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THE PRESENT STATUS OF OTOLITH RESEARCH AND APPLICATIONS

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Preface

The collected papers in this report are the outcome of a 3 day workshop held by the research and application of ageing methodology cell of the European Fish Ageing Network. This was the first of three workshops that were intended to review, discuss and communicate advances in the application and understanding of otolith microstructure, chemistry and novel ageing methods. By reviewing current understanding of otolith formation and ageing techniques the first workshop was intended to help identify areas where further research was required.

Research has shown that interpretation of otolith structures and chemistry has often been underpinned by false assumptions. In the first section of this report we review the mechanisms of otolith formation and highlight areas where our limited understanding constrain interpretation of structures and chemistry. The second section of the report deals with several novel applications to annual age estimation, including radiometric dating, other otolith chemistry approaches, lipofuschin and microstructure. The final section contains two papers dealing with certain aspects of the analysis of age structure and age estimation. In the time since this workshop report was written there have been further developments in several fields discussed. Some of these recent developments can be found in the proceedings arising from the Second International Otolith Symposium held at Bergen in 1998, that will be published shortly.

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The regulation of otolith formation and its significance to age determination, size back-calculation and elemental composition

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1. Otolith formation

Teleost otoliths are calcareous structures involved in mechanoreception (Popper & Hoxter, 1981). They are bathed in endolymph within a semi-permeable membrane of the inner ear. Otoliths are formed extracellularly from the crystallisation of calcium carbonate in the aragonite form onto an organic matrix template composed largely of a keratin-like protein, otolin, which is rich in aspartate and glutamate residues (Degens *et al*. 1969; Watabe *et al*., 1982; Morales-Nin, 1987a; Mugiya, 1996). Otoliths exhibit a range of incremental structures that are often formed regularly over time scales ranging from sub-daily to annual. Unlike skeletal calcium, which may be mobilised for homeostasis (Simpkiss, 1974), otoliths do not appear to be subject to mineral resorption except under extreme stress (Mugiya & Uchimura, 1989). Consequently, otoliths appear to be highly suitable for age estimation. Applications of otolith increment counts include age estimation for assessment of population structure, size at age determination, estimation of hatch date analysis, mortality estimation and pattern analysis for identification of life-history events, year-class and sex determination.

Given that otoliths function as baro-receptors, it is generally assumed that they must increase in size in proportion to fish growth so as to maintain the integrity of the information it provides about acceleration and stability. As otoliths continue to accrete under the most variable conditions of somatic growth there has been great interest in the potential use of otoliths as a chronological record of an individual's growth. Many studies have found highly significant correlations between the width of annual and daily growth increments and fish size. Otolith-fish size relationships have been used to back-calculate growth down to a daily level, in order to infer individual growth histories and assess whether size selective mortality has occurred (Campana & Jones, 1992).

The continuous and daily deposition of material has also led to considerable interest in the otolith's potential as a chronological record of the environmental chemical exposure of individual fish. Otolith chemistry has been used in the spatial discrimination of fish stocks, which is presumed to reflect differences in environmental water chemistry. Together with microstructure, otolith chemistry has also been used to infer the timing of fish movements between different water masses. Despite the considerable potential of otolith structure and chemistry for fishery studies, the application of this methodology is constrained by our limited understanding of otolith formation. This lack of understanding limits interpretation and may result in population applications being underpinned by false assumptions. This paper reviews our

current understanding of the regulation of otolith formation and highlights areas where further research is needed.

2. Incremental structures

Although age determination is central to most fisheries assessment methodology and many aspects of fish biology, our understanding of the process affecting the periodicity of increment formation in calcified structures is relatively limited. Whilst an understanding of the influences on increment periodicity is not essential for their use as chronometers (Casselman 1987), it could help to give greater certainty in otolith interpretation (Williams and Bedford 1974) and reduce the continual need for validation under a natural range of environmental conditions (Casselman, 1987).

Fish ageing depends on visible changes in otolith growth. The growth patterns of most interest are at three levels of resolution:

- primary increments, enabling resolution of days
- seasonal bands, enabling resolution of several months or growth season
- annual increments, enabling resolution of years

The mechanisms which produce these visible patterns are slightly different, although at the basic level they are the result of variations in the relative calcium content of the increments or bands (Dannevig, 1950; Morales-Nin, 1987a).

Primary increments are formed from the successive deposition of a mineral rich and a matrix rich, mineral deficient layer around a nucleus (Fay, 1980; Watabe *et al*., 1982; Morales-Nin, 1987a; Mugiya, 1987; Zhang and Runham, 1992a). Various terms have been given to the two layers forming these primary increments. A review of otolith terminology at the first international symposium on otolith research proposed that the terms L- and D-zones, for the mineral and matrix rich layers, respectively (Anon., 1996). These terms refer to the bipartite appearance of the increments, L- and D-zones appearing light and dark, respectively when viewed under transmitted light. The difference in chemical composition of the two zones also leads to a different appearance under scanning electron microscopy following acid etching. This terminology will therefore be used in the following review. In many fishes the deposition of a primary increment occurs over a day, so producing a recognisable daily increment (Pannella, 1980).

Annual increments, also termed annual marks or checks, rings or annuli, are often distinguishable. Most temperate and many tropical species exhibit annual increments, comprising an opaque and translucent zone within their otoliths. In addition to, The two zones differ in terms of the width of primary increments, the thickness and size of aragonite crystals (Morales-Nin, 1987a), the frequency of growth discontinuities and organic layers (Mugiya *et al*., 1985), the ratio of calcium carbonate and protein matrix (Mugiya 1984; Casselman 1974; 1980; 1987) and elemental ratios (Kalish, 1989; 1991). The combination of these factors leads to differences in optical density of the two zones.

3. Increment periodicity

3.1 Primary (daily) increments

The large literature on daily increments has led many researchers to infer that primary increments can be assumed to be formed daily (see Gjosaeter *et al*., 1984). However such an assumption is invalid for a number of reasons. Otolith increment deposition may not be daily or easily discernible in all species (Geffen, 1982a; McGurk, 1984; Alhossaini & Pitcher, 1988; Morales-Nin, 1992). Inter-observer comparisons have shown that otolith structures are often interpretated differently by different readers (Campana and Moksness 1991). Primary increments may not be

deposited daily until some time after hatching (Geffen, 1987). Sub-daily increments and discontinuities in the increment record may occur (Campana & Neilson, 1985). The daily deposition of increments generally appears to cease in adult and/or juvenile life-history stages of long-lived fish (Pannella, 1971; 1980). In some cases this apparent cessation in the daily periodicity might be related to the formation of very thin growth increments below the detection limit of the light microscope (Morales-Nin, 1988; Morales-Nin & Ralston, 1990). However, ultrastructural investigations have also demonstrated that primary increments are not deposited daily in some species (Volk *et al*., 1995). Clearly then, the interpretation of microstructural growth patterns in wild fishes requires an understanding of the physiological process and regulation of otolith accretion and the environmental factors that influence it (Campana & Neilson, 1985). For otolith primary increments to be of use in age determination, the processes involved in their regulation either must be synchronised to cyclical environmental events, or must possess an endogenous circadian rhythm, entrained to a diel environmental cycle (Geffen, 1987). In addition, increment formation must be independent of somatic growth. Experiments have shown otoliths continue to accrete even when somatic growth has naturally ceased (Brothers, 1981; Wright *et al*., 1990) or has been artificially restricted (Mosegaard *et al*., 1988). This continuity may be related to differences between the growth of sensory systems such as the inner ear and other parts of the body.

Exogenous influences on primary increment periodicity

Several studies have examined the relation between increment formation and specific environmental factors and a number of possible synchronising factors have been proposed. Pannella (1980) suggested that increment periodicity may be related to the number of peaks in feeding activity. Feeding frequency has been reported to influence increment periodicity in some species, for example, *Oncorhynchus tshawytscha* (Neilson & Geen, 1982), *Pleuronectes platessa* (Alhossaini & Pitcher, 1988), but not in others; *Lepomis macrochir* (Taubert & Coble, 1977), *Oncorhynchus nerka* (Marshall & Parker, 1980), *Platichthys stellatus* (Campana, 1983), *Salmo salar* (Wright *et al*., 1992). Moreover, starved fish often continue depositing increments at a daily rate (Taubert & Coble, 1977; Marshall & Parker, 1982; Campana, 1983; Wright *et al*., 1990). Thus there appears to be little evidence to support Pannella's hypothesis of a relationship between increment periodicity and peaks in feeding activity.

Otolith growth is sensitive to temperature in a number of species (Brothers, 1978; 1981; Mosegaard *et al*., 1986; 1988) and Brothers (1978; 1981) proposed that temperature fluctuations are a major factor influencing increment formation in temperate stream fishes. Light-dark cycles appear to be necessary for daily increment formation in larval *Lepomis macrochir* (Taubert & Coble, 1977) and *Fundulus heteroclitus* (Radtke & Dean, 1982). Campana & Neilson (1985) suggested that such a dependence on light-dark transitions may be age mediated, as light-dark cycles appear to be essential for daily increment deposition in the larval but not the juvenile stages of plainfin midshipman, *Poricthys notatus* (Campana, 1984). However, other larvae exhibit primary increments under constant light suggesting there also phylogenetic differences influencing sensitivity to environmental cycles (A. Geffen, unpubl. data).

Growth increments of microstructure and thickness similar to those found in shallow water fish and with rhythmical groupings, are found in deep-sea fish in the absence of light and feeding daily rhythms (Morales-Nin, 1980; Lombarte & Morales-Nin, 1995; Morales-Nin *et al*., 1996). In these species small variations in tidal currents along the slope or vertical migrations of planktophagous preys might act as an daily zeitgeber.

Endogenous regulation and entrainment

If increment periodicity is controlled by an endogenous circadian rhythm then increment deposition would be expected to continue in the absence of entraining stimuli, although the

absence of an entraining stimulus would be expected to eventually lead to a divergence from a daily deposition rate. Several studies have shown a continued daily increment deposition rate in the absence of one potential entraining stimulus such as light-dark transition. Information has come from several reports of a continued daily rate of increment formation in juvenile fish held under constant light (Campana, 1984) or darkness (Radtke & Dean, 1982) or in the absence of cyclical variations in light, temperature or feed frequency (Wright *et al*., 1991). Whilst the study of Wright *et al*. (1991) on juvenile salmon is the only one to consider all three key entraining cues, food intake may have varied cyclically even when multiple feeds were given since studies of feeding intensity in this species have found a diurnal variation in feeding activity (Rawlings & Talbot, 1993). Consequently, environmental manipulation experiments do not provide unambiguous experimental evidence of an endogenously regulated cycle of increment formation. Moreover, no study has demonstrated a divergence from one increment per day as might be predicted when there is no entraining stimulus, although this may reflect the short (<30 day) duration of experiments.

From a review of environmental manipulation experiments Campana & Neilson (1985) proposed that the endogenous circadian rhythm controlling otolith accretion was entrained to photoperiod, but could be masked by sub-daily temperature cycles or feeding patterns. Investigations into diurnal rhythms have demonstrated that it is possible to artificially alter the entraining cue. For example, Spieler and Noeske (1984) found that feeding schedule, when it provides a consistent cue, overrides the effect of photoperiod to entrain cyclic activity in goldfish. So it is possible that in environmental manipulation of feeding events or an enhanced temperature cycle could provide an artificial zeitgeber.

Support for light-dark transitions as a means of entrainment has come from ultrastructural and radio-labelling experiments. Tanaka *et al*. (1981) demonstrated that in *Tilapia nilotica* (L.), the order of formation of the L- and D- zones was dependent on photoperiod, as a reversal of the light-dark cycle was found to induce a reversal in the order of the two layers. Using radiolabelled calcium (^{45}Ca) to study in vivo otolith calcification in goldfish, Mugiya and coworkers (Mugiya *et al*., 1981) found an apparent diel cycle in calcification, associated with photoperiod. However, these experiments were flawed because no consideration was given to the effect of isotopic equilibration on ^{45}Ca incorporation. Nevertheless, a latter in vivo experiments, involving juvenile Atlantic salmon that were subject to an isotopic equilibration period, did demonstrate that otolith calcification was entrained to dark-light transitions (Wright *et al*., 1992). Radiolabelling experiments have also demonstrated diel cycles of both calcification and organic matrix formation, associated with photoperiod, within isolated sacullae (Mugiya *et al*., 1981; Mugiya, 1987).

3.2 Regulation of annual increment periodicity

At present the regulation of annual increment formation in otoliths is not well understood, although it is commonly assumed that they are related to seasonality in growth or to reproductive cycles. However, there is debate as to how sesonality mediates its effect on the formation of annual increments. One view is that seasonal variation in otolith formation is related to cyclical physiological changes within the fish, such as the onset of reproductive activity or the accelerated somatic growth that occurs in spring (Johnson 1983; Fowler 1990). Alternatively, it has been suggested that the physiology of otolith formation is independent of the other somatic and reproductive processes taking place within the fish, and is an independent physiological response to environmental variation (Loubens 1978; Fowler and Doherty 1992). Evidence for and against these hypotheses is generally in the form of correlations in the timing of the different processes and is generally weak and insufficient to reject either hypothesis. Whilst in some species the formation of the opaque zone coincides with time of year when fish are reproductively active,

opaque zone formation is also generally seen in the juvenile stages of many fish species (Johnson 1983; Fowler, 1990). Further, it is difficult to envisage how reproductive activity could directly affect otolith composition since although reproducing females have elevated plasma calcium concentrations, this is in the form of protein bound calcium which will not affect calcium ion levels in the endolymph (Kalish, 1991). As yet no experimental studies have been conducted to distinguish between them.

The season of formation of opaque and hyaline zones may change during development and in relation to the geographical distribution. For instance in *Gadus morhua* from the North Sea the opaque zone forms earlier in the southern extremes of this species range and becomes progressively later further north. Within each stock the younger fish, begin to lay down the opaque zone up to 4 months before the older fish. The spawning occurs when the hyaline zone is well under the formation process. The temporal delay in opaque zone formation increases with age (Williams & Bedford, 1974). The time of translucent-zone formation in *Sebastes entomelas* from the U.S. Pacific coast has been found to vary in relation to sex, geographical area and year (Pearson, 1996). In this species a link between temperature and hyaline zone formation is apparent although other temperature-related factors, such as food availability or nutrient content of the prey, may also be important. Reproductive activities apparently are not related to the time of translucent-zone formation. Further evidence of a temperature related zone formation was found for several species of acanthurids from eastern Australia (Choat & Axe, 1996). Recapture of tetracycline marked fish showed that the formation of opaque zones corresponding to summer water temperature rise.

The evolution of the marginal increment along the year and the zone formation in marked specimens kept in aquaria also indicated a temperature-related zone formation for *Albula vulpes* (Crabtree *et al*., 1996). In this species zone definition and width appear related to the temperature cycles. For example, in warm areas the narrow well defined translucent zone is related to the season of minimal water temperatures where as from the more tropical and less seasonal parts of the species distribution the zones may appear diffuse and difficult to interpret.

4. Otolith accretion rate

4.1 Measures of fish size and otolith size

Concurrent fish and otolith growth may be studied in a number of ways. When otolith growth has been studied in short term experiments, measurements of otolith linear dimensions have often been used, whereas both weight and length measures have been used to compare fish and otolith growth in field studies. Short term experiments often show a linear relation between otolith radius and fish length. However, field material and long term experiments often show non linear relationships between otolith size and fish size. The power function $O=aL^b$ of otolith linear dimension O versus fish length L, has often show b to be < 1 for juvenile and adult fish (Mosegaard *et al*. 1988). Conversely, in larvae the rate of increase may be greater or smaller than one depending on species or even population (greater than one: Battini *et al*. 1995). Several researchers have suggested that since the anatomical role of the otoliths are to provide mass-to-mass information, the only functional relationship should be that between otolith mass and fish mass. Nevertheless, the entire technique of backcalculating fish growth is based on the establishing a usable predictive relationship between linear growth of fish and otolith.

4.2 Somatic and otolith growth

The existence of a functional relationship between otolith growth and fish growth is so fundamental to studies of fish populations that the reasons for this relationship have scarcely been examined. It has frequently been assumed that because there is often a highly significant correlation between otolith size and fish size that the accretion rate of primary increments is functionally related to somatic growth rate (Rosenberg and Haugen 1982; Penny and Evans 1985; Barkman and Bengston 1987; Post and Prankevicius 1987; Radtke 1989). This commonly held view of a proportional or allometric relationship between otolith size and fish size still appears in more recent theoretical work (Xiao 1997). However, a large number of investigations have shown time related effects on otolith accretion rate where fast growing individuals have relatively smaller otoliths at equal fish size (Templeman and Squires 1956; Krivobok and Shaturnovsky 1976; Taubert and Coble 1977; Marshall and Parker 1982; Wilson and Larkin 1982; McGurk 1984; Miller and Storck 1984; Boehlert 1985; Neilson *et al*. 1985; Penney and Evans 1985; Rice *et al*. 1985; Post and Prankevicius 1987; West and Larkin 1987; Mosegaard *et al*. 1988; Reznick *et al*. 1989; Secor and Dean 1989; Secor *et al*. 1989; Wright *et al*. 1990; Secor and Dean 1992; Hare & Cowen 1995; Choat and Axe 1996;).

