. Images of the same otolith in thin transverse section observed in compound microscopy. (**a**) Scale bar = 100 μm. (**b**) Detail of (**a**). Scale bar = 20 μm (photos K. Diop & A. Malam Massou).

3.2.3 Stress

Stress of different origins acts on the otolith with a reduction of calcium deposition and produces marks of different intensities. Stress, like reproduction, is a preponderant factor that could lead to changes in the structure and growth of otoliths (Campana & Neilson 1985). There are many sources of stress for fishes, including capture, manipulation in artificial conditions, human or natural environmental pressures, inter- and intra-specific aggression, which, like all environmental changes, can be recorded as checks (discontinuities) inside the structure of the otolith. Pannella (1980) has given a precise but unverified definition of different checks (so called "growth discontinuities") produced by different stresses: "they range from small gaps that grade into interrupted sequences to major unconformities that can be traced continuously along the entire sagitta. The magnitude of the hiatus reflects the importance of the event in the life history of the fish that caused it." He has listed numerous possible causes for growth discontinuities ranging from extreme physiological stresses to starvation, migration or reproduction stresses.

Handling of fish undoubtedly acts as a stress factor during otolith growth. The effect most commonly cited in the literature is that of fish handling for otolith marking (e.g., tetracycline labelling with appropriate dosage). The trauma caused by this marking disturbs the regular deposition of primary increments for 3 to 4 days before the restart of regular deposition (Hoedt 1992, Arai et al. 2000a, Panfili & Tomás 2001, Massou et al. 2002, Massou et al. 2004b). Massou et al. (2004a) have shown that confinement stress could lead to checks in the otolith structure of tilapia *Oreochromis niloticus* (Figure 11). Their results suggest that there is a disruption in the deposition of primary increments during periods of confinement inducing physiological changes like an increase of plasma concentrations of cortisol, a stress hormone, and a reduction of somatic growth. A rapid modification of ambient temperature can also act as a stressor, modifying physiological responses with higher muscle heat shock protein levels and plasma cortisol levels (Nakano et al. 2004). This may reflect significant energetic costs of these stress responses and induce slower growth rates in otoliths.

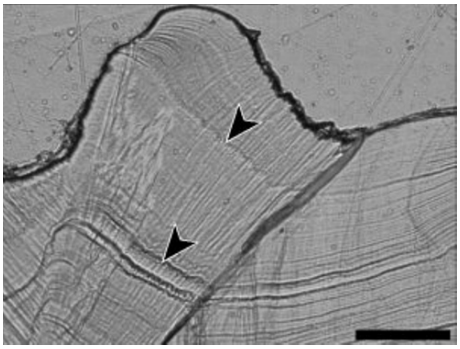

Figure 11. Checks (*arrows*) induced by confinement stress in the otolith of *Oreochromis niloticus*. Image of thin transverse section observed along the sulcus area on the ventral face in compound microscopy. Scale bar = 100 µm (photo K. Diop).

Table 1 summarises specific growth marks associated with life-history traits for tropical species. In conclusion, almost all major life-history or environmental perturbation events encountered by tropical fish may be reflected inside the otolith structure in the form of different marks. Although there are general rules concerning the interpretation of these marks, the responses also seem to be very individual among fish, even for the same species. These marks can be assumed to be reference points in cases such as strong environmental transitions or settlement and metamorphosis. Some difficulties reside in the precise description of these marks in the otolith with the aim of using them as indicators but the information that they contain remains invaluable for managers of natural populations. They can record all steps of developmental stages such as birth, first feeding, metamorphosis, habitat changes and different external stresses. These are taxon-specific and especially useful for back-calculating birth dates, time at settlement or transition between environments.

4 Experimental manipulation of otolith microstructure

The experimental manipulation of otolith microstructure can be undertaken at two levels: (1) the definitive incorporation of new substances or elements inside the structure; and (2) the modification of the growth rate of the structures. The first level includes labelling with fluorescent markers or the incorporation of elements or radionuclides. The second generally uses the manipulation of exogenous factors such as light and temperature to create intrinsic marks. These marks can then be assumed to be references for further analysis.

