THE GROWTH CHARACTERISTICS OF SPARID OTOLITHS

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JUDY BRENDA LANG

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ABSTRACT

The periodicity of formation of growth increments in the otoliths of South African sparids was validated by the oxytetracycline labelling technique. Intramuscular injections of oxytetracycline at a dosage of 250mg/kg marked the otoliths of laboratory held juvenile sparids, while a dosage range of 50 - 100mg/kg oxytetracycline, injected intramuscularly, marked adult sparids in the field. Laboratory held, larval sparid otoliths were marked by immersion for 24hrs a solution of 100-150mg/l alizarin complexone. Both daily (microstructural) and annual (macrostructural) growth increments were identified in the otoliths.

The microincremental pattern of deposition in the sparid otoliths conformed to the general pattern of otolith structure. Sparid otoliths consisted of a central opaque nucleus composed of multiple primordia. Surrounding this nucleus were daily increments which decreased in width as the distance from the nucleus increased. Both check rings and subdaily increments were visible throughout the otolith. Minor environmental changes did not affect microincremental deposition.

Narrow opaque and wide hyaline annual growth zones were identified in sectioned sparid otoliths. Scanning electron microscope analysis of the annual growth zones revealed that microincrements within the opaque zone were narrowly spaced with prominent discontinuous phases. This resulted in the greater optical density and higher protein content of the zone. The hyaline zone was composed of widely spaced daily increments with prominent incremental phases accounting for the translucent nature of this zone. Opaque zone formation in the otoliths of many South African sparids was found to occur primarily during periods of reproductive activity and was shown to be indicative of slow otolith growth. The hyaline zone was formed after the spawning season, representative of fast otolith growth.

The results of this study have resolved much of the controversy surrounding the rate of growth and time of formation of the opaque and hyaline growth zones in South African sparid otoliths.

CHAPTER 1. INTRODUCTION

The interpretation of the pattern of growth zones in scales, otoliths, spines and rays, vertebrae and other bony structures, also known as osseochronometry, is the most generally accepted method for age determination in fish. In these tissues patterns of variable optical intensity are formed in response to seasonal changes in the environment such as temperature and photoperiod, changes in metabolic rate during reproductive activity or starvation and to endogenous rhythms (daily, monthly or annual). Accurate age interpretation depends on correctly associating a recurrent pattern in the hard tissue under examination with factors which influence the rate of growth or metabolism of the fish.

In South Africa, otoliths have been extensively used to age sparid fishes (Hecht & Baird 1977; Nepgen 1977; Coetzee & Baird 1981; Buxton & Clarke 1986; 1989; 1991; 1992; Pulfrich & Griffiths 1988; Smale & Punt 1991; Mann 1992; Buxton in press), which are important to both the recreational and the commercial linefisheries off the South African coast (Smale & Buxton 1985; Clarke & Buxton 1989; Penney, Buxton, Garratt & Smale 1989; van der Elst 1989). Even though otoliths are the most common structures used in age determination (Six & Horton 1977), considerable confusion surrounds the interpretation of the growth zones (Pannella 1974; Williams & Bedford 1974; Buxton 1986; Buxton & Clarke 1986; 1989). This confusion arises because the physiological processes governing zone formation in otoliths, and other hard tissues, are not well understood (Blacker 1974). The accuracy of age estimates is dependent on validating the study. No direct validations of sparid age estimates have been done, although marginal zone analysis has provided indirect validation. The importance of a direct validation of age interpretation for South African sparid otoliths has therefore often been stressed (Hecht & Smale 1986; Buxton & Clarke 1989).

Age assessment from otoliths is highly subjective (Weatherly &

Gill 1987) and until the interpretation of the growth patterns are validated, the determined age is only considered an estimate of the true age. If the zones are indistinct, variable in appearance or coalesce as a result of decreased growth rate at higher ages, the age interpretation is difficult and produces highly variable results. Such problems have been experienced in some sparids (Hecht & Smale 1986; Smale & Punt 1991). In order to decrease this variability it is imperative that the validity of the age estimates be directly determined. It has been suggested that validation should be an essential and routine part of every study that involves the extraction of age data from otoliths (Beamish & McFarlane 1983; Weatherly & Gill 1987). However, only indirect methods, which help to verify the interpretation rather than provide direct validation, have been used in South African sparids. With the exception of Pulfrich and Griffiths (1988), who used daily increment analysis, validation of most South African sparid ageing has been achieved through marginal zone analysis (Coetzee & Baird 1981; Buxton & Clarke 1986; 1989; 1991; 1992; Mann 1992; Buxton in press).

The accuracy of an age estimate can be tested by labelling otoliths. Labelling is usually performed using wild fish which are captured, tagged, injected with the label (e.g. a chemical fluorochrome) and released. The label, which is deposited at all sites of calcification, provides a precise temporal and spatial mark in the otolith. When the fish are recaptured, the otoliths can be removed and examined for the label to ascertain if they contain the presumed number of growth zones that correspond with the known time at liberty. Although labelling techniques have been available for many years (Weber & Ridgway 1962), to date, attempts to chemically mark South African sparid otoliths in order to rigorously validate the ageing convention used have proved unsuccessful (Buxton & Allen 1989).

One of the problems associated with otolith interpretation is the confusion surrounding the deposition of the annual growth zones. This encompasses three major areas: i) the rate of growth

represented by each zone, ii) the time of formation of each zone and iii) the chemical composition of each zone.

The aim of this study was to investigate the correlation between otolith zone deposition and somatic growth in sparids. Key objectives were :

i) To determine an accurate labelling technique for sparid otoliths.

ii) To use this labelling technique to validate the periodicity of increment formation.

iii) To investigate the optical characteristics and chemical composition of the otolith growth zones.

CHAPTER 2. LITERATURE REVIEW

History of fish ageing

The importance of determining the age of fish as a tool in fisheries science is undisputed (Bagenal 1974; Summerfelt & Hall 1987). Assessment and management of environmentally and economically important species requires a sound data base of this and other life history parameters. However, information necessary to determine population structure is often lacking due to difficulties inherent in both the determination of age and subsequent calculations of growth rate, mortality and age at maturity (Radtke, Kinzie & Folsom 1988). These difficulties make rational resource management decisions problematic. Accurate fish age data are essential for the calculation of growth, which, in conjunction with mortality rates and a measure of recruitment, provide the three most important population rate functions essential for the proper assessment and management of fisheries (Summerfelt & Hall 1987).

Aristotle documented the earliest studies on the ageing of fish, by Greek fishermen on tunas nearly 2000 years ago, in his "Historia Anumalium" (Bell 1964 in Radtke & Morales-Nin 1989). The first account of reliable age determination from fish vertebrae was recorded in 1759 by Hederstrom (Ricker 1975), while otoliths were first used for age determination by Reibisch in 1899 (Carlstrom 1963). Since the late 19th century, age related studies have been an important part of any detailed investigation of the biology and stock assessment of a fish species (Carlstrom op. cit.).

Age determination

Growth in fish is a continuous process inversely related to age but is responsive to both internal and external environmental factors (Weatherly & Gill 1987). Cyclical variation in the somatic growth of fish, caused by internal factors (e.g.

spawning and stress) and external factors (e.g. temperature, food availability and photoperiod), is known as plasticity (Weatherly 1990). This plasticity affects the formation of calcified tissues and is registered as growth zones in the hard parts of fish (Radtke, Targett, Kellerman, Bell & Hill 1989). For this reason features interpreted in otoliths as age marks can only arise from time-dependent changes in the physiological growth of the fish (Gauldie & Nelson 1990). The interpretation of growth zones which appear on the hard parts of fishes is the most frequently used method of age determination. Scales, opercular bones, fin rays, vertebrae and even eye lenses are all structures that are used in this respect (Bagenal & Tesch 1978), but by far the most important and commonly used structures are the otoliths (Six & Horton 1977).

Otoliths

Unlike scales and other hard structures, otoliths do not appear to be susceptible to resorption during periods of physiological stress (Campana & Neilson 1985; Casselman 1990), and they undergo no chemical changes once formed (Campana 1983). Otoliths are particularly useful in species where no other hard structures are suitable for age determination, for example the bluefin tuna, Thunnus thynnus thynnus (Radtke & Morales-Nin 1989).

Teleosts generally have three pairs of otoliths: the sagittae, asterici and lapilli, located in the membranous labyrinth of the inner ear on either side of the brain (Lowenstein 1971). Except for fishes of the order Cypriniformes and Siluriformes, the sagittae are used for ageing because they are the largest of the three otoliths (Hecht 1979). The primary functions of otoliths are the maintenance of balance and the mechanical component of the sound transduction mechanism responsible for hearing in fish (Gauldie & Nelson 1990). The shape of the otolith is generally species specific (Hecht 1978) and determines the particular sound frequencies for which the otolith acts as a transducer (Gauldie

1988a).

The structural and chemical composition of otoliths can give rise to a number of different patterns, each with a specific origin and function. Analysis of the structural and chemical differences in these patterns can provide a retrospective life-history profile of the individual, as well as a recording of environmental changes. From a management perspective, these data are invaluable to the understanding of the processes underlying growth rates and recruitment (Radtke 1989a).

Otoliths are crystalline structures composed of the aragonite morph of calcium carbonate (Irie 1955; Carlstrom 1963) in its twinned form (Gauldie & Nelson 1988). These needle shaped crystals radiate in three dimensions, from a central area, through an unevenly distributed organic matrix of protein termed otolin (Degens, Deuser & Haedrich 1969). The long axis of the crystals are orientated nearly perpendicular to the outer margin of the otolith (Irie 1955; Mugiya, Watabe, Yamada, Dean, Dunkelberger & Shimizu 1981). Incremental growth occurs through differential deposition of calcium carbonate and protein in the otolith such that a series of bands is formed which are widely, and often differently, interpreted as indicators of the age of the fish (Gauldie & Nelson 1990).

Macrostructural analysis

It is assumed that a regular pattern in the deposition of organic and inorganic material in the otolith forms recognizable zones which can be related to time. The first zones in otoliths interpreted to have temporal significance were termed annual growth zones. When an otolith (whole or sectioned) is viewed under low magnification with either reflected or transmitted light, it shows a pattern of light and dark zones similar to those laid down in the trunk of a tree. It is the appearance of these zones, their relative rates of deposition and their chemical composition which have been the focus of much debate

since the 1960's.

Table 1 gives a comprehensive overview of otolith interpretation from 1947 - 1989. Work from 1947 to 1964 determined that the hyaline zone was composed primarily of calcium carbonate and represented fast growth. However, from 1964 onwards a great deal of confusion arose concerning the interpretation of the zones. Christensen (1964) deduced that the proteinaceous band was located between the opaque and hyaline zones while Baird (1970) and Botha (1971) found the opaque zone to be representative of fast growth. Blacker's (1974) review of otolith structure supported this interpretation which was followed by most authors after 1974. The conventional interpretation of otoliths was later questioned when Buxton (1987) found the hyaline zone to be representative of fast growth in a number of South African sparids.

Problems associated with otolith interpretation

By 1974 there was already some confusion surrounding the chemical composition and rate of deposition of the two zones as well the relevant terminology. Pure chemical data appeared to be consistent with the view that the opaque zone was composed primarily of protein (Irie 1957; 1960; Mugiya 1964a) but this interpretation was confused by the application of the burning technique and the effects of different lighting conditions (Christensen 1964; Blacker 1974). For this reason it was necessary to determine how much confusion stemmed from the inversion of terms and how much was due to genuine differences in the seasonal growth pattern of different species from different areas.

The abundance of synonymous terms used to describe the optical appearance of the growth zones resulted in many apparent contradictions. These terms included light, dark, narrow, wide, opaque, hyaline, translucent, fast, slow, white, black and transparent. It is important to note that these terms refer to

the visual appearance of the otolith zones and do not necessarily describe their chemical composition. A variety of structures including opaque and hyaline annuli, check rings, daily growth rings and subdaily rings are all usually found within the same otolith and the use of different structures in the determination of age can produce inconsistent results (Gauldie & Nelson 1990).

To understand the apparent contradictions it is important to understand how growth zones are formed. The overall size of the otolith is determined by the rate of crystal deposition which in turn is determined by similar metabolic processes that effect the overall size of the fish itself (Gauldie 1988a; Casselman 1990). The rate of growth of the otolith is therefore a function of the metabolic rate of the fish (Gauldie & Nelson 1990). Temperature, food availability and general physiological state all affect the rate of growth of fish. Recent work on the microstructure of otoliths has shown that growth occurs through the differential deposition of calcium carbonate and protein during an endogenous circadian rhythm (see review by Campana & Neilson 1985). Incremental growth is primarily calcium carbonate which has hyaline optical properties, while discontinuous growth appears opaque and is mostly organic (Tanaka, Mugiya & Yamada 1981; Campana & Neilson 1985). During periods of slow growth the width of the daily incremental phase is narrower and the relative proportion of protein is high (Campana & Neilson 1985). Because discontinuous growth appears opaque, the slow growth zone should appear opaque. Increased incremental growth on the other hand will result in decreased opacity due to the higher calcium content (Buxton 1987).

In unburnt transverse otolith sections the incremental (fast) growth is hyaline (translucent) and appears as a wide, dark zone when viewed under reflected light. The discontinuous (slow) growth appears as a narrow white band when viewed with the same technique. This relationship can be seen in whole otoliths from smaller fish but is not clearly visible in larger whole otoliths because the increased thickness of the otolith results in an

increase in the opacity of the whole otolith. In whole otoliths the opaque zone often appears to be wider than the hyaline zone. Slight charring of the otoliths is a quick and easy technique that can be used to determine the location of the protein dominant zone in the otolith. In burnt otoliths the opaque zone turns brown, indicating a higher protein content (Dannevig 1956; Christensen 1964; Campana & Neilson 1985; Buxton & Clarke 1989). Because an opaque or hyaline band only becomes visible once the change in zone composition becomes great enough to produce a change in optical density, the distinction between a formed or a forming band can add to the general confusion associated with interpreting otoliths (Pannella 1974).

Many of the interpretive problems have probably resulted from the different optical properties of whole and sectioned otoliths as well as the characteristic appearance of the growth zones under different lighting conditions. The failure by many authors to state clearly the techniques used increases the confusion. An opaque zone is optically dense and inhibits light passage. It will reflect light and appear white or lighter than the hyaline zone which will absorb light and appear dark. These optical characteristics are reversed under transmitted light (Fig. 1). In this thesis **opaque** (optically dense) and **hyaline** (translucent, transparent) zones will be used to represent the two forms of annual growth.

It is important to note that the confusion surrounding the interpretation of the relative rate of growth in different zones of the otolith does not invalidate the estimate of age as long as zone formation can be validated and accurately related to a time period (Buxton 1987).

Microstructural analysis

Macrostructural opaque and hyaline zones result from patterns of daily microstructural increment formation. Although annual zones are still routinely used in age determination, particularly

of older fish, they are augmented by the study of daily increments. Degens et al. (1969) first photographed and used daily growth increments in his determination of the molecular structure and composition of fish otoliths, but it was Pannella (1971) who recognized their daily periodicity. Daily increments have since been documented by a number of other workers and are routinely used in ageing studies of larval and juvenile fish (Brothers, Matthews & Lasker 1976; Wild & Foreman 1980; Radtke & Targett 1984; Wilson 1988; Radtke et al. 1989; Schultz 1990). Although the methodology of visualizing microincrements in adult fish is time-consuming and tedious, the process can yield valuable data not accessible in any other way (Radtke, Fine & Bell 1985). These concentrically formed increments have considerable potential to both increase the precision of age determination and may provide a dated record of ecological and physiological events through the lifetime of an individual fish. Daily rings in otoliths have, for example, been used to determine the temperature histories (Radtke 1989a; Radtke, Townsend, Folsom & Morrison 1990) and migration patterns (Radtke et al 1988; Radtke & Morales-Nin 1989) of various fish species.

Daily growth increments in otoliths are bipartite structures consisting of a relatively wide zone of calcium carbonate embedded in a protein matrix, and an adjacent narrower band composed primarily of protein (Campana & Neilson 1985; Wright 1991). Mugiya et al. (1981) and Tanaka et al. (1981) found calcium carbonate deposition in the otolith to slow down at sunrise, thereby allowing the formation of the protein zone. Incremental growth, therefore, occurs through differential deposition of calcium carbonate (aragonite) and protein (otolin) over a 24 hour period (Campana & Neilson 1985). The relative amounts of calcium and protein deposited in the daily increments varies in relation to somatic growth during the life of a fish. These variations in the daily increments result in the formation of wide seasonal growth zones with the different visual properties termed opaque and hyaline.

Daily increments are not always evident in all species or at all ages. Pannella (1971) found that in temperate species the number of microincrements deposited annually decreased with age and that the decline became more pronounced with the onset of sexual maturity. Various environmental factors such as photoperiod, temperature and food availability have also been found to influence the deposition of the daily rings (Brothers et al. 1976; Taubert & Coble 1977). In addition, the number of rings may be overestimated through the production of more than one ring per day or underestimated through poor otolith preparation (Taubert & Coble 1977; Beamish & McFarlane 1983). The formation of check rings in the daily increment sequence can record periods of physiological stress but may also be produced with a lunar periodicity. These check rings can further confuse the interpretation of daily and annual growth zones (Brothers et al. 1976; Campana 1984a) and verification of the age related increment zone production is essential for any age assessment study. Experimental study of the environmental factors which may influence the deposition of increments in the otolith is necessary to understand the full extent of the influence of both internal and external factors on the formation of increments (Radtke & Dean 1982).

Validation of ageing techniques

In spite of the important role of estimating fish age in fisheries management, validation of ageing conventions has proved difficult. Validation is defined by Beamish & McFarlane (1983) as "proving that a technique is accurate". The conventional techniques for ageing have been universally applied in the belief that the technique "is a simple one which can be applied without technical knowledge" (Van Oosten 1941 in Beamish & McFarlane 1983, p. 735). A review by Beamish & McFarlane (op. cit.) revealed that out of 500 papers published on fish ageing between 1907 and 1980, only 17 had actually validated age estimates for all age groups. The consequences of errors in ageing can be relatively minor if the units of age determination are small e.g.

daily. However, if the units are annual, even minor errors can have serious management implications.

Three methods can be applied in the validation of daily growth increments. Firstly, laboratory reared larvae of known age provide the best material with which the frequency of increment formation may be determined (Brothers et al. 1976; Barkman 1978; Radtke & Dean 1982). The number of days elapsed since hatching can be compared with the rings counted on the otoliths of these fish and from this the frequency of ring deposition can be determined. For many species, rearing the larvae from birth through the juvenile stage is difficult or impossible and alternative methods of validation are required. Secondly, the change in the mean number of increments formed over time in fish captured in the wild and held in captivity may be used. The number of rings in fish killed immediately after capture is compared to those in fish maintained in captivity, assuming that all the fish are of the same age at capture (Wilson 1988). A variation of this technique is the sequential sampling of a population in which neither age-selective mortality or migration is assumed to occur (Radtke 1989b). Thirdly, a time mark can be introduced into the otolith growth sequence (Brothers 1990). A distinguishable mark, identifying the growth increment at that particular time, can be used as a reference point from which further growth increments can be counted. This technique can be applied to either laboratory held or wild fish and uses either chemical or intrinsic marks.

Indirect validation of annual growth zones can be achieved through marginal zone analysis (Manooch 1982), analysis of length frequency modes, monitoring of strong year classes or through the comparison of back-calculated lengths with observed lengths at age (Beamish & McFarlane 1983). However, direct validation can only be achieved through mark-recapture studies with marked otoliths or through long-term laboratory studies (Weatherly & Gill 1987).

It is clear that broad application of otolith interpretation techniques, without complete comprehension of otolith structure and formation, can lead to many erroneous conclusions (Radtke 1987). In order to accurately interpret the depositional increments in otoliths it is important to understand the physiological mechanisms involved in the formation and growth of both increments and otoliths, as well as the factors which influence these processes.