In a number of cases individual somatic and otolith growth rate have been studied experimentally under varying food conditions. In most studies a linear regression with a significant positive intercept provided a good fit for the relationship between otolith radial accretion rate and growth rate of fish length. Therefore, in all cases where zero or negative somatic growth rate was studied, otolith accretion rate was relatively high. Some results from previous studies that have been either estimated or recalculated from published figures are presented here as percentage of maximum otolith growth rate at zero fish growth rate in the experiment: (percentage of maximum :31% - Volk *et al*. 1984; 25% - Alhossaini and Pitcher 1988; and decreasing from 100% to 71 - Molony and Choat 1990; c. 18% - Sogard 1991; 8% - Zhang and Runham, 1992b).

Although most studies suggest that some otolith accretion is obligate and partly independent of fish somatic growth rate, some studies have also reported an absence of otolith growth when somatic growth occurs during specific developmental stages (Geffen 1995, A. Folkvord unpubl.). Consequently, at low somatic growth rates the variation in otolith accretion rate among

individuals would be expected to lead to a high variability in the relationship between fish size and otolith size. Therefore the evidence is against any direct functional relationship between somatic growth rate and otolith accretion rate.

4.3 Temperature effects on otolith accretion rate

Temperature has well known effects on both fish growth (i.e. Elliott 1979) and on otolith structural formation (i.e. Simkiss 1974). However, experimental studies relating controlled temperature effects to concurrent otolith and somatic growth are relatively scarce. Nevertheless, the available information from different species are consistent and indicate that increasing temperature leads to increasing otolith accretion rate for fish at equal size and/or age and in excess of any effects from increased somatic growth due to higher temperatures (Mosegaard and Titus 1987; Mosegaard *et al*. 1988; Radtke 1989; Zhang and Runham 1992; Hoff and Fuiman 1993; Gauldie 1996). Results with less strictly controlled temperatures show the same pattern of larger otoliths at fish size with higher temperatures (Secor and Dean 1989) In contrast, to the slow response between otoliths and changes in somatic growth rate, temperature seems to immediately alter otolith deposition (Neilson and Geen 1985; Mosegaard *et al*. 1988; Mosegaard and Titus 1987; Brothers 1990). It has been suggested that geographical variations in temperature may lead to different otolith size-fish size relationship among populations of the same species (Lombarte and Lleonart 1993), and may also to some degree explain differences in otolith morphology leading to different otolith area - fish size relationship in different geographical areas (Smith 1992).

Although temperature appears to have a major influence on both the reaction rate and the average accretion rate of otoliths, some studies of natural populations increment widths show that correspondence with temperature variation is only partial (Greenberg and Brothers 1991) or that otoliths may form at nearly constant rates despite a seasonal increase and later decline of natural temperatures (Wright *et al*. 1990). Further, time series analysis showed a high positive auto-correlation in otolith daily increment width, whereas no significant effects of small variations in temperature could be demonstrated (Morales-Nin *et al*. 1995; range 1.6^{o}C).

4.4 Variation in composition of accreted zones in relation to temperature and optimal feeding conditions

Protein is a minor but varying part of the otolith composition (Degens *et al*. 1969). The D-zone (the optically dense part of the primary increments) is high in organic fibres (Zhang and Runham 1992a; Morales-Nin 1986). At a greater scale opaque zone formation contain higher amounts of organic material than translucent zones (Dannevig 1956). Under starvation conditions otolith increments may appear faint due to a more inconspicuous formation of the D-zones (Rice *et al*. 1985; Eckman and Rey 1987; Titus and Mosegaard 1991). In salmonid yolk sac fry which would be expected to have no nutritional problems, increasing temperatures above 10oC lead to wider but increasingly transparent otoliths with almost indiscernible D-zones (Mosegaard and Titus, 1987), also in ad lib. feeding juveniles increasing otolith transparency was observed with increasing temperatures (Mosegaard *et al*. 1988). Since growth rate shows an optimum response to temperature, super-optimal temperatures lead to a decrease in the scope for growth and conditions that could be defined as a state of physiological starvation even at relatively high feeding rates (Elliott 1979). The formation of translucent otolith material may therefore in general be associated with states of decreased growth efficiency (Mosegaard 1986).

4.5 Physiological determinants of accretion rate

In terms of relative size, the mineral rich L-zone forms the major component of daily otolith accretion. The rate of accretion of the L-zone appears to varies diurnally. Radio labelled calcium experiments both *in vitro* (Mugiya *et al*. 1981) and *in vivo* (Wright *et al*. 1992) indicate a peak in accretion rate in salmonids during early day-time hours following lights on. Direct observations of diurnal variations in thickness of the outermost increment show a distinct maximum of otolith accretion rate from 7 to 13 hours after lights on in *Tilapia* (Tanaka *et al*. 1981; Zhang and Runham 1992a).

A correspondence between otolith formation rate and metabolic rate of the brain parts was suggested by Mosegaard (1986). The increase in otolith accretion rate with temperature (Mosegaard and Titus 1987; Mosegaard *et al*. 1988; Hoff and Fuiman 1993) is much more similar to the increasing trend of metabolism than to the optimum curve of somatic growth rate. Wright (1991b) provided more direct evidence for a metabolically driven otolith formation rate from a close relationship between daily increment width and individual oxygen consumption rate, despite a very poor relationship between the amount of otolith accreted and the individual somatic growth rate during the same period. Altered feeding conditions, like starvation, have a rapid influence on growth rate but only gradually change standard metabolic rates according to feed-back adjustment mechanisms from changed protein turn-over rates (Houlihan *et al*. 1988).

Since some component of the metabolic rate appears to influence otolith accretion rate, periods of starvation would only be expected to lead to a gradual decline in increment widths, as has been found in a number of experimental studies (Neilson and Geen 1985; Eckman and Rey 1987; Molony and Choat 1990; Umezawa and Tsukamoto 1991; Bradford and Geen 1992; Zhang and Runham 1992b; Molony 1996).

The observations of otolith accretion rate as a function of metabolism and the amount of organic material incorporated reflecting net protein synthesis may be combined into a conceptual model of otolith structural formation (Table 1).

HIGH ENERGY INTAKE

INCREASING TEMPERATURE ----->

Macro-zones	Narrow	Wide	VeryWide
Appearance	Opaque	Opaque	Translucent
Primary increments	Narrow	Wide	Very Wide
Appearance	Distinct	Distinct	Vague
somatic-otolith relation	correlated	correlated	uncoupled

LOW ENERGY INTAKE

INCREASING TEMPERATURE -------->

Macro-zones	Narrow	Wide	Very Wide
Appearance	Translucent	Translucent	Translucent
Primary increments	Very Narrow	Wide	Wide decreasing
Appearance	Absent	Vague	Vague
somatic-otolith relation	variable	correlated	increasing uncoupling

With a combination of intermediate metabolism and a good nutritional condition, the rate of protein synthesis will scale to metabolic rates leading to an apparent coupling between the rates of somatic growth and otolith accretion with a relatively high amount of organic content. Formed during a defined period this will macroscopically appear as a relatively wide opaque otolith growth zone, microscopically daily increments will appear relatively wide, optically dense, and exhibit well defined D- and L-zones.

At very high metabolic rates (often associated with super-optimal temperatures) energy intake will not meet respiratory requirements and growth rate will not scale to metabolism and otolith accretion rate. Otolith formation rate will be high with a low organic content due to a rapid mineralisation. Therefore otolith formation during a defined period will macroscopically appear as wide translucent zone, microscopically daily increments will appear wide, optically translucent, and exhibit very faint D- and L-zones, under restricted energy intake feedback regulation will lower metabolic levels and gradually decrease daily increments.

Even without energy expenditure on activity or SDA-related respiration there seems to be a minimum metabolic threshold with temperatures approaching zero. Otoliths zones formed at low temperatures without food intake would according to the hypothesis be translucent and narrow, whereas depending on the possibility to process food intake, protein synthesis even if low, might be substantial compared to standard metabolism and allow for the formation of opaque and relatively narrow otolith zones.

5. Physiological regulation of otolith formation at sacculus level

5.1 Otolith calcification

Otolith calcification is limited by the number of nucleation sites provided by the matrix (Crenshaw, 1982; Mann *et al*., 1983) as well as physico-chemical conditions at the otolith surface. Therefore the rate of matrix production by the matrix producing cells of the sacculus (see Saitoh & Yamada, 1989; Wright, 1990) will ultimately determine the rate of otolith calcification. Further, the isolation of a soluble matrix capable of regulating the rate of mineral deposition in otoliths suggests that the variation in the production of this protein could regulate the rate of mineralisation (Wright, 1991; Sasagawa & Mugiya, 1996).

Investigations of isolated sacullae have indicated that endolymph calcium ion concentration is dependent on intracellular active transport that is sensitive to plasma calcium concentration (Mugiya & Yoshida, 1995). Similarly, recent work has also demonstrated that proton secretion

through the sacculus is driven by an energy dependent (Na-ATPase) mechanism that is sensitive to plasma pH (Payan *et al*., in press). Consequently changes in plasma ion concentration would be expected to have a direct effect on that in the endolymph. However, the precise mechanism by which plasma calcium and pH induces changes in the physico-chemical conditions at the otolith surface is not clear. This is because the sensory kinocilia bathed by endolymph are sensitive to changes in Ca^{2+} concentrations well below the solubility product needed for calcification (Hubbard *et al*., 1969; Wright, 1990). Therefore, it is necessary to explain how ion levels are elevated at the otolith surface above the background concentrations found in the endolymph. Calcareous spherules have been observed in close association with the otolith surface of a number of fish species (Dale, 1974; Wright, 1990). These are formed and secreted from the otolithic membrane and are transported to the surface of the otolith within the fibrous sub-cupular meshwork (Wright, 1990). It is likely that **t**hese calcospherules are be involved in mineralisation as has been proposed for similar structures found in association with molluscan shell (Arnold, 1992).

5.2 Relation between increment and other physiological rhythmicity

Diurnal rhythmicity in otolith calcification may be mediated by a diel variation in plasma chemistry as Mugiya (1984) and Wright *et al*. (1992) found a parallel diel decline in otolith calcification and total and free plasma calcium concentration. Further, Mugiya (1984) found a seasonal reversal in the rhythm of otolith calcification associated with a reversal in the diurnal plasma calcium cycle. Campana (1983) demonstrated that a stress induced depression in branchial uptake of calcium, which presumably lowered plasma calcium concentration, resulted in a decline in otolith calcification in coho salmon, *Oncorhynchus kisutch*. Wright *et al*. (1992) found that an induced depression in plasma calcium led to a net loss of calcium from the mineralising otolith increment, which indicates that a lowering of the calcium ion concentration at the otolith surface had occurred. Mugiya & Takahashi, 1985 also found evidence for a diel decline in plasma pH and total CO_2 that paralleled that of the otolith.

Whilst there may be a periodical ionic limitation to otolith calcification, this alone cannot explain reports of a diel variation in matrix secretion (Mugiya, 1987; Wright, 1990) or the formation of matrix rich layers (see Watabe *et al*., 1982; Morales-Nin, 1987; Mugiya, 1987; Wright, 1990). The distribution of matrix- mineral in the otolith appears to occur in two phases. The first one associated to the twinning plane of the basic aragonite crystal (Gauldie & Xhie, 1995). Twinning has been proposed as a stabilizator of crystal polymorphisms and increases the growth rate of the crystal (Smith, 1974). The second phase of the matrix-mineral association appears as the dense band of fibers corresponding in size and orientation to the D-zone of the primary increment (Morales-Nin, 1987). This observation is consistent with the diel variation in insoluble matrix protein indicated by radio-labelling experiments (Mugiya and 1985). The two phases of the protein matrix may have different roles, the first to provide a template for crystal growth and the second to stabilize the otherwise soluble (Wright *et al*., 1992) and thermo-dynamically unstable aragonite morph (Gauldie & Xhie, 1995; Mann *et al*., 1983). Consequently, it is necessary to consider the regulation of both ion concentrations and matrix production in the periodic deposition of L- and D-zones. Given the correlation between otolith calcification and plasma ion concentration, the concentration of certain ions in the plasma may have a direct effect on matrix cell secretion or may covary with some other signalling factor.

6. Endocrine regulation of otolith accretion

As plasma calcium concentration is regulated by hyper- and hypo-calcemic hormones (Dacke, 1979), diel changes in the plasma concentration of these hormones may be indirectly involved in the periodic decline in otolith calcification. Growth hormone (STH) may also be involved since

hypophysectomy has been found to cause a reduction in otolith growth (Mugiya, 1990) or otolith demineralisation (Simmons, 1971), and otolith mineralisation in hypophysectomised fish can be restored by injection of pituitary extract (Simmons, 1971). Such hormonal regulation could influence both ion transport and matrix production in the sacculus. Studies on shell mineralisation in the snail, *Lymnaea stagnalis* (Dogterom *et al*., 1979; Dogterom & Doderer, 1981) have indicated that a growth hormone may regulate shell formation by controlling both the secretion of calcium binding protein and calcium ion concentration at the mineralising surface.

Entrainment to light-dark cycles suggests the involvement of the pineal-hypophysial complex. Endocrine secretion displays a circadian periodicity in many animals and through the intermediary of metabolic rate, ultimately controls most physiological processes (Simpson, 1978). Endocrinological studies have demonstrated diurnal variations in the levels of several hormones in the plasma of teleosts (Matty, 1985), including thyroxine (T_4) (Eales *et al*., 1981) a hormone known to influence skeletal growth and calcification in rainbow trout (LaRoche *et al*., 1966). Many metabolic processes are also known to fluctuate diurnally, including heart rate (Priede, 1978) and oxygen consumption (Ali, 1964). Given, the apparent relationship between oxygen consumption and otolith accretion (Wright, 1991b), future investigations may benefit from an examination of the influence of diurnal variations in metabolism on increment formation.

7. Otolith Elemental composition

7.1 Elemental constituents

It is generally believed that trace elements found in fish otoliths accumulate because of substitution of calcium by other divalent cations such as Mg^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} and smaller monovalent cations, such as Li^{+} (Fritz *et al*. 1990). However, it has also been suggested that Mg^{2+}, and probably larger cations and anions can be incorporated by becoming entrapped within the crystal lattice, as crystal inclusions (Fritz *et al*. 1990, Rosenberg 1991).

The mechanism of trace element accumulation in fish otoliths has not been well described, especially in contrast with accumulation pathways in other fish tissue or the calcareous tissues of invertebrates. There are at least two means by which elements, including calcium, can be incorporated into the accreting otolith. Trace elements may be combined with the calcospherules within the epithelial cells and transported to the otolith surface. Structural evidence has been shown for this mechanism by SEM examination of shell (Arnold 1992) and histochemical studies of labyrinth and otoliths (Wright 1990).
In addition, calcium carbonate can precipitate directly out of solution and trace elements in the endolymph fluids can be incorporated into the growing otolith in this manner. Calcium reaches the endolymph primarily from the plasma (Kalish, 1991; Wright *et al*. 1992), and it is likely that other trace elements can also follow this path.

The incorporation of inorganic ions by both cellular secretion and fluid precipitation has been described for bivalve shells (Fritz *et al*. 1990), and specific differences in the pattern of accumulation have been ascribed to the behaviour of the different elements in terms of whether they can move directly into the extrapalladial fluid which bathes the growing shell, or whether they are metabolised and move into the shell by cellular secretion within spherules.

When elements precipitate onto the otolith from extracellular fluid, they may be expected to have a more homogeneous distribution on the growing surface. However, this has been shown not to be the case for barium, which can crystallise directly onto the shell formation layer in discrete barite crystal ($BaSO_4$) clusters (Fritz *et al*. 1990). Elements which are deposited in the otolith from cellular secretion may also be incorporated as discreet crystals, or homogeneously if there is direct ionic substitution for calcium in the crystal lattice.

7.2 Regulation of elemental incorporation rate

The accumulation of trace elements in fish otoliths depends on a number of factors, including the concentration in the environment, bioavailability, the physiological state of the individual fish (affecting the exchange rate between the external and internal environments), the mechanisms of different species for detoxifying different metals, the growth rate of the individual fish (affecting the rate of accumulation of otolith material), and the affinity of the calcium carbonate otolith for different elements. As otoliths grow faster in faster-growing individuals, it is more likely that the rate of trace element accumulation will be higher in situations which facilitate fish growth. Where environmental conditions can be shown to result in reduced growth rates (Nash 1985, Nash 1988), otolith composition could actually show reduced levels of trace elements.