Table 1. Examples of otolith marks associated with life-history events (excluding hatching and first events after birth) for tropical fish species. Literature review not exhaustive.

L-H event	Otolith mark	Species	References
Metamorphosis	Mark and specific growth rate	*Anguilla celebesensis*	Arai et al. (2000a), Arai et al. (2003)
	Check and specific growth rate	5 *spp. Anguillidae*	Marui et al. (2001)
	Specific growth rate	*Heklotsichthys castelnaui*	Thorrold (1989)
	Metamorphic bands	Labridae (2 species)	Searcy and Sponaugle (2001)
	Accessory primordia	Myctophidae (55 species)	Linkowski (1991)
	Specific growth rate	*Vinciguerria nimbaria*	Tomás and Panfili (2000)
Parturition	Extrusion check	*Sebastes jordani*	Ralston et al. (1996)
Reproduction	Checks	*Oreochromis niloticus*	Massou et al. (2004b)
Settlement	Mark classification, specific growth rate	44 spp. (Great Barrier Reef)	Wilson and McCormick (1999)
	Breakpoint-transition, specific growth rate	38 spp. (Great Barrier Reef)	Brothers et al. (1983)
	Settlement mark, specific growth rate	38 spp. (Great Barrier Reef)	Wilson and McCormick (1997)
	Settlement mark	*Acanthurus triostegus*	McCormick (1999)
	Settlement mark (discontinuity)	Chaetodontidae	Fowler (1989)
	Check	*Chromis atripectoralis*	Thorrold and Milicich (1990)
	Check	*Ctenochaetus binotatus, Scarus rivulatus*	Lou (1993)
	Settlement mark	*Thalassoma bifasciatum, Halichoeres bivittatus*	Victor (1982)
	Settlement mark (check)	*Lentipes concolor*	Radtke et al. (2001)
	Accessory growth centres	*Lota lota*	Fischer (1999)
	Increment settlement transition	Lutjanidae	Zapata and Herron (2002)
	Check	*Lutjanus griseus*	Allman and Grimes (2002)
	Reference mark	Pomacentridae	Wellington and Victor (1989)
	Check	*Pomacentrus coelestis*	Thorrold and Milicich (1990)
	Settlement mark	*Pomacentrus vaiuli*	McIlwain (2002)
	Settlement mark	3 reef spp.	Wellington and Victor (1992)
	Transition mark	*Thalassoma bifasciatum*	Masterson et al. (1997)
	Metamorphic band, transitional mark	*Thalassoma bifasciatum*	Sponaugle and Pinkard (2004), Sponaugle et al. (2006)
Sex change	Check	*Parapercis cylindrica*	Walker and McCormick (2004)
Stress	Specific growth rate	*Abudefduf vaigiensis*	Nakano et al. (2004)
	Check	*Oreochromis niloticus*	Massou et al. (2004a)

4.1 FLUORESCENT LABELLING

The incorporation of a specific fluorescent dye, acting as a marker, into the mineralising surface of the otolith is one of the most common methods used in validation experiments. Several markers have been used but compounds of the tetracycline family have been used mainly for tropical species (Wild & Foreman 1980, Speare 1992, Choat & Axe 1996, Fowler & Short 1998, Thompson et al. 1999, Panfili & Tomás 2001, Grandcourt 2002, Massou et al. 2002, Schwamborn & Ferreira 2002) although calcein (Wilson et al. 1987, Leips et al. 2001) or alizarin complexone (Arai et al. 2000a, Al Husaini et al. 2001, Sugeha et al. 2001, Morioka & Matsumoto 2003, Meifsjord et al. 2006) have also been used. All these markers have the capacity to emit a specific fluorescence under ultraviolet light. The chemical marks are incorporated into otoliths after intra-peritoneal or intra-muscular injection, immersion of the fish in a solution or incorporation of the chemicals into food. Injection doses vary between 25 and 75 mg tetracycline per kg of body weight. The immersion doses and duration of the baths vary with the fluorescent dyes used and fish species (Wright et al. 2002a). The mechanism of incorporation into the otolith structure is rather complex and not very well understood since few experiments have been done to elucidate the incorporation processes. Nevertheless, once the marker is inside the body, its calcium-chelating properties make it become directly integrated into the mineralising surface of the otolith. The non-incorporated fraction is excreted by metabolism after a few minutes or hours (Meunier 1982). This clearly explains the systematic observation of a single fluorescent band after marking, which sometimes can diffuse across a few increments. Multiple incorporations are possible by repeated administration of the dye spaced over a few days or longer (Tsukamoto 1988, Meifsjord et al. 2006).