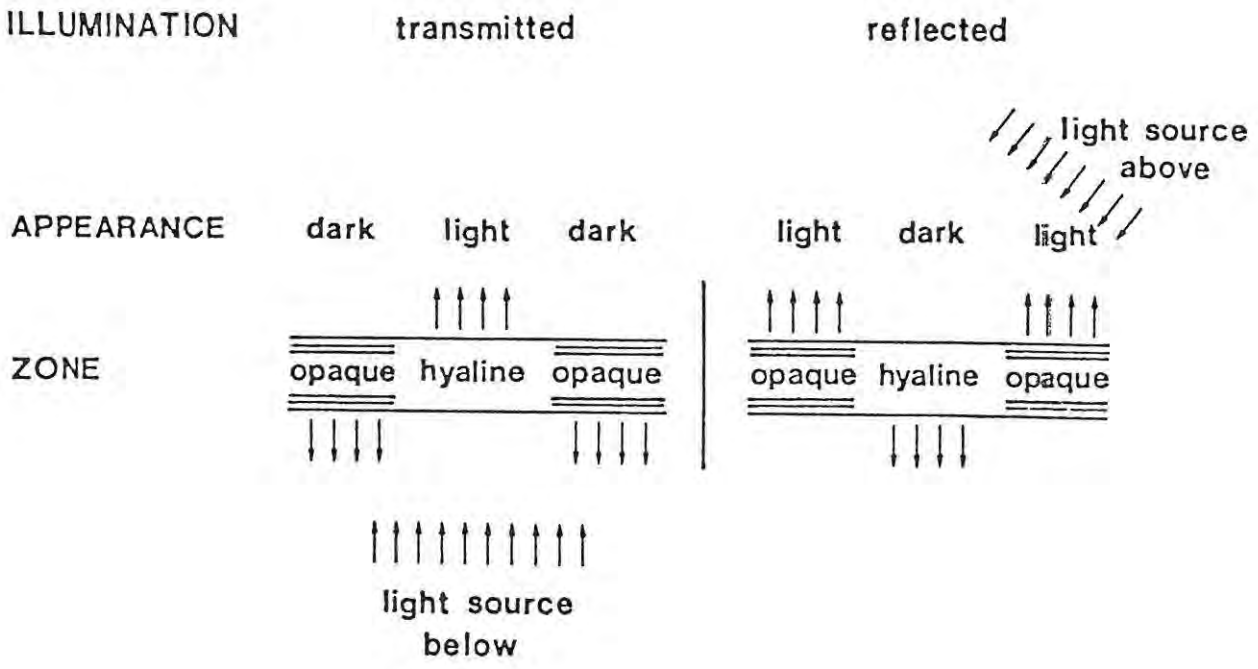

Figure 1 The appearance of opaque and hyaline zones viewed by transmitted and reflected light. (Arrows indicate direction of light. Opaque zones do not allow the passage of light. Hyaline zones permit light passage.) (after Buxton 1986).

TABLE 1. Different interpretations of annual growth zones in the otoliths of fish.

SOURCE	FISH (where stated)	COMPOSITION OF FAST GROWTH ZONE	COMPOSITION OF SLOW GROWTH ZONE
Molander 1947	Plaice	Hyaline (Calcium carbonate)	Opaque
Dannevig 1956	Cod	Hyaline (Calcium carbonate)	Opaque (Protein)
Irie 1957	Argyrosomus Pseudociaena Lateolabrax	Hyaline (Calcium carbonate)	Opaque (Protein)
Mugiya 1964a	Kareius Carassius Salmo & Limanda	Opaque (Protein)	Hyaline
Christensen 1964	Solea	Protein found between opaque and hyaline zone	
Baird 1970	Sardinops	Opaque	Hyaline
Botha 1971	Merluccius	Opaque (Calcium carbonate)	Hyaline (Protein)
Blacker 1974	Review	Opaque	Hyaline (Protein)
Coetzee & Baird 1981	Cheimerius	Opaque	Hyaline
Thomas 1983	Sardinops	Opaque	Hyaline
Buxton & Clarke 1986	Pachymetopon	Opaque	Hyaline
Pulfrich & Griffiths 1988	Pachymetopon	Opaque	Hyaline
Japp 1990	Genypterus	*Opaque	Hyaline
Buxton & Clarke 1989,1991	Sparidae	*Hyaline (Calcium carbonate)	Opaque (Protein)
Buxton in press	Chrysoblephus	*Hyaline (Calcium carbonate)	Opaque (Protein)

* Sectioned otoliths examined.

CHAPTER 3. GENERAL METHODOLOGY

Laboratory experiments

Juvenile sparids including blacktail, Diplodus sargus capensis, zebra, D. cervinus hottentotus, streepie, Sarpa salpa and Cape stumpnose Rhabdosargus holubi, were chosen for the study because they are abundant in intertidal rockpools and estuaries in the Eastern Cape, are easily captured, hardy and adapt well to laboratory conditions. Specimens between 8 and 200mm caudal fork length (FL) were collected using throw and seine nets from the Kowie River and from intertidal rock pools in Port Alfred between June 1989 and April 1991. Fish were allowed to acclimatize to laboratory conditions for a minimum of three weeks prior to the commencement of any experiments.

Fish were maintained in aerated, recirculating biologically filtered aquaria with a retention time of approximately two hours. Tanks were cleaned regularly by siphoning out debris. The fish were maintained under a 16:8 light:dark regime and fed once daily on a mixture of chopped pilchard, chopped squid, open, whole mussels (Perna perna) and a formulated dry flake feed (TetraMin). The temperature in the tanks varied between 15°C and 24°C. Salinity remained between 32 and 35ppt. Any fish with signs of the protozoan ichthyoparasite Cryptocaryon irritans (Grabda 1991) were immediately treated with a $CuSO_4$ solution, (1g $CuSO_4$/1l distilled water, 250ml solution/75l tank water).

Otolith preparation

At the end of each experiment fork length (0.01mm) and weight (0.1g) were recorded before both sagittal otoliths were removed. Ultrasound was used to remove adhering tissue from the otoliths which were stored dry, in the dark, until viewing. Both the lapilli and the asterisci were examined initially, but these proved to be too small for detailed analysis. The right sagittal otolith was subsequently used in all analyses except in cases

where the right otolith was not available and the left was used. Examination of both otoliths in this and previous studies indicated no difference in internal structure between the two otoliths (Boehlert & Yoklavich 1984; Molony & Choat 1990).

Whole otolith analysis

Sagittal otoliths from 261 adult D. sargus capensis and 241 D. cervinus hottentotus were collected between May 1989 and December 1991 in the Tsitsikamma National Park (TNP), a marine reserve extending 5.6km offshore and covering a 60km stretch of coast off the south-east coast of Southern Africa. Otoliths were measured to establish whole otolith growth in relation to fish growth. The otoliths were weighed (to 0.001g) and measurements of otolith length and width were made (to 0.01mm) using vernier calipers (Fig. 2). All measurements were made as close as possible to the nucleus. The size of the otoliths was related to fish fork length by regression analysis. Whole otoliths were examined under a microscope at low power with transmitted light both prior to and after burning over a low intensity spirit flame (Buxton & Clarke 1986). Care was taken not to char the otolith as this obscured the internal structure.

Preparation of otolith sections

Whole otoliths from fish less than 15mm were viewed without any preparation as the daily increments were clearly visible. The otoliths were mounted, sulcul side upward, on glass microscope slides in the synthetic mountant and clearing agent dibutyl-phthalate-polystyrene-xylene (DPX) and were viewed under transmitted light.

Otoliths from fish 15 - 25mm were embedded, anti-sulcul side upward, in clear casting resin on a glass slide and were ground to a plane where the peripheral growth increments were most visible (Steffensen 1980). The otoliths were first ground by hand with 600 grit aluminium oxide grinding paste followed by 300 grit

lepigate illumina paste. Otoliths were then polished on a Struers rotating polishing machine to ensure even polishing on the entire surface of the otolith. Fifteen micrometer diamond spray was used for the first 20 - 30 minutes of polishing, followed by 5um diamond spray for 10 minutes. The above process yielded the best polished surface, without loss of the outer edge of the otolith. The otoliths were checked regularly during the grinding processes to ensure that the thin edges of the otoliths were not ground away. Between each grinding or polishing step the slides were esonified to clear away adhering grit. Problems were encountered during the grinding process as the exact plane of grinding necessary to locate the nucleus was difficult to determine. Clearing media such as oil of wintergreen or microscope immersion oil were used to enhance the clarity of the sections.

The otoliths from all fish larger than 25mm were embedded in clear casting resin and sectioned on a conventional twin blade diamond saw (Rauck 1976). Otoliths were sectioned both transversely and longitudinally through the nucleus to determine the plane of sectioning which would produce the most visible growth increments. Transverse sections of 0.3mm were selected for further analysis as, in longitudinal sections, the growth increments were very compact and could not be counted. The sections were cemented to glass slides with DPX. During viewing the sections were covered in immersion oil.

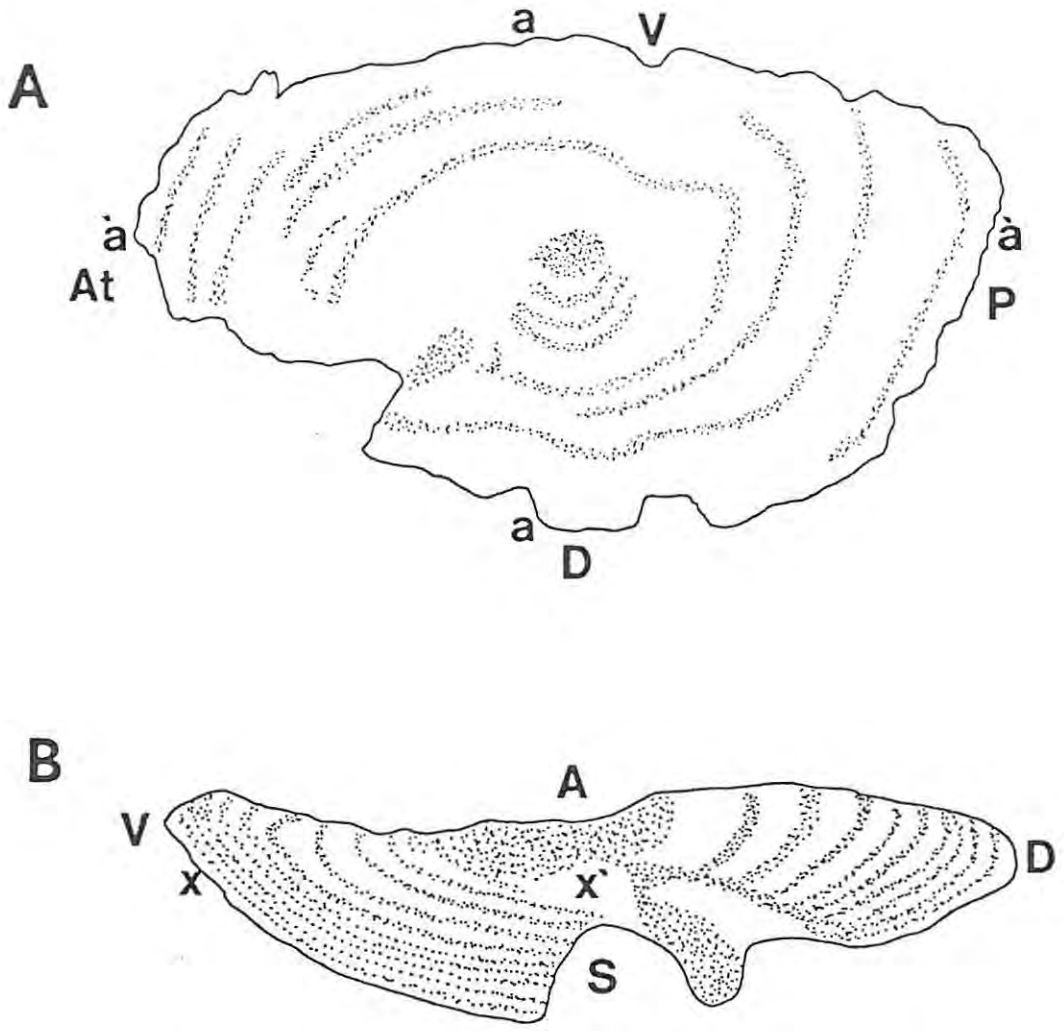

Figure 2 A: Schematic diagram of a typical whole sparid otolith. The dorsal (D), ventral (V), sulcul (S), antisulcul (A), anterior (At) and posterior (P) regions are marked. Otolith width a - a and length a'-a' are given. B: Typical sectioned otolith showing positions of the regions marked in A and the dorsal free growth line x-x'. (Terminology after Hecht 1987 and Gauldie 1990)

Scanning electron microscopy

Scanning electron microscope analysis (SEM) has the advantage of providing much higher magnifications and resolution than a light microscope. Otoliths for SEM examination were sectioned using the twin blade diamond saw technique, mounted in resin on glass slides and polished as described above. The sections were initially etched with 0.1M Hydrochloric acid (HCl) or 6% EDTA (ethylene-diamine-tetra-acetate) buffered to a pH of 6.5 with sodium hydroxide. EDTA is a calcium chelator which removes calcium carbonate and leaves the protein portion of the

increments intact resulting in protein ridges. Hydrochloric acid, on the other hand, removes the protein-rich areas leaving calcium carbonate ridges. Otolith sections etched with HCl revealed clear increment series when viewed under SEM. Increments in sections etched with EDTA were generally not very clear and HCl was selected for further etching. After etching the sections were washed, dried, photographed under transmitted light and mounted on SEM stubs with an epoxy glue. They were then sputter coated with gold and examined using the scanning electron microscope at magnifications of 100 to 6000 x.

Optical otolith examination

A daily growth microincrement was regarded as a bipartite structure consisting of a narrow opaque band and a wide hyaline band only visible when viewed under high power magnification. These microincrements were examined using a Zeiss phase contrast microscope (<630x) with a resolution limit of 0.5 - 1um. An annual growth zone was identified as a bipartite structure consisting of an opaque and hyaline band when viewed under low power magnification (<70x) using a binocular stereo-microscope with transmitted and reflected light.

For the enumeration of daily growth increments, counts were made from the nucleus along the dorsal free growth line (Fig. 2). This was generally the region where the most rings were visible. Some of the smaller otoliths showed a linear sequence of growth increments from the center to the edge, but the majority displayed scattered patches of ring systems which were combined to represent the total growth history. In most cases concentric features were present which guided the counting from one part of the otolith to the other. Micrographs were taken in series and were compiled into montages to facilitate daily increment enumeration. Daily increments were distinguished from subdaily increments on the basis of the following criteria : i) increments of similar visual prominence to adjacent increments, ii) similar increment widths to adjacent increments and iii) no merging of

the increments in the immediate area (Campana 1984b). Due to growth differences in different areas in the otolith, annual and daily ring counts were not made along the sulcul or antisulcul axes (Gauldie 1990).

The daily increments were enumerated three times for each otolith without prior knowledge of the fish length or of previous estimates. If two counts were the same or within two rings of each other, the count was accepted. In cases where all counts differed, the middle count was taken unless the discrepancy was greater than 5 rings, in which case the otolith was discarded. Daily increment width was determined from photographic analysis.

To enable enumeration of the increments beyond the fluorescent band for daily increment validation, the position of the fluorescent band relative to the growth increments visible under white light was determined. Each otolith was photographed in a paired sequence (400 ASA KODAK colour film), once under bright field illumination and once under UV light, without adjusting otolith position or focus. Where fluorescence was very bright both UV and white light could be combined in one photograph. The area to be counted was selected by scanning the margin of each otolith to find the position where the greatest number of distinct rings could be seen between the innermost fluorescent increment and the edge. The number of microincrements between successive fluorescent marks or from the fluorescent mark to the margin of the otolith were counted and correlated with the number of days elapsed between treatment and recapture. This procedure was followed for both laboratory and field injected fish. In the older fish the microincrements could not be distinguished and the position of the fluorescent band relative to annual growth zones was examined.

CHAPTER 4. OTOLITH LABELLING EXPERIMENTS

Introduction

A number of chemical markers have been used in otoliths with varying degrees of success. These markers include strontium (Behrens-Yamada, Mulligan & Fairchild 1979; Hurley, Odense, O'Dor & Dawe 1985), oxytetracycline (Weber & Ridgway 1962; 1967; Wild & Foreman 1980; Campana & Neilson 1982; 1985; Beamish & McFarlane 1985; Lipinski 1986; Buxton & Allen 1989; Babaluk & Craig 1990), calcein (Wilson, Beckman & Dean 1987), calcium 45 (Mugiya *et al.* 1981), alizarin complexone (Tsukamoto 1988) and acetazolamide (Ralston & Miyamoto 1983). Of these, oxytetracycline and alizarin complexone are the most common and reliable chemical markers.

Oxytetracycline is one of a range of tetracycline antibiotics which all become incorporated into the growing margins of calcified tissues. Oxytetracycline can be administered by injection (Wild & Foreman 1980; Campana & Neilson 1982), by addition to the diet (Choate 1964; Weber & Ridgway 1967) or by total immersion of the fish in water containing the drug (Campana & Neilson 1982; Hettler 1984; Schmidtt 1984). Localization of tetracyclines is a result of their ability to bind first to the protein and then to the calcium in actively calcifying regions (Lipinski 1986). Oxytetracycline bands in the calcifying areas become visible under ultraviolet light as fluorescent yellow-gold bands due to UV absorption in the 360mu range. Although oxytetracycline has been used on a number of fish species, *Thunnus albacares* (Wild & Foreman 1980), *Platichthys stellatus* (Campana & Neilson 1982), *Anoplopoma fimbria* (McFarlane & Beamish 1990) and *Pomacentrus mollucensis* (Fowler 1990) amongst others, its use in sparids has been limited to a mark-recapture study in the TNP on *Chrysoblephus laticeps*, *C. cristiceps* and *Petrus rupestris* (Buxton & Allen 1989). To date no otoliths from the above study have been examined for fluorescence.

Alizarin complexone, a colorimetric indicator dye, is similar

to oxytetracycline in its ability to become incorporated into actively calcifying tissues. It is visible within the otolith under ultraviolet light as a red mark (Tsukamoto 1988). Although alizarin complexone has not been applied by injection, it has been used in solution for the immersion marking of eggs and larvae (Tsukamoto op. cit.).

Materials and Methods

Commercially available oxytetracycline hydrochloride (OTC) dissolved in a stabilizing agent at a concentration of 50mg OTC/ml was used in this study. Each bottle of OTC was checked for fluorescence before administration. Commercially available alizarin complexone (ALC) (1,2-Dihydroxyanthraquinone-3-yl-methylamine-N,N-diacetic Acid) was used for the immersion trials.

Otoliths from all injected specimens were prepared according to the general methodology and were examined and photographed at a magnification of 16 - 630x under both white and ultraviolet transmitted light on a Zeiss fluorescent photomicroscope equipped with Neoflour objective lenses. The filter combination was 48-77-09 with a BP 450-490mm exciter filter, an FT 510 chromatic beam splitter and an LP 520mm barrier filter. Using this filter combination, the green auto-fluorescence visible on the margin of all otoliths was clearly distinguishable from the yellow fluorescence induced by the OTC. The otoliths from all experimental fish were stored in the dark as the intensity of the fluorescent band weakens on exposure to direct light.

1. Immersion experiments

As larval and small juvenile sparids are difficult to inject, the fish were immersed in dilute solutions of OTC. Oxytetracycline was dissolved in sea water to dilutions of 100 and 200mg OTC/l. Fish (<30mm FL) were immersed in each solution for 24hrs under either light or dark conditions. After OTC administration the fish were transferred to their original

tanks where they were monitored constantly until they began feeding (usually within one day).

In the second immersion experiment, fish (<30mm FL) were immersed in solutions of either 100mg/l or 150mg/l ALC dissolved in sea water. Immersion times ranged from 12 to 24hrs (Tsukamoto 1988) under light conditions. A further ten juveniles were immersed in the 100mg/l solution for 24hrs in the dark. After immersion, the fish were transferred to their original tanks.

As a control, ten fish (<30mm FL), were transferred to a separate sea water tank for 24hrs after which they were returned to the original tanks.

2. Intraperitoneal, intramuscular and dietary administration

A number of experiments were conducted to determine which injecting technique and OTC dosage would yield the clearest fluorescent mark on the otolith, without adversely affecting growth.