8. Recommendations for future research

An understanding of the interaction between environmental and endogenous regulation of increment periodicity would greatly benefit from identification of the signalling factors regulating calcium transport and matrix production in the sacculus. Work on isolated sacculi may provide a direct means of testing the importance of potential stimulatory factors.

The relationship between seasonal zone formation and environmental influences requires further investigation to determine whether zones are formed directly in response to a cyclical environmental factor, such as temperature or indirectly through physiological processes such as growth, that are influenced by environmnetal conditions. Comparative investigations of marginal increment analysis and environmental factors in species with a wide latitudinal range could help to identify important environmental factors.

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RADIOMETRIC AGEING

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The basic principle

The most frequently used method of radiometric age estimation in fish involves the measurement of the $^{210}Pb/^{226}Ra$ disequilibria in the otoliths. The isotopes of ^{226}Ra and ^{210}Pb are part of the natural decay chain of ^{238}U. Two characteristics of the isotopes are crucial for their use in radiometric ageing. Both isotopes are of natural occurrence in seawater but Ra is much more soluble than its parent or daughter isotope. Ra is a proxy for Ca and is taken up through the food chain and deposited in the otoliths along with calcium. The decay of ^{226}Ra to ^{210}Pb is such that it is sensitive for ages of up to about 100 yr.

For shorter lived fish species the $^{228}Th/^{228}Ra$ disequilibria has been used. The principles are the same but because the decay from ^{228}Th to ^{228}Ra is shorter it is only useful for fish up to about 10 yr old.

$^{210}Pb/^{226}Ra$ disequilibria

Whole otoliths

The technique was first used to confirm longevity in the rockfish *Sebastes diploproa* by Bennett *et al*. (1982). It is important to recognise that in this study the authors were not using radiometric techniques to determine the precise age of the fish. The starting point for the study was the controversy over age determination in this species resulting from counting growth zones on whole or sectioned otoliths. Whole otolith readings gave maximum age estimates of 30 yr while sections gave estimates of 80 + yr. There was general agreement ± 2yr between the two methods in otoliths with up to about 20 growth zones. The reason for the discrepancy is that there appears to be a change in growth pattern of the otolith such that beyond a certain age otoliths may grow little in length or width but instead grow in thickness. Such a phenomenon is not confined to Scorpaenid fishes. For example, the so called 'collar' seen in sections of otoliths collected from large *Coryphaenoides rupestris* (a deep-water macrourid) also represents a change in growth pattern (Bergstad, 1990). Bennett *et al*. (1982) set out to resolve the problem of which readings to use for age determination of older fish by using the $^{210}Pb/^{226}Ra$ disequilibrium method.

They divided the otoliths into four 'age' groups corresponding to whole otolith or section otolith age. Groups one and two corresponded with ages of 1-2 and 8 respectively and were aged by counting growth zones in whole otoliths. Group three were aged by both whole otolith and section counts and were 20-22 yr with a discrepancy between the methods of no more than 2 yr. The fourth group consists of two otoliths where there was a large discrepancy between the methods; 30 yr for whole otoliths and 82 and 86 yr for sectioned otoliths.

Otoliths were pooled for each group to give 1 g material for analysis (22 for smallest otoliths to 2 for the largest). ^{210}Pb was assayed as -emitting ^{210}Po which should have been with 5% of secular equilibrium with ^{210}Pb by the time the otoliths were assayed. ^{226}Ra assayed by ^{222}Rn emanation technique.

To obtain an estimate of age from the ^{210}Pb/^{226}Ra ratio a knowledge of the mass growth rate of the otolith as function of the number of growth-zones is required. Using data from 246 males they calculated the relationship between otolith mass and 'age', physical dimensions and weight. These were used to construct simple 2-phase linear growth models. Two distinct growth models were obtained depending on whether whole or sectioned age was used.

The authors make two main assumptions:

(1) that initial Ra and Pb activities deposited in each growth zone are invariant with time.

(2) There are no losses or gains of Ra or Pb except by radioactive decay or growth. In this respect the following quote is important: "Loss of the noble gas ^{222}Rn or other intermediate members of the decay chain between ^{226}Ra and ^{210}Pb from these aragonitic otoliths is believed to be negligible since ^{226}Ra and ^{210} distributions measured in aragonitic corals by Dodge and Thomson (1974) are not compatible with significant loss of ^{222}Rn or the other nuclides in the decay chain".

The other implied assumption is that the uptake of ^{226}Ra is in constant proportion to the mass accumulation rate of the otolith. If, as seems probable in this study, the ^{226}Ra specific activity is the same between samples then it would be appropriate to use the mean activity. If they are different then it would be more appropriate to use the specific activity for each sample. The ^{210}Pb/^{226}Ra ratios were calculated using both scenarios.

The results show that the radiometric age calculated using a simple two phase growth model for the three youngest groups supported the age determined by counting growth zones in whole or sectioned otoliths. In the fourth group the estimated radiometric age agreed with counts of the growth zones revealed in sections.

The next attempt to use ^{210}Pb/^{226}Ra disequilibria to verify fish age was by Fenton *et al*. (1990) who studied the deep-water blue grenadier (*Macruronus novaezelandiae*) from Australian waters. They cite some of the reasons why validation is difficult for deep-water species; tagging is not possible, growth is too slow for modal analysis, otolith annuli are too narrow and complex to allow marginal increment or edge analysis and the fisheries are too recent to be able to follow strong year groups.

Fenton *et al*. (1990) reiterate the 3 assumptions which are fundamental to radiometric ageing.

(1) Both ^{226}Ra and ^{210}Pb are taken into the otolith at a rate that is always in a constant ratio to the rate of mass increase of the otolith.

(2) The rate of uptake of ^{226}Ra significantly exceeds that of any ^{210}Pb; i.e. allogenic ^{210}Pb is much less than ^{226}Ra and can therefore be distinguished from radiogenic (authigenic) ^{210}Pb.

(3) No losses or gains of ^{226}Ra or ^{210}Pb occur after uptake, other than by radioactive decay or ingrowth.

Any violations of assumptions 1 and 2 can be identified in the analysis, especially if material from juveniles is available. These authors, like Bennett *et al*.(1982), believe that assumption 3 is valid "based on the observations of numerous authors (reviewed by Veeh and Burnett 1982) that ^{226}Ra and ^{210}Pb distributions in modern aragonitic corals are not compatible with any significant loss of ^{222}Rn, or other nuclides, in the ^{238}U decay chain between ^{226}Ra and ^{210}Pb." This aspect will be dealt with in more detail below. Fenton *et al*. (1990) remark that the closed system and constant uptake assumptions have been shown to be invalid in shark vertebrae (Weldon *et al*., 1987).

Fenton *et al*. (1990) stress the importance of the analytical techniques required for handling such low levels of activity and describe their methods in some detail and at the same time point out some differences between their background rates and those of Bennett *et al*. (1982). Once again it is -emitting ^{210}Po that is being measured and the authors point out that ^{210}Po can accumulate at high levels in marine tissues. Elaborate procedures were adopted to remove sources of allogenic ^{210}Po from the tissue surrounding the otolith but the results show that this material is very difficult to remove. When measuring such low levels of ^{210}Po complete removal is essential.

In this study otoliths with the same number of annuli were pooled to give 1 g of material (30 for 0+ fish to 2 for 21+ yr). The results showed that ^{226}Ra specific activity decreased with increasing age as determined by annuli counts. There was no increase in ^{210}Po with increasing age. The fact that ^{226}Ra did not accumulate in the otolith at a constant rate throughout its life meant that the radiometric method could not be used to verify age. The reason for the non-constant uptake of ^{226}Ra is probably because the young live in shallow water where high levels of ^{226}Ra are to be expected while the adults live on the continental slope where ^{226}Ra levels are thought to be lower.

Fenton *et al*. (1990) conclude that "the failure of the ^{210}Pb/^{226} radiometric method to determine the age of blue grenadier could be attributed largely to a major change in habitat that occurs between the juvenile and adult stages of the fish. The result, although a negative one in terms of age determination, is valuable in that it indicates a type of species that could not profitably aged by this method (i.e. a species known to have a major change in habitat during it life cycle)."

Attempts have also been made to age another deep-water fish, the orange roughy (*Hoplostethus atlanticus*), using ^{210}Pb/^{226}Ra disequilibria (Fenton *et al*. 1991). Whole otoliths were used and the method was similar to that used by Fenton *et al*. 1990. In this species the specific activity of ^{226}Ra was similar for all size groups of otolith although a weak upward trend was apparent which the authors suggest might result from decreasing protein concentrations with age. To convert to ages the rate of growth of otolith mass must be known or estimated. A constant linear rate of otolith growth was used for fish of fish length up to about 34 cm (maturity) followed by an arbitrary rate of growth equal to 45% of the pre-maturity value. Using these assumed growth rates radiometric ages of about 32 yr were obtained for fish at first maturity and of 77 to 149 yr old for fish of 38 to 40 cm. The radiometric ages obtained for the smallest fish were comparable with, but greater than those validated by Mace *et al*. (1990) using length mode analysis (up to 3 yr). The authors recognise that the growth models are arbitrary but consider that the ages obtained are reasonable. They point out that the need for growth models can be largely avoided if otolith cores are used (Campana *et al*.,1990) but suggest that because of their shape it would be difficult to extract cores from orange roughy otoliths.

Smith *et al* (1995) revisited radiometric age determination in orange roughy. They point out that the radiometric ages determined by Fenton *et al*. (1991) were considerably greater than the maximum age of 42 yr estimated by Mace *et al*. (1990) who counted annuli on the surface of the otoliths. Smith and Robertson (1992) argued that the otoliths from larger fish were too thick for this technique to be reliable and suggested that otolith sections might be more useful. Smith *et al*. (1995) have sectioned otoliths and estimated ages of up to 125 yr by counting the annuli. They then used these ages to calculate new otolith mass growth rates. As a result changes were made to the pre-maturity growth rate and the post-maturity growth rate was estimated at 65% of the pre-maturity rate which compares with 45% used by Fenton *et al*.(1991). There was also a reduction of the age of maturity from 32 yr to 25 yr. The substitution of these new variables into the equation resulted in reduced radiometric age estimates compared to those given by Fenton *et al*. (1991). These new ages were consistent with maximum recorded age of 125 yr derived from annuli counts in sectioned otoliths.

One of the assumptions for radiometric analysis is that the ratio of ^{210}Pb remains constant relative to ^{226}Ra throughout life. By comparing the Pb content of juvenile and adult otoliths any change in uptake of allogenic ^{210}Pb should be detected. Smith *et al*. (1995) used inductively coupled plasma mass spectrometry (ICPMS) to measure stable lead in juvenile orange roughy and the values obtained were compared with previously published levels for Pb in the otoliths of adult fish from the same area. There was a large variation in Pb content of both juvenile and adult otoliths but the mean values suggested some increase over the lifetime of the fish. However, in view of the uncertainties no change was made to this variable in the calculation of radiometric age

All these methods of radiometric ageing using whole otoliths depend on an assumed rate of otolith mass growth. This introduces an element of circularity in the argument because growth of the otolith infers a knowledge of age. Francis (1995) has also reanalysed the data of Fenton *et al*. (1991) to illustrate two alternative approaches that avoid making assumptions about otolith growth. He first derives a series of radiometric equations and in doing so found a number of inconsistencies in the interpretation of some variables used by other researchers. To estimate the most probable age he chose trial values of the two otolith mass growth rates and used these to calculate the expected age of each sample from its mean otolith mass. He then used these expected ages to calculate expected activity ratios. The deviation between expected and observed activity ratios were calculated as weighed sum of squares (S) and the process was repeated until minimum value of S was obtained. Using this technique the age estimates obtained were broadly similar to those obtained by Fenton *et al*.(1991). There were much larger differences between samples and wider confidence limits in the new analysis. Changing the estimate of the age at which the growth rate switches from one phase to the other had a relatively small effect on age estimates. Francis also used a simulation procedure to calculate the lower bound of the maximum age which for the orange roughy was 84 yr. The major limitation of the technique is that it does not take into account between-individual variability in otolith-mass growth.

^{210}Pb/^{226}Ra disequilibria have also been used to validate otolith increment age estimates in another Australian deep-water fish, the warty oreo (*Allocyttus verrucosus*). The small size and bi-lobed nature of the otoliths precluded the use of cores and necessitated the pooling of large numbers of whole otoliths to obtain 1 g of material. Otoliths were pooled on the basis of fish length, otolith weight, sex (all female) and date of collection. The radioanalysis for ^{210}Pb and ^{226}Ra was as described by Fenton *et al*. (1991). In addition stable Pb, Ba, Sr and Ca were measured in samples from juvenile, pre-maturity and post-maturity fish. The Pb:Ba ratios did not

change significantly between juveniles and older fish which suggests an insignificant change in the uptake-activity ratio.

Although there was no significant difference in ^{226}Ra specific activity between samples, the ^{210}Pb/^{226}Ra ratios were calculated using the actual ^{226}Ra activities for each sample. Age estimates using a single linear model for otolith mass growth gave a maximum age of 174 yr for a pooled sample of otoliths from the largest fish. A two phase groeth model reduced the age estimate for the same sample to 132 yr. These high radiometric age estimates support the ages determined by ncrement counts.

Otolith cores

One method of avoiding the problems associated with the assumption of constant uptake of parent and daughter radionuclides in proportion to the mass accumulation rate of the otolith is to extract the core of the otolith. This method was used by Campana *et al*. (1990) to verify ages determined by annuli counts in *Sebastes mentella*. Some of the assumptions involved in this study are also given in Smith *et al*. (1991). The age of the otoliths was estimated by counting annuli and then the otoliths were placed in one of seven age categories. A grinder was used to extract rectangular blocks centred on the core of the otolith. These cores were pooled to give 1 g of material for each age category. The method of analysis was essentially similar to those used by Bennett *et al*. (1982). Because the ^{210}Pb/^{226}Ra ratio is measured only from the core, which represents the first year of life, there is no need to use otolith growth models in the estimation of age. The results suggest that *Sebastes mentella* off Atlantic Canada live to a least 75 years which confirms the ages estimated from annuli counts.

Kastelle *et al*.(1994) used otolith cores from the sablefish (*Anoploma fimbria*) to validate age estimates based on growth zone counts on broken and burnt otoliths. The otoliths were placed into 4 age categories: 1 yr, 9-11 yr, 14-23 yr and 24-34 yr. The one year old otoliths were used whole and in all the others the core was ground out . The requirement for 1 g of material meant pooling of cores within age groups. This usually meant 83- 141 otoliths for each age category.

The analytical techniques were as described by Bennett *et al*. (1982). The ^{226}Ra specific activity was significantly different between the four age categories and therefore the radiometric ages were calculate using the activity value for each sample. The radiometric ages were in general agreement but consistently lower than the ages determined by annuli counts. It was noted that sablefish accumulate higher levels of isotopes than other species. The authors believe that these high activity levels may be explained by biological and environmental factors. ^{226}Ra can be incorporated from a variety of sources such as the water, food, and sediments and therefore the geographical area, vertical distribution and food transfer efficiency are all important factors. Sablefish are one of the fastest growing epipelagic juvenile fishes and therefore probably have a rapid uptake of Ra.

The authors consider their results in relation to the three main assumptions listed above. They consider the possible loss of ^{222}Rn but believe that the work on corals argues against such a loss. If there were such a loss it might account for the lower observed ages. The use of cores makes it unnecessary to use otolith mass growth models. They comment on the large differences in ^{226}Ra activity between samples and consider that, because the factors controlling uptake are complex and poorly understood, they may be real. They conclude that the conservative approach to radiometric ageing is to use otolith cores and the specific ^{226}Ra activity for each sample.

Comparison of whole otolith and otolith core age estimation

A interesting study on age determination of tropical fish highlights some problems of radiometric ageing (Milton *et al*., 1994). The growth zones in the otoliths of tropical fish may not be laid down annually and are often difficult to interpret. In the Lutjanid fishes there was a considerable discrepancy between the number of rings counted in whole and sectioned otoliths, the latter have the higher numbers. The purpose of this study was to provide an independent estimate of age. Three different species of Lutjanid were examined and the analysis was carried out using grouped otoliths. The grouping was done on the basis of reproductive state, sectioned age, similar weight of otolith and size of fish. The analyses were done on pooled samples of whole otoliths and also pooled otolith cores. The radiometric age was estimated using a single linear growth model for otolith mass. In all three species the radiometric age estimates were most similar to whole otolith ring counts. There was considerable variability in both ^{226}Ra specific activity and ^{210}Pb/^{226}Ra ratio between samples and it was not consistent between species. In one species the age estimates from intact samples of juvenile otoliths were higher than both whole and sectioned otolith ring counts. This was thought to be the result of allogenic ^{210}Pb uptake as indicated by high Pb/Ba ratios. The levels of Pb and Ba in the otoliths was measured by ICPMS. These lutjanid fishes live for less than 10 years and Milton *et al*. (1994) point out that this is the first use of ^{210}Pb/^{226}Ra disequilibria in short lived tropical species. They consider that their methods would have tended to overestimate age and the only factor that might lower the radiometric age estimate might be loss of ^{222}Rn. Milton *et al* (1994) consider the evidence for Rn loss and consider that "significant loss of radon from otoliths is extremely unlikely as previously suggested from empirical studies (Fenton and Short, 1992).