4.2 THERMAL MANIPULATION

Temperature fluctuations have an immediate effect on otolith accretion rates and thus lead to discernible marks inside the structure. These marks are in the form of changes in the width of increments or as zones more or less dense optically (Volk et al. 1999) that result in incremental patterns distinct from those caused by natural fluctuations. Thermal marking can be applied to mark thousands of fish with minimal effort to create "bar codes" with microstructures. This technique has been used mostly to mark the otoliths of larvae or juveniles but not adults. The idea is to use short-term temperature manipulations to mark juvenile fish otoliths by altering the appearance of D-zones and L- zones in one or more primary increments to produce a clear pattern of events. The production of a visible mark can be done with a temperature depression of only a few degrees for a few minutes or hours (e.g., 3.5°C and 4 h for salmon, Volk et al. 1994). Although this technique has mostly been applied to mark salmonids (Volk et al. 1999), it has been shown that otoliths of fish from non-temperate environments can also be marked with this technique. A change of temperature from 19–21ºC to 28ºC or 16ºC was enough to mark the otoliths of the subtropical species Chinese sucker (*Myxocyprinus asiaticus*) (Song et al. 2008b). The combination of cold and warm temperatures in time lapses of 6–36 h created distinct patterns. Details regarding the facilities necessary to carry out thermal marking can be found in Volk et al. (1999).

4.3 MANIPULATION OF OTOLITH ELEMENTAL COMPOSITION

Otoliths grow by successive deposition of increments, of which the L-zone is a band rich in calcium carbonate crystals in the form of aragonite crystals. The composition of the L-zone can be altered to create a distinct chemical mark. The aragonite group is composed of carbonate minerals with a single divalent cation of radius > 1.00 Å. Carbonates which are isostructural with aragonite include strontianite ($SrCO_3$), cerussite ($PbCO_3$) and witherite ($BaCO_3$) classified by increasing single divalent cation radius. It is possible, therefore, to manipulate the elemental composition of the otolith aragonite by replacing Ca in the crystal lattice by other elements such as Sr, Pb or Ba.

Injections of $SrCl_2$ have been used to chemically mark otoliths of juvenile southern bluefin tuna, *Thunnus maccoyii* (Figure 12) (Clear et al. 2000), whereas strontium chloride baths have been used to mark the otoliths of tropical clupeids (sprat, *Spratelloides delicatulus* and herring, *Herklotsichthys quadrimaculatus*) (Milton et al. 1993) and juvenile barramundi (*Lates calcarifer*) (Milton & Chenery 2001). The Sr mark produced by a Sr-bath was found in the otolith of juvenile barramundi 3 days after the Sr-bath (Milton & Chenery 2001). In all cases, the Sr mark was easily detected using a scanning electron microscope (SEM) fitted with a backscatter detector or Energy Dispersive Spectroscopy (EDS). Nonetheless, caution is needed when marking freshwater fish by immersion as it has been shown that discrimination of strontium against calcium is a function of an osmoregulatory mechanism and increases in waters with low salt concentrations (Rosenthal 1960).

Barium chloride ($BaCl_2$) has been successfully used to mark offspring of coral reef species and Ba isotope ratios in the otolith cores of settlers have permitted larval dispersion to be followed in the environment (Thorrold et al. 2006, Almany et al. 2007). Pb has not been used to mark otoliths, although its proclivity to replace Ca in the crystal lattice has also made it an ideal candidate for marking otoliths and monitoring perturbations such as the exposure of fish to polluted environments (Spencer et al. 2000) or the occupation of estuaries by diadromous or migrating fish (Bath et al. 2000). Alternative chemical species used to mark otoliths are lanthanides such as lanthanum, samarium and cerium (Ennevor & Beames 1993) whose mark in the otoliths of coho salmon (*Oncorhynchus kisutch*) was still detectable 10 months after marking.