Each fish was weighed to the nearest 0.01g, measured to the nearest millimeter and injected with the appropriate dosage, a procedure which lasted approximately 30 seconds. A Hamilton syringe, with 0.001ul calibrations, was used to inject fish requiring very low dosages, while larger dosages were administered with a diabetic syringe with calibrations of 0.01ml. Control fish were injected with a saline solution.

a. A sub-sample of fish (30-200mm FL) were injected intraperitoneally (IP) with OTC doses ranging from 20-1000mg/kg.

b. Intramuscular (IM) injections of OTC with dosages ranging from 50-2000mg/kg were administered to fish ranging in size from 30-200mm FL. These injections were administered to the mid-body region directly below the dorsal spines. Each fish received between one and three IM injections over an eight month period.

c. Dietary administration of OTC was given to fish of 30-200mm FL which had been starved for two days. Fish were fed for four consecutive days on pilchard or mussel which had been soaked in 20ml of 50mg/ml OTC for 24hrs. The fish readily ate all of the food offered but the exact dosage received by each fish could not be determined accurately.

d. To determine the rate of uptake of OTC into the otolith, sixteen fish (average 65mm FL) were injected intramuscularly with doses of 250mg/kg OTC. After injection two fish were killed at time intervals of 6, 12, 24, 36, 48, 60 and 72 hours.

3. Field experiments

Adult Roman, C. laticeps (>200mm FL), are resident in the TNP and are easily caught (Buxton & Allen 1989). For this reason they were selected for the field experiments conducted between 1989 and 1991. Once captured, each fish was placed on a foam rubber mat to minimize injury and fork length was measured to the nearest millimeter. A plastic anchor tag was inserted below the second dorsal spine and, when necessary, the swimbladder was deflated using a hypodermic needle to relieve barotrauma (Buxton & Allen op. cit.). Using the length/ weight relationship for C. laticeps (Buxton in press), fish weight was estimated and each fish was given an intramuscular injection of 50 - 100mg OTC per kg body mass and returned to the water (Buxton & Allen op. cit.). Total time from capture to release averaged about 2 minutes. Type D tags, obtained from the National Tagging Program of the Oceanographic Research Institute, were used as identification of individual fish was required. The tagged fish were recaptured six to 12 months later using handline and spear fishing techniques.

Results and Discussion

1. Immersion experiments

1.1 Oxytetracycline

Although post treatment survival was good (>96% after 5 days), no positive results were obtained from the attempts to label juvenile sparid otoliths by immersion in OTC. While immersion of very small juvenile and larval fish in dilute solutions of OTC has been found to be a successful marker for a number of species (Hettler 1984; Schmidtt 1984; Tsukamoto 1985; Thorrold 1988; Fowler 1989; Tzeng & Yu 1989), the results of this experiment, as well as those of Radtke et al. (1988) and Brothers (1990), indicate that immersion may not be an effective technique for marking all marine species. As oxytetracycline chelates with calcium and magnesium in seawater before it binds with the calcium in bone forming tissue, its availability for absorption in otoliths is reduced (Campana, S., Bedford Institute of Oceanography, Dartmouth, Nova Scotia, pers. comm.; Hettler 1984). This chelation may have reduced the uptake of OTC in the sparid otoliths examined.

No distinguishing features were visible in the otoliths of the control specimens at the time corresponding to the retention in a separate tank.

1.2 Alizarin complexone

Alizarin complexone produced a distinct scarlet band in the otoliths of all specimens immersed for 24 hours in a seawater solution of either 100mg/l or 150mg/l ALC under both light and dark conditions (Fig. 3). Immersion for 12 hours in either solution did not yield positive results. The experimental animals did not react adversely to the immersion and began feeding soon after transferral to the holding tanks. No mortalities resulted from these treatments.

The results of this and other studies indicate that alizarin complexone may be used in the marking of otoliths from larval and very small juvenile marine species where OTC administration by injection is not feasible (Tsukamoto 1988; Tsukamoto, Kuwada, Hirokawa, Oya, Sekiya, Fujimoto & Imaizumi 1989). The immersion of larvae and juveniles allows large populations to be marked without individual handling. This technique could be used in large scale marking programs in areas such as the TNP in order to expand our understanding of larval sparid distribution and growth.

2.Intraperitoneal, intramuscular and dietary administration

TABLE 2. Uptake of OTC in sparid otoliths using three different labelling techniques. N = number of fish marked.

Technique	N	Result
IP injection	55	-
Feeding	20	-
IM injection	191	+ (dosage dependent)

+ Clear fluorescent band visible under ultraviolet light.
- No fluorescent band visible.

Many previous studies involving the use of oxytetracycline as a chemical marker in otoliths found that intraperitoneal injections were the most effective means of administration (Weber & Ridgway 1962; Kobayashi, Yuki, Furui & Kosugiyama 1964; Campana & Neilson 1982; Mugiya & Muramatsu 1982; Ralston & Miyamoto 1983; McFarlane & Beamish 1987). For this reason intraperitoneal injection of OTC was the first method used to mark the larger juveniles. Despite using a wider dosage range than that used in any previous studies, no definable fluorescent bands were produced in any of the sparid otoliths examined (Table 2). The failure of this

technique may be attributed to two factors. Firstly, due to the tendency of OTC to bind first to protein and then to calcium (Lipinski 1986), protein present in the gut may bind with the OTC thereby decreasing its incorporation into the calcifying regions. Secondly, absorption of OTC from the peritoneal cavity is slower than from muscle tissue (A. Bowker - Veterinary Surgeon, 17 Park Rd., Grahamstown, pers. comm.). This will reduce the efficiency of its uptake into the otolith and thereby reduce the visibility of the fluorescent band.

The administration of OTC in the diet of the fish is thought to eliminate stress associated with immersion or injection of the fish (Weber & Ridgway 1962; 1967; Choate 1964; Odense & Logan 1974). Despite the success achieved with this method, the results of this study, as well as those of Brothers (1990), indicate that dietary administration of OTC does not always produce a fluorescent band (Table 2). Much of the OTC may have leached out of the soaked food as it was placed in the water for consumption. This would decrease the amount of OTC ingested by the fish and may account for the lack of fluorescence. In addition, doubt has been expressed as to the efficacy of OTC uptake from the gut due to the presence of calcium in the diet (Weber & Ridgway 1967). It has been suggested that the calcium and other divalent and trivalent metallic cations will chelate with the OTC and reduce absorption of the OTC (Weber & Ridgway op. cit.). This could also have affected the uptake of IP injected OTC, accounting for the limited OTC uptake in sparids.

Intramuscular injections at dosages of 250 - 300mg/kg produced clear fluorescent bands in the otoliths of more than 80% of the fish injected. Associated mortality rates increased with higher levels of OTC, the optimum being 250mg/kg (Fig. 4). Despite dosages of up to 2000mg/kg saline solution, no increase in the mortality rate of the control specimens was noted, the volume of the solution not adversely affecting the survival of the fish. In the control specimens only auto-fluorescence was visible around the periphery of otoliths. The intensity of the

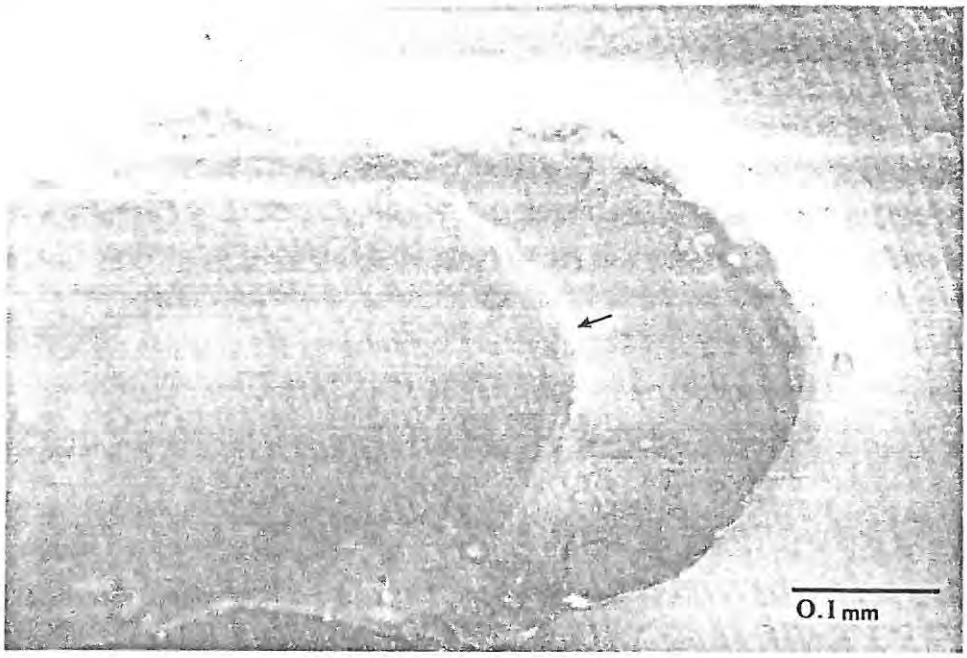

Figure 3 Alizarin complexone mark in the otolith of a D. sargus capensis juvenile (57mm FL). The mark was induced by immersion for 24hrs in the dark at a time when the fish was 18mm fork length. Growth after immersion amounted to 117 days.

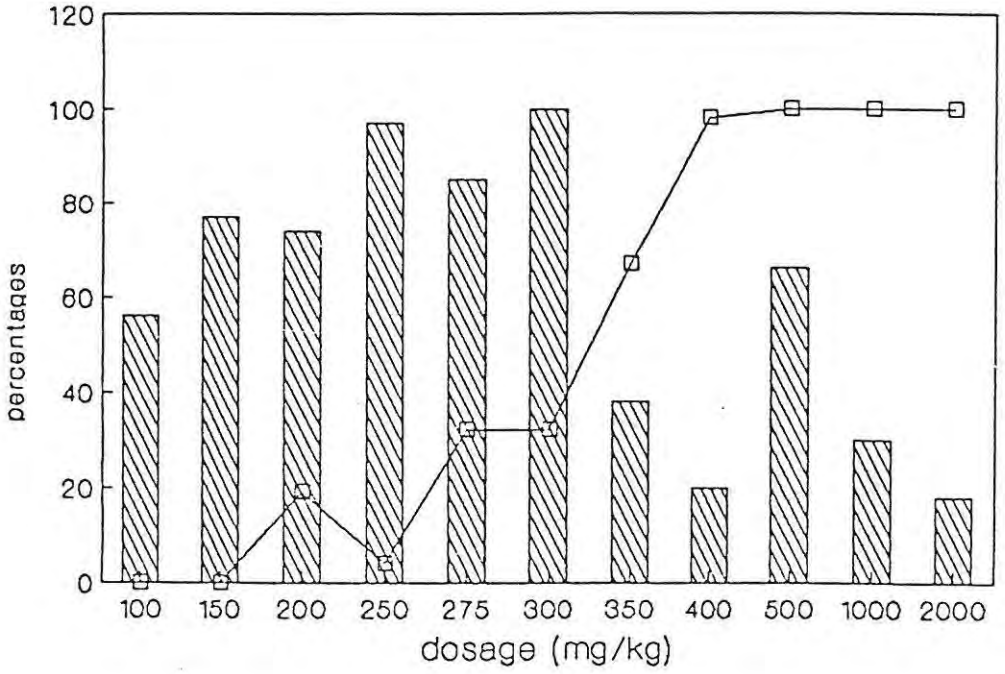

Figure 4 The proportion of fish exhibiting clear fluorescent bands after intramuscular injection at successively higher dosages (Bar graph). The percentage mortality after five days in the same experiment (Line graph).

fluorescent band in injected fish was dosage dependent - the higher the dose the more intense the fluorescence (within the acceptable tolerance limits) (Fig. 5).

Although IP injections were first used in the administration of OTC, recent work, including this study, indicate that IM administration is possibly more effective (Yoklavich & Boehlert 1987; Simoneaux & Warlen 1987; Radtke et al. 1989; Radtke & Hourigan 1990). Intramuscular injections eliminate the chance of puncturing a vital organ as the injection is made directly into the muscle and the rich blood supply to the muscle tissue ensures that the OTC is absorbed directly into the bloodstream without chelation.

The IM dosages found to be effective in this study were substantially higher than those of other workers (Simoneaux & Warlen 1987; Radtke et al. 1989; Radtke & Hourigan 1990) and it is apparent that there is a great deal of interspecific variability with regards to the effective OTC dosage. Interspecific and size related differences in metabolic rate can affect the uptake of OTC (Campana & Neilson 1982). The small size of the specimens used in this study may account for the high dosages required. This highlights the importance of a pilot study during which the optimal dosage and administration technique is determined (McFarlane & Beamish 1987).

A light fluorescent band was visible in 50% of the sparid otoliths within 12hrs of OTC administration while 100% of the otoliths had clear fluorescent bands after 24hrs. To be effective as an accurate time marker, OTC must be incorporated in the otolith shortly after administration (Babaluk & Craig 1990). A study of time elapsed between application of the drug and the appearance of the fluorescent band is important as any lag period may seriously alter the daily increment count interpretation (Campana & Neilson 1982). The known lag period of 24 hrs (Campana & Neilson 1982; Babaluk & Craig 1990) was confirmed by the results of this study.

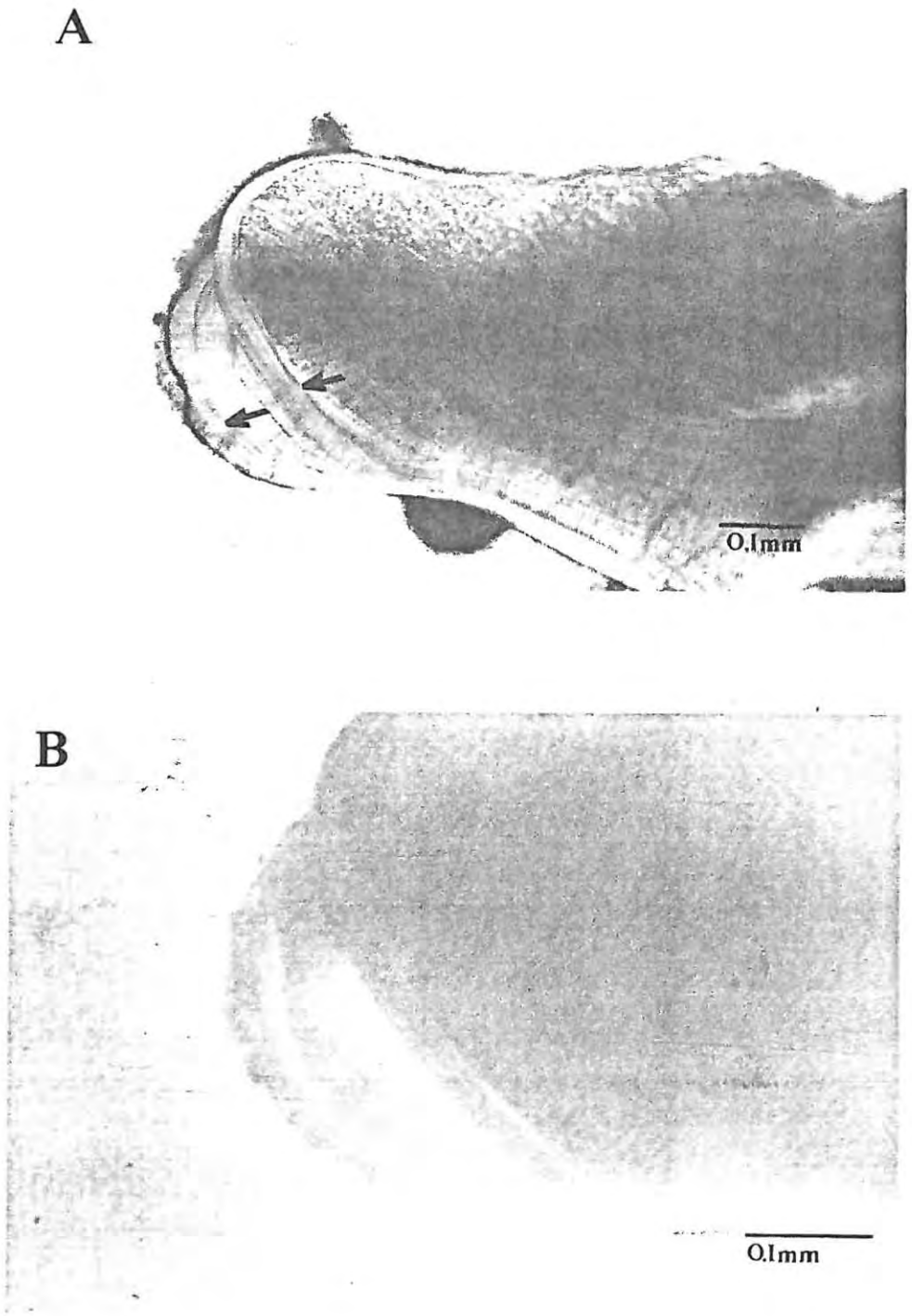

Figure 5 A) Photomicrograph pair of the otolith of a D. cervinus hottentotus (90mm FL) injected intramuscularly with OTC at dosages of 150, 275 & 200mg/kg on day 32, 68 and 134 after capture, respectively.
B) The same otolith under UV light. Check rings (arrows) coincident with each fluorescent band clearly visible.

Residual OTC remains in the body and can be incorporated into the otolith over a period of days subsequent to administration. For this reason, fluorescent bands do not always span only one microincrement. The fluorescent band in sparid otoliths spanned two to six microincrements. Campana & Neilson (1982) and Brothers (1990) found tetracycline bands of 14-20 increments in juvenile starry flounders and one to three increments in salmonid fry respectively, while Wild & Foreman (1980) found total incorporation of OTC within a day. The differences in OTC incorporation time may be attributed to differences in metabolic rate. In smaller fish, with a higher metabolic rate, the band is narrow as the OTC is quickly metabolized, while in larger fish the fluorescent band appeared wider. These metabolic differences may be both species and size specific (Campana & Neilson 1982).

Immediately after OTC administration, fish sank to the bottom of the tank and remained motionless for up to 30 seconds, after which they either began swimming slowly or died. The surviving fish usually displayed some evidence of short-term stress, including cessation of feeding for two or three days. After this, the fish either died or began feeding, in which case they then remained in good condition until the experiment ended. The short term stress evident after the administration of OTC has also been noted by a number of other workers (Hettler 1984; Radtke & Hourigan 1990). The control specimens also displayed the initial symptoms of stress after injection with saline solution but recovered more rapidly than those injected with OTC.

Not all of the otoliths which had fluorescent bands could be used for daily increment validation because daily increments adjacent to the fluorescent band in some otoliths were less distinct than normal (Fig. 6). This phenomenon has been recorded by other authors (Hettler 1984; Yoklavich & Boehlert 1987). The tetracycline range of antibiotics causes a disruption of the mineralization of teeth and bones in man and other animals but the exact effects of OTC on the normal growth and metabolism of fish, especially those already stressed by capture, are not

known (Mugiya & Muramatsu 1982; Smith 1984). The presence of a check ring (see Fig. 5) associated with the fluorescent band in 46% of the otoliths indicates that the stress associated with injection is sufficient to disrupt the mineralization process. The formation of this check cannot however be solely attributed to the effect of OTC as a similar check ring was found at the position corresponding to the time of injection in many of the control fish. Although it appears that OTC does have a slight effect on increment deposition in sparid otoliths, this effect was limited and could not be distinguished from the effects of laboratory conditions on increment deposition. An OTC induced otolith label can therefore be used in the accurate enumeration of daily increments in sparid otoliths.

3. Field experiments

Ten of the 303 C. laticeps released in the TNP were recaptured. Average time at liberty was 255 days with a maximum of 525 days. All the otoliths from C. laticeps marked in the field showed clear fluorescent bands (Fig. 7). Intramuscular injections at dosages of 50-100mg/kg OTC were, therefore, effective for the marking of adult sparids in the field.

The effective dosage for marking adult sparids was slightly higher than that recommended for adult skipjack and yellowfin tuna (Wild & Foreman 1980). The range of dosages that will produce a fluorescent mark is quite wide and may indicate that dosages close to the optimal for adult C. laticeps would be suitable for other sparid species in future mark-recapture studies.