The results show that for relatively short lived species whole otoliths and cores give similar radiometric age estimates using a simple single-phase linear otolith growth model.They also demonstrate the usefulness of Pb/Ba ratios concerning assumptions of initial activity.

Vertebrae

Welden *et al*. (1987) used ^{210}Pb in an attempt to determine the age of four species of shark. They measured the Pb in the isolated growth bands in calcified vertebrae. However the results were very variable and probably invalid because two of the assumptions necessary for valid radiometric ageing were violated. Uptake of ^{210}Pb was not constant but increased with increasing size of fish, probably as a result of change of diet with age. There was also some evidence that the closed system assumption for Ca and by analogy Pb is not valid for shark vertebrae

A critical review of the methodology

West and Gauldie (1994) have written a critical review of age determination by the ^{210}Pb/^{226}Ra disequilibria method. Their review is based on the studies by Bennett *et al*. (1982), Fenton *et al*. (1990, 1991) and Campana *et al*.(1990). They have three main concerns about the method.

(1) They believe that it is probable that ^{222}Rn diffuses out of the otolith.

(2) No authors have quantified the sources and sinks of ^{210}Po, ^{210}Pb and ^{226}Ra or any intermediate product.

(3) The estimated radiometric ages depend on an assumed otolith mass growth-in- time model` typically derived from some other ageing method thus involving a circular argument.

^{222}Rn Loss

^{222}Rn is an intermediate product between ^{226}Ra and ^{210}Pb with a half-life of 3.825 days and because it is a gas it could emanate from the otolith. West and Gauldie are critical of the literature on corals which are cited in support of the chemically closed system and indeed believe that some of the results are consistent with a 25% loss of ^{222}Rn. They believe that the crystalline structure of fish otoliths and the presence of interstitial spaces means that the escape of ^{222}Rn is possible and highly likely. Using the data of Fenton *et al*. (1991) and assuming a 16% loss of ^{222}Rn they estimate that age estimates could increase from 46.7 to 62 yr for one sample and 156 to 469 yr for another.

Sources

West and Gauldie (1994) briefly review some literature on ^{210}Pb in the food chain and its uptake by fish and point out the difficulties of measuring allogenic Pb. They also note that it is Po and not Pb that is being measured and that none of the authors consider allogenic sources of Po. They consider that if the ratio of allogenic ^{210}Pb to allogenic ^{226}Ra increased during the formation of the otolith then the age estimates would be too high.

Otolith mass growth

Some problems associated with otolith mass growth models discussed by West and Gauldie (1994) have already been dealt with in this review and some of the alternatives to avoid the circular arguments have been described. While agreeing that the use of cores gets over the problem of growth models and reduces need to demonstrate constancy of uptake of Ra and Pb over the lifetime of the fish, West & Gauldie (1994) suggest that Campana *et al*.(1991) underestimated age. This is because in cutting rectangular cores only 52% of the material analysed was from years 1-5.

West and Gauldie (1994) conclude by stating that the "failure to test or meet essential assumptions, and critical dependence on the otolith mass growth-in-time model adopted, disqualifies current ^{210}Pb/^{226}Ra disequilibrium techniques as a means of validating teleost ages. Further experimental and theoretical studies of the properties of otoliths are might hold promise of providing corrections for ^{222}Rn losses. Similarly increased effort in understanding the biochemistry and physiology of the otolith will improve our understanding of the sources and sinks of radionuclides. Radionuclide disequilibrium methods hold great promise in ageing studies, but that great promise is matched by technical difficulties that have yet to be resolved."

^{228}Th/^{228}Ra Disequilibria

Smith *et al*. (1991) report on some preliminary age radiometric estimations using whole otoliths from the flying fish (*Hirundichthys affinis*) and the silver hake (*Merluccius bilinearis*). The results suggested that ^{228}Th/^{228}Ra could be useful for fish which live up to a maximum of 10 yr. Campana *et al*. (1993) used this method for a much more detailed study of the flying fish (*Hirundichthys affinis*). This important species of the Caribbean has no annual rings on scales or otoliths and all the assessments are based on an assumed longevity of less than 2 yr. Both whole otoliths and cores were used in this study and otolith volume rather than weight was used for the otolith growth model. The relationship between otolith volume and fish size was used to develop a relationship between otolith volume and daily age. In a departure from previous

studies the cubic version of the von Bertalanffy equation was used to model otolith growth. The results of the analysis of both whole otoliths and cores were consistent with adult flying fish being about 1 yr old. The estimated ages also fitted with known spawning periods. The authors consider that as a result of the uncertainties in measuring ^{228}Ra due to its activity being close to background levels the possible errors in age estimation are fairly large. Because of their short life span and the fact that all stages live in the same pelagic environment problems with otolith mass growth models are minimal. Nevertheless the authors consider that the use of non-linear models have some important advantages. One advantage of the ^{228}Th/^{228}Ra decay series is that there is no gaseous intermediate. The authors consider that this method could have considerable use for short-lived tropical species if the radium assay could be improved.

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Otolith microchemistry in Fishes: Application to stock discrimination and temperature record

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"This is a preliminary version of a paper which is going to be submit in a scientific journal".

Otolith chemistry

Early studies of the chemical composition of otoliths (Dannevig 1956, Degens *et al* 1969) described two main components: an inorganic component containing aragonitic calcium carbonate constituting up to 90% of the otolith, and an organic component, composed primarily of protein. Since the early 1980's interest in otolith composition has increased, and a variety of applications and analytical tools have been evaluated (Tables 1 and 2). Recent research has demonstrated the potential value of otolith composition analysis for information about individual life-histories. This potential is based on two assumptions:

(1) otoliths are metabolically inert, unlikely to be resorbed, and grow throughout the life of the fish (Campana and Neilson 1985);
(2) the calcium carbonate and other elements which make up 90% of the otolith are mainly derived from the water and the food, and the incorporation of different elements is influenced by environmental factors and physiological factors.

Until recently, otolith microchemistry has not had routine applications because of the difficulties and expense of the analytical techniques. As the number of applications has increased, it has become obvious there are a number of constraints, some due to the analytical tools, and some due to biological effects on elemental incorporation (Fig. 1)

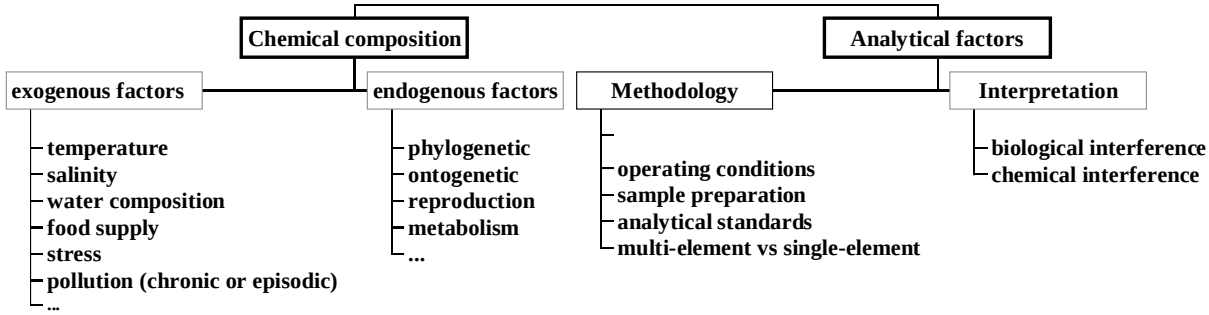

Figure 1. Factors involved in otolith composition and analysis.

There are three categories of elements in the inorganic material of the otolith, defined by their concentrations in weight (Gunn *et al*. 1992, Edmonds *et al*. 1994, and Table 1):

- one major element: Ca (40%),
- minor elements (> 200 ppm) for example: Na (~ 2500 ppm), Sr (~ 2000 ppm), K (~ 500 ppm),

- trace elements (< 200 ppm), for example: Mg, Fe, Ni, Cu, Ba, Cl, Pb...

Oxygen isotopes can also provide useful informations.

Sufficient data exists on the concentrations of certain elements to provide some indication of accumulation patterns across fish species and chemical families (Table 3). Virtually nothing is known about the trace element composition of the organic matrix, and there is no data on the affinity of elements to the organic, as opposed to the inorganic, fraction.
The incorporation of the minor and trace elements during otolith formation is not a simple process, and probably varies for the specific elements (Campana and Gagné 1994, Jones 1994, Thresher 1994). Biological factors such as genetics, metabolism, growth rate, life history stage, sex, and physiological condition are important, as are environmental (abiotic) factors such as temperature, salinity, water composition and food composition (see section 3 and Table 1).

Analytical tools and limits

Analytical techniques are broadly divided into surface analysis techniques which can provide composition information on specific locations or spots on the otolith, and solution-based techniques which provide global composition information, usually integrated over the whole otolith. Some techniques provide measure of the concentrations of several elements simultaneously (multi-element techniques) and others are only capable of measuring the concentrations of a single element at a time. The selection of the most appropriate technique for a particular otolith application depends on several criteria such as the nature of the required information, the temporal and spatial resolution required for a particular application, and the limits of detection (LOD) in relation to expected concentrations.
Surface techniques utilize x-ray based methods which are based on the interactions of the different elements when radiated. Analytical (and observation) techniques use the interaction of a primary energy particle beam (photons, electrons, particles, ions,...) with the sample. The nature, structure and composition of the sample produce modifications of the primary energy beam. The modifications result in the emission of the secondary beam, and this signal is interpreted to give structural and composition information. Information related to chemical composition is obtained by spectrometry of X-ray, electrons, and ions (Ruste and Bresse 1995, Eberhart 1997). Techniques for spot analysis of local variations in otolith chemical composition are based on this principle, and include EDS, WDS, PIXE, Ion Microprobe, and SIMS (see Table 2 for expansion of acronyms).

Solution-based, or whole otolith, analysis utilizes tools based on the principle of atomic spectroscopy which has its origin in the flame test in which many elements can be identified of the characteristic colour their salts give to a flame. This group of techniques is varied and highly developed, and allow the reliable determination of a wide variety of elements present at very low levels: 1 ppm (part per million) and even 1 ppb (part per billion) or less with very sophisticated procedures (Metcalfe 1987). These spectrometric methods include AAS, AES, and Mass Spectrometry (see Table 2 for expansion of acronyms). Stable isotopes of oxygen and carbon are measured using mass spectrometers.
The development of Inductively Coupled Plasma as a ion source for a mass spectrometer has resulted in hybrids techniques such as ICP-MS and ICP-AES. ICP-MS has fewer problems than ICP-AES with wavelength interferences, and better detection limits. The coupling of a laser ablation microprobe operating in the far UV linked to a good quality microscope and an ICP-MS has resulted in a new potentially powerful technique which can provide local data on the spatial distribution of many elements within the otolith.

Numerous techniques are available for the analysis of otolith microchemistry (Table 2), and several have been used sufficiently to be considered routine (Table 3). There is an growing literature giving comprehensive accounts of these techniques (e.g. Ruste and Bresse 1995, Benoit *et al*. 1996).

Resolution in otolith microchemistry should be considered on several scales; there is the question of physical resolution as in the minimal spatial dimension of the analysed zone in case of local analysis. In this case, spatial resolution is the equivalent of temporal resolution since the location of the sample spot on the otolith is linked to a point in time experienced by the individual fish. Two dimensions have to be taken into account, the depth and the area.

The level of resolution in terms of the composition data (i.e. how fine a level of resolution is needed for the biological question, e.g. how small a temperature difference is detectable, how big a salinity difference is detectable) is related to the limits of detection (LOD) which correspond to the minimum detectable concentrations. LOD depends on the signal intensity, the intensity of the continuous background and the "noise" which corresponds to the random fluctuations of the emitted signal. LOD varies among the different techniques. Moreover, for a given technique, the LOD depends on several parameters such as (1) sample nature (2) elemental atomic number (3) operating conditions and (4) elemental concentration.

The use of sophisticated tools to detect elements present in very low concentrations involves specific preparation procedures. There are preparation constraints imposed by the need to reduce sample contamination, as well as those constraints imposed by the specific analytical tool used. For global (whole otolith) analysis, otoliths are generally dissolved in suprapure nitric acid diluted 10 to 50 fold. Two things are important to consider: (1) the minimum sample size required, and (2) the otolith dilution factor which determines the actual LOD. The constraints imposed by surface analysis differ between techniques used. As an example, EDS, WDS and ion microprobe require a highly polished, mirror-flat surface with no defects. Other techniques such as LA-ICPMS can be used with little preparation beyond sectioning and cleaning.

Very few studies have focused on methodological topics. Gunn *et al*. (1992) reviewed the principles of using Electron Probe MicroAnalysis (EPMA) for otolith composition analysis, and emphasized the need to avoid surface irregularities and contamination and provided the first quantitative comparison of the detection capabilities of EDS and WDS. Comparing between EDS and WDS capabilities to measure Sr, Na and K, these authors concluded that EDS is not sensitive and precise enough to provide suitable data for otolith composition analysis. They also tested and discussed the effects of WDS operating conditions (spot size, voltage, current intensity and acquisition time) on data quality.

More recently, an international otolith composition experiment has been carried out with the aims of comparing the potentialities of the four main tools EDS, WDS, LA-ICPMS and PIXE and estimating differences among laboratories using similar instrumentation. The main conclusions from this work are the following (Campana *et al*. 1997):

- There is no single instrument to be preferred for otolith microchemistry. Some minor elements such as Na and K are only measured by microprobes (EDS and WDS) whereas trace elements are best measured with LA-ICPMS or PIXE. As expected, EDS shows the highest LOD and the poorest precision.

- There are differences in accuracy, precision and LOD among laboratories using the same instrument. Such differences may be partly due to laboratory-specific operating conditions. There is thus a need for inter-calibration among laboratories involved in a common project.

- The development of certified reference materials for otoliths is critical for further development of otolith microchemistry applications.

Applications

Otolith microchemistry analysis has concentrated on 2 major types of applications:

- the analysis of differences in otolith composition for distinguishing among different populations or stocks,
- the analysis of variations in composition across the otolith section for reconstructing the individual environmental history. Variations in composition may reflect environmental conditions such as temperature, salinity, or pollution, and major events during the life cycle such as metamorphosis, spawning or stress.

Stock discrimination

Several studies have shown the applicability of otolith microchemistry analysis for stock discrimination purposes (Table 1; Edmonds *et al*. 1989, Edmonds *et al*. 1991, Campana and Gagné 1994, Edmonds *et al*. 1994, Thresher *et al*. 1994, Campana *et al*. 1995, Severin *et al*. 1995). Most of these studies have used ICPMS or ICP-AES tools for their analyses. Mulligan *et al*. (1987) first investigated the potential of using otolith composition for stock discrimination using an EDS microprobe but it is known now that most of the elements used for discrimination were actually below the detection limit for this analytical tool (Gunn *et al*. 1992). It is still worthwhile noting that statistically significant levels of discrimination were made with up to 78% accuracy in the Mulligan *et al*. study. WDS has also provided acceptable data for stock separation, despite the low detection limits for most trace elements (Tables 1 and 2). Kalish *et al*. (1996) used ICP-AES analysis which is considered to be very sensitive for trace elements, but could not separate between the two presumed stocks of *Macruronus novaezelandiae* around New Zealand. There is therefore some discrepancy between the ability of poor detection limit techniques to give usable results, and the inability of sensitive techniques to provide supporting data for presumed stock differences. The difference in results could be due to numerous factors, including (1) the lack of true stock separation between the fish examined, (2) the lack of distinguishing features in the otolith and (3) otolith chemistry may be influenced by factors other than environmental conditions and these factors may mask any composition signal due to stock differences. Some stock discrimination studies have shown that the chemical composition of the otolith is much less sensitive to environmental conditions than previously thought (Thresher *et al*. 1994). As a result of the sometimes conflicting evidence, present and future otolith microchemistry studies must address the interaction between the complexity of analytical tools and that of the otolith chemistry.

Most studies of stock discrimination by otolith microchemistry have relied on statistical multivariate analysis (PCA, etc) of the concentrations of a large sample of elements (Table 1). This is a procedure similar to the process of stock discrimination by allozyme frequencies, or morphometrics, though there has been no effort to consider any problems with the statistical procedures, which might have been discussed in the genetics literature.