Experiments with ^{45}Ca have also demonstrated the use of radio-labelled calcium to mark the otoliths of goldfish *Carassius auratus* (Mugiya et al. 1981, Ichii & Mugiya 1983, Mugiya & Uchimura 1989). Intraperitoneal injection of ^{45}Ca into rainbow trout showed that ^{45}Ca deposition in the otoliths occurred within 3 h (Mugiya 1974). The otoliths of various tropical species (zebra fish, *Danio rerio*, white cloud mountain fish, *Tanichthys albonubes*, and guppy, *Poecilia reticulata*) were marked with similar results using radiostrontium (^{90}Sr), taking advantage of the fact that strontium, fed or injected, accumulates in otoliths as in other calcified tissues (Rosenthal 1957, 1960).

In summary, it is recommended marking the otoliths of tropical fish with tetracycline injection or immersion rather than alizarine, calcein or strontium chloride since it produces a brighter mark than other fluorescent dyes and the detection of the mark is cheaper compared to that of strontium chloride, which requires electron microscopes (Hernaman et al. 2000). Nevertheless, the use of tetracycline constitutes an

environmental issue since it does not degrade in the environment and is not suitable for human consumption if accumulated in the fish tissues. The trade-off between these issues should lead the researcher to make a decision regarding which marker to use.

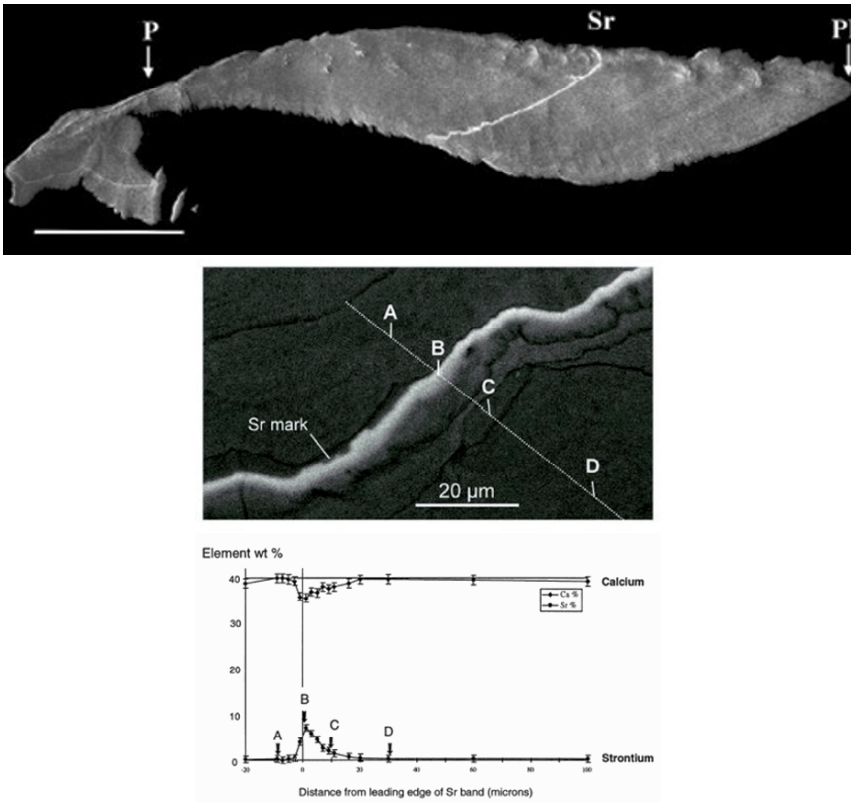

Figure 12. Backscattered SEM image showing a clear strontium mark apparent as a bright band on a section of *Thunnus maccoyii* otolith, along the primordium (P) – post-rostrum (PR) axis (scale bar = 1 mm). The region analysed for the Sr and Ca levels is magnified. The graph indicates the measured variation in Sr and Ca along the transect: points A–D indicate representative positions along the transect to enable comparisons (Source: Clear et al. 2000).