The results of this study, as well as those of Cass & Beamish (1983) and McFarlane & Beamish (1987) show that higher dosages are required to mark small laboratory held fish than larger wild fish. A possible reason for this may be that smaller fish are able to tolerate larger doses of OTC without experiencing an increased mortality. This effect may be due to an increased

metabolic rate in the smaller fish, resulting both in the rapid incorporation of OTC into the otolith and excretion of excess OTC more rapidly. For this reason care should be taken when extrapolation of dosages from laboratory to field conditions is undertaken.

A great deal of interspecific variation exists in the effect of OTC on fish. There is no universally acceptable method of administration or of dosage required to produce an effective fluorescent mark in the otoliths. For South African sparids the dosages and administration methods described in this study provide a base-line for future age validation studies.

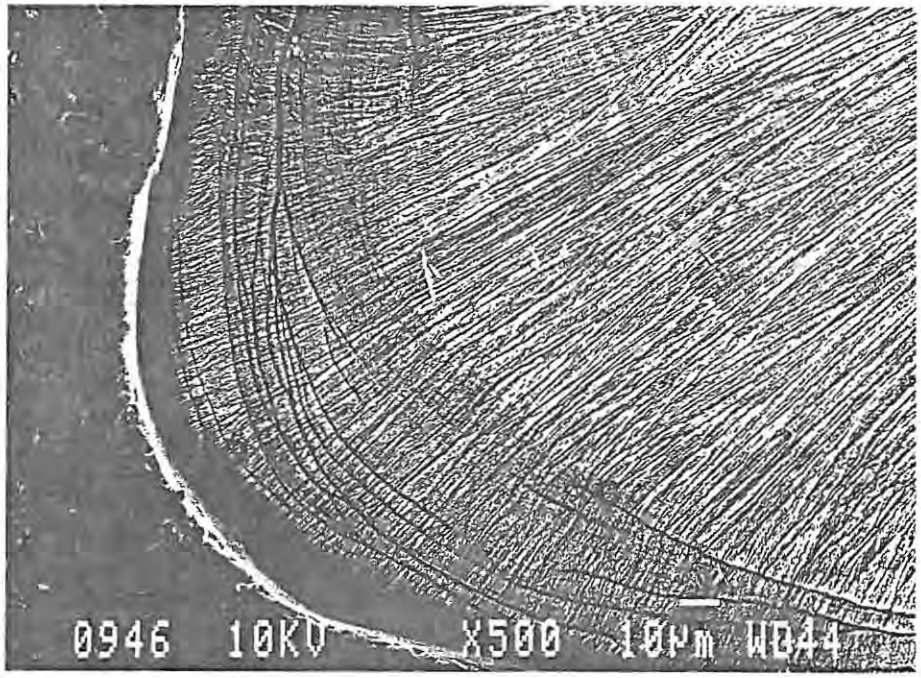

Figure 6 Check ring (c) associated with the time of capture and OTC administration (250mg/kg) (O) of a 140mm D. sargus capensis. Note the decrease in visual clarity after capture as well as the increase in frequency of the check rings.

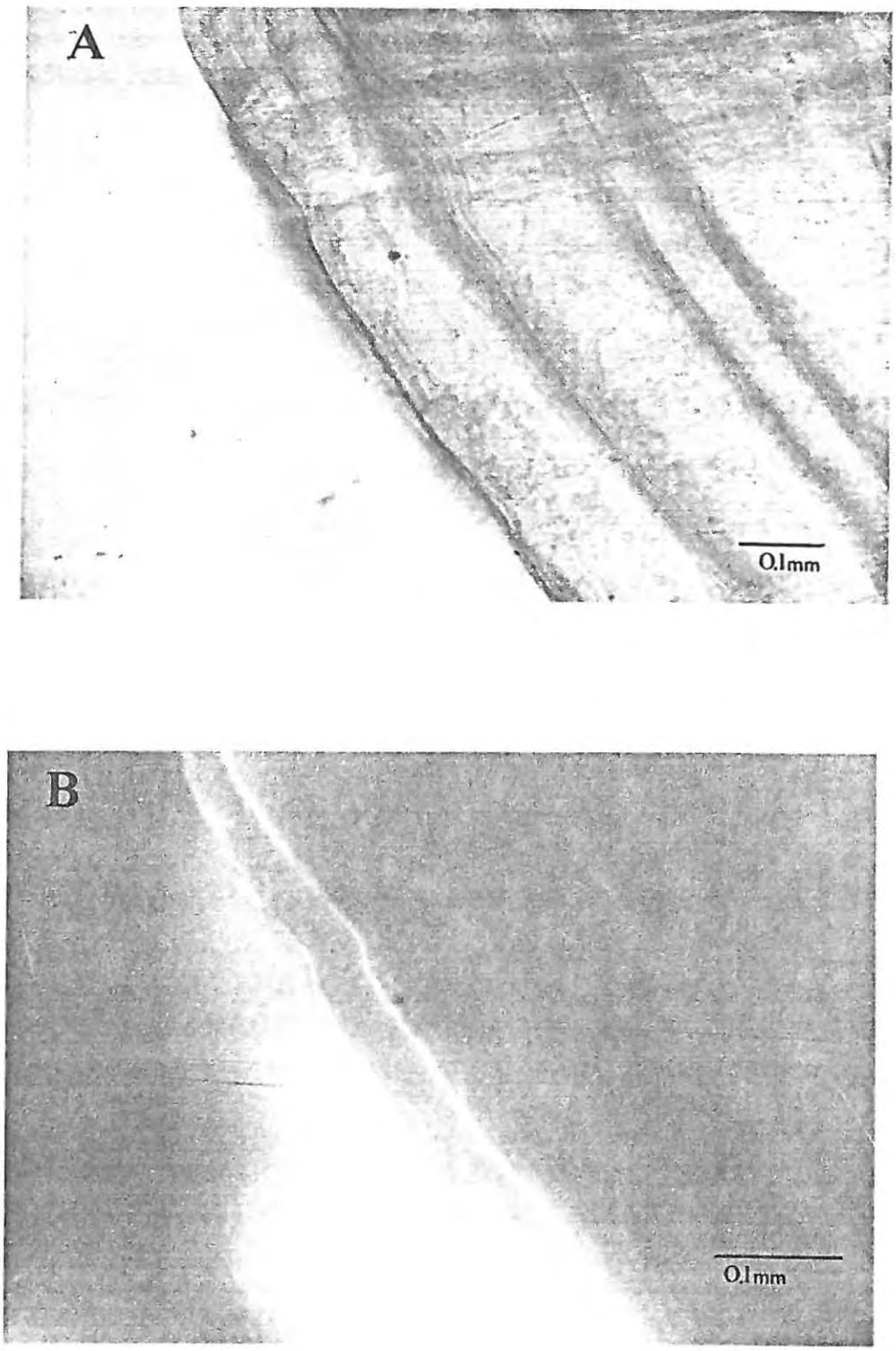

Figure 7 Comparative photomicrographs of the otolith of a C. laticeps (345mm FL) injected with 50 - 100mg/kg OTC in February 1991 and recaptured in November 1991. A) Under transmitted visible light and B) under UV light.

CHAPTER 5. OTOLITH STRUCTURE

5.1 Daily increment validation

To investigate the periodicity of the microincrements, the relationship between the number of growth increments laid down after the fluorescent band was formed and the number of days between the OTC administration and death were determined (Fig. 8). A one-way ANOVA using a Scheffe multiple range test ($p < 0.0001$; df (1,30)) indicated that one increment was deposited daily. Counts were only made from readable otoliths and in most cases every single growth increment was not always visible. Counts of daily rings after the fluorescent zone which were inconsistent with the number of days after administration were found predominantly in the fish which stopped feeding for a few days after OTC administration.

Known age larval D. sargus capensis, collected in the Kowie River (A. Whitfield - JLB Smith Institute, Grahamstown), were used to confirm that one microincrement was deposited each day in the larvae of this species.

An examination of the otoliths from 100 D. sargus capensis showed that the relationship between daily increment counts and fork length was exponential (Fig. 9), ($R^2 = 68.63\%$, $Y = \exp(-0.0539 + 0.774X)$. Daily increment counts were not made from fish older than two years as in these older fish the daily increments towards the edge of the otolith were very closely spaced and not easily discernible.

5.2 Annual increment validation

Annual periodicity of the zones was validated by examination of the macrostructural opaque and hyaline zones under both fluorescent and low power white light. Growth in the otolith beyond the fluorescent band (measured in um) was well correlated

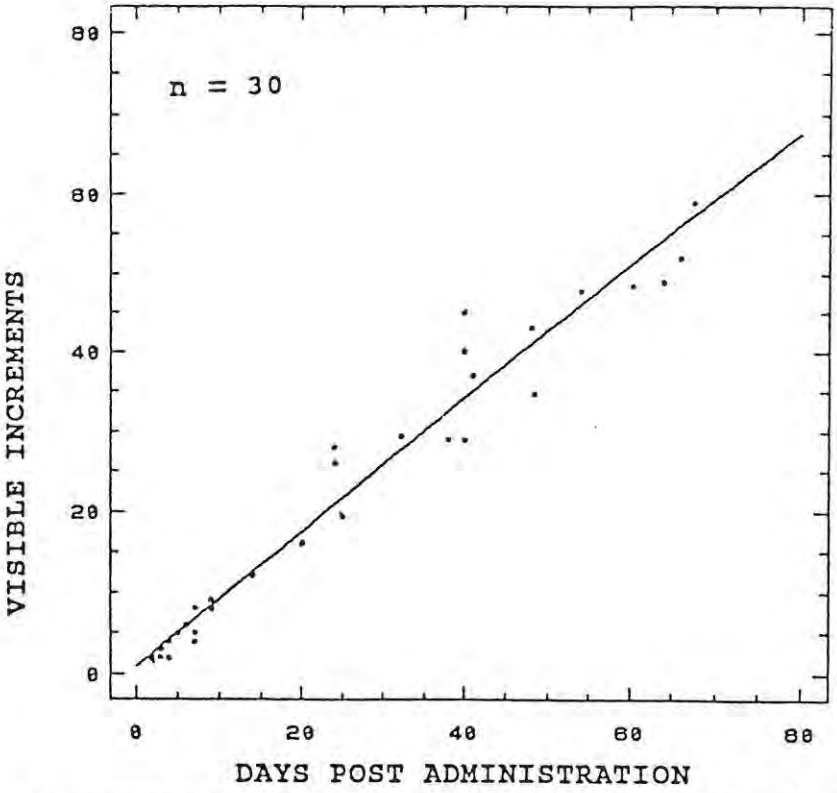

Figure 8 Relationship between the days subsequent to IM injection with OTC and the number of increments visible after the fluorescent band in 30 D. sargus capensis.

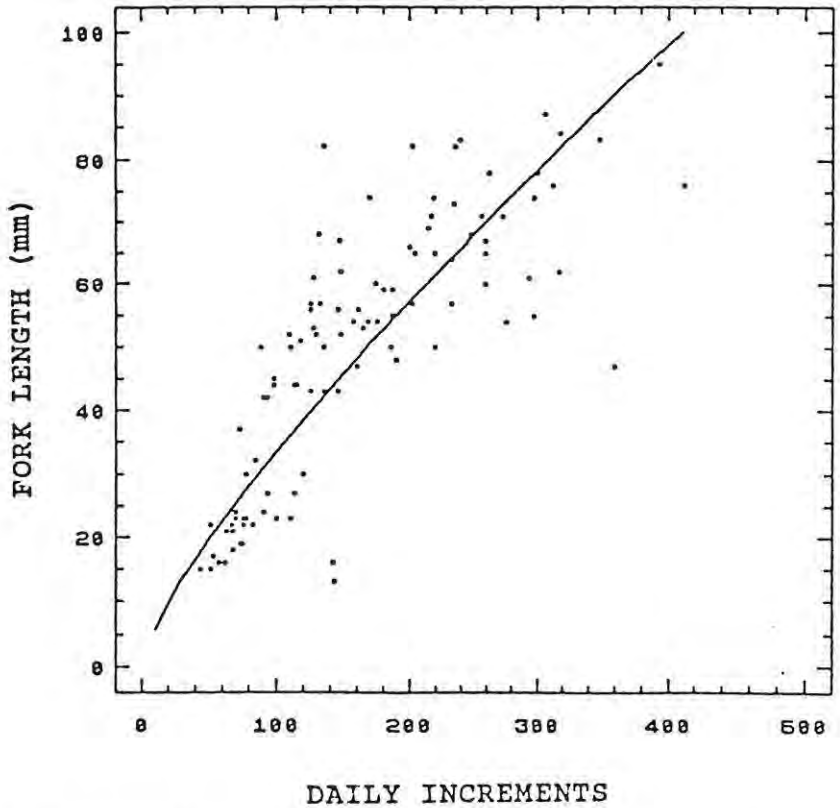

Figure 9 Regression of fork length (mm) on age as determined by daily increment enumeration. D. sargus capensis <180mm. N = 100.

with the time at liberty (One-way ANOVA test $p < 0.0001$; df (1,9); $F = 182.668$).

To investigate the time of formation of growth zones in sparid otoliths the position of the fluorescent band relative to an opaque or hyaline zone was noted. This was correlated with the date of injection and the formation of annuli documented by Buxton (in press) (Appendix 1). The nature of the marginal zone was also correlated with the date of recapture. A good correlation existed between the observed and expected growth zones at both the position of the fluorescent band and the margin of the otolith (Table 3) (see Fig. 7).

TABLE 3. Observed and expected chemical composition of tagged and recaptured C. laticeps otoliths based on the seasonal distribution of hyaline and opaque zones determined by marginal zone analysis by Buxton (in press).

Tagged	Zone appearance		Recaptured	Marginal zone	
	Expected	Observed		Expected	Observed
May 1990	Hy	Op/Hy	Nov. 1991	Op	Op
May 1990	Hy	Hy	Feb. 1991	Op/Hy	Op/Hy
May 1990	Hy	Hy	Nov. 1990	Op	Op
Nov. 1990	Op	Op	April 1991	Hy	Hy
Feb. 1991	Op/Hy	Hy	Nov. 1991	Op	Hy
Feb. 1991	Op/Hy	Op/Hy	Nov. 1991	Op	Op
Feb. 1991	Op/Hy	Op	Nov. 1991	Op	Op
Feb. 1991	Op/Hy	Hy	Nov. 1991	Op	Hy
Feb. 1991	Op/Hy	Hy	June 1991	Hy	Hy

Op - Opaque zone
Hy - Hyaline zone
Op/Hy - Opaque/Hyaline interface

5.3 Microstructural otolith analysis

To determine the microstructural features of sparid otoliths, 238 otoliths from various species including D. sargus capensis, D. cervinus hottentotus, C. laticeps, S. salpa and R. holubi were examined using light microscopy. A further 82 otoliths were examined under SEM. Daily increment series were discernible in 146 (61%) of the otoliths examined under light microscopy and in 53 (65%) of the otoliths examined under SEM.

Examination of the otoliths revealed a number of characteristic features. In very small fish (<30mm FL) the nucleus was clearly visible and multiple primordia (usually 3) were present making the exact position of the first ring difficult to discern (Fig. 10). Growth occurred in an almost spherical manner around each primordium, however, as growth continued, the increments consolidated and became continuous. In the central region of the otolith widely spaced daily increments were visible, while in the rest of the otolith the bands were closely spaced (Fig. 10). No hatch check ring could be identified. Each daily increment visible under SEM consisted of a wide lightly etched incremental band and a narrower, deeply etched discontinuous band (Fig. 11).

Some of the otoliths, especially those from the smaller fish, displayed a clearly visible sequence of growth increments from the centre to the edge of the otolith, but the majority displayed scattered patches of ring systems (Fig. 12). A clear check ring was often visible within the daily increment series corresponding to the time of capture of the fish. Between this check and the edge of the otolith the daily increments generally appeared less distinct and were spaced closer together (see Fig. 6). This area also had more random check rings than the area of deposition prior to capture, making the enumeration of daily increments after capture especially difficult. Fish which died soon after OTC administration did not have any discernible daily increments after the fluorescent band and in many cases the band was hardly visible as it merged with the margin of the otolith.

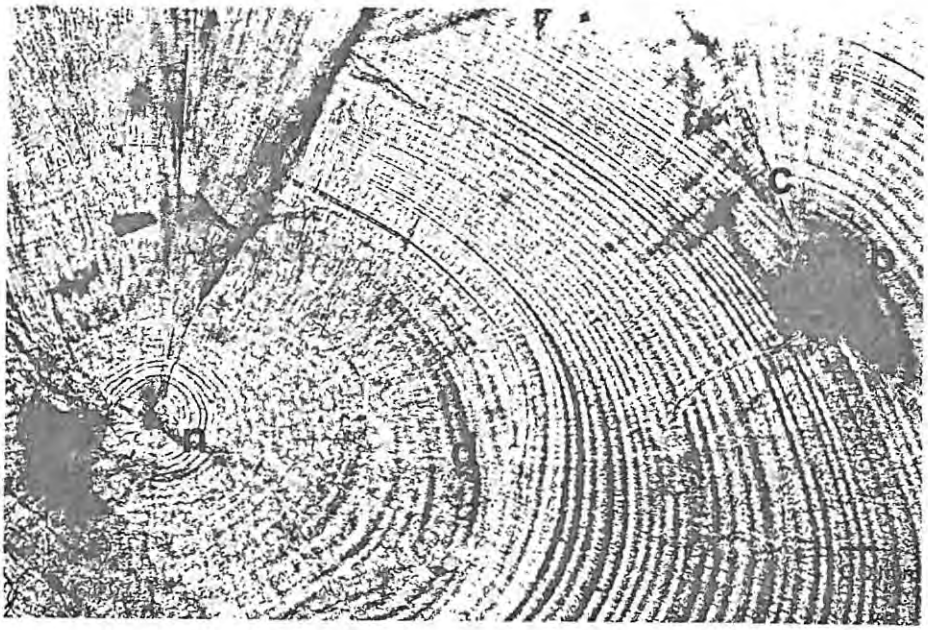

Figure 10 Daily growth increments in the sagitta of a 24mm D. sargus capensis. Nuclear area with wide daily increments visible (n). Growth around an accessory primordium (p). The distinct dark increment (d) and the change over (c) in depositional pattern.

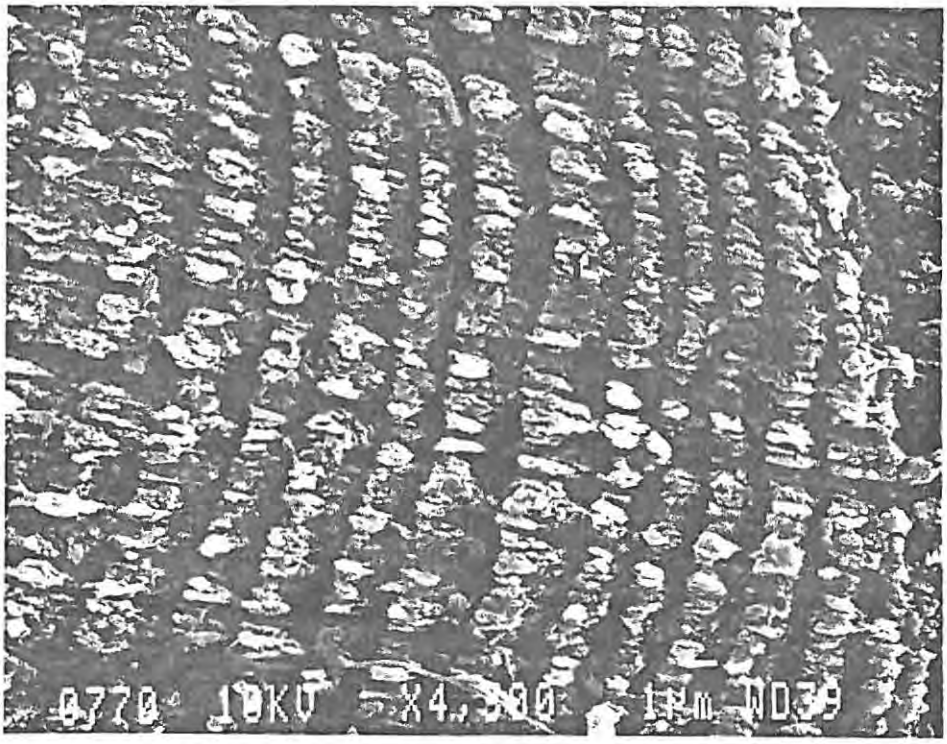

Figure 11 Scanning electron micrograph of the sectioned otolith from a 145 mm R. holubi showing clear daily increments just beyond the immediate nuclear area. I is the incremental band, D is the discontinuous band.