The elements which have proved reliable for stock comparison in a number of different species and studies are Na, K, Sr, S (Table 1). The analysis is often done with a dissolution of the whole otolith. At this level, 2 problems arise: (1) the decrease of the concentrations with the relative increasing sizes of the fish (Edmonds *et al*. 1994, Severin *et al*. 1995) and (2) the isolation of otolith parts to discriminate specific stages of the life (Kalish *et al*. 1996). The mean sizes of the fish indicated in the stock discrimination studies show often rather small variations between the populations (few centimeters) but differ systematically. If fish age and/or length differentiate

already most of the compared locations (Severin *et al*. 1995), extreme cares should be given in analyzing the results of microchemistry. Changes in concentration with fish size can be significantly correlated and proportionally important (Edmonds *et al*. 1994): some authors have proposed corrections of element concentration for fish (otolith) size (Edmonds *et al*. 1989, 1994). One other proposed solution is to extract and prepared a constant amount of otolith powder for analysis (Edmonds *et al*. 1989). The isolation of different otolith parts for microchemical analysis is also very difficult due to the concavo-convex compressed otolith shape. Only one study (Kalish *et al*. 1996) has intended to do that but it was unable to discriminate between the presumed stocks.

No field study has compared the concentration of elements in the otolith with concentrations in the fish's environment. This seems to be a general lack in otolith microchemistry studies, but may be the result of difficulties in trace element analysis in seawater This data does exist for a number of laboratory studies, but the actual correlation of otolith concentrations with water and food concentrations has been rather ignored in the literature. (i.e. the data is presented but not directly reported or discussed).

In the future, special attention has to be paid at several level in the stock discrimination for otolith microelements: the homogeneity of the fish sizes between locations, the extraction of otolith parts for the analysis, the knowledge of environmental physico-chemical information.

Ecology (life history, migration, environmental history)

As mentioned above, there appear to be many factors affecting otolith elemental composition which can be exogenous (water chemistry and physic, food resources, stress, pollution) or endogenous (ontogeny, genetic, metabolism). The fact that all these factors may interact largely increases the complexity of elemental incorporation mechanisms (Radtke and Shafer 1992). Numerous studies have tried to document past environmental conditions encountered by field-caught fish of various species from various geographical areas (Table 1).

salinity

Strontium concentration in water is known to vary with salinity. In addition, the Sr concentration in seawater is relatively constant, but Sr in freshwater may vary because of geology, weathering and hydrographic conditions. The large differences between the Sr concentrations in seawater and freshwater support the use of otolith Sr/Ca ratios to study anadromous or catadromous migrations in different species (Radtke 1989, Kalish 1990,. Secor 1992, Otake *et al*. 1994, Tzeng and Tsai 1994, Limburg 1995, Radtke *et al*. 1995, Halden *et al*. 1996). There is a good consensus on the relationship between Sr/Ca ratio and salinity. Numerous verification studies have been recently achieved (Table 1). As an example, Secor *et al* (1995) showed that juvenile striped bass reared at six salinities presented sagittae Sr/Ca ratios positively related to salinity. Moreover, these authors showed that fish exposed to increasing then decreasing salinity gradient present a gradual rise and decline in sagittae Sr/Ca ratio which directly correspond to experimental salinity changes.

temperature

The investigation of changes in the temperature experienced by fish have focused primarily on measuring variations in the otolith Sr/Ca ratio and oxygen isotope ratios. Early conflicting evidence for a simple relationship between temperature and otolith Sr/Ca led to a rapid increase

in experimental studies to get a better understanding of the mechanisms involved in elemental incorporation. Many studies have incorporated both field-caught and laboratory- reared samples. However, this approach has failed to provide clear evidence for a simple relationship, applicable to a range of species from different temperature regimes, between the temperature experienced and otolith Sr/Ca.

Many studies have assumed a direct relationship between temperature and Sr/Ca ratios (Radtke 1989, Radtke and Morales-Nin 1989, Townsend *et al*. 1989, Townsend *et al*. 1992, Tzeng 1994). On cod otoliths from individuals laboratory-reared between 5 to 14 °C, Townsend *et al*. (1995) found a negative exponential relationship between Sr and T°C with a high coefficient of determination. According to them, relative temperature histories of individual field-caught larvae can be reconstructed from the egg stage to the time of capture on the condition that the chemical analyses follow the daily growth increments. Fowler *et al* (1995a, 1995b) found temperature effects on otolith elemental composition stronger than salinity effects. Arai *et al*. (1995) ascertained that concentration of trace elements such as Mn, Fe, Zn and Sr appear to increase in proportion with temperature. To investigate thermal history in deepwater species, Gauldie *et al* (1995b) chose a mixed approach regressing Sr counts on $\delta^{18}O$ measurements which led to consistent results. However, the temperature effect remains quite unclear and the existence of a direct relationship has been contradicted by various studies (Kalish 1989, Kalish 1991, Hoff and Fuiman 1993). According to Sadovy and Severin (1992, 1994) this relationship could be indirect through the augmentation of the body growth rate. In a recent study, Gallahar and Kingsford (1996) did not find any relationship between Sr and T°C on *Girella elevata* reared between 19 to 28 °C. There were no difference as well in Sr/Ca patterns of juvenile American shad reared at 12.5 and 22 °C (Limburg 1995).

The ratio of the oxygen isotopes $^{18}O/^{16}O$ deposited in the carbonate portion of marine organisms has been extensively used in the geological sciences to estimate temperature conditions during the life time of the depositing organisms. The use of fossil and living coral and foraminifera is a standard tool in determining climate variation over geological periods and the literature on the subject is enormous. Recent papers give examples of the wide scope of these studies including analysis of the oscillation in El Niño events in the Pacific using reef-building corals (Wellington and Dunbar 1995), global temperature changes based on biogenic silica (Shemesh *et al*. 1992) and estimation of warming in interglacial periods in the Aegean based partly on fossil foraminifera (Aksu *et al*. 1995).

The application of these techniques depends on the assumption that the oxygen in biogenic material is deposited in equilibrium with the surrounding medium. The ratio of ^{18}O increases as the temperature in the surrounding water mass decreases. This assumption has been vigorously tested over a long period of use. Further studies such as those by Mook and Vogel (1968), Degens *et al*. (1969), Kalish (1991), Wefer and Berger (1991) confirm the basic premise. The wide range of organisms studied would appear to imply that the deposition of oxygen in isotonic equilibrium is a universal occurrence in marine biogenic material and also probably in freshwater environments (Stuiver, 1970, Fritz and Poplawaski, 1974). Not all studies are in agreement. A detailed analysis of brachiopods from a wide geographic distribution including Antarctica, Japan and Norway concluded that there were differences in ^{18}O ratios depending on the part of the shell sampled (Carpenter and Lohmann 1995). Calcite from primary layers and specialised areas such as shell hinges were found to be depleted in ^{18}O compared with other “secondary” shell layers. It is also likely that different carbonate materials such as aragonite and calcite may have varying ^{18}O concentrations (Jones *et al*. 1983, Grossman and Ku 1986).

The use of oxygen isotope ratios as a proxy for a temperature record provides a means of analysing environmental conditions and growth over the life history of individual organisms. A wide range of species has been studied. Paetzold *et al*. (1987) studied the growth patterns of the bryozoan, *Pentapora foliacea*. The temperature signal indicated clear annual growth checks and allowed age to be validated. Jones *et al*. (1983) recorded growth checks in the Atlantic bivalve, *Spisula elliptica* corresponding to the winter bottom temperature of 7-10°C. Weidman and Jones (1994) and Witbaard *et al*. (1994) found annual patterns in ^{18}O in the bivalve *Arctica islandica* which were consistent with the observed growth bands on the shell. Weidman and Jones (1994) were also able to confirm that for this species, the ^{18}O was in isotopic equilibrium with the ambient seawater. Annual growth rates and age of giant clams (*Tridacna spp*) were obtained by analysing the seasonal variation in ^{18}O from shell samples (Romaneck *et al*. 1987, Paetzold *et al*. 1991).

Studies on fish have used ^{18}O ratios in otolith aragonite mainly to look at temperature in relation to general environment of the species (Devereux 1967, Degens *et al*. 1969, Radtke 1984, Meyer-Rochow *et al*. 1992). The temperature signal has also been considered as a mean of assessing differences in migratory behaviour (Nelson *et al*. 1989) or changes in depth distribution with age especially for deep water species (Mulcahy *et al*. 1979, Gauldie *et al*. 1994, 1995b). The development of microsampling techniques (Dettman and Lohmann 1993) together with micro-mass spectrometry, has allowed detailed studies to be undertaken on individual otoliths. Samples can be obtained with a 10µm resolution (Weidman and Jones 1994) and this means that seasonal variation in ^{18}O ratios can be used to validate growth bands in the same way as previous work with bivalves.

Oxygen isotope analysis has shown more reliable relationships to temperature, although the inclusion of laboratory testing is lacking in this area. O_2 ratios within the otoliths seem to respond to temperature variation in a reliable manner, but most evidence indicates that the disequilibrium factors applied to fish are inappropriate, since temperature estimates in the literature are rarely accurate.

diet and water composition

Sr enriched seawater leads to higher otolith Sr/Ca ratios as well as Sr enriched diet (Mugiya and Tanaka 1995, Gallahar and Kingsford 1996). Limburg (1995) observed a significant rise in Sr/Ca ratios when juveniles of American shad were weaned from a freshwater plankton diet to an artificial diet. However, larval and juvenile red drum fed on "normal" and Na, Mg, K and Sr supplemented diets, showed little or no dietary effect on otolith elemental incorporation Hoff and Fuiman (1995)

Ontogeny

Both growth and ontogenic events produce clear changes in otolith composition. A significant change in Sr/Ca ratio was observed in elver otoliths during their migration from open ocean to coastal water (Otake *et al*. 1994, Tzeng and Tsai 1994). It has not been established whether changes in water chemistry or physiological changes due to ontogeny were most important in determining otolith composition. Otake *et al*. 1994 argued that ontogenetic changes, in particular a likely glycosaminoglycans (GAG) breakdown during metamorphosis would reduce the Sr absorption from the seawater, leading to lower Sr otolith concentrations. Tzeng (1996) observed that the Sr/Ca ratios in elvers otoliths were much lower, even when held at 35 ‰, during the metamorphosis. A preliminary study on sole larval transport towards the coastal and estuarine nurseries showed a decrease in Sr/Ca ratios concomitant to metamorphosis (Amara 1995).

Rather than reflecting an environmental variation as first hypothesised, this phenomena may be due to an ontogenetic change in Sr incorporation (Pontual *et al*., in preparation)
According to Fuiman and Hoff (1995) annual cyclic trends in sodium and potassium concentrations in otolith correspond to cyclic sea water temperature changes. It is likely that the observed relationship is an indirect one since these authors suggest that the variations in Na an K concentrations may be induced by reproductive activity.

The question of Sr/Ca change with age and the consequences of using variations in Sr/Ca ratios for age verification has also been investigated in different ways. An increasing Sr incorporation with distance from the core has been shown (Gallahar and Kingsford 1992, Fuiman and Hoff 1995). Mugiya and Satoh (1995) found a close correlation between D- zones (SEM observations) and peaks of Sr/Ca ratios in the otoliths of goldfish kept in Sr-enriched seawater. Age estimation of deep water species are hampered by the difficulties of traditional validation experiments. Analysing orange roughy sagittae, Gauldie *et al*. (1995a) found similar cyclic patterns of microincrement widths and Sr concentration suggesting the potential of correlating otolith composition information with optically visible structures as a means of indirect validation.

Conclusion

Studies of otolith microchemistry show very great potentials but there are still four levels of uncertainty which cause problems in the application of otolith microchemistry in general, and for stock separation specifically:

- inexperience with the analytical tools. This comes, not from failures of the operators, but because many of these tools have only recently been used for biogenic carbonate material. There is only a small body of experience to draw on for assessment of the effects of the otolith composition on the measured concentrations, or the behaviour of otolith material when presented in different phases to the different analytical tools.
- inexperience with otolith material, such that we have little knowledge of the interaction between the different elements which can influence their relative incorporation rates, and little knowledge of the pathways of accumulation for different elements which could influence not only the concentrations, but also the spatial distribution of these elements within the otolith. We have not yet begun to interpret existing data on differences between co-occuring species (but see Kalish 1989), or between geographically separated populations of the same species.
- inexperience with the mechanism of otolith formation, which means that we cannot yet evaluate the significant/consequences of observed differences in composition, whether within the otolith, between individuals, or between stocks.
- lack of international standard reference material.

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Table 1. Otolith microchemistry studies in the litterature (non exhaustive). AAS, Atomic Absorption Spectrometry; AES, Atomic Emission Spectrometry; EDS, Electron Dispersive Microprobe; FAB-SIMS, Fast Atom Bombardment - Secondary Ion Mass Spectrometry; ICP-AES, Inductively Coupled Plasma - Atomic Emission Spectrometry; ICPMS, Inductively Coupled Plasma Mass Spectrometry; ID-ICPMS, Isotope Dilution - Inductively Coupled Plasma Mass Spectrometry; PIXE, Proton Induced X-ray Emission; WDS, Wave-length Dispersive Electron Microprobe.

Authors	Species	Geographical zone	Application	Tool	Method	Analysed elements
Review / Methodology						
Campana et al. 1997	*Micropogonias undulatus*	rearing (Canada)	methodology / review	EDS, WD PIXE, ICPMS	transect on sagittal section	Mg, Na, Ca, K, Fe, Ni, Cu, Pb
Gunn et al. 1992	*Nemadactylus macropterus* *Thunnus maccoyii*	?	methodology /review	EDS, WDS	transect on transverse section	Ca, Na, Sr, K, S, Cl
Radtke and Shafer 1992	review	review	review	review	review	review
Sie and Thresher 1992			methodology	PIXE		
Stock discrimination						
Campana and Gagné 1994	*Gadus morhua*	Eastern coast (Canada)	stock discrimination	ICPMS	acid otolith dissolution	B, Mg, Si, Ca, Sc, Ti, Cr, M Ni, Cu, Zn, Ga, As, Br, Rb, Ru, Rh, Cd, Cs, Ce, Ba, Eu,
Campana et al. 1994	*Gadus morhua*	East coast (Canada)	stock discrimination	ICPMS	transect on sagittal section	9 elements
Campana et al. 1995	*Gadus morhua*	East coast (Canada)	stock discrimination	ID-ICPMS	acid otolith dissolution	Li, Mg, Zn, Sr, Ba, Pb
Edmonds et al. 1989	*Chrysophrys auratus*	Western coast (Autralia)	stock discrimination	ICP-AES	acid otolith dissolution	Na, Mg, Si, P, S, K, Ca, Fe,
Edmonds et al. 1991	*Hoplostethus atlanticus*	Eastern coast (Tasmania)	stock discrimination	ICPMS	acid otolith dissolution	K, Mg, Na, S, Sr, Ba, Cd, C
Edmonds et al. 1994	*Pagrus auratus* *Sardinops sargax*	Western coasts (Australia)	stock discrimination	ICP-AES	acid otolith dissolution	Na, K, Sr, Mg, S, P, Ba
Kalish et al. 1996	*Macruronus novaezelandiae*	Southern coasts (N Zealand)	stock discrimination	ICP-AES	acid otolith dissolution	Na, P, S, K, Ca, Cu, Zn, Sr
Mulligan et al. 1987	*Morone saxatilis*	Chesapeake Bay (Marylar USA)	stock discrimination	EDS	transect on sagittal section	Si, Al, Cl, S, Na, K, Mn, Cu, Ra, V
Proctor et al. 1995	*Thunnus maccoyii*	Western and Southern coa (Australia, South Africa)	stock discrimination	WDS PIXE	transect on transverse section	Ca, Na, Sr, K, S, Cl
Severin et al. 1995	*Theragra chalcogramma*	Gulf of Alaska / Bering Sea	stock discrimination	WDS	transect on sagittal section	Na, Mg, P, S, Cl, K, Ca, Sr
Thresher et al. 1994	*Nemadactylus macropterus*	South-western coa (Autralia), Tasmania	stock discrimination	WDS	transect on transverse section	Ca, Na, Sr, K, S, Cl
Environmental history						