5 Procedural considerations

5.1 TRADE OFFS AND QUALITY ASSURANCE

The study of otolith microstructure demands more effort than any other standard method (e.g. ageing), both in terms of time and cost. The financial investment in materials, equipment and preparation time depends on the type of information that one

seeks from microstructural examination. For example, any study of the fine details within the microincrements at high magnifications (above ×2000) requires the use of a SEM because this exceeds the resolution limit of any optical compound microscope. The simple study of life history events, however, mainly relies on observations made with a compound microscope, as details of microincrements can be seen at between ×400 and ×1000 magnifications.

The analysis of the results of experimental otolith manipulation (e.g., fluorescent labelling, thermal manipulation, elemental composition) requires different observation techniques than those used for otolith microstructure examination. Compound microscopes are used for thermal or light-cycle manipulation, UV lamps for fluorescent labelling, and electronic probes for elemental composition. Table 2 lists the comparison of the investment and analysis costs for the different equipment involved in such studies. Techniques used for otolith composition analysis remain the most expensive.

Quality assurance when studying otolith microstructure is directly linked to the instrumentation used. The main constraining factor in compound microscopy is the optical resolution available. The theoretical limit of resolution is about 0.3 μm (Campana 1992). The widths of some microincrements are below this limit and the use of a SEM is thus essential (Morales-Nin 1988). SEM gives the best level of quality assurance provided that the sequence of sample preparation is accurately followed (sectioning, polishing, etching and sample coating).

5.2 SAMPLING DESIGN

The aim of the study will determine the methods to be employed. Only light microscopy is required when the goal is to observe growth micro-increments for age validation, life history events, etc. In some tropical species during certain slow growth periods, however, very thin increments may be laid down on the otolith (Morales-Nin 1988) and when viewed with a compound microscope these would appear as thin translucent bands without structures. It would be necessary in such cases to observe some otoliths of different ages with SEM. This tool is also recommended for microstructural studies and for observation of growth discontinuities to determine if they correspond to periods of several days.

The number of otoliths to be observed will depend on the methods used because SEM time and sample preparation are costly. It is advisable in general to always select one otolith from the same side of the fish and keep the other otolith of the pair for additional observations or as a backup. Time available for the study is also important, as cutting and polishing an otolith might take one hour or more and observation of microstructures is time consuming. Thus, at the beginning of any study, available time and money resources should be considered to define the number of otoliths to be examined. It is advisable to have extra otoliths because frequent losses occur during otolith preparation. It is advisable to examine males and females separately when sexual dimorphism is present or when mature fish are studied. Similarly, when various life phases are studied, it is necessary to increase the number of otoliths examined in the transition sizes (for instance at settlement). The asymptotic otolith growth in adult fish might make it difficult to prepare sections comprising all the increment sequence and extra otoliths might need to be examined to get all required information.

Table 2. Different relative costs of initial investment, analysis and effort (time required) for the preparation and operation of the indicated equipment. X is simply an indicative relative measure and not equal to any monetary value or specific amount of time. ***Legend:*** UV, ultra-violet; SEM, scanning electron microscopy; EDS, energy dispersive microprobe; ICPMS, inductively coupled plasma mass spectrometry; WDS, wavelength dispersive microprobe.

Equipment	Investment	Analysis	Effort	Study
Compound microscopy	X	0	X	Life history events Growth Annulus-age estimates Marginal increment analysis Thermal manipulation Light manipulation
UV light microscopy	XX	X	X	Labelling manipulation
Confocal microscopy	XXX	X	X	3D structure
Raman spectrometry	XXX	XX	XX	3D structure
SEM	XXXX	XX	XXX	Ultrastructure
EDS	XXXXX	XXXX	XXX	Manipulaton of elemental composition
WDS or ICPMS	XXXXXX	XXXXX	XXXXX	Manipulation of elemental composition

5.3 DATA ANALYSIS ISSUES

Microstructural observation is generally descriptive and does not require data analysis. For increment measurement see Vigliola and Meekan (Chapter 6) and for counting Fowler (Chapter 3) and Sponaugle (Chapter 4).

