THE PERIODICITY AND FORMATION OF OTOLITH INCREMENTS IN *SALMO SALAR* AND *GASTEROSTEUS ACULEATUS*

Peter John Wright

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ABSTRACT

Teleost otoliths are calcareous structures deposited within the otic sacculi and are involved in mechanoreception. They are composed of aragonite crystals and a proteinaceous matrix. Accretion occurs through the successive deposition of a mineral rich and mineral deficient layer. In many fishes these two layers are laid down over a day, so producing a recognisable daily increment. This study investigated the formation of otolith increments and the influence of intrinsic and extrinsic factors on increment periodicity and size in two species of freshwater fish, the parr of Atlantic Salmon, *Salmo salar* and the three-spined stickleback, *Gasterosteus aculeatus*.

A daily periodicity of increment formation, under a natural range of photoperiod and temperature, was confirmed for both species, by sequential sacrifice of fish of known age and fish with marked otoliths.

Histological, histochemical and scanning electron microscopy techniques were used to investigate the formation of otoliths. Calcospherules (2-8μ) were found in dense layers over the apical surface of certain epithelial cells and on the otolith surface, suggesting that these structures were involved in otolith formation. Four types of non-sensory epithelia were identified within the sacculus; one of these (designated type II cells) appeared to secrete a fibrous material, containing sulphated glycoprotein, that was involved in crystal growth. A higher density of matrix fibres were found in the mineral-

deficient layer than in the mineral-rich layer of the otolith increment. It was suggested that the high density of fibres has a limiting effect on crystal growth.

A soluble calcium-binding protein, capable of inhibiting calcification *in vitro*, which may have been involved in the periodic inhibition of otolith mineralisation, was extracted from the otolith.

The influence of extrinsic factors on increment periodicity was investigated in a series of experiments in which light, temperature and feeding frequency were systematically varied. Daily increments continued to be deposited even under constant conditions, indicating that increment deposition was endogenously controlled. However a 6L:6D photoperiod regime induced two indistinct increments per day in the otoliths of both salmon parr and sticklebacks.

The effect of light/dark transitions on increment formation was investigated further in a series of *in vivo* ^{45}calcium uptake experiments on salmon parr for a range of natural photoperiod regimes. These experiments demonstrated that a change from a net positive to a net negative flux from the otolith occurred at around three to five hours prior to dawn. A return to a net positive flux of calcium on to the otolith coincided with the dark:light transition indicating that the rhythm of increment formation was entrained to the light-dark cycle.

An analysis of variations in total calcium concentrations in the plasma of salmon parr suggested that calcium levels declined around the time of no net deposition on to the

otolith, although a regular cyclical decline was not evident in the limited data set. The possible influence of plasma calcium concentration on otolith calcification was investigated by examining the effect of induced hypocalcemia on *in vivo* ^{45}calcium uptake on to the otolith. Induced hypocalcemia led to a decrease in the calcium concentration of the plasma and a net calcium efflux from the otolith, thus suggesting a positive relationship between plasma calcium concentration and otolith calcification.

A comparison of somatic and otolith growth in salmon parr that grow throughout autumn and winter and smolt in their first year (S1) and those which almost cease feeding in autumn and smolt in their second year (S2), indicated an uncoupling of the two processes in S2 parr. However increment width was found to be positively correlated to oxygen consumption, in both S1 and S2 salmon parr, indicating that otolith accretion was related to metabolic rate. Consequently the uncoupling observed, may be explained by a decline in somatic growth during a metabolically favourable period.

The results of this study were discussed in relation to previous concepts of the process and control of otolith increment formation.

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Chapter 1

GENERAL INTRODUCTION

1.1 USE OF CALCIFIED TISSUES IN STUDIES OF FISH GROWTH

Age determination is central to fisheries research (Bagenal, 1974) and is relevant to most aspects of fish biology. The most reliable means of determining age in fishes for more than two hundred years has been provided by growth increments in calcified tissues (Bagenal, 1974). Growth increments are produced by alternate periods of fast and slow growth and are associated with changes in the relative proportions of calcified and non-calcified constituents (Love, 1970; Mugiya, 1964). These may reflect various environmental or internal influences (see Simkiss, 1974; Bilton, 1974) but where factors responsible vary with a regular periodicity the increments produced can be used to estimate age.

In addition to using increment number to assess age increment width can be used to backcalculate somatic growth, provided that the growth of calcified tissues is proportional to somatic growth (Bagenal & Tesch, 1978). Further, life-history transitions and environmental changes experienced by an individual may be recorded in calcified tissues as such events can induce structural changes in the otoliths.