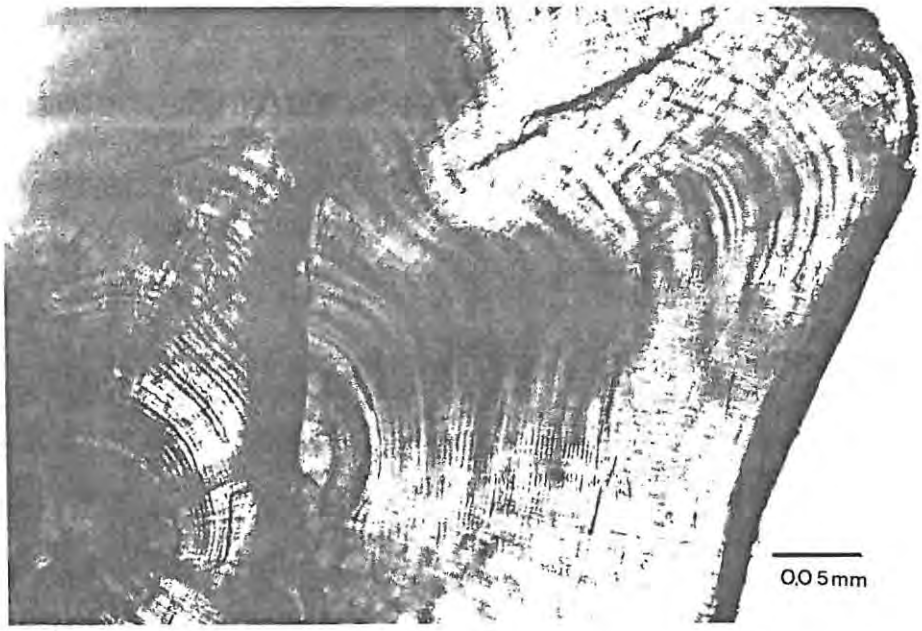

Figure 12 Scattered areas of daily growth increments from a sectioned 50 mm D. sargus capensis otolith.

Otoliths from control fish killed immediately after capture in most cases displayed a clearer set of edge increments and exhibited a more regular pattern of check rings.

The presence of subdaily rings in the otoliths was noted in many fish less than one year old. These rings appeared as very narrow bands between the more sharply defined, thicker, daily increments (Fig. 13). Differential focusing of the otolith under light microscopy, so that the subdaily rings were out of focus, helped to ensure that only the daily increments were counted.

5.4 Annual growth zone analysis

Macrostructure

Whole, unburnt otoliths from D. sargus capensis, D cervinus hottentotus and C. laticeps larger than 200mm FL, when viewed under transmitted light, appeared opaque with narrow hyaline

zones. When the same otoliths were burnt the lighter, unburnt areas appeared narrow, corresponding to the hyaline zones, while the deeply burnt, wide areas corresponded to the opaque zones. Whole otoliths from fish 90 - 130mm FL appeared primarily opaque when viewed under transmitted light. However, when these otoliths were burnt, a clear light zone was visible adjacent to the opaque nucleus. In 38% of the otoliths examined from D. cervinus hottentotus distinct surface sculpturing was present and the ridges were consistent with the external appearance of the opaque zones (Fig 14), however, otoliths from the other species examined showed very little or no surface sculpturing.

Burnt and unburnt, sectioned otoliths from a variety of sparids including D. sargus capensis, D. cervinus hottentotus (Mann 1992), C. laticeps (Buxton in press), Cymatoceps nasutus, Sparodon durbanensis and Pachymetopon grande (Buxton & Clarke 1989; 1991; 1992 respectively), when viewed under transmitted light with a light microscope, showed a concentric pattern of broad hyaline and narrow opaque zones (Fig. 15A). In burnt, sectioned otoliths the darkly burnt nuclear area was followed by the juvenile ring which appeared as a narrow dark band just outside the first area of hyaline growth. Although the juvenile ring was not always visible in all sections, care was taken not to interpret this as the first annulus.

The distinction between the opaque and hyaline zones from some of the otolith sections was unclear due to the close spacing of the zones and the presence of incomplete or very narrow zones. The width of the zones decreased towards the edge of the otolith in older fish (Fig. 15B). This decrease in band width with increasing age often results in the 'stacking' of the growth zones making the interpretation of age in older fish very difficult (Blacker 1974).

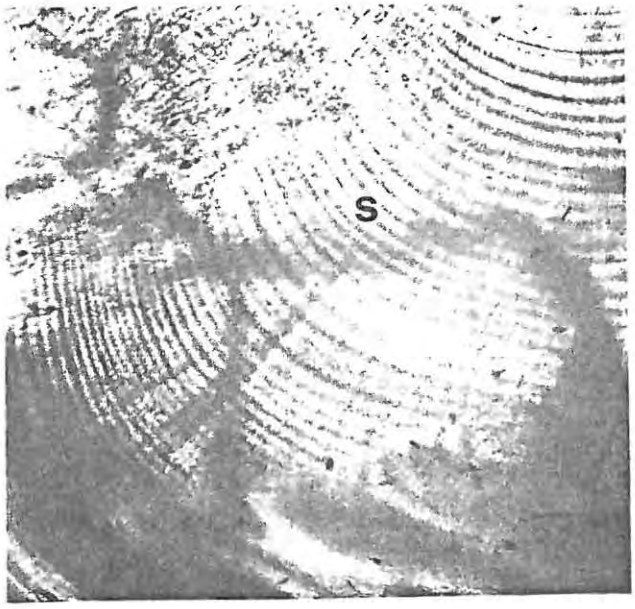

Figure 13 Otolith microstructure showing subdaily rings (s) found between the daily rings of a 60mm D. sargus capensis.

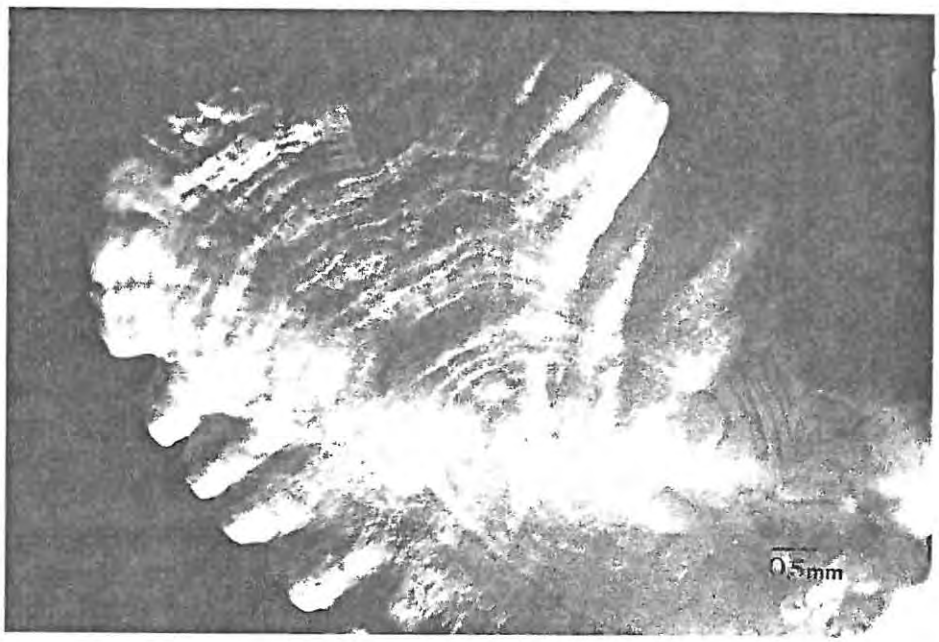

Figure 14 Photomicrograph of a 262 mm D. cervinus hottentotus unburnt otolith showing clear surface sculpturing.

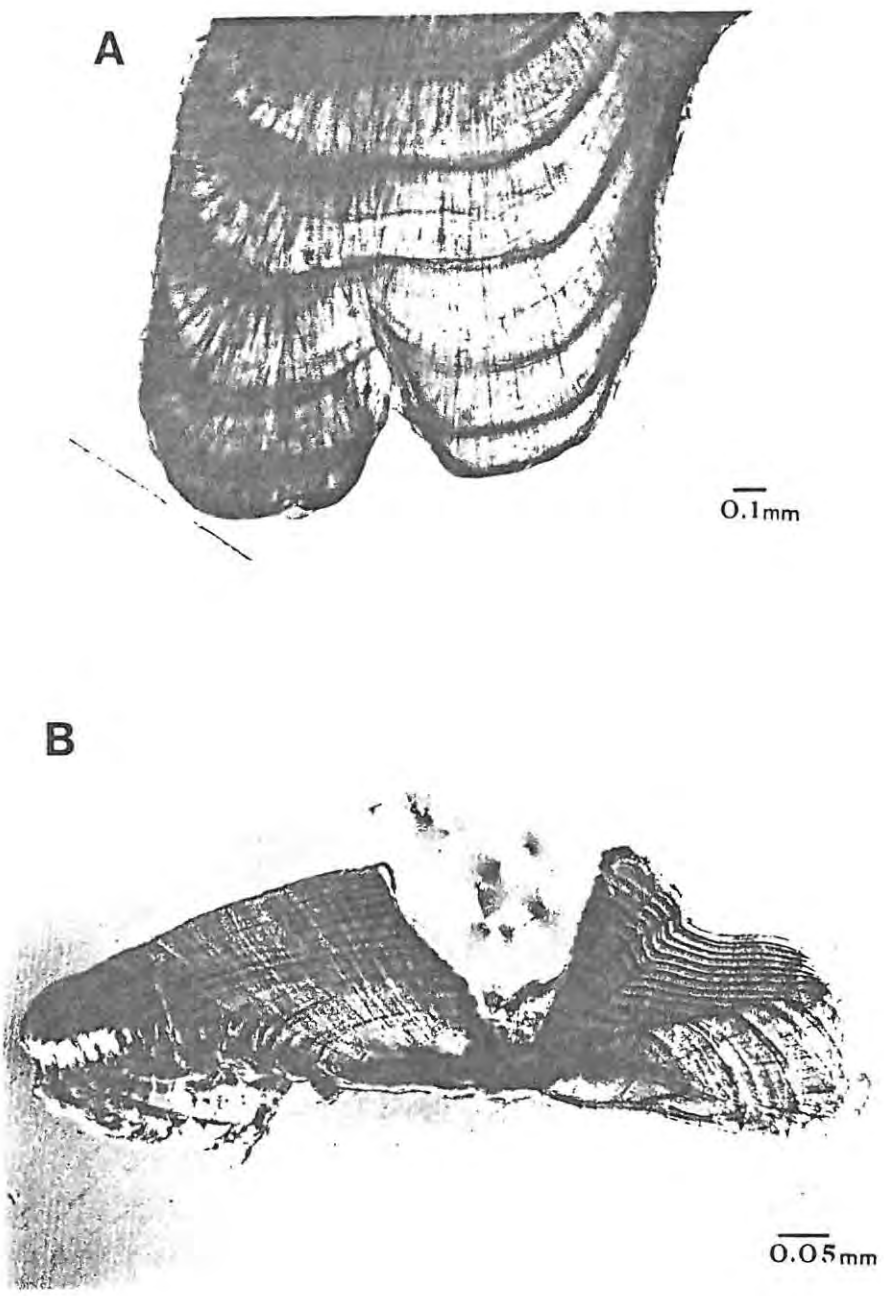

Figure 15 A: Photomicrograph of a typical burnt, sectioned sparid otolith viewed under transmitted light. (D. cervinus hottentotus, 285mm FL).
B: Photomicrograph of the otolith from a 760mm S. durbanensis showing the decrease in the width of the annual zones towards the margin of the otolith.

Microstructure

Using scanning electron microscopy the daily increment series within the annual growth zones of the otoliths from larger fish were visible. Otolith microstructure was characterized by a series of daily growth increments interrupted by prominent, deeply etched check rings (Fig. 16). Where the check rings were exceptionally deeply etched they were also visible under high power light microscopy. Regular check rings only became clearly defined in the first opaque zone. Enumeration of the daily increments between the check rings showed that the checks were deposited at intervals of 12.5 or 24.8 days, with more clearly defined check rings visible in the opaque zones than in the hyaline zones. In captive D. cervinus hottentotus and R. holubi, check rings appeared more frequently towards the margin of the otoliths. The checks were often incomplete and many appeared to merge or 'disappear' into other checks (Fig. 16).

Using a one-way ANOVA a significant difference in the width of daily increments within the hyaline and the opaque zones was found ($p < 0.0001$; $df = (1,78)$, $F = 28.236$). Increments in the opaque growth area close to the nucleus averaged 3.5um +/-0.41um while those outside the immediate nuclear region, even in the hyaline zone, averaged 1.25um +/- 0.62um (Fig. 17). Daily increments in the opaque zones had an average width of 0.66um +/- 0.39, however large differences in increment width were found within each zone. As the otolith increased in size daily increment width decreased in both the opaque and hyaline zones. Daily increments were not visible throughout each zone.

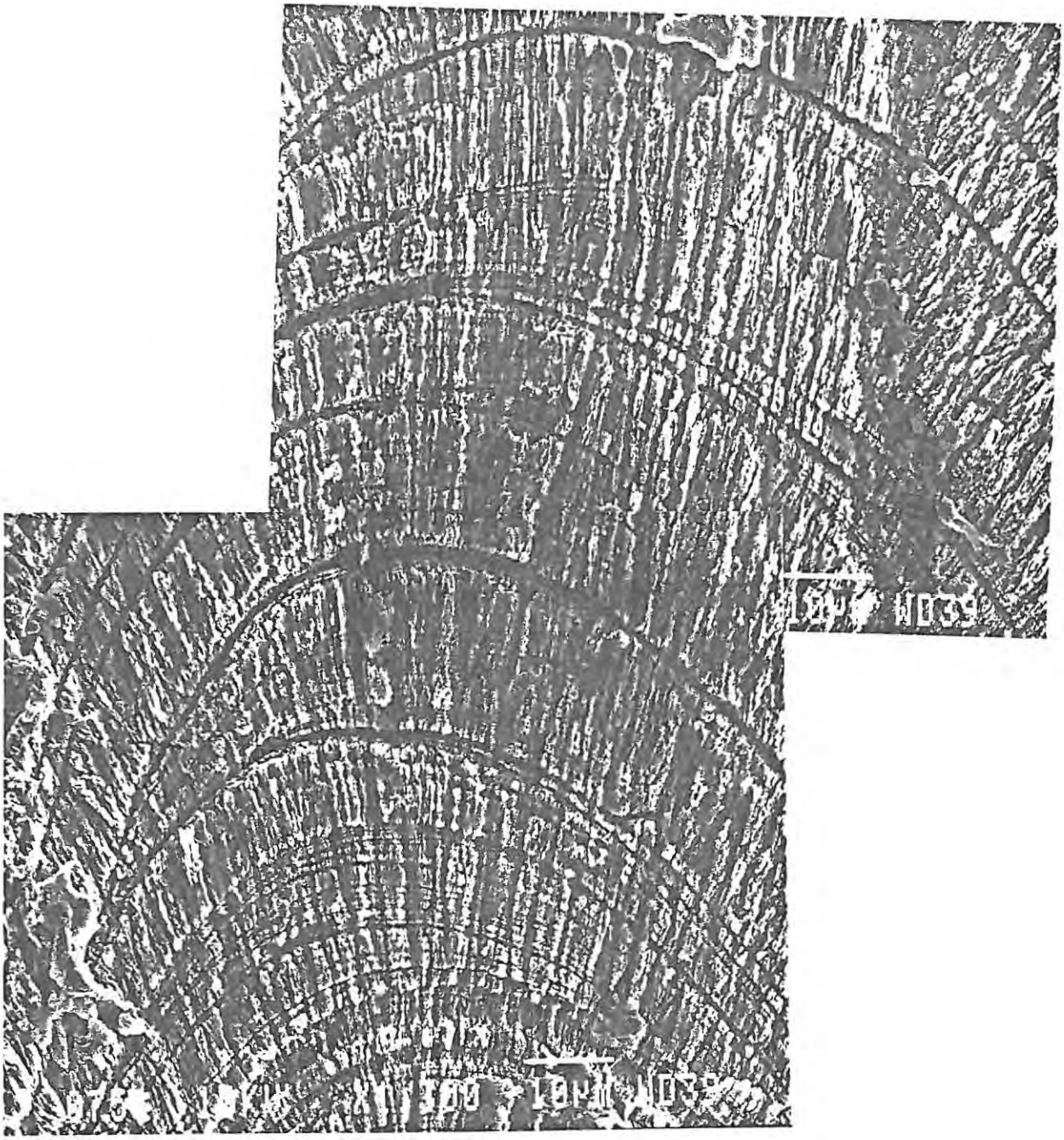

Figure 16 Otolith microstructure of a 236 mm D. sargus capensis. The regular series of daily increments is interrupted by the prominent, deeply etched checks. Check rings merge towards the edges of the otolith (m).

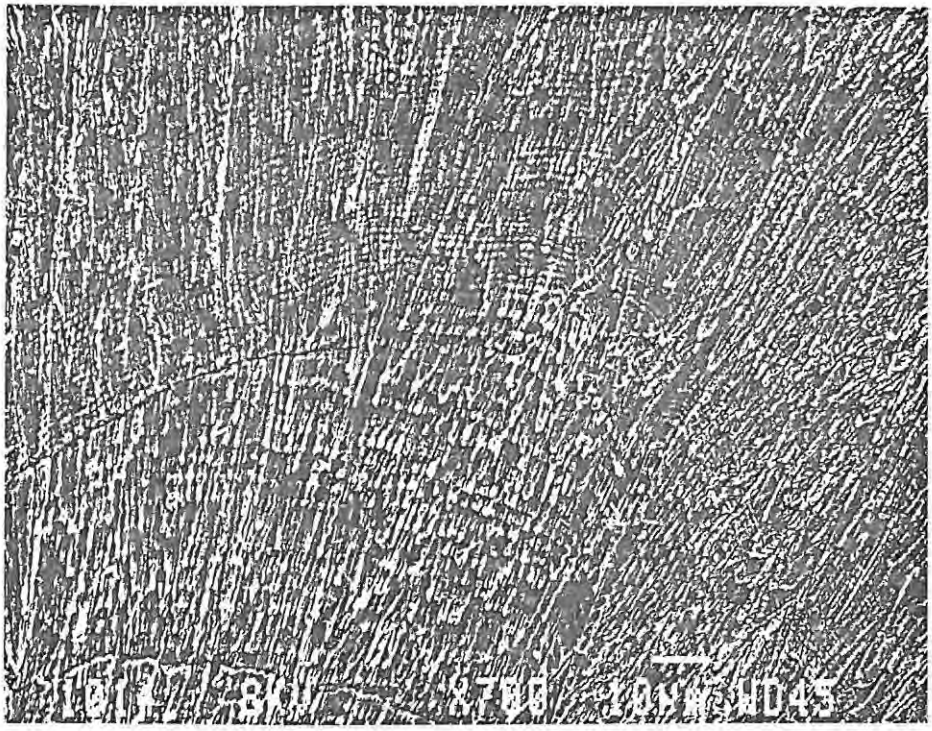

Figure 17 SEM micrograph of a sectioned otolith from a 248 mm C. laticeps showing clear daily increments in the region of change between the opaque and the hyaline zones. Widely spaced daily increments in the hyaline (H) zone, narrow increments in the opaque (O) zone.

5.5 Otolith growth

5.5.1. Relationship between fish size and otolith size

The size of the whole otolith of D. sargus capensis and D. cervinus hottentotus increased proportionally with fork length. The relationships between otolith width, mass and length and fish fork length for D. sargus capensis and D. cervinus hottentotus are given in Figure 18 and 19 respectively.

5.5.2 Otolith growth patterns

Considerable variation in growth occurs in the different areas of the otolith. Measurement of the distance between the edge of the otolith and the fluorescent band revealed that maximal growth occurred along the dorsal free growth edge. Minimal growth was found to occur in the antisulcul region (Fig. 20).