5.4 FUTURE PERSPECTIVES

The microscopes traditionally used, based on light or electrons, are being improved both in their features (optical capacity and quality of the lenses) and with the use of accessory devices such as image analysis systems. These have increased the resolution power and quality of light microscopy, which is now also more user-friendly due to the availability of cameras (digital, with increased resolution). Moreover, PC-based analysis systems help recording, improving, filtering and analysing images, as well as helping in the observation and interpretation of otolith structures displayed on a screen by more than one observer. Thus, new developments concern both the observation devices (microscopes) and the rapidly evolving image treatment systems.

Modern SEM microscopes also now work at ambient or low pressures (Environmental SEM, ESEM), allowing observation of biological samples without metallic coating of the surface. This could be an advantage when otolith microstructure examination is coupled with analysis of chemical composition, since any coating would alter the surface of the otolith. Future studies will certainly use the latest instrumentation (such as Atomic Force Microscopy) to make progress in the analysis of otolith growth at the microincrement level and provide insights into the interaction between aragonite crystals and proteins at the nanoscale.

6 Technical recipes

6.1 TECHNICAL PREPARATION OF OTOLITH MICROSTRUCTURES

Preparation techniques for otoliths are universal and not specific for tropical or temperate species. The examination of otolith microstructure necessarily implies some kind of preparation to reveal its internal structure except if observed with Laser Scanning Microscopy (Lagardère et al. 1995). Preparation usually requires sectioning the otolith along a particular plane. This plane has to be chosen prior to any further manipulation. There are three main planes of sectioning, following the general orientation of the fish: sagittal (vertical longitudinal), transverse and frontal. The sagittal plane can be used for small-sized fish for which both the core and the edge of the otolith can be reached easily. It is more difficult for larger fish with otoliths with a concave-convex or even more complex shape, in which case the transverse section plane is recommended for a standard observation of internal structures.

Whole otoliths usually should be embedded in a suitable block of material prior to sectioning (Box 1) since it is difficult to manipulate the otoliths directly. There are a lot of embedding media available but generally polyester resins or related resins (e.g., epoxy, thermoplastic cement) are used for structure examination. A complete table with the standard mounting materials is available in McCurdy et al. (2002). The internal structure of embedded otoliths can be revealed with two main techniques of sectioning, the first using low speed saws (Box 2) and the second using hand grinding.

The otolith sections can be viewed directly for microstructure examination under light microscopy after appropriate embedding, sectioning and polishing (Box 3). Nevertheless, in order to reveal more precisely the differences in internal crystallisation, acid (Box 4) or proteinase (Box 5) etching is used to accentuate the differentiation between L-zones and D-zones. Etching is also an obligatory step before discriminating zones by staining (Box 6) or examining the structure at high magnification by scanning electron microscopy (SEM). In SEM, the electrons require a high vacuum environment to assure an aligned beam sputtered onto the sample surface and the sample must be electrically conducting to avoid electron clouds on the sample surface that would compromise the quality of observation (Box 7). Consequently, the otoliths must be dried and coated to increase the aragonite conductivity. Techniques to remove the aragonite and preserve the structure of the organic matrix are required when the organic matrix is to be observed. Technical recipes for each step of otolith preparation mentioned above are provided in Boxes 1–7 below (see also Fowler, Chapter 3 and Spanaugle, Chapter 4 in this volume).

7 Observation

7.1 SCANING ELECTRON MICROSCOPY (SEM)

The SEM is a surface topographic examination tool generally used for otolith studies. The instrument offers an over 300-fold increase in the depth of field when compared with the highest quality compound microscope, which is reflected in the superb 3D

images it provides at very high magnification (> ×10000). After etching the otolith surface, it reveals the internal crystalline structure as well as the orientation of the crystals and the protein matrix structure (L-zones and D-zones, respectively). Studies of beam-specimen interaction using the SEM can provide useful information about the chemical composition at the specimen surface, as well as the crystallographic, magnetic, and electrical characteristics of the specimen. For example, for microchemical analysis purposes the SEM is coupled to an EDS (Energy Dispersive Spectrometer).