Amara 1995	*Solea solea*	Bay of Biscay	migration (salinity ontogeny)	WDS	Local analyses on sagittal section	Ca, Na, Sr, K
Arai et al. 1994	*Pagrus maj* *Sebates schegeli*	rearing and wild-caug (Japan)	environmental condition	PIXE	no preparation	Sr, Fe, Mn, Zn
Arai et al. 1995	*Pagrus major*	rearing (Japan)	environmental history (temperature)	PIXE	transect on whole otolith	Mn, Fe, Zn, Sr
Cheng and Tzeng 1996	*Anguilla japonica*	rivers (Taiwan, Chi Japan)	migration (salinity)	WDS	transect on sagittal section	Sr/Ca
Farrell and Campana 1996	*Oreochromis niloticus*	rearing (Canada)	environmental history (diet and water composition)	liquid scintillation counter	acid dissolution	Sr, Ca
Fowler et al. 1995a	*Micropogonias undulatus*	rearing	methodology (temperature, salinity)	ICPMS	acid otolith dissolution	Li, Mg, Al, P, Cl, Ca, Mn, Zn, As, Rb, Sr, Ba, Pb
Fowler et al. 1995b	*Micropogonias undulatus*	rearing	methodology (temperature, salinity, ontogeny)	LA-ICPMS	transects on sagittal sections	B, Mg, Ca, Fe, Ni, Co, Cu, Ba, Pb
Fuiman and Hoff 1995	*Sciaenops ocellatus*	Port Arensas (Texas, USA)	seasonal variations, age	WDS	transects on transverse sections	Ca, Sr, Na, K and Sr/Ca
Gallahar and Kingsford 1992	*Girella elevata*	New South Wales Coa Australia	age	WDS	transect on sagittal section	Ca, Sr, Fe
Gallahar and Kingsford 1996	*Girella elevata*	rearing (Australia)	environmental history (temperature, Sr enriched wa and diet)	WDS	transect on sagittal section	Sr/Ca
Gauldie et al. 1986	*Oncorhynchus tshawytcha*	rearing (Canada)	environmental history (temperature)	AES	acid otolith dissolution	Fe, Mn, Zn, P, Na, Sr
Gauldie et al. 1993	*Macruronus novaezealndiae*		age estimation	PIXE	transects	Sr, Ca, N, C, O
Gauldie et al. 1994a	*Hyperoglyphe antarti* *Beryx splendens*		methodology	SERS	otolith acid dissolution	Ca, Sr
Gauldie et al. 1994b	*Beryx splendens* *Coryphaenoides profundiculus*	deep water	migration	mass-spectrometer	transect along sagitta	Oxygen isotopes
Gauldie et al. 1995a	*Hoplostethus atlanticus*		age estimation	PIXE	map on transverse section	Sr
Gauldie et al. 1995b	*Trachyrincus murr* *Coryphaenoides mediterraneus*	deep water	environmental history (temperature)	PIXE	map on transverse section	Sr
Halden et al. 1995	*Salvelinus alpinus*	freshwater river (Canada)	migration (salinity)	PIXE	transect on transverse section	Sr
Hoff and Fuiman 1993	*Sciaenops ocellatus*	wild caught and rearing	environmental history, age (temperature)			
Hoff and Fuiman 1995	*Sciaenops ocellatus*	rearing	environmental history (salinity, temperature)	AAS WDS	acid otolith dissolution transect on transverse section	Mg, P, Sr, Na, Ca, ratios
Kalish 1989	*Arripis tru* *Macruronus novaezealndiae*	wild caught and rearing	seasonal variation (temperature, growth, reproducti	WDS	specific sites on transverse sectio	Sr, Na, K, S Ca ratios
Kalish 1990	*Salmo trutta*	Derwent river (Tasmania)	migration (salinity)	WDS	transect on transverse section	Sr, Na, K, S, Ca, ratios

Kalish 1991	*Pseudophycis barbatus*	Variety Bay (Tasmania)	seasonal variation	WDS	specific sites on transverse sectio	Ca, Na, K, Sr, S
Kalish 1992	*Artipis trutta*	Marion Bay (Tasmania)	environmental history (salinity, temperature)	WDS	transect on transverse section	Ca, K, S, Na, Sr, ratios
Limburg 1995	*Alosa sapidissima*	rearing / Hudson river (US.	migration (salinity)	WDS	transect on sagittal section	Sr/Ca
Meyer-Rochow et al. 1992	*Mugil cephalus*	New Zealand	environmental history	mass-spectrometer	transect along sagitta	Oxygen isotopes
Mugiya et al. 1991	*Carassius auratus*	rearing	environmental histo (trace elements)			Al, Cd, Ba, Mn, Fe, Ni, Cu,
Mugiya and Satoh 1995	*Carassius auratus*	rearing Japan	growth	EDS	transect on sagittal sections	Sr/Ca
Mugiya and Tanaka 1995	*Carassius auratus*	rearing Japan	environmental history Sr-enriched water, temperature	AAS EDS	specific sites on sagittal section	Sr/Ca
Nelson et al. 1989	*Retropinna retropinna*	New Zealand	migration	mass-spectrometer	whole sagitta	Oxygen isotopes
Otake et al 1994	*Anguilla japonica*	wild-caught (West Maria Islands and Japan)	migration metamorpho (salinity)	WDS	transect on sagittal section	Sr/Ca
Radtke 1989	*Fundulus heteroclitus*	rearing	environmental history (temperature)	AAS WDS	acid otolith dissolution transect on sagittal section	Sr/Ca
Radtke and Targett 1984	*Notothenia larseni*	South Georgia Island (USA	ageing (temperature)	WDS	transect on sagittal section	Sr/Ca
Radtke and Morales-Nin 198	*Thunnus thynnus thynnus*	Mediterranean Sea	environmental history (temperature)	WDS		Sr/Ca
Radtke et al. 1990	*Clupea harengus*	rearing	environmental history (temperature)	WDS	transect on sagittal section	Sr/Ca
Radtke et al. 1995	*Salvelinus alpinus*	Labrador and Spitsbergen	migration (salinity)	WDS	transect	Sr/Ca
Radtke et al. 1996	*Gadus morhua*	Norway	environmental history (temperature)	mass-spectrometer	whole otolith	Oxygen/Carbon isotopes
Rieman et al. 1994	*Oncorhynchus nerka*	Redfish Lake (Idaho, USA)	migration (salinity)	WDS	transect on sagittal section	Sr/Ca
Sadovy and Severin 1992	*Haemulon plumieri*	coasts (Puerto Rico, No and South Carolina)	environmental history, growth (temperature)	WDS	transect on transverse section	Sr/Ca
Sadovy and Severin 1994	*Epinephelus guttatus*	coasts (Puerto Ri Bermuda)	environmental history, growth (temperature)	WDS	transect on transverse section	Sr/Ca
Secor 1992	*Morone saxatilis*	Chesapeake Bay (Marylar USA)	life history (migration) (salinity)	WDS	transect on transverse section	Sr/Ca
Secor et al. 1995	*Morone saxatilis*	rearing / Chesapeake Ba Massachusetts (USA)	methodology: migrati (temperature, salinity)	WDS	transect on transverse section	Sr/Ca
Seyama et al. 1991	*Lutjanus sebae*	West coast (Australia)	ageing	FAB-SIMS	transect on transverse section	Sr, Na, K, Ca
Toole and Nielsen 1992	*Microstomus pacificus*	rearing (Oregon, USA)	methodology	WDS	transect on sagittal section	Sr/Ca

			(temperature)			
Toole et al. 1993	*Microstomus pacificus*	Oregon coats (USA)	metamorphosis, migration (salinity)	WDS	transect on sagittal section	Sr/Ca
Townsend et al. 1989	*Clupea harengus*	Gulf of Maine (USA)	environmental history (temperature)	WDS	transect on sagittal section	Sr/Ca
Townsend et al. 1992	*Clupea harengus*	rearing	environmental history (temperature)	WDS	transect on sagittal section	Sr/Ca
Townsend et al. 1995	*Gadus morhua*	rearing / Georges Ba (Canada)	environmental history (temperature)	WDS	transect on sagittal section	Sr/Ca
Tzeng 1994	*Anguilla japonica*	rearing / river (Taiwan)	environmental history (temperature)	WDS	transect on frontal section	Sr/Ca
Tzeng 1996	*Anguilla japonica*	rearing	environmental history (salinity)	WDS	transect on sagittal section	Sr/Ca
Tzeng and Tsai 1994	*Anguilla japonica*	rivers (Taiwan)	environmental history, migration (salinity)	WDS	transect on frontal section	Sr/Ca

Table 2. Analytical tools and characteristics mainly used in otolith microchemistry.

Application	Technique	Acronym	Information	Resolution, limit of detecti elemental range	Comments
SD EH AV	Energy Dispersive Spectrometry	EDS (or ED-EM)	- quantitative elemental analy - X cartograp - profile of concentration	- x : 0.5 µm to 10 µ - z : 0.5 µm to 10 µ > 1000 p B-U	spot sample (solid sample) associated to SEM or TEM
SD EH AV	Wawelength Dispers Spectrometry	WDS (or WD-EM)	- quantitative elemental analy - X cartograp - profile of concentration	- x : 0.5 µm to 10µ - z : 0.5 µm to 10 µ 100-1000 p B-U	spot sample (solid sample)
	X-Ray fluorescence analysis	XRFA	quantitative elemental composition	- x : a few c - y : a few 10 µ 1 to 100 p F-U	- quantitative whole otol analyis (solid samp - quantification with calibrat curve
AV	Secondary Ion Mass Spectrometry	SIMS	- elemental analy - molecular analy - isotopic analysis	- x : 0.1µm to 0.5 n - z : 1 nm to 10 nm < ppm to 100 p mass 1 to 500	- depth profil - traces analy - solid samples
SD AV	Particule Induced X-ray Emission	PIXE	- elemental analysis	- x : 10 µm to 1 - z : 1-10 µm 1 to 1000 p H-U	- quantification with standa samples
SD EH	Inductively Coupled Plasma-Ator Emission Spectrometry	ICP-AES (or ICP-OES)	- elemental analysis	<1 ppb to 30 ppb	solution based samples
SD EH	Inductively Coupled Plasma-M Spectrometry	ICP-MS	- elemental analy - isotopic analysis	< p Li-U	-trace analysis on solution ba samples
SD EH AV	Inductively Coupled Plasma-M Spectrometry with Laser Ablation	LA-ICPMS	- elemental analy - isotopic analysis	- x : 20 µ - z : 20 µ 1 p Li-U	spot sample
	Atomic Emission Spectrometry	AES (Flame, arcs a sparks)	- mono elemental analysis	- x : n - z : 0.1 mm Li-U > 5 ppb	liquid or solid samples
	Atomic Absorption Spectrometry	AAS (Flame , Furnac	mono elemental analysis	0.1 ppb to 500 p (flame) 0.005 ppb to 50 p (furnace)	solution based samples
AV	Mass spectrometry	MS (?)	quantitative stable isotopes	(0.5°C)	micro samples needed

SEM Scanning Electron Microscopy,
TEM Transmission Electron Microscopy
x : spot sample area
z : sample penetration depth

Table 3. Elemental composition of otoliths from the litterature (mean in ppm ± SD, and range). ICP-AES, Inductively Coupled Plasma - Atomic Emission Spectrometry; ICPMS, Inductively Coupled Plasma Mass Spectrometry; ID-ICPMS, Isotope Dilution - Inductively Coupled Plasma Mass Spectrometry; WDS, Wave-length Dispersive Electron Microprobe, NGAS Neutron Activation Gamma Spectrometry.

Species and author(s)	Tool	Li	Na	Mg	Al	Si	P	S	Cl	K
Chrysophrys auratus (Edmonds et al. 1989)	ICP-AES		2188 ± 41 514-654	8 ± 1 5-10		18 ± 16 0-43	111 ± 22 77-172	174 ± 49 90-257		389 ± 53 278-464
Gadus morhua (Campana et al. 1995)	ID-ICPMS	0.4-0.6 ± 0.02-± 0.0		14-19 ± 0.5-± 1.1						
Hoplostethus atlanticus (Edmonds et al. 1991)	ICP-AES		2289 ± 142 1976-2514	2 ± 1 0-3				190 ± 36 127-274		188 ± 17 165-230
Macruronus novaezelandiae (Kalish et al. 1996)	ICP-AES		2814 ± 169 2470-3380				164 ± 44 91-300	274 ± 180 39-890		418 ± 86 268-681
Nemadactylus macropterus (Thresher et al. 1994)	WDS		3331 ± 122 2680-4240					421 ± 76 220-1220	255 ± 72 0-1230	729 ± 72 280-1630
Pagrus auratus (Edmonds et al. 1994)	ICP-AES		2100-2400 - -	4-20 - -			10-200 - -	10-450 - -		350-450 - -
Sardinops sargax (Edmonds et al. 1994)	ICP-AES		2000-2600 - -	5-15 - -			140-250 - -	100-600 - -		400-500 - -
Theragra chalcogramma (Severin et al. 1995)	WDS		3484-5314 - -	0-142 - -			827-4034 - -	0-560 - -	0-587 - -	536-1221 - -
Thunnus maccoyii (Gunn et al. 1992)	WDS		2916 ± 117 1590-3790					392 ± 75 210-790	272 ± 72 10-1170	442 ± 67 230-750
Parma microlepis *Acheorodus viridis* (Dove et al.1997)	ICPMS		100	1183	0.15					
Carrassius auratus (Mugiya et al.1991)	EDS				yes					
Gadus morhua (Protasowick and Kosior 198										
Scomber scombrus (Papadopoulou et al. 1978)	NAGS									
Scianeops ocellatus *Hoff and Fuiman 1995*	WDS		2.60	3900						
Scomber scombrus (Grady and Jonson	AAS									
Pleuronectes platessa (Geffen et al. in press)	ICPMS			33.7 ± 7.5	10.4 ± 1.1					
Merlangius merlangus (Geffen et al. in press)	ICPMS			19.9 ± 2.7	17.3 ± 2.9					

4. Microstructural validation of annual increments

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The idea of using microstructural analysis to validate the periodicity of annual increments in fish otoliths was already indirectly suggested by Pannella (1971), who pointed out the fact that around 360 microincrements were present in one opaque and one translucent macroscopic band in the otoliths of *Urophicis chuss* and *Merluccius bilinearis*. Pannella actually was trying to validate daily increments from annual ones and not the opposite. Brothers *et al*. (1976) confirmed the correspondence between age determinations obtained by annuli and daily ring counts and proposed the use of daily marks as a mean of accurate age determination for fast growing species in their first years of life.

Taubert and Coble (1977) produced first scientific evidence to the observation that an annulus is produced by deposition of narrower daily rings. In temperate species daily deposition can be very compressed or arrested in cold periods (Campana and Neilson, 1985) and an annulus often appears at light microscopy as a discontinuity preceded by increasingly narrow increments (Victor and Brothers, 1982). Counts of daily rings between successive annuli allowed Victor and Brothers (1982) and Taubert and Tranquilli (1982) to validate annual increments by means of microstructural analysis. Campana and Neilson (1985) reviewing the papers by Pannella (1971), Taubert and Tranquilli (1982) and Victor and Brothers (1982) stated that in general it is questionable the use of microstructure beyond the first year of life particularly in temperate zones. Warnings have been raised also in the case of tropical fishes because of possible interruptions in daily periodicity after the onset of maturity (Ralston and Miyamoto, 1983) and in general in old fishes (Ralston and Williams, 1988). Further reviews on the problem of daily periodicity of deposition in adult fishes, which is a prerequisite for the use of daily rings for validation of annuli, are given in Campana and Jones (1992) and Geffen (1992).

The use of microstructural analysis for the validation of annual increments has been applied to various fish species despite the caveats on the daily periodicity of increments and the technical limitations due to tedious and difficult preparations and analysis (Morales-Nin, 1992; Geffen,1992). SEM and/or light microscopy and different procedures in the preparation of the otoliths and the analysis of results have been used by various authors.

Powell (1982) estimated the age at first annulus formation in young of the year of *Paralichthys dentatus*. Wenner *et al*. (1986) made a similar study to determine the age at the first annulus formation in black sea bass, *Centropristis striata*, by counting by light microscopy the increments from the core to the first translucent zone. Padovani Ferreira and Russ (1992) counted assumed daily increments from the nucleus to the beginning of the first opaque zone to indicate that the formation of the first annulus occurred in the first year of life of the serranid *Plectropomus maculatus*, annual periodicity was then validated through tetracycline marking. McPherson

(1992) used assumed daily increments of one to two years old fishes to further validate (in addition to marginal increment analysis) the annual periodicity of annuli in *Scomberomorus commerson*. Yosef and Casselman (1994) used validated daily increments to determine the timing of deposition of the first annulus and to discriminate between the two recruitment cohorts in a Tilapia with two separate spawning seasons per year.

Validation of annuli in small pelagic fishes has been obtained by Morales-Nin (1988) by plotting the age in days versus the age in years (obtained by annuli counting) in peruvian anchoveta (*Engraulis ringens*). Hoedt (1992) validated the first annulus in the tropical anchovy *Thryssa hamiltoni* counting increments in otolith sections from the nucleus to the first seasonal ring (narrower increments) in adult individuals: daily ring counts were also useful to distinguish between checks and annuli. A similar result was obtained by Waldron (1994) for the south african anchovy (*Engraulis capensis*) by means of SEM preparations.

A rather different approach has been developed for bluefin tuna (*Thunnus thynnus*) by Radtke (1984) who used sequential etching and SEM observation to count daily increments between presumed annuli from the edge of the otolith section inwards: the number of microincrements between succesive major increments corresponded to an annual periodicity. Slight variations to this technique (e.g. start counting from the core) has been applied to temperate fishes as oyster toad fish (*Opsanus tau*) (Radtke *et al*. 1985), tropical fishes as damselfish (*Dascyllus albisella*) (Hill and Radtke, 1988) and to several antarctic fishes (Radtke, 1984, 1990; Radtke and Targett, 1984; Radtke *et al*. 1989,1993; Radtke and Hourigan, 1990). In the studies on the oyster toad fish and the damselfish changes in daily increment width during the year were found to correspond to the seasonal pattern of deposition of opaque and translucent zones. On the contrary in the antarctic fish studies daily ring counts were used directly to age adult fishes because the macroscopic zones (opaque and translucent) were not considered by the authors to show an annual periodicity although, sometimes (e.g. Radtke, 1990) the results obtained were comparable with those obtained by other authors counting annuli.