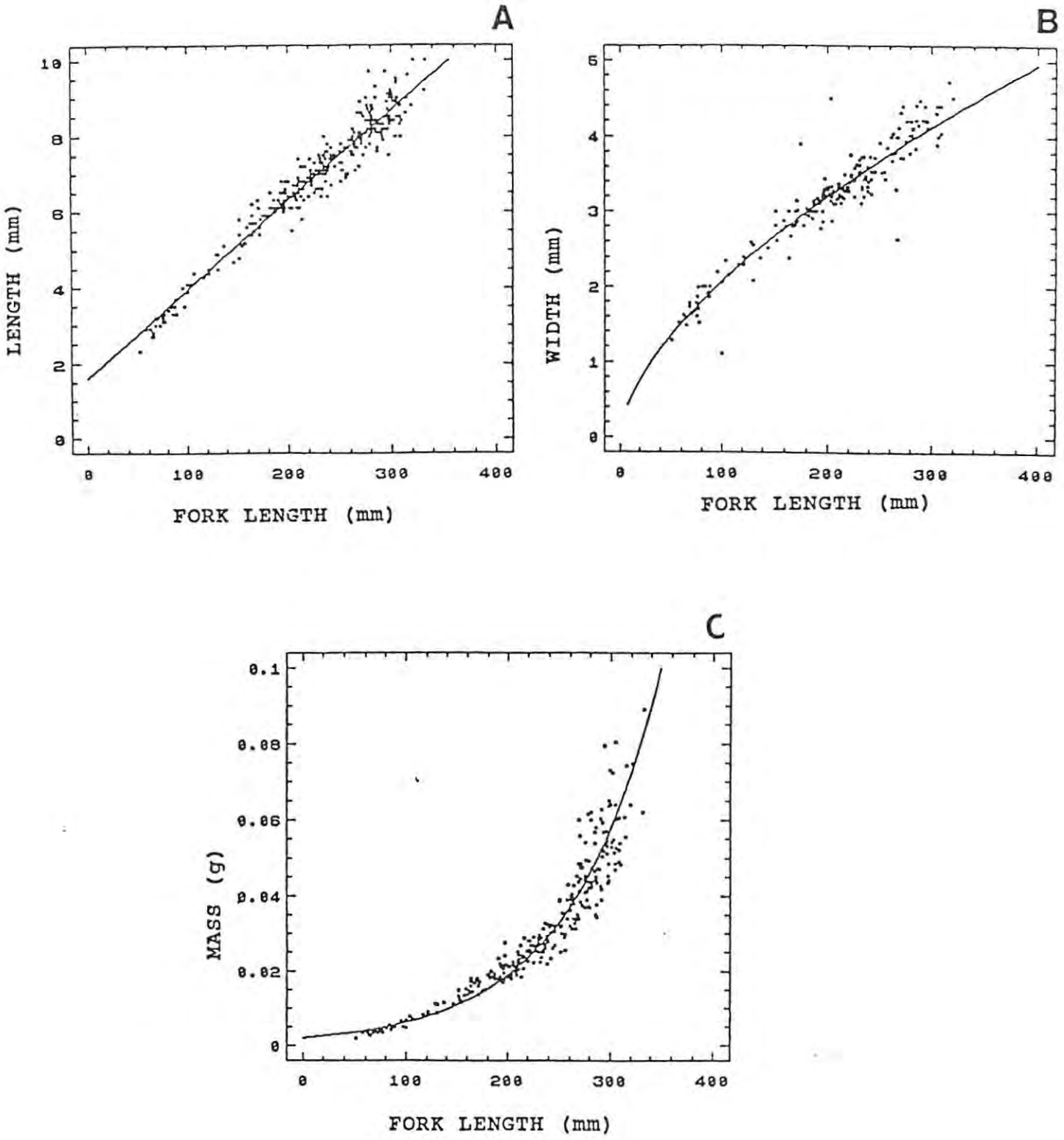

Figure 18 Relationship between fork length and otolith A) mass, B) width and C) length for 261 D. sargus capensis sampled in the TNP from 1989 to 1991.

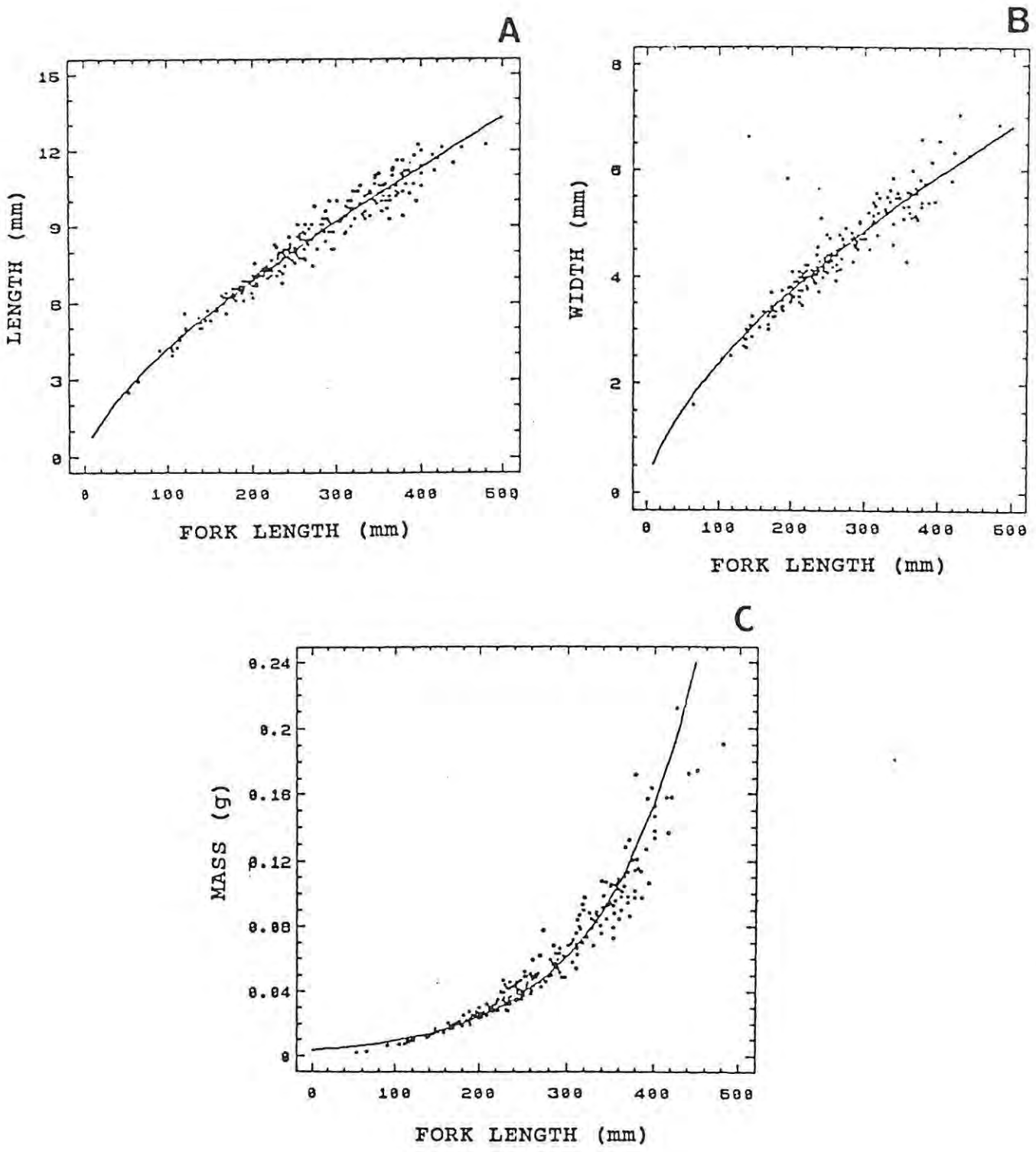

Figure 19 Relationship between fork length and otolith A) mass, B) width and C) length for 241 D. cervinus hottentotus sampled in the TNP from 1989 to 1991.

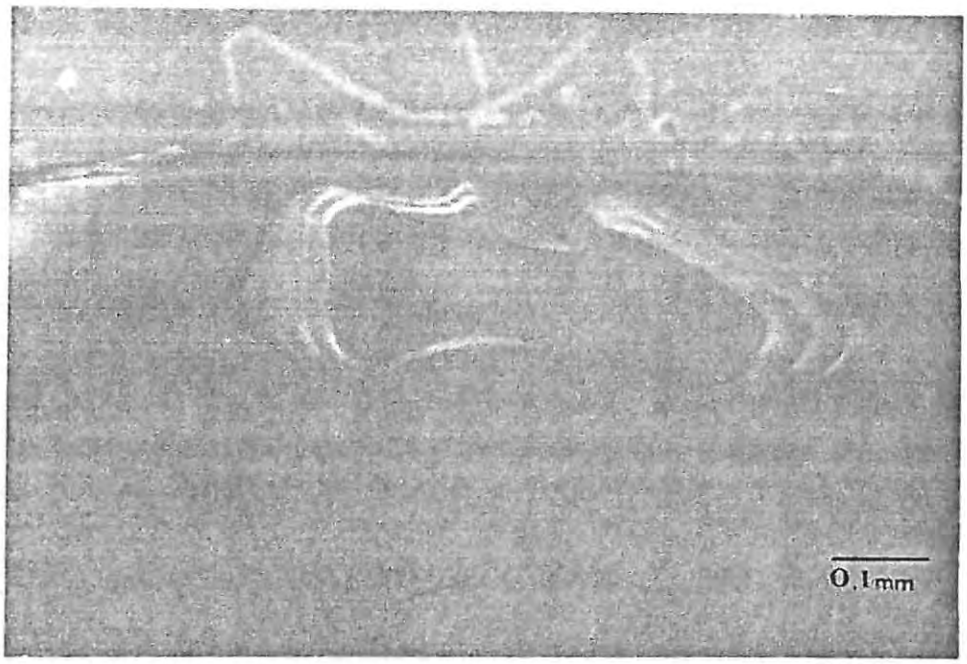

Figure 20 Photomicrograph, taken under ultraviolet light, of a 80mm D. sargus capensis. The three fluorescent bands visible around the whole otolith demonstrate growth differences in the different areas of the otolith. Specimen injected with 400mg/kg, 275mg/kg and 200mg/kg on days 40, 66 and 70 after capture, respectively.

Discussion

As already stated, incremental growth in otoliths occurs through differential deposition of calcium carbonate and protein over a 24hr period and adjacent calcium carbonate and protein bands represent one growth increment. When viewed under the scanning electron microscope, after light acid etching, the calcium carbonate band (incremental band), appears as a wider, lightly etched band while the discontinuous band, composed primarily of protein, appears narrower and more deeply etched (Watabe, Tanaka, Yamada & Dean 1982). The daily increments visible in sparid otoliths under both light and scanning electron microscopy conformed to this generalized pattern of otolith microstructure. Broad incremental and narrow discontinuous increments were clearly visible in most of the otoliths prepared for daily increment analysis. Under light microscopy the incremental band had hyaline optical properties while the discontinuous band appeared opaque.

Microincrements were shown to be deposited on a daily basis in the young of sparid species examined. It would be expected that the same number of rings as days elapsed would be visible after the fluorescent zone, however, this was not always true. Reasons for this include the effects of OTC on the growth of the otolith and the different environmental conditions encountered in the laboratory. Stress exhibited by the fish after OTC administration could have affected daily increment formation because many of the treated fish stopped eating for up to three days after administration. The variation between the increment counts and the days elapsed is well known (Campana & Neilson 1982; 1985; Molony & Choat 1990) and, in addition to the physiological factors mentioned, may arise from the variability associated with reader error or poor otolith preparation (Steffensen 1980; Campana, Gagne & Munro 1987). The level of resolution available from a light microscope may not be sufficient to resolve the indistinct zones (Jones & Brothers 1987) and the need to detect the fluorescent band precludes the

use of SEM to increase resolution unless a check ring coincided with the band.

The annual periodicity of the macrostructural growth zones was validated by the mark-recapture study. One narrow opaque and one wide hyaline zone is deposited annually in the otoliths of the sparids examined. The exact width of the fluorescent band in the adult fish was difficult to discern due to the very close spacing of the daily increments within the growth zones. The otolith growth subsequent to the fluorescent band increased with increasing time post-treatment. Otolith growth in adult C. laticeps does not appear to be affected by the marking procedure. However, care should be taken when extrapolating growth estimates from tagged fish as it has been shown that the presence of an external tag seriously affected growth in tagged sablefish, Anoplopoma fimbria (McFarlane & Beamish 1990).

The observed nature of the growth zones deposited at the time of OTC marking correlated well with that expected from the marginal zone analysis done by Buxton (in press). The findings of this study substantiate the view that marginal zone analysis provides a good indirect method for the validation of growth zone periodicity in sparid otoliths. It also validates the interpretation of the time of zone formation determined by Buxton (op. cit.).

The microstructure of the juvenile sparids was similar to the generalized pattern found in other juvenile fish otoliths (see reviews by Campana & Neilson 1985 and Beamish & McFarlane 1987). Features such as multiple primordia, subdaily increments, scattering of increment groups and check rings are all commonly noted in otolith microstructure. This indicates that the basic structure of most otoliths is similar, but that there are wide interspecific variations. These variations arise from differences in the timing of increment deposition, the rate of increment deposition, the effect of internal and external variables on increment deposition and the interpretation of the annual growth

zones formed by changes in daily increment width.

Daily increment series in sparid otoliths were interrupted by many prominent, deeply etched, check rings. The majority of these check rings occurred with a regular periodicity while others were either randomly distributed within the otolith or could be associated with a stressful event, such as capture or OTC administration. The presence of check rings within the daily increment pattern has been noted by a number of workers (Campana 1983; Morales-Nin 1987a; 1987b; Gauldie 1987; 1988a; 1988c; 1990). Although it has been suggested that the checks may represent areas of otolith resorption (Mugiya & Uchimura 1989), it has been shown that while calcium deposition is considerably reduced during check formation no calcium resorption occurs (Campana 1983; Yoklavich & Boehlert 1987). The stress induced by laboratory conditions appears to be responsible for the greater frequency of check rings visible near the otolith margins in the laboratory held specimens compared to the wild fish (Morales-Nin 1987a). Once an accurate correlation has been made between event and the production of a check ring, short-term stressful events such as starvation or cold shock may be used to mark sparid otoliths in laboratory studies (Brothers 1990).

The rhythmic growth patterns found in the otoliths of a number of species (Morales-Nin 1989; Pannella 1974; Campana 1984a) were also evident in the sparid otoliths examined. The decrease in calcium required to produce the regular series of check rings may be under endogenous control, probably linked to environmental cues such as tidal cycle or cyclical temperature changes (Campana 1983). The formation of random checks in the wild may be related to natural stressful events in the life-history of the fish (e.g. spawning) or to sudden environmental changes (e.g. rapid temperature change during cold upwellings). The appearance of more check rings in the opaque zones of sparid otoliths has not been previously noted in other species and may be related to stressful events such as spawning which often occur during opaque zone formation. Although it has been suggested that naturally

occurring check rings may be used to record ecological and physiological changes (Radtke et al. 1988), the difficulty in distinguishing between different check rings and associating them with their causes precludes this possibility in sparid otoliths.

The presence of juvenile, split or incomplete rings and of zone stacking in the otoliths of older fish examined in this as well as in other sparid ageing studies (Buxton & Clarke 1991; Smale & Punt 1991), emphasizes the need for accurate validation of increment periodicity as well as the vital importance of accurate otolith reading. It is imperative that sparid otoliths are read a number of times by more than one experienced reader to ensure accurate age determination.

The nucleus of all sparid otoliths examined appeared opaque, regardless of the viewing technique. This initially appears contradictory as early growth is fast and should appear hyaline. Otolith growth originates from a small nuclear region with subsequent 3-dimensional growth occurring around the whole nucleus. Due to the effects of growth restriction in the sulcul and antisulcul regions, the daily increments in these areas are spaced closer together than in the dorsal and ventral regions causing the nuclear region to appear opaque.

Although Gauldie (1988b) proposed that the appearance of opaque zones within the otolith may be caused by the visual effects of surface sculpturing, no such correlation was found in the sparid otoliths examined. Surface sculpturing was only visible in a small percentage of the D. cervinus hottentotus otoliths examined, while all of the otoliths had visible growth zones. The zones visible in sparid otoliths are therefore produced by differences in the optical density within the otolith and are not due to the optical effects of surface sculpturing.

Annual opaque and hyaline zones visible in otoliths under low power light microscopy are formed by changes in the calcium to

protein ratio (Mugiya 1964b; 1990) and are due to seasonal variations in the width of daily increments (Radtke 1984; Radtke et al. 1985). The wider annual zones in sparid otoliths, the hyaline zones, are composed of wide daily increments with prominent incremental phases. The incremental phase has hyaline optical properties and is composed primarily of calcium carbonate (Casselman 1974). As the daily increments in the opaque zones are spaced closer together this zone appears optically more dense relative to the adjacent zone. The discontinuous bands of the daily increments in the opaque zone are closer together producing a protein-dominant zone. This interpretation was substantiated by burning whole otoliths. The opaque zone appeared more darkly charred, indicative of a higher organic content (Dannevig 1956).

Although a few studies have shown no consistent relationship between otolith or body size and growth rate in very young fish (Neilson & Geen 1985), most studies on older fish support the hypothesis that growth rate affects the relative size of the otolith in a consistent manner (Blacker 1974; Mosegaard, Svedang & Taberman 1988; Reznick, Lindbeck & Bryga 1989; Casselman 1990; Gauldie 1990). The suggestion that daily increment width provides a record of growth in juvenile fish (Radtke 1984; Barkman & Bengston 1987; Roland Pitcher 1988; Fowler 1989) has been substantiated by the results of this study. The width of each increment varied in accordance with its distance from the nucleus and its position relative to an opaque or hyaline zone. As expected, the daily increments were wider in younger fish than in older fish, due to a decrease in size specific growth rate (Radtke & Dean 1982; Radtke & Targett 1984). The narrow daily increments found in the opaque zone indicate that in sparid otoliths the opaque zone is representative of slow growth while the wide spacing of the microincrements in the hyaline zone is representative of fast growth. This finding in relation to the interpretation of the growth zones of South African sparid otoliths confirms the conclusions drawn by Buxton (1987) and Buxton & Clarke (1989), (1991), while negating those of Buxton & Clarke (1986) and Pulfrich and Griffiths (1988) and will be dealt

with in more detail in the general discussion.

The large degree of variation found in the measurements of increment width may be due to differences in the plane of sectioning (Radtke 1987) or differences in the axis along which the measurements were made. Although every attempt was made to ensure that the otoliths were sectioned through the nucleus and that measurements were only made along the dorsal free growth axis, slight variations in these factors probably account for the observed differences.

The suggestion that otolith growth differs locally (Irie 1960; Fowler 1989; Gauldie 1990) is supported by the results of this study as maximal growth occurred along the dorsal free growth line while minimal otolith growth occurred in the antisulcul regions. Due to the restriction of growth in the sulcul and antisulcul regions, caused by the constraints of otolith shape and position in the skull, the rings in these areas provide a censored version of the increments (Gauldie 1990). Growth along the dorsal free growth axis is unrestricted and probably represents the maximum growth of the otolith (Gauldie op. cit.). In future sparid ageing studies increment enumeration should only be done along the dorsal free growth axis to ensure accurate age estimation.

The measurements of whole otolith width, length and mass confirm the results of the sectioned otoliths with respect to the localization of growth. In both species, otolith mass increased exponentially with fish length. This indicates that the otolith continues to grow even after fish length has reached an asymptote. The linear relationship between otolith length and fish length in D. sargus capensis and the power relationship for otolith width verify the suggestion that maximal growth occurs along the longitudinal axis. In D. cervinus hottentotus both the above relationships can be described by a power curve, producing the slightly rounder otoliths.

From the above discussion a number of features concerning the structure of the sparid otoliths examined have emerged:

1) Sparid otoliths are composed of microstructural increments deposited with a daily periodicity.
2) Periodic and stress induced random check rings are found within the daily incremental series.
3) The width of the daily increments varies in relation to both the age of the fish and the season of deposition.
4) Otolith growth differs locally with maximum growth occurring longitudinally and otolith mass increasing exponentially with fish length.
5) Annual opaque and hyaline growth zones are visible in the otoliths of adult fish. Narrow daily increments are found in the opaque zone while the microincrements in the hyaline zone are wider.