Box 1. Embedding otoliths for sectioning

1. Clean and dry the otolith before embedding.
2. Tag the otolith and embedding mould.
3. Mix carefully one unit of resin (e.g., polyester) by volume or weight and 1% catalyst (e.g., for polyester).
4. Leave the mixture for 2 min to let the largest bubbles escape.
5. Pour a layer of resin into each individual mould (flexible rubber moulds are preferred).
6. Wait for polymerisation that takes at least a few hours (e.g., 4 h for polyester), preferably in a dry oven (30°C). Avoid humid environments for polymerisation.
7. Put the otolith to be embedded over the solid resin layer of the mould.
8. Mix carefully another portion of resin with 1% catalyst.
9. Leave the mixture for 2 min to let the largest bubbles escape.
10. Pour over the otolith until it is covered by the mixture.
11. Turn over the otolith in its mould under the resin to eliminate air bubbles.
12. Orient the otolith precisely in the mould for further preparation (e.g. sectioning).
13. Wait for complete polymerisation over a minimum of several hours (e.g., 24 h for polyester), if possible in a dry oven (30°C), before further manipulation.
14. Remove the embedded otolith from the mould and tag the resin block.

Box 2. Sectioning the otolith

1. It is best to use a slow-speed rotary diamond saw.
2. Use an appropriate liquid media as a cutting fluid (e.g., water is preferred with polyester resin).
3. Mark guidelines for sectioning directly on the resin block holding the embedded otolith.
4. Lock the embedded otolith on the specific support saw and orient it as required to cut in the preferred section plane.
5. Cut the section, being sure not to destroy desired features (e.g., core).
6. If a slice is necessary, adjust the level with the saw micrometer taking into account the thickness of the sectioning tool (e.g. about 300 μm for diamond disc) and make the second cut to produce the section of desired thickness.
7. Dry the section.

Box 3. Grinding and polishing the section

1. Use grinding after sectioning or, for smaller otoliths, use grinding instead of sectioning.
2. Grinding generally is done by hand with wet abrasive paper (grit grades between 120 and 1,200).
3. Polishing follows grinding and generally is done by hand with polishing cloths with different grades of alumina pastes (from 3 µm to 0.3 µm) or diamond powder.
4. Start grinding with a coarser grit paper until the approximate position of the surface is attained and then carefully fine-tune the surface with finer grit paper.
5. Similarly, start polishing with a coarser paste and finish with finer paste.
6. Use random movements when grinding and polishing to avoid systematic distortion of the preparation plane or systematic scratches.
7. Frequently check the state of the ground or polished surface under a microscope to ensure desired features (e.g., the otolith core) are not being damaged or removed.
8. Rinse the preparation in clean water between stages of grinding or polishing.
9. At the end of the process clean the preparation in an ultrasonic bath.

Box 4. Acid etching

1. Prepare acid solution (generally 1–5% EDTA or 1–5% hydrochloric acid).
2. Plunge the preparation to be etched (e.g., otolith section) into the acid solution.
3. Measure the time of acid attack (generally between 1 and 5 min). The speed of etching depends on the temperature of the acid solution, the cooler the solution the slower the reaction.
4. Check the etching process under a binocular or a compound microscope.
5. Thoroughly rinse the etched preparation with distilled water (for a few minutes).
6. Leave the preparation to dry (open air or dry oven at 30°C).
7. Check the etched surface and restart at point 2 (above) if necessary.

Box 5. Proteinase etching (from Shiao et al. 1999)

1. Prepare proteinase K buffer: 10 mg of proteinase K, 0.1 ml of Tri–HCl (pH=8.0, 1 M), 0.5 ml of sodium dodecyl sulphate (SDS), 0.02 ml of NaCl.
2. Digest the otolith section with the solution in a 1.5 ml microtube at 45°C with gentle shaking.
3. Depending on species and temperature, the reaction time varies from a few minutes to a few hours.
4. Check the otolith surface under a compound microscope until optimal etching .
5. Leave the preparation to dry in an oven after digestion.
6. If SDS crystallizes on the surface, use 75% alcohol to dissolve it.