Morales-Nin (1989) and Morales Nin and Ralston (1990) counted in otoliths of tropical fishes the number of daily rings in one presumptive annual period formed by one fast growth zone (thick increments) and one slow growth zone (thin increments). Counts were performed by SEM and mean values obtained were not significantly different from the number of days in a year. Annual periodicity was further validated by marginal increment analysis of seasonal zones (Morales-Nin, 1992).

Microstructural validation of annual increments has been applied successfully also to abyssal fishes (Wilson, 1988) under the assumption of daily periodicity of primary microincrements. It can be therefore concluded that this technique can be used in a wide variety of situations provided that there is reasonable evidence of daily periodicity in microincrements deposition; that it is possible to detect at microscopic level a starting and a final point for the count of the increments inside one presumptive annual period and that it is possible to detect minor checks as false by microstructural observations. The technique appears to be more easy to apply in tropical fish species than in temperate ones because of the difficulty of detecting daily increments in slow growth (narrow or absent increments) zones in the otolith. The fact that daily increments have been found in anctarctic as well as in abyssal fishes might signify that the applicability is more determined by specific (and unknown) physiological characteristics of a single species than broad ecological factors.

The reason why zones appear different at the macroscopic level is due to changes in their microstructure and chemistry. Taubert and Coble (1977) produced the first scientific evidence that an annulus is produced by deposition of narrower daily rings. In temperate species daily deposition can be very compressed or arrested in cold periods (Campana and Neilson, 1985) and

an annulus often appears at light microscopy as a discontinuity preceded by increasingly narrow increments (Victor and Brothers, 1982). Those zones of the otoliths of Dicentrachus labrax produced during periods of fast growth were represented by thick micro-crsytals, while for periods of slow growth the microcrystals were shorter and more compactly spaced (Morales-Nin, 1987). In *Lutjanus kasmira* it has been proved the formation each year of one zone composed by thin increments with numerous discontinuities or checks, and one zone composed of thicker increments with rhythmic growth patterns. The transition from one zone to the other is generally abrupt without a transition growth phase (Morales-Nin & Ralston, 1990).

These microstructural characteristics result in a different chemical composition of both zones. The opaque zones are richer in lamellar structures and thick organic bands than the hyaline zones (Mugiya *et al*., 1985). These differences in organic matrix abundance may be both related to developmental stage and to temperature (Morales-Nin, 1986). In polar and temperate regions, annual temperature cycles have bee proposed to explain some of the observed seasonal variation in otolith chemistry (Radtke), but other authors have diasagreed with this interpretation (Kalish, Savoy). Rates of otolith calcification (Wright, 1990) and increment width (Mosegaard *et al*, 1988) are temperature sensitive. These relative chemical differences have been used to increase the contrast between zones by means of burning or staining the otolith. The translucent zones appearing darker due to their relative higher protein content.

Depending of otolith preparation (sections, whole otoliths) and observation (magnification, ilumination) techniques, discrepancies results may be obtained due to the interpretation of different non equivalent morphological structures (Gauldie, 1994). The presence of secondary growth structures provides an additional source of uncertainty.

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The use of fluorescent age pigments for ageing fish

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In the 80s, "lipofuscin" or "age pigment" assay was proposed as an alternative or an emphasis to conventional morphometric age estimation techniques in Crustaceans (Ettershank 1983, 1984). These pigments are formed under normal conditions in cells as the result of polymerization reactions between oxidized lipids and proteins (peroxydation). Lipofuscin molecules are not digested by the lysosomes but accumulate instead (Ettershank 1983). They are highlighted by histological preparations but are also characterized by containing numerous autofluorescent components (Schiff bases and flavins), ranging in emission color from blue to yellow (Hammer 1988; Hill and Womersley 1991).

Techniques for measuring lipofuscin are mainly based on solvent-extracted fluorophores and spectrofluorometric assay. The use of term "lipofuscin" is currently disputed due to the differential properties of fluorescence in tissue extracts compared to histologically observed preparations (Hill and Womersley 1991). Following these authors, the term "fluorescent age pigment" or "FAP" will be preferred to describe age-related fluorophores which have been measured spectrofluorometrically compared to "lipofuscin" or "ceroid" which should be reserved for describing age pigments observed histologically.

In the late 80s and beginning of 90s the potential of fluorescent age pigments has been investigated to follow the physiological age of fishes (Hammer and Rao 1987; Hammer 1988; Hill and Radtke 1988; Mullin and Brooks 1988; Vernet et al. 1988; Hill and Womersley 1991, 1993; Girven et al. 1993). In spite of these few studies no recent research have been undertaken in the topic in comparison to crustaceans. This must be due to the lack of clear applications for age estimation in fish and methodological problems for such analyses. Few problems and limits have been enumerated by Hill and Womersley (1991): the necessity of calibration with known-age individuals, long term analysis to take into account ontogenic changes, extraction procedures for fluorophores and a lack of method standardization.

Principles and methods

In order to use FAP for ageing, it is necessary to quantify the level accumulated in tissues. Two methods are used for the quantification of lipofuscins: spectrofluorometric assay from whole tissue samples after extraction of fluorophores and epifluorescent microscopy on tissue sections. The first one has been the most widely adopted for a range of taxons (crustaceans, fishes...) whereas the second has only been used for crustaceans. Each technique works only on post-mitotic tissues such as brain, heart, skeletal muscles. Brain is the most widely used tissue.

Spectrofluorometry

All the variants of this method are derived from the protocol of Ettershank (1983, 1984) who has himself modified the method of Fletcher et al. (1973) using spectrofluorometric assay for measurement of blue emitting fluorophores. Most of the literature experiments have worked on part of the post-mitotic tissues and only one has used whole animals to quantify FAP (Nicol 1987).
The FAP extraction procedures can be resumed as follow (Ettershank 1983; Hill and Womersley 1991):

- maceration of tissues in spectroscopic-grade chloroform/methanol mixture (concentrations of these solvents are variable),
- ultrasonication,
- mixing with magnesium chloride solution,
- centrifugation.

Chloroform extracts are then assayed in fluorescence spectrophotometer. The dissolved FAP molecules are characteristically fluorescent with excitation maxima around 350-380 nm and emission maxima around 440-470 nm (Hammer 1988).

FAP concentrations are expressed either as relative fluorescence intensity (RFI) or as whole organ % fluorescence (%FL):

$$\mathrm{RFI} = \frac{\mathrm{Luminescence}_{\mathrm{sample}}}{\mathrm{Luminescence}_{\mathrm{standard}}} \times \frac{\mathrm{vol}_{\mathrm{solvent}}}{\mathrm{weight}_{\mathrm{sample\ dry}}} \times 100$$

$$\%\mathrm{FL} = \frac{\mathrm{luminescence}_{\mathrm{sample}}}{\mathrm{luminescence}_{\mathrm{standard}}} \times \mathrm{vol}_{\mathrm{solvent}} \times 100$$

Fluorescent microscopy

Sheehy (1990a, 1990b) was the first who introduced the potential of morphological lipofuscin as an index of age in crustaceans. His method is based on the treatment of tissue sections and the quantification using alternative fluorescent microscope and image analysis techniques. Sections are prepared histologically in a simple manner: tissues are fixed in neutral buffered formaldehyde and unstained serial 6 µm sections are made for the observations under microscope. The sections are then examined under epifluorescence microscope with a 450 to 535 nm excitation filter: lipofuscin is detected by its characteristic yellow-orange autofluorescence (Sheehy 1990a; Sheehy et al. 1996). For lipofuscin quantification, images of the section are recorded and treated semi-manually: brightly fluorescing lipofuscin granules are discriminated from the background using a manual greyscale thresholding. The results are expressed as a percentage volume fraction (%VF).

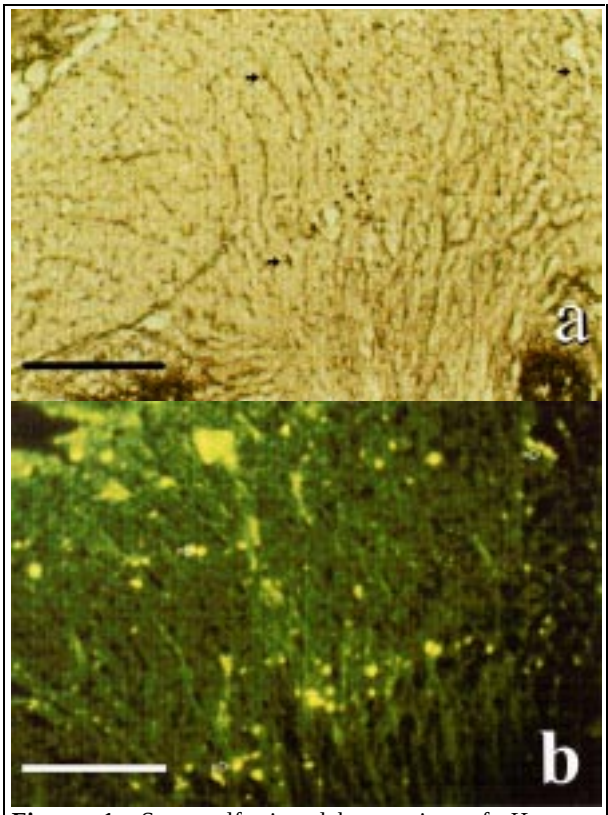

Figure 1. Same olfactive lobe section of *Homarus americanus* viewed under transmitted light after staining with Soudan Black (a) and under fluorescent light (b). Arrows show three FAP particles in the section. Barre = 100 µm (From de Kerros 1996).

It is often necessary to verify if the observed fluorescence corresponds to lipofuscin granules prepared histologically (i.e. by staining with Soudan Black and/or hematoxylin-eosin complexone, Fig. 1) (de Kerros 1996; Sheehy et al. 1996).

This method has been improved for several crustacean species until recently (Sheehy 1990a, 1990b, 1992; Sheehy et al. 1994, 1995, 1996; de Kerros 1996) but never for fish species.

Problems and limits

FAP quantification in tissues is not easy and numerous problems have been listed in the literature. Before quantifying FAP, preparation procedures of material can lead to numerous bias in ulterior analysis (Hill and Womersley 1991). Most of these bias have been underlined with spectrofluorometry instead of histological observations.

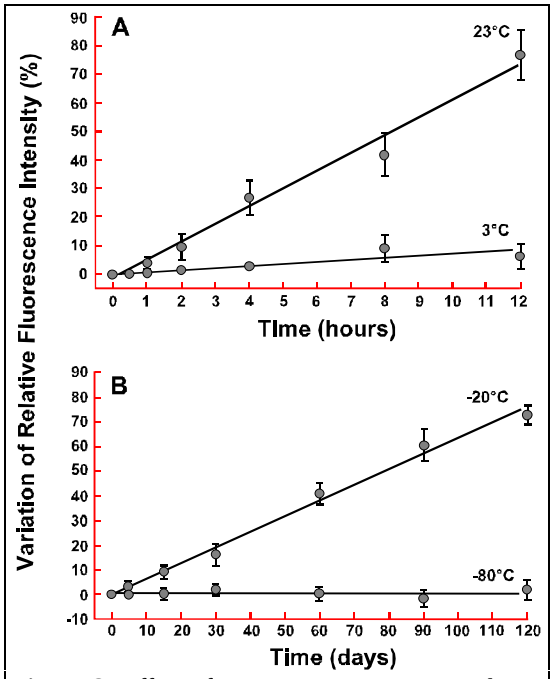

Figure 2. Effect of storage temperature on relative fluorescent intensity in skeletal muscle (mean and standard deviation). (A) Short-term storage at 23 and 3°C; (B) Long-term storage at -20 and -80°C. (From Hill and Womersley 1991).

Preparation procedures

Working on spectrofluorometrical analysis of native FAP in post-mitotic tissues of the teleost *Oreochromis mossambicus*, Hill and Womersley (1991) have demonstrated that:

- FAP-like fluorophores increase in vitro in brain, heart and muscle tissues and their extracts with increased storage temperature (-20°C and above) and time. This increase is linear with time (Fig. 2). Storage temperature of -80°C has to be respected.
- Sonification of homogenates greatly enhances this effect and generates other non-native fluorophores in sample solution. It also conducts to a shift in emission/excitation maxima.
- Fluorescence assay temperature also affects the results and some phase extracts contain large amounts of fluorescent flavin contaminants.

The variations observed in FAP concentrations are then submitted to different preparations constraints and it will be difficult to separate the variation due to native-FAP and those due to FAP-like substances. Nicol (1987) has clearly shown for crustacean that fluorescence readings depend on the previous treatment of the specimens: fluorescence decreases with conservation in formalin, ethanol and freezing. Environmental temperatures affect FAP levels in brain and heart (Hill and Womersley 1993). Moreover FAP accumulation seems to depend also on the feed quality and each organ exhibits tissue specific FAP accumulation profile (Hammer and Rao 1987).

The fluorescent microscopy method has not yet conducted to many criticisms. This is probably due to the fact that almost all works have been carried out by the same research team. Nevertheless the image processing technique used in this method is not well argued in the whole literature (Sheehy 1990a, 1990b, 1992; Sheehy et al. 1994, 1995, 1996). It seems rather a manual (and so more or less subjective) quantification tool than a semi-automatic or automatic one as image processing softwares are able to do nowadays. Besides that point, not any studies have really used specific image processing software for the quantification.

Relation FAP vs age

More than applications of the FAP methodologies to estimate the age of organisms, and particularly of fishes, published experiments have tried to show relationships between FAP contents and age (or size) of individuals. Mullin and Brooks (1988) estimated that variations in spectrophotometrically measured FAP are too important to give correlation with age in fish larvae. Taking care of the preparation procedure problems mentioned above, Hill and Womersley (1991) have shown positive correlation between FAP accumulation and chronological age of fish (Fig. 3). Nevertheless, the brain fluorescence is very variable for individuals of same age (Fig. 3). Given a value of RFI or % of whole brain fluorescence, it seems very difficult or even impossible to estimate an age as precise as otolith microstructures can do, at least during the first year of growth (Fig. 3).

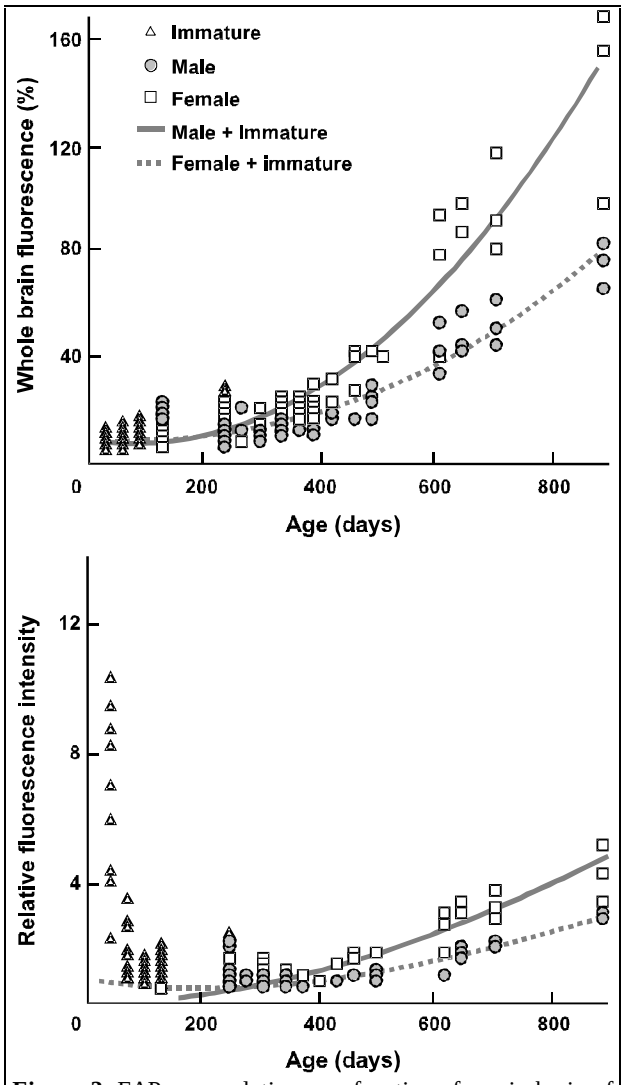

Figure 3. FAP accumulation as a function of age in brain of *Oreochromis mossambicus*. (From Hill and Womersley 1991).