CHAPTER 6. INFLUENCE OF ENVIRONMENTAL FACTORS ON DEPOSITION

Introduction

Environmental variables such as temperature, food availability and photoperiod all have the potential to influence otolith increment deposition (Campana & Neilson 1985). Although it is agreed that an endocrine driven, endogenous circadian rhythm, entrained during early life by some external variable is responsible for daily increment deposition (Tanaka et al. 1981), the full extent of the influence of external factors on the formation of increments has not yet been determined (see reviews by Campana & Neilson 1985; Jones 1986; Beamish & McFarlane 1987).

Both the rate of deposition and width of microincrements are affected by intrinsic factors such as age, sexual state and responses to stress, as well as by external environmental factors. In the wild the exposure of the fish to the above variables is unknown. Despite this it was necessary to investigate the influence of these factors on increment deposition to facilitate accurate interpretation of the increments. The aim of this study was not to force depositional changes through the imposition of extreme environmental changes but, with the exception of photoperiod, to examine the effects of slight environmental changes, well within their natural range, on increment deposition. As extreme photoperiod changes would not be encountered naturally this factor was used to investigate the importance of photoperiod in the maintenance of the endogenous rhythm which governs increment deposition.

Materials and Methods

Juvenile sparids including D. sargus capensis, D. cervinus hottentotus, S. salpa and R. holubi, ranging in size from 40-150mm FL, were captured and maintained in the laboratory under the conditions described in Chapter 3. Prior to the initiation of

each experiment acclimated fish were injected intramuscularly with 250mg/kg OTC to provide a marker in the otolith from which further increment deposition could be monitored.

Three parameters, photoperiod, temperature and feeding periodicity, were used to determine the effect of certain environmental conditions on the deposition of daily increments. Only one environmental variable was changed in each experiment.

a) Photoperiod

To test the effect of photoperiod on increment deposition in otoliths, ten fish were held for 20 days under a 16:8 light:dark (L:D) regime. Following this they were held for a further 40 days under a 24:24 L:D photoperiod. Photoperiod was then returned to 16:8 L:D for the following 20 days.

b) Feeding regime

The effect of feeding on the formation of daily increments was examined by comparing the otoliths of 15 fish fed once daily to those of another 15 fed regularly throughout the day. The average size of the fish prior to the experiment was the same in both groups. This experiment ran for 20 days.

c) Temperature

To examine the effects of temperature change on the deposition of microincrements, water temperature for 15 fish was raised overnight by 4° to 28° C. After 40 days they were returned to control conditions for a further 20 days.

On the completion of each experiment, the otoliths were removed and prepared for both light and scanning electron microscopy according to the general methodology.

Results and Discussion

A difference in the daily increment pattern was observed in fish transferred from a 16:8 L:D photoperiod cycle to a 24:24 L:D cycle (Fig. 21). The width of the incremental and discontinuous phases of each microincrement was measured separately as initial examination suggested that there may be a difference in increment phase width between the two photoperiods. A one-way analysis of variance using a Scheffe multiple range test showed that there was a significant difference in the width of both the incremental and discontinuous phases between the two photoperiods (incremental phase: $p < 0.004$, df (1,17), $F = 11.026$; discontinuous phase: $p < 0.0003$, df (1,18), $F = 19,521$). A check coincident with OTC administration was noted in some of the otoliths while in others a check corresponding to the time that the photoperiod was returned to normal was noted.

Results concerning the effect of photoperiod on the deposition of daily increments are contradictory. In this study the alteration of the light dark cycle from 16:8 L:D to 24:24 L:D had a limited effect on the formation of daily increments in young sparids. Similar results were found by Neilson & Geen (1982) in Onchorhynchus tshawytscha and Campana & Neilson (1982) in Platichthys stellatus, while Taubert & Coble (1977), Tanaka et al. (1981), Radtke & Dean (1982), Campana (1984b) and Mugiya (1987) working on the embryos, larvae and juveniles of a number of species, found that significant differences in microincrement deposition were induced through changes in photoperiod. The laboratory reared fish used in the three latter studies were very young (average <130 days) and exposure to photoperiod changes took place under laboratory conditions before the entrainment of a natural endogenous rhythm. The fish used in this study, as well as those used by Campana & Neilson (1982), were wild caught and older than four months. The photoperiod change was not sufficient to alter the established natural rhythm of the older, wild caught fish.

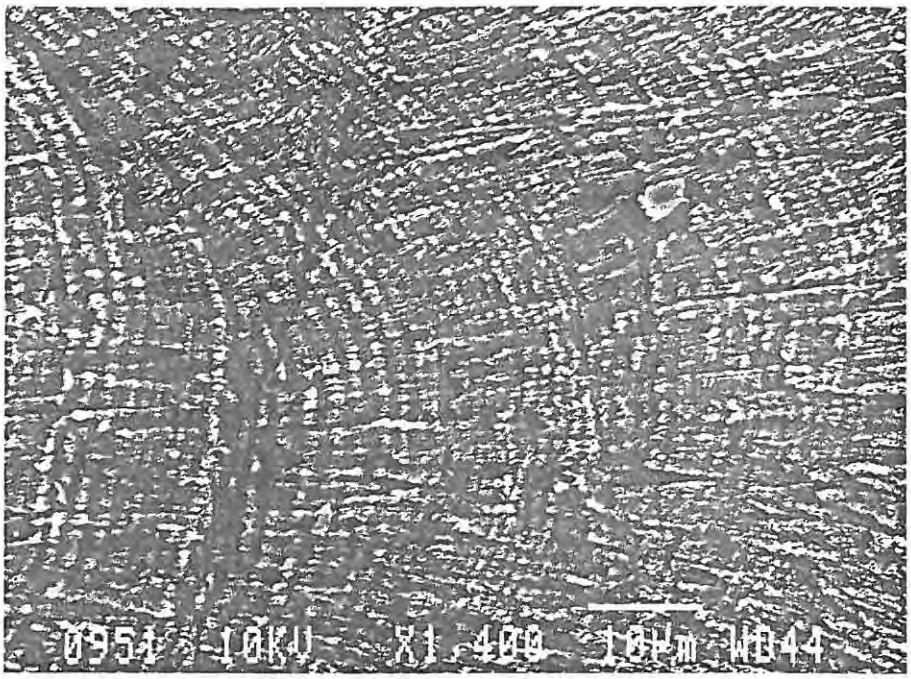

Figure 21 Scanning electron photomicrograph of the otolith of a D. cervinus hottentotus (93mm FL) exposed to a 24:24 L:D and a 16:8 L:D photoperiod. Clear check ring (c) coincident with the change in photoperiod from 16:8 to 24:24.

The slight increase in increment width found under the 24:24 L:D regime may be accounted for by the increased photoperiod. The dark phase was considerably longer under the experimental regime resulting in the greater significance in the differences in the discontinuous phase. The stress induced by the sudden alteration in photoperiod back to the 16:8 L:D regime may have been sufficient to cause the formation of a check ring in the otolith. A check may also have been present at the first change in photoperiod but may have been confused with one caused by OTC administration.

No significant difference in the pattern of deposition or the width of daily increments was noted in fish exposed to different feeding regimes (Fig. 22). The suggestion by Campana (1983) that multiple feedings may induce the formation of subdaily increments was not confirmed for young sparids. Although Neilson & Geen (1982) and Eckmann & Rey (1987) noted a response to changes in ration level, no feeding related change in increment deposition was noted by Taubert & Coble (1977) and Tanaka et al. (1981) in tilapia.

Although the responses noted by Eckmann & Rey (1987) were visible within one to three days of change, Molony & Choate (1990) and Neilson & Geen (1985) both noted a lag period of up to three weeks before changes in increment width became detectable. The duration of the feeding trial in this study may not have been sufficient to enable the detection of minor differences in increment width.

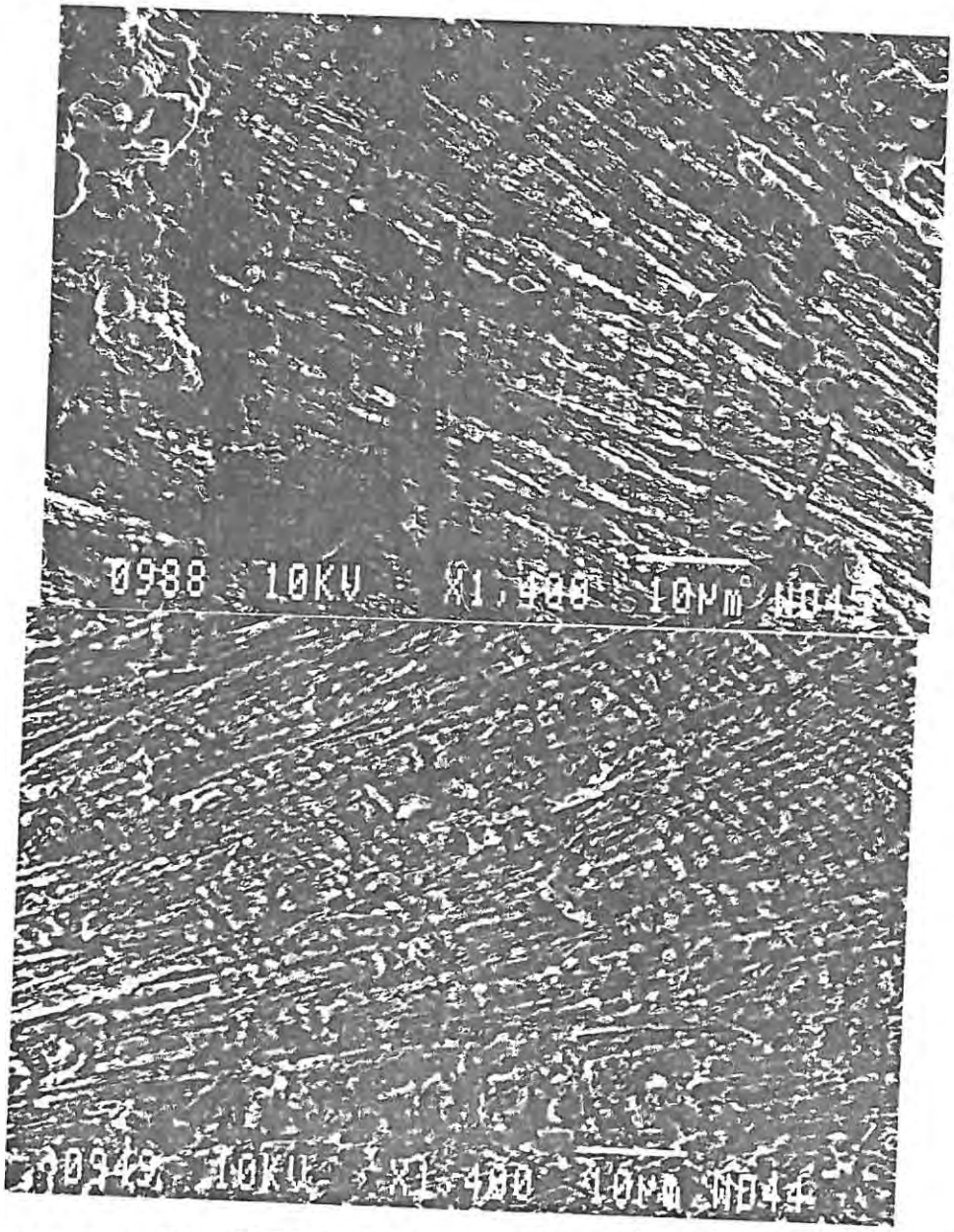

Figure 22 No difference in the microincremental depositional pattern visible in the otolith of a D. sargus capensis exposed to a multiple feeding regime (A) and another D. sargus capensis fed once daily (B).

The temperature change to which the young sparids were exposed did not influence either the rate of deposition or width of the daily increments. A check ring coincident with the sudden temperature change was, however, noted in 46% of the otoliths examined. Other workers have found no inhibition of daily increment formation under conditions of constant high or low temperatures (Taubert & Coble 1977; Campana & Neilson 1982; Neilson & Geen 1982; Radtke & Dean 1982), except where low temperatures have resulted in the cessation of growth. The temperature change used in this experiment may not have been sufficient to alter the established daily increment deposition cycle. Although sudden temperature changes in the natural environment may induce the formation of a check ring, naturally occurring long-term high or low temperatures are unlikely to alter natural increment formation.

Environmental changes have different effects on the formation of daily increments in adult and larval fish. In adult fish, preconditioned to the natural environment, the altered environmental conditions can only mask the effects of the established circadian rhythm, perhaps through altering the metabolic rate. Alternatively the exposure of younger fish (embryo or larval) to altered environmental conditions before the establishment of a circadian cycle is most likely to influence increment deposition (Campana & Neilson 1985). The fish used in these experiments were beyond the stage of entrainment by environmental factors and, as the endogenous rhythm had already been established in the fish, the effects of the environmental changes were not sufficient to mask the cycle of deposition. The formation of a check due to the sudden change in water temperature may be used to intrinsically mark otoliths in future validation experiments.

The effects of laboratory conditions were visible as a lower degree of visual contrast in the daily increments after capture in otoliths of both the experimental and control fish (see Fig. 6). Although the increments were visible, the visual definition

of the daily increments was not as clear as in the wild fish. This decrease in the visual clarity of the microincrements was initially attributed to the effects of OTC on the growth of the otolith however, as the microincrements deposited after capture were equally unclear in both the experimental and the control fish it appears that the environmental conditions in captivity produced this effect. Visual contrast in otolith increments is largely a function of environmental conditions as constant temperatures, such as those usually found under laboratory conditions, appear to reduce increment contrast and render growth patterns less clearly defined (Campana & Neilson 1985; Alshuth 1988; Townsend, Radtke, Morrison & Folsom 1989). The absence of normal cyclical activities (e.g. diurnal feeding cycles and migrations) in captive fish may also affect the visual contrast of the daily increments.

These results indicate that daily increment formation in the otoliths of juvenile sparids remains constant under a variety of experimental conditions. This supports the suggestion that unnatural photoperiod, feeding or temperature regimes are not likely to alter the depositional pattern in otolith microstructure in juvenile fish preconditioned to a natural environmental regime (Campana & Neilson 1985). The microincremental patterns visible in wild fish are therefore unlikely to be significantly altered by natural environmental changes.

CHAPTER 7. ELECTRON MICROPROBE ANALYSIS

Introduction

The primary chemical constituent of otoliths, calcium carbonate, can be contaminated by a number of trace elements, primarily strontium. Strontium (Sr) has the same +2 valence as calcium (Ca) and can therefore substitute for calcium and become incorporated into the aragonite crystal (Townsend *et al.* 1989). Strontium/calcium ratios in aragonitic corals have been negatively correlated to temperature at the time of calcium deposition (Smith, Buddemeier, Redalje & Houck 1979). However, it has been shown that, in addition to temperature, the species of coral, growth rate, metabolism and other biological factors also affect the incorporation of Sr in the aragonitic matrix (Kalish 1989). Strontium/calcium ratios measured in the seasonal growth zones of otoliths of a variety of fish species were found to be inversely related to temperature (Radtke & Targett 1984; Radtke 1989a; Radtke & Morales-Nin 1989; Townsend *et al.* 1989). However, Kalish (1989) disputed this and found no evidence for a linear relationship between Sr/Ca ratios and temperature in blue grenadier (*Macruronus novaezelandiae*) and Australian salmon (*Arripis trutta*). To confirm the timing of zone formation determined with OTC labelling, differences in the calcium profiles and Sr/Ca ratios in the seasonal growth zones of sparid otoliths were investigated.

Materials and Methods

A wavelength dispersive JEOL 733 electron microprobe was used to analyse the strontium and calcium present in the otolith. Otoliths used for electron microprobe analysis were first esonified to cleanse them of any adhering tissue, mounted in clear epoxy resin and sectioned with a twin blade diamond saw. The sections were mounted on glass slides with a small amount of epoxy resin, ground with 5um alumina oxide and polished with 15um, 5um and 1um diamond spray. The sections were buffered on a

cellulose disc with 1/4um diamond spray. This process ensured that the surface of the otolith was extremely smooth prior to analysis with the electron microprobe, as any inconsistencies on the otolith surface would result in large diffractions of X-rays leading to analytical errors. The specimens were vacuum coated with a thin layer of carbon to further dampen X-ray diffraction and to increase electron conductance.

Analyses were made across sections of the otolith that contained clear annual zones visible under the light microscope. Spot analyses were executed at 10um intervals with a beam size of 10um. Probe current and accelerating voltage were 15nA and 15kV respectively. Long counting times (50 sec) and the relatively low current were used to prevent damage to the otolith surface. When shorter counting times and higher probe currents were employed, burn depressions were left on the otolith surface.

After microprobe analysis, the samples were photographed under light microscopy to establish a correlation between the area probed and the resultant Sr/Ca profile.

Results and Discussion

Initial work indicated that pure calcium analyses were ineffective due to the high levels of background calcium throughout the otolith. No correlation could be found between the amount of calcium present and the presence of either an opaque or hyaline zone.

Twenty Sr/Ca analyses were performed on ten otolith sections. The analyses of only one section provided any clear correlation between the Sr/Ca profile and the presence of an opaque zone (Fig. 23 & 24). In the section where a clear correlation was visible an increase in the Sr/Ca ratio was found coincident with the appearance of the opaque zone while the Sr/Ca ratios were lower in the hyaline zones (Fig. 23).

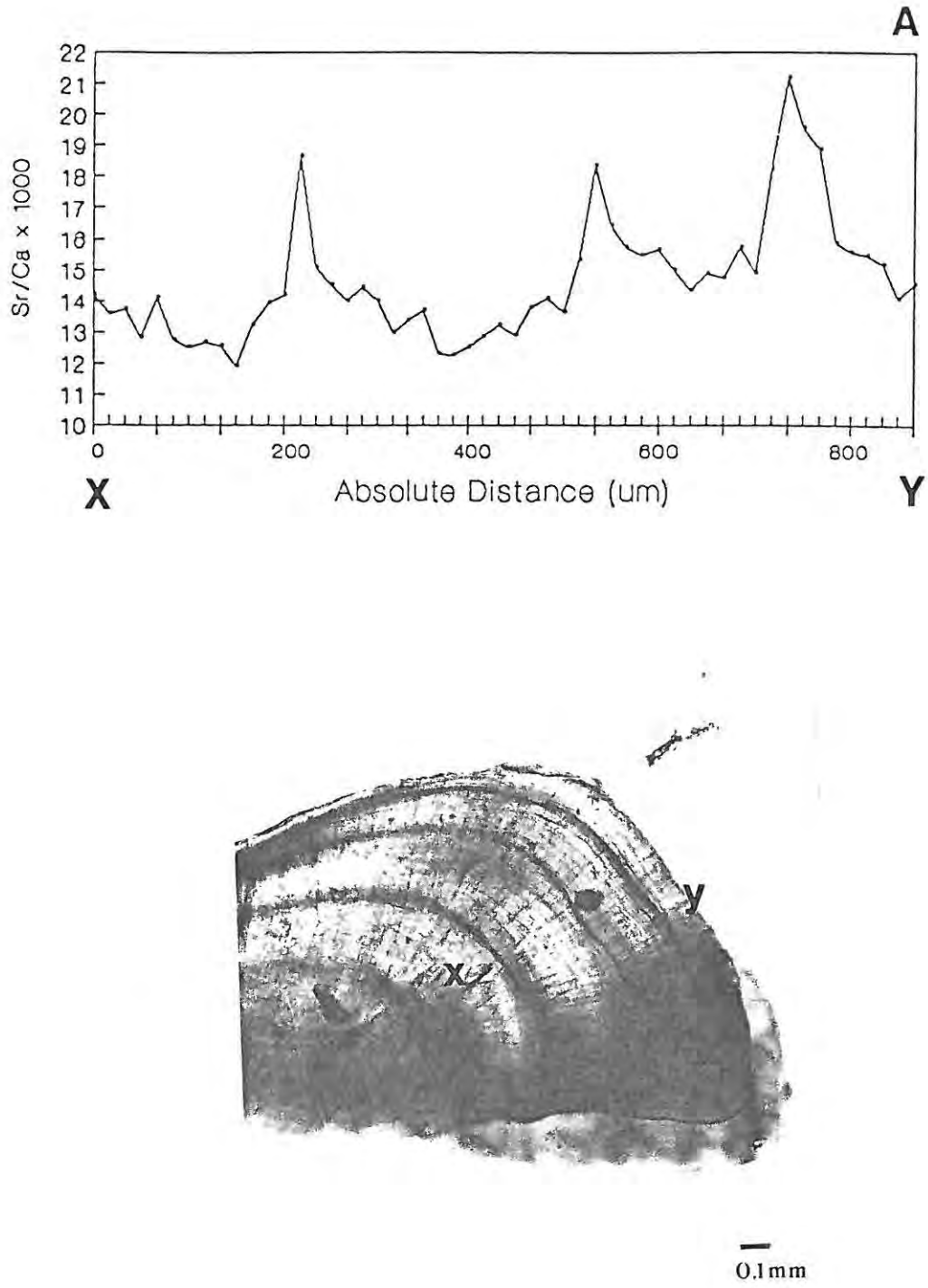

Figure 23 A: Sr/Ca ratio profile determined by wavelength dispersive electron microscopy. B: Area scanned X to Y as indicated on the photomicrograph. Otolith section from a 240mm (FL) D. sargus capensis. Distance measured is absolute distance along the otolith section. Sr/Ca ratios multiplied by 1000 for presentation.