Box 6. Staining (from McCurdy et al. 2002)

1. Prepare staining solution (e.g., 1% Toluidine blue).
2. Plunge the preparation to be stained (e.g., otolith etched section) into the staining solution (or put a drop of stain on the etched section).
3. Measure the time of staining (generally between 1 and 5 min).
4. Rinse the stained preparation carefully with distilled water. Be careful as the stained surface is sensitive to further manipulation.
5. Dry the preparation (open air).
6. Check the stained surface and restart at point 2 if necessary.

Box 7. Surface coating for SEM

1. Dry the sample for 6–8 h at low temperature. Keep it protected from dust.
2. Mount the sample on the SEM sample holder or stub, using carborundum paint, thermoplastic glue or double-sided tape, with surface to be observed uppermost.
3. A thin line of colloidal silver should run from the otolith surface to the stub to avoid electric charges on the otolith during observation.
4. Sputter the otolith surface with gold (100 Angströms) or carbon (10–50 Angströms).
5. Keep dry and dust free until observation.

The electron beam used as "illumination" that is directed onto the specimen surface is accelerated with voltages of 1–30 kV. The interaction between the beam and the specimen generates the image. Specimen-beam interaction, in addition to producing backscattered and secondary electrons, also produces photons, specimen currents, Auger electrons and X-rays, which are characteristic of the elements excited by the beam in the specimen matrix. While in principle any signal generated can be used to produce an image, in practice it is the low-energy secondary electrons released from the sample that are most commonly used. Using surface emitted electrons, rather than those passing through the specimen, as in TEM, surface images revealing some three-dimensional quality can be obtained.

The impression of the 3D surface image is the result of the distribution of light and dark areas. This distribution is accounted for largely by the incident beam which generates more collectable secondary electrons per unit area when it strikes a sharply curved edge or sloping surface than when it hits a flat surface. The contours of biological specimens facilitate the production of such a differential effect. Where the surface is smooth, tilting the sample at an angle to the probe will enhance the desired variation in collected secondary electrons. More subtle effects involving the manner in which structures lying above a primary surface either deflect or absorb the probe electrons also come into play in the process. In any case, the "shadows" seen are a true representation of the 3D character of the specimen surface under study. This is why etching is a pre-requisite of otolith observation under SEM.

Depending on the beam accelerating potential used, which is generally around 15 kV for otolith observation, the ratio between the low-energy secondary to high-energy

backscattered electrons will be higher. Higher voltages, although giving more penetration into the sample surface, result in electrical charges due to the low conductivity of aragonite.

7.2 RAMAN SPECTROMETRY

Raman spectrometry can be useful to describe the nature of the otolith layers within the crystal lattice. It measures the Raman effect, which is the inelastic scattering of light by matter. When a photon of visible light interacts with a molecule, it can be elastically scattered (conserving its energy), it can be inelastically scattered losing energy (Stokes scattering) or it can be inelastically scattered gaining energy from the molecule (anti-Stokes scattering). The measurement of such vibrational energy in otolith studies provides information about the metal and carbonate bonds within the crystal lattice of the otolith. A laser excites the sample surface and the Raman effect and the resulting distortion of the crystal lattice are recorded as energy spectra which are characteristic for each polymorph of $CaCO_3$. The volumes excited by the laser can be as small as 20 μm^3. The main use of Raman spectrometry in otolith studies is thus the identification of the $CaCO_3$ polymorph deposited, aragonite, calcite or vaterite (Gauldie et al. 1997, Tomás & Geffen 2003). The readability of the otolith and its chemical composition will vary greatly depending on which polymorph is precipitated (Brown & Severin 1999, Tomás & Geffen 2003) and so it is necessary to verify that the $CaCO_3$ polymorph precipitated is aragonite prior to interpretation.

7.3 CONFOCAL MICROSCOPY

Confocal laser microscopy provides images of the internal structure of samples without preparation for as long as these fluoresce. It can therefore only be used for the observation of microincrements if these are first marked with a fluorescent dye. Confocal microscopy has been applied to detect the internal oxytetracycline mark in the otoliths of larval and juvenile stripped-bass (*Morone saxatilis*) (Secor et al. 1991), to study the ontogenetic changes in the 3D structure of otoliths of various temperate species (Lagardère et al. 1995) and to make observations of the fluorescently labelled saccular membrane of herring (*Clupea harengus*) embryos (Tytler et al. 2002).

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