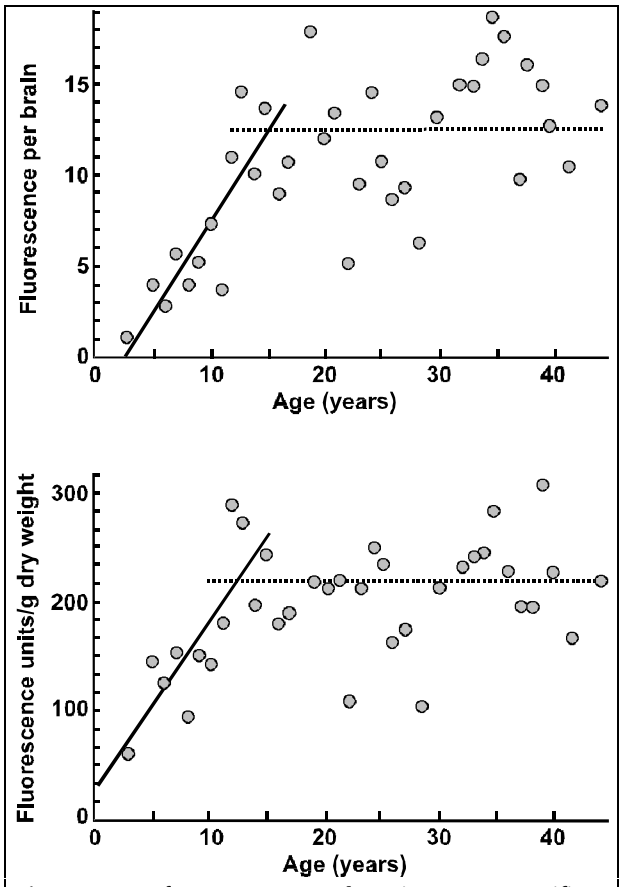

Figure 4. Lipofuscin in Dover sole, *Microstomus pacificus*, brain as a function of age. (From Vernet et al. 1988).

Most of the applications of lipofuscin accumulation technique in ageing fish have been carried out on young stages (Hammer 1988; Mullin and Brooks 1988) or on few years old specimens (Hill and Womersley 1991). Hill and Radtke (1988) finally showed important variations in FAP quantities for a damselfish during the first 7 years of its life (comparable to Fig. 3). For a old living fish (Dover sole), Vernet et al. (1988) demonstrated that concentration of lipofuscin increased over a wide range of lengths and with estimated age of 15 years but did not increase with older fish (Fig. 4). Moreover variations recorded by these authors are

considerably large (Fig. 4). All these results show the extreme complexity of the FAP analysis for age estimation application. Until now, no universal method has been improved in that way.

Perspectives

We have seen that the FAP quantification by spectrofluorometric method is submitted to numerous non endogenous variations. It has also never been used as a real application technique to estimate the age of fishes. Few works in this field are necessary to conclude if this method is really relevant for ageing.

The future ways of research in this field could be conduct on the histological analysis of post-mitotic tissue sections observed under epifluorescence. Actually the simple preparation of tissues seems to have less bias effects on the FAP quantification but further investigations could validate those suppositions. The quantification could also be automatized using a specific image processing software (similar to those used in the interpretation of calcified tissues). The specific development of an FAP quantification software could very simple and a such tool could reduce totally the interpretative subjectivity when observing the fluorophores. Moreover the ageing could be done routinely with light preparation of material.

The FAP methods for ageing fishes are at their beginning of development and they are still promising. Such tool associated with classical analysis of calcified structures and/or length frequencies analysis could contribute in an amelioration of the age estimations for management purposes.

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SECTION C) Statisitical analysis of age structure

AGE AND GROWTH DETERMINATION BASED ON LENGTH FREQUENCY METHODS: THE CURRENT STATE OF THE ART

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INTRODUCTION

Three basic characteristics for fish stock assessment are the mean length at each age, the distribution of lengths and the distribution of ages. Usually the distribution of lengths are the easiest to determine, needing only to take a large random sample of the stock and measuring the fish. Thus, many attempts have been made to infer the age distribution, the mean length at age and the growth parameters from the length distribution.

Length based methods for fish growth determination were firstly applied by Petersen to determine age-groups in *Zoarces viviparus* (1892). He developed two methods, the Petersen method and the modal class progression. The first one is based upon the identification of modes in a single length frequency and the attribution to them of a relative age. The second method requires a series of length frequency compositions, the modal classes in each length frequency are linked to the consecutive length frequencies in such a way that the growth in length along time is represented.

Almost at the same time Pearson (1894) developed the first statistical treatment of the problem of distinguishing overlapping component distributions for two components. Hasselblad (1966) first described the method for several components and made the computer program. However, the more widely used methods (Harding, 1949; Cassie, 1954), before the introduction of computers, were based on graphic approaches.

The appearance of modes in a length frequency plot depends upon the normality of each age group lenght distribution and a combination of the distances between means, the magnitudes of the variances, the proportion of the population in each age group, and the overall sample size. Polymodality is therefore an unreliable guide to underlying age groups. The mathematical form of the component size distributions must be assumed before any analysis more sophisticated than Petersen´s is possible (Macdonald and Pitcher, 1979). However, these methods are best applied to species with short recruitment periods and fast growth rates to avoid excessive overlapping between contiguous age classes. In this contribution we revise briefly the currently applied methods for length frequency analysis and made some comments in their suitability. A comprehensive bibliography is included for a more extensive review.

1-METHODS FOR MODAL ANALYSIS

METHODS BASED ON NORMAL SIZE DISTRIBUTIONS

If we assume that each component of the length distribution is normally distributed, each component can be identified by successive plotting of the distribution on normal probability paper. Working from one side of the distribution to the other, the components are graphically identified and removed in turn. Various methods of this type are described by Buchanan-Wollaston and Hodgson (1929), Harding (1949), and Cassie (1954). Elaborations of this method, which depend on determining inflection points in further derived graphs, have been published. The most widely employed in fisheries research is Bhattacharya's (1967). Who, based in the work of Buchanan-Wollaston and Hodgson (1929), Oka (1954) and Tanaka (1962), transformed the length frequencies into Gaussian components.

The logarithms of the consecutive length frequencies are subtracted and the accumulative frequencies are plotted in the y-axis and the lengths in the x-axis on probability paper. A series of linearly grouped points appear with clear inflection points between them. Each linear series is attributed to an age group and the inflexion points to the intermediate lengths between contiguous age groups. Using an iterative method the mean length and deviation for each age group are identified.

Pauly and Caddy (1985) simplified the calculation by transforming the linear relationships into a parabolic function. Their interactive computer graphic method is included in the ELEFAN programme.

Although the computer assisted programmes allow to carry the Bhattacharya method quickly, a great deal of imagination is necessary to identify the linearly associated clusters of points. When the length frequency is not clearly polymodal it is quite possible for two workers to obtain different results.

NONGRAPHICAL STATISTICAL METHODS

The nongraphical statistical methods that have been applied to mixture estimation problems include moments, maximum likelihood, and various minimum-distance methods. The method of moments is of historical importance (Pearson, 1894) but has low efficiency when more than two components are present in the mixture. Since then, the identification of the components in a distribution mixture have been studied extensively by statisticians. Recent developments were made by Macdonald (1975), Clark (1976), Odell and Basu (1976). McNew and Sommerfelt (1978) discussed difficulties than can occur when the distribution of lengths at each age is not normal as Hasselblad (1966) assumes.

2-VON BERTALANFFY PARAMETERS DETERMINATION FROM LENGTH FREQUENCY DATA

Pauly and David (1981) developed an integrated method based on both Petersen approaches. The length frequencies are plotted in a time series (if only a length frequency is available it is repeated along a time axis), then the modes are identified and a von Bertlanffy growth curve is fitted. The modes corresponding to supposed age groups are identified by means of a transformation of the length frequency that converts it in positive and negative values. The transformation consists for each

length interval, of subtracting from 1 the result of dividing the frequency value by a mobile average of the 5 contiguous frequencies. A set of growth parameters are interactively calculated trying to maximise the number of positive values hit in relation to the total number of positive values present in the length frequency (Rn = goodness of fit value). This procedure is roughly similar to fit a r^2 value.

Shepherd (1987) developed a method conceptually similar to Pauly and David's. This method, as well as ELEFAN and Projection matrix method (Projmat), are included in the package LFDA (Holden and Bravington, 1992). This package allows to maximise the estimates and determines the probability of the results.

A method developed by Fournier *et al*. (1990), MULTIFAN, combine the modal analysis and direct estimation of growth parameters.

3. Methods integrating length frequency data and age structure

Macdonald and Pitcher (1979) showed how some variations of Hasselblad's maximum likelihood algorithm can be applied to fish stocks. They developed an iterative computer program that uses maximum likelihood and biological information (mean length at age, number of age groups, s.d., variance) identified the components of the length distribution. Once the theoretical distribution based on the information imput is fitted to the observed data, the parameters are calculated interactively minimising the discrepancy between both. The different algorithms that can be used are discussed by Macdonald and Pitcher (1979). The age stratified subsample necessary to obtain the biological information is much more reduced than the age sample necessary to calculate the population parameters. The sampling effort can also be concentrated in the older age groups where the length overlapping is greater. However, the method is sensitive to sampling. An adequate sampling is necessary to obtain an adequate representation of the size or number smaller groups.

A single procedure to determine the percentage of fish at each age, the mean length and standard deviation (s.d.) in length at each age, as well as the von Bertalanffy growth parameters, was developed by Schnute and Fournier (1980). The procedure require that the means and/or s.d. should conform to a growth pattern. To include the von Bertalanffy growth structure in the procedure, the growth parameters should be reformulated (Schnute and Fournier, 1980). More biological structure leads to less ambiguity, due to the restriction of solutions which are biologically meaningful.

Macdonald and Green (1985) developed the MIX interactive program for fitting mixtures of distributions using Macdonald and Pitcher (1979) and Schnute and Fournier (1980) approaches. This program alternates between constrained direct-search optimisation and fast iterative calculations. Given the number of components in a distribution mixture, the estimation of the proportion of fish in each age-group, the modal means and standard deviations and their standard errors are calculated. The best fit is determined by the minimisation of the χ^2 value between the observed and calculated length distributions. Although the method is not graphical, an interactive screen is included to visualise the fit of the results to the data.

Morgan (1987) using the transformed von Bertlanffy equation with length as the dependent variable developed by Kirkwood (1983), applied age data on the ELEFAN calculations. The best combination of growth parameters is which maximises the goodness of fit determined for both the transformed length at age data and the length frequency distributions. This approach was incorporated into ELEFAN V and the LFSA software package.

4- METHODS USING OTOLITH SIZE FREQUENCY DATA

Otolith growth is conservative to fish growth giving rise to larger and heavier otoliths in slow growing than in fast growing fish of the same size (e.g. Templeman & Squires, 1956). This will lead to an age effect in the otolith size-fish size relationship that also in some cases have been employed for age determination purposes (Pawson, 1990). Also without information on fish length, otolith weight, length, and width were found to be excellent multivariate predictors of rockfish age (Boehlert, 1985).

Most analyses of fish length frequency have been combined with readings of annual structures in otoliths or other hard parts, for mutual confirmation of the methods. In some cases length frequencies from time series have been analysed (e.g. Morales-Nin, 1991). Very few studies, however, have focused on the analysis of otolith size frequency data. Fletcher (1991) found and later validated (Fletcher, 1995) a much higher modal resolution when comparing analyses of otolith weight frequency with fish length frequency in pilchard.

5- SUGGESTIONS FOR FURTHER RESEARCH

The obvious extension of the presented methodologies would be to use time series information, when available, on size frequency data for both otolith and fish morphometric measurements combined. By analysis of the progression of deviations from constant or running median values, individual year groups might be identified and utilised in further analysis of growth or age structure.

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2. Multivariate analysis and data structure

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The structure of the otolith increment data require special considerations during statistical analysis. The statistical techniques employed depend on field of study, and a short list of possible applications of otolith microstructure information is listed below together with appropriate statistical methods.

1) Analysis of increment number at age, validation studies (linear regression analysis)
2) Analysis of increment width pattern, otolith growth (multivariate and repeated measures analysis, discriminate function analysis)
3) Back-calculation of fish growth (linear and non-linear regression analysis)

In the following, the first section (validation) will be briefly discussed, but the main emphasis will be on aspects of increment width data. Aspects of statistical properties of back-calculation algorithms is not treated here, but treatments of this subject can be found in Campana (1992), Francis (1995), Zivkov (1996).

Aspects of validation

Cell 4 in EFAN deal specifically with aspects of validation. In this section only two statistical concerns will be dealt with. These are:

- Problems due to non-homogenous error. A common finding when plotting increment number versus age is the absolute increase in deviation of data points from the common regression line with increasing age. This corresponds to a situation where the absolute ageing error is not constant. Efforts should be made to standardise the magnitude of errors over the entire range to comply to the underlying assumptions of regression analysis.
- Problems due to aliasing. The error structure of the increment number versus age relation is skewed at young ages (few increments) due to the inherent censoring caused by increment counts being non-negative. Omitting data on the youngest fish from the regression relation can be a way of avoiding this bias. The results from the age restricted regression should be compared with the overall regression to check if the slopes are significantly different.

Aspects of otolith increment width data structure:

The increment widths of an otolith represent an array of data which are to some extent interrelated. When analysing the size of cumulative radii, the correlations of consecutive radii

sizes are evident. Correlation have also been shown to be present when individual consecutive increment widths are compared. These interrelation is caused by:

- The increment widths being dependent on the choice of sector (and in otoliths of adult fish also section) read (Campana, 1992). Inconsistencies in choice of sector read, e.g. largest possible radius, will result in a scaling compared to readings done on along a different sector.
- General otolith deposition pattern. The correlation structure of increment data is important for specification of growth models (Weisberg, 1993). Increment widths tend to increase and decrease gradually from previous increment widths due to inertia in the deposition process. A sudden stop in the food supply does not generally cause an immediate stop increment formation. This will generate a autocorrelation of the increment widths, where the degree inertia will determine the magnitude of the correlation.
- Limitations and simplifications in the reading procedure. Some custom otolith reading programmes estimate individual increment widths as averages from several increments creating additional similarity between successive increment width measures. The resulting error structure of the width data will be affected. This type of increment width averaging is common for otolith regions where the readability is limited. The inner increments widths can be below the resolution limit of light microscopy, and a common value is often used until individual increment widths can be read (Andersen and Moksness, 1988). A continuous increment pattern thus often consists of a combination of actually measured increment widths, interpolated increment widths and pre-determined increment widths.
- Reading error. If an otolith is read more than once, the number of increments and their respective widths are negatively correlated since an incorrect underestimation of increment number will overestimate increment widths and vice versa. A common observation during otolith reading is also the shift in increment location due to focusing. In this instance an overestimated increment width on one hand may lead to an underestimation of the following increment width and vice versa.

In addition to the problems linked to the reading and measurements of available otoliths, it is essential to question the representativeness of the otoliths used in the study. A non-random sampling of larvae from the population under investigation will result in a biased otolith sample available for analysis. This can be due to selective mortality or avoidance of larvae to sampling gear. Unless this is an explicit part of the investigation in progress, no statistical methods can counteract the problems of an inappropriate sample. Another issue that is of great concern is the exclusion of data (i.e. otoliths). This may have been done for several reasons, one of the most common being that the otolith in concern was difficult to read with desirable precision. The risk of selectively excluding otoliths from fish with special growth characteristics should warrant special attention, and exclusion of otoliths should be kept at a minimum.

<u>Recommendations:</u>

- The data structure of the increments widths should be analysed with multivariate statistical techniques. A repeated measures ANOVA or a MANOVA (multivariate ANOVA), can both take into account the inherent autocorrelations, but the MANOVA is generally preferred due to less rigorous assumptions about the underlying data structure (Chambers and Miller, 1995). The effect of using univariate techniques without correcting for data dependence, will be inflated

degrees of freedom in the statistical tests, yielding artificially high precision and thus increased likelihood of type I error.

- Investigations regarding the correlation structure of increment widths of individuals reared under varying environmental conditions should be carried out.
- Special attention should be paid to scaling algorithms that combine several independent readings from the same otolith. In many instances this will involve the combination of readings with varying increment numbers, and thus require some splitting/joining of increments to achieve a common increment pattern (Methot, 1981). In cases where the sector read is of varying size, the use of relative increment widths (to the total radius f. ex.) may provide the needed basis for estimating a common otolith growth pattern.
- From a statistical point of view, single readings of individual otoliths would yield a relatively simple error structure suitable for analysis.
- Computer intensive techniques can be an alternative statistical method when the underlying assumptions of traditional statistical methods cannot be met. Bootstrapping can yield confidence intervals of increment widths and check sizes (e.g. Anderson, 1995).
- A proper larval sampling strategy is essential. A clear definition and documentation of which are the population units under investigation is required to be able to make correct between sub-population or temporal within population comparisons. A different growth pattern obtained from two samples collected at different times and places, can be either due to differences between sub-populations or differences within a sub-population (caused by selective mortality or gear selectivity).

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