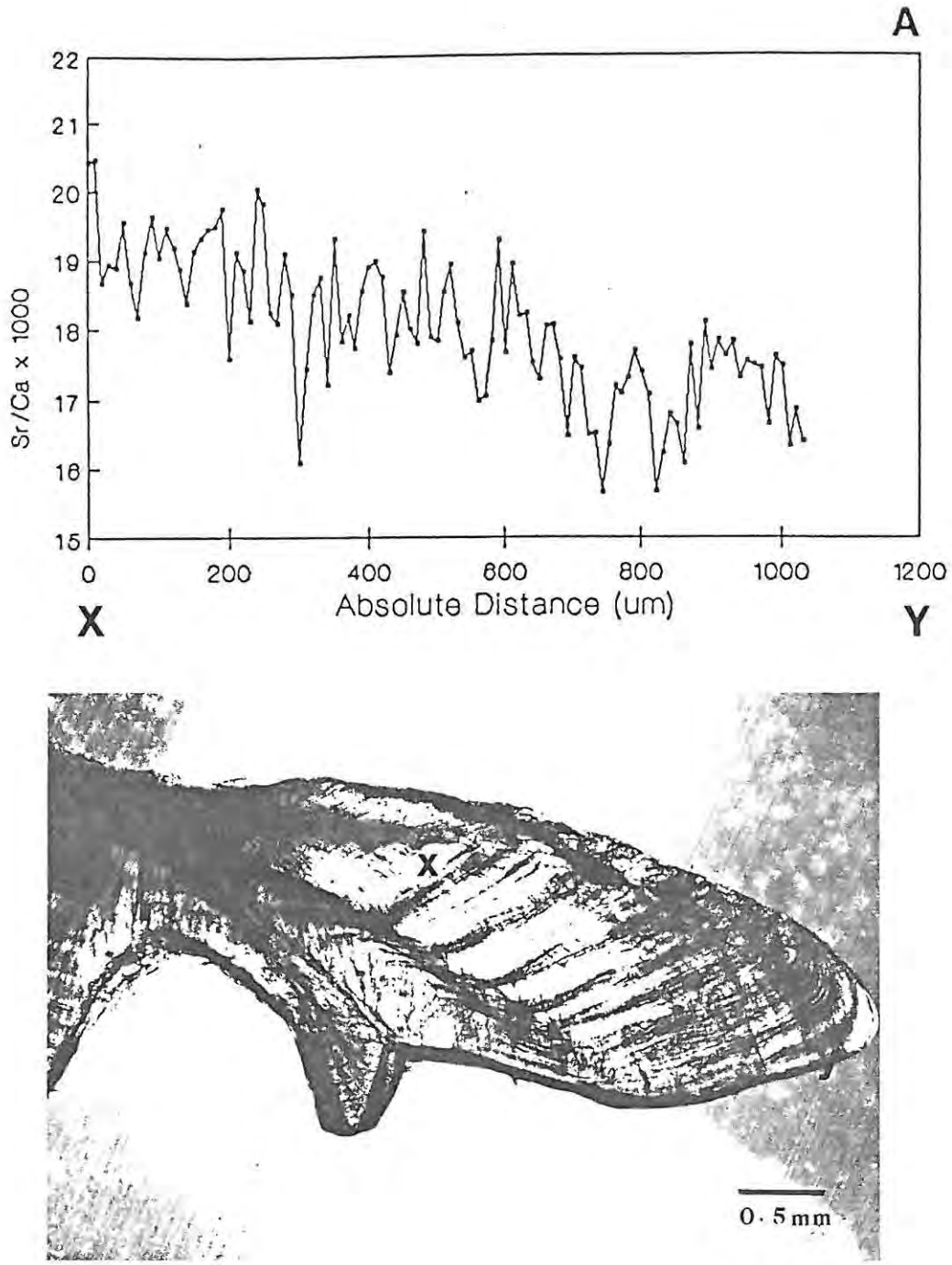

Figure 24 A: Sr/Ca ratio profile determined by wavelength dispersive electron microscopy. B: Area scanned X to Y as indicated on the photomicrograph. Otolith section from a 262mm (FL) D. cervinus hottentotus Distance measured is absolute distance along the otolith section. Sr/Ca ratios multiplied by 1000 for presentation.

Attempts to confirm the time of formation of the growth zones using strontium/ calcium microprobe analysis were inconclusive. Although four out of five analyses performed on one section produced a strong correlation between the position of the opaque zone and the Sr/Ca ratio, the results from all other sections were poor. This indicates that, while the technique is accurate more emphasis should be placed on the selection of the otolith sections for analysis, only sections with clearly defined opaque and hyaline zones should be used.

Smith et al. (1979), Radtke & Targett (1984) and Townsend et al. (1989) all found the Sr/Ca ratios in otoliths to be negatively correlated with temperature. In this study the highest Sr/Ca ratios were noted in the opaque zones. If the interpretations of the above authors are correct, the data suggests that this zone was deposited at a time when ambient water temperatures were low. This result appears contradictory to those presented in Chapter 5 where the opaque zone was found to be deposited primarily during summer. The lack of wide seasonal temperature fluctuations (max. variation 5° C per annum) found off the south east coast of southern Africa due to the occurrence of cold upwellings during summer (Schumann, Perrins & Hunter 1982; Shannon 1989), may have influenced the the Sr/Ca ratios. The upwelling events noted during summer may have been sufficient to alter the Sr/Ca ratios thereby producing the Sr peaks found in the opaque zones. A pronounced seasonal temperature difference appears to be necessary to influence the incorporation of strontium in the otolith. The single section which produced a strong correlation between the Sr/Ca ratio and growth zone may have been exposed to the high temperature differences required to induce the incorporation of strontium. This technique may be more effective in areas where clear seasonal temperature differences arise such as the west coast of South Africa and may be used to verify the negative correlation found between hyaline zone formation and temperature in the pilchard, Sardinops ocellata (Thomas 1983).

Kalish (1989) and Radtke (1989a) have pointed out that there are a variety of factors, in addition to temperature, which may influence strontium levels in marine organisms. These include biochemical fractionation, environmental variables, mineralogy, water chemistry, species, growth rate and metabolism. The limited correlation between the appearance of an opaque or hyaline zone in the sparid otolith and Sr/Ca ratios in the other otoliths examined may be attributed to some of these variables. The regional temperature differences noted above may also have influenced the Sr/Ca ratios obtained.

Further experimental work in order to accurately correlate Sr/Ca ratios and temperature is required before this technique can be used to its full potential. Once the exact correlation between Sr/Ca ratios in sparid otoliths and environmental temperatures have been experimentally determined, the electron microprobe technique of chemical analysis has the potential to improve our understanding of otolith structure and composition.

CHAPTER 8. GENERAL DISCUSSION

There are two primary criteria which must be satisfied before otoliths can be used for the determination of age and subsequent growth rate of any fish species. The first is that a recognizable pattern must be seen in either the whole, sectioned, burnt or stained otolith. Secondly, the features recognized must be formed at a constant frequency, and must be proportional to fish growth (Campana & Neilson 1985). Only once these two criteria have been satisfied can an otolith be reliably used as an age determinator (Williams & Bedford 1974). The assignment of a particular time period to a regular feature in the microstructure of an otolith is often done in the absence of rigorous verification (reviewed by Beamish & McFarlane 1983). Without validation of the periodicity of the otolith features age determinations become questionable.

Microscopically, sparid otoliths conformed to the accepted pattern of otolith microstructure published in the literature (see Campana & Neilson 1985 and Beamish & McFarlane 1987 for reviews). Sparid otoliths consisted of a central opaque nucleus composed of multiple primordia. Surrounding the initial narrow increments around the nucleus were wide daily increments which decreased in width as the distance from the nucleus increased. This was in contrast to the results of Campana *et al.* (1987) where increment width was found to increase with age (up to 50 days). The majority of the otoliths from larger fish did not display a visible sequence of daily increments from the center of the nucleus to the edge. This common phenomenon results from the presence of multiple primordia and uneven grinding during preparation.

Both check rings and subdaily increments were visible throughout the otolith. The check rings appeared to have different origins as both periodic and random check rings were noted. Periodic check rings appeared at intervals of 12.5 or 24.8 daily increments while the random checks could often be

associated with a stressful event such as capture. Both such check rings have been previously noted in the literature (see review by Campana & Neilson 1985). Subdaily increments have also been noted by other researchers (Taubert & Coble 1977; Campana & Neilson 1982; Eckmann & Rey 1987) and although both temperature and feeding frequency have been implicated in their formation (Neilson & Geen 1982), the exact nature of their deposition remains obscure.

Exposure of the fish to mild variation in environmental conditions showed that, in young sparids preconditioned to the natural environment, minor environmental alterations in temperature, feeding frequency and photoperiod were not sufficient to alter the established endogenous rhythm of microincremental deposition. These results have confirmed speculations by Neilson & Geen (1982) and Campana & Neilson (1982; 1985), who suggested that environmental changes will not alter the pattern of increment deposition in preconditioned fish. However, extreme environmental variables have the potential to influence otolith deposition.

The growth rates of the two zones in the otolith can be inferred on the basis of their appearance in relation to daily increments (Radtke 1984). It was suggested as early as 1974 that the protein zones (opaque) represented periods of slow growth (Williams & Bedford 1974). Although this interpretation has undergone many revisions (Blacker 1974), the results of this investigation support the original interpretations. In sectioned sparid otoliths the opaque zone appears narrower than the hyaline zone. When examined under SEM this zone was found to be composed of closely spaced daily increments consistent with slower growth. The hyaline zone appeared wider and was composed of wide daily increments representative of fast growth. This correlation between otolith and somatic growth has confirmed the suggestion by Buxton (1987) that opaque zone deposition in sparid otoliths was representative of slow somatic growth while the wider hyaline zone represented fast growth. This interpretation is also in

accordance with the microstructural composition of otoliths reviewed by Campana & Neilson (1985).

The growth rate represented by opaque and hyaline growth zones has been differently interpreted in the literature. In studies on whole otoliths, wide opaque zones are apparent between narrow hyaline zones (Buxton & Clarke 1986). The wide opaque zones are usually interpreted as being representative of summer growth (Geldenhuys 1973; Hecht & Baird 1977; Buxton & Clarke 1986). However, when an otolith is sectioned and viewed under transmitted light, opaque zones appear as narrow bands between wide hyaline zones (Buxton & Clarke 1992; Buxton in press). Careful examination showed that the opaque zones of whole otoliths are equivalent to the hyaline zones of sectioned otoliths. Wide opaque and narrow hyaline zones are therefore an optical illusion caused by the thickness of the otolith (Buxton 1987). The source of the confusion surrounding the relative growth rate of the two zones stems from the early studies on whole otoliths (Nepgen 1977; Hecht & Baird 1977; Coetzee & Baird 1981; Buxton & Clarke 1986). Through marginal zone analysis the opaque zone (whole otoliths) was found to be formed during summer, it appeared wide and was therefore interpreted as being representative of fast growth. The high proportion of hyaline zones found during winter suggested to many researchers that the hyaline zone was representative of slow growth (Botha 1971; Geldenhuys 1973; Hecht & Baird 1977; Buxton & Clarke 1986; Thomas 1985; Morales-Nin 1986). By comparison, in sectioned otoliths the deposition of the opaque zone appears to coincide with spring and summer growth (Mann 1992; Buxton in press).

Table 4 summarizes the season of opaque zone deposition, as determined by marginal zone analysis, for a number of South African sparids. With the exception of Buxton & Clarke (1991; 1992) and Smale & Punt (1991), the opaque zone in South African sparid otoliths appears to be deposited during the spring and summer months, coinciding with the breeding season of these species (Brownell 1979).

TABLE 4 Period of opaque zone formation in various South African sparid species.

Author	Species	Time of Opaque Zone Formation	Method
Nepgen (1977)	*P. blochii*	Summer	W, R.
Nepgen (1977)	*A. argyrozona*	Summer	W, R.
Hecht & Baird (1977)	*P. laniarius*	August - May	W, R.
Coetzee & Baird (1981)	*C. nufar*	July - February	W, R.
Buxton & Clarke (1986)	*P. aeneum*	September-February	W, R.
Pulfrich & Griffiths (1988)	*P. blochii*	Summer	W, R.
Buxton & Clarke (1989)	*C. nasutus*	October-December	S,T,R.
Buxton & Clarke (1991)	*S. durbanensis*	June-September	S, T.
Smale & Punt (1991)	*P. rupestris*	Indeterminate	S,T,R.
Buxton & Clarke (1992)	*P. grande*	June - November	S, T.
Mann (1992)	*D. sargus capensis*	August - October	S, T.
Mann (1992)	*D. cervinus*	August - December	S, T.
Buxton (in press)	*C. laticeps*	September-February	S,T,R.
Buxton (in press)	*C. cristiceps*	September-February	S,T,R.

Method used to view otoliths
W - Whole otolith examined
S - Otolith section examined
R - Reflected light
T - Transmitted light

Hyaline zone formation occurs following the spawning season of these species. Intuitively it may be expected that somatic growth will be highest during periods when other energy demanding processes are low. Gonadal development and spawning are costly energetic processes and Simkiss (1974) has shown that less calcium is available for somatic growth during periods of reproductive activity. For this reason it is to be expected that during periods of peak reproductive activity, when very little calcium is available for otolith growth, a zone low in calcium, with a high protein content, will be produced.

In light of the findings of this work a number of published studies can be re-interpreted. Botha (1971), working on whole otoliths of Cape hake, M. capensis, suggested that the opaque zone represented a fast growth period between spawning seasons and that the hyaline zone was a period of slow growth during spawning. The observation by Botha (op. cit.) that peak feeding takes place immediately after spawning however indicates that the interpretation presented in this study is more plausible.

The lack of direct validation of the timing of zone formation by Morales-Nin (1986; 1987b) of the growth zones in Cape hake otoliths has led to a misinterpretation of their relative growth rates. Morales-Nin (1987b) accepted the interpretation of fast and slow growth determined by Botha (1971) and used this as the basis of her microstructural interpretation. She found that the microincrements in the fast growth zones (which she interpreted as opaque) were broader and more dense than the increments in the slow growth zones (termed hyaline). If the interpretation of growth zones given in this study are correct it would appear that while Morales-Nin (1987b) has accurately represented the pattern of wide daily increments in the fast growth zone she has

misinterpreted this as being the opaque zone.

Similarly, Buxton & Clarke (1986) examined whole otoliths from the sparid (Pachymetopon aeneum) and found wide 'opaque' zones (fast growth) deposited during summer and narrow 'hyaline' zones (slow growth) deposited during winter. Reproductive activity in this species was recorded during summer and from the interpretation of otolith growth shown in this study, it would be expected that this would coincide with a narrow growth zone. The reason for this apparent anomaly is not clear, however it is suggested that marginal zone analysis done on whole sparid otoliths be reviewed using sectioned otoliths due to the difficulty in determining the nature of the marginal zone in whole otoliths.

The results of this study can be used to explain the anomalous timing of opaque zone deposition in P. grande (Buxton & Clarke 1992). Marginal zone analysis showed that in this species the opaque zone was deposited during winter. Peak reproductive activity in P. grande, unlike other sparids, does not coincide with the increased temperatures and photoperiod associated with summer (Buxton & Clarke 1992). Instead, peak spawning occurs between late summer and early winter. This change in the timing of reproduction may be sufficient to influence the formation of the opaque zone in the otolith and further supports the suggestion that opaque zone formation appears to be closely associated with reproductive activity in sparids.

From the oxytetracycline labelling experiments the periodicity of the growth zones in the otoliths was shown to be annual. This result provided a direct validation of the annual periodicity of growth zones in C. laticeps and confirms the indirect validation

(marginal zone analysis) done on these species (Buxton in press). In addition, the seasonality of zone deposition determined by Buxton (in press), through marginal zone analysis of C. laticeps otoliths, has been confirmed by the results of this study. The position of the fluorescent band corresponded well with the expected growth zone at the time of OTC deposition (Appendix 1)). The opaque zone was deposited primarily during summer (September - March) followed by a season of hyaline zone formation (April - August) (Buxton 1990; in press). As the marginal zone analysis reveals percentages of edge zones, a perfect correlation between zone and season of formation cannot be expected. Unfortunately, a large number of fish were marked in February which coincides with the end of opaque and the beginning of hyaline zone deposition (Appendix 1).

While this interpretation was consistent for many of the sparids studied, it should not be applied uncritically to other species. It is strongly recommended that, where possible a direct validation of the periodicity of the growth zones be incorporated into all age studies. Furthermore, the importance of more than one experienced reader for accurate interpretation of growth zones is stressed. This is especially important in sparid otoliths from the older year classes where split rings, stacking and indistinct zones confuse the readings and tend to produce overestimates of fish age.

For the labelling of sparid otoliths, the OTC administration techniques and dosages determined in this study provide a baseline for future mark-recapture studies. Recommended dosages for adult sparids are 50 - 100mg/kg. Even a small scale mark-recapture program of this nature can provide the direct validation necessary for confident age estimation.

In conclusion, the accurate chemical labelling technique for sparid otoliths determined in this study enabled the validation of the annual periodicity of growth zones in a number of South African sparid species. A greater insight into sparid otolith depositional structure has been achieved through the analysis of both the optical and chemical characteristics of the otolith. The correlation between otolith and somatic growth rate has been achieved through both daily increment analysis and a mark-recapture study, resolving much of the current confusion surrounding this topic.

This study would be incomplete without recommendations for future studies on South African sparid otoliths.

1. Further mark-recapture studies should be undertaken in the TNP on a wider variety of sparids. Marking should preferably be done throughout the year to obtain a more comprehensive overview of the times of zone formation. Although it appears likely that all the sparids examined have a similar pattern of otolith formation, due to the effect of species specific differences, validation is required especially for species which do not conform to the suggested pattern.

2. Annual increment counts should only be made along the dorsal free growth axis or along the ventral axis as increments in the sulcul region are generally not complete.

3. In long-lived sparids, where stacking is evident in the otoliths, the marginal zone analysis should only be done on the sectioned otoliths of younger fish where the edge zone can be easily interpreted. However, care should be taken to ensure that

an unbiased sample is obtained.

4. The first opaque zone visible in sparid otoliths is generally not indicative of the first year's growth and should not be interpreted as such. Validation of this can be obtained by OTC marking of juvenile fish followed by daily increment analysis or with the use of known age fish.

5. Experimental work in order to determine the exact correlation between temperature and the Sr/Ca ratios in sparid otoliths is essential if this technique is to be used during further analysis of sparid otoliths. Once this correlation has been determined the technique promises to be effective in future studies on otolith depositional structure.

To conclude:... " We shall not cease from exploration and the end of all our exploring will be to arrive where we started and know the place for the first time."

(The Four Quartets by T.S. Elliot)

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APPENDIX 1

Marginal zone analysis of the monthly percentage frequency of hyaline zones in the otoliths of Chrysoblephus laticeps sampled in the Tsitsikamma and Port Elizabeth areas between 1983 and 1986. (Adapted from Buxton in press).