Annual growth patterns, termed annual marks, annual rings or annuli, are often distinguishable within calcified tissues. Annuli have been found in nearly all large

calcified tissues, including vertebrae, opercula, cleithra, spines, scales and otoliths (see Bagenal & Tesch, 1978 for review). Otoliths, which are calcareous structures within the inner ear, and scales are the most frequently used for age determination. Scales have the advantage that their removal does not require killing the fish. However studies of calcium metabolism have shown that the calcium contained in scales can be resorbed in times of stress (see Simkiss, 1974), resulting in the loss of growth information. Unlike scales, the calcium in otoliths does not appear to be freely exchangeable with the body fluids (Simkiss, 1974; Ichii & Mugiya, 1983) and otoliths may continue to accrete under conditions of food deprivation (Marshall & Parker, 1982, Campana, 1983a). Thus under many circumstances otoliths provide a more reliable means of age determination than do scales.

1.2 DAILY OTOLITH INCREMENTS

An important advance in age determination studies in recent years has been the suggestion (Pannella, 1971) and subsequent verification (Brothers *et al.*, 1976) of daily growth increments in the otoliths of some teleosts. Daily increments in otoliths are now known to be a widespread phenomenon, present in taxa from both freshwater and marine habitats and in species distributed from the polar regions (Townsend, 1980; Townsend & Shaw, 1982) to the tropics (Ralston, 1976; Victor, 1982; Gjosaeter *et al.*, 1984). This discovery has enabled fishery biologists to study the age composition and growth of early life-history stages and

of short-lived species. Daily increments have been used to study larval cohorts (Miller & Storck, 1984; Isely & Noble, 1987; Savoy & Crecco, 1987; Thorrold & M^{C}B. Williams, 1989), thus promoting studies of recruitment. Daily ageing has also made it possible to construct age-growth curves for short-lived species, which is of particular benefit in studies of the many tropical species with a protracted spawning season whose growth cannot easily be studied by length-frequency analysis (Struhsaker & Uchiyama, 1976; Gjosaeter, 1982; Wright *et al.*, 1990).

Otolith radius is often significantly correlated with body length (Wilson & Larkin, 1982; Volk *et al.*, 1984) indicating that the width of daily increments may provide a means of back-calculating daily growth within individuals. Otolith microstructure may provide an accurate record of life-history transitions and environmental changes experienced by young fish as such events often lead to changes or interruptions in daily increment deposition. This has enabled researchers to determine the time of hatching (Radtke & Dean, 1982; Savoy & Crecco, 1987; Radtke, 1989), of metamorphosis (Brothers & McFarland, 1981) and settlement (Victor, 1982) in some species. Further changes in the fishes' environment may also be recorded such as the time of migration from freshwater to seawater in anadromous and estuarine species (Neilson *et al.*, 1985).

The increasing number of reports of daily increment formation has led many researchers to infer that otolith increments are always formed at a daily periodicity (see

Gjosaeter *et al.*, 1984 for examples). However such an assumption is invalid for a number of reasons. Firstly, otolith increment deposition may not be daily in all species (Geffen, 1982a; M^{C}Gurk, 1984; Alohassini & Pitcher, 1988). Secondly, the daily deposition of increments generally appears to cease in adult and/or juvenile life-history stages of long-lived fish (Pannella, 1971; 1980). Further, the interpretation of microstructural growth patterns in wild fishes requires a knowledge of the factors that may affect the production of one increment per day (Gjosaeter, 1982; Campana & Neilson, 1985). Whilst the deposition of increments in a rhythmic fashion could be a mark of a daily event and increment width possibly a measure of growth, the validity of this technique depends on the influence of external and internal factors on the formation of otolith increments, and this has rarely been tested.

Environmental variables such as photoperiod (Taubert & Coble, 1977; Radtke & Dean, 1982) and temperature (Brothers, 1978; 1981) and patterns of food availability (Neilson & Geen, 1982) may influence increment production in some species, although no universal influence has been identified. In cases where increment deposition does not appear to be daily, a positive relationship between increment deposition rate and somatic growth has been indicated (Geffen, 1982a; Alhossaini & Pitcher, 1988).

In order to resolve these problems it has become clear that an understanding of the physiological process and regulation of otolith accretion and the environmental

factors that influence it, is needed (Gjosaeter, 1982; Campana & Neilson, 1985). The main aim of this study is to investigate the mechanisms of increment formation in two freshwater fishes.

1.3 STRUCTURE AND FUNCTION OF THE OTOLITHS

Otoliths lie within the otic sacs of the inner ear, which in fishes are paired structures embedded in the cranium on either side of the head close to the midbrain. The gross anatomy of the labyrinths and the structure of the labyrinthine mechanoreceptor organs are known from many fishes (Lowenstein, 1971). Each ear is a complicated structure of canals, sacs and ducts filled with endolymph, a fluid with special viscous properties (Figure 1.1). Teleosts have three semicircular canals, each incorporating a bulbous expansion, the ampulla, occluded by a jelly-like flap or diaphragm, the cupula. The otoliths are contained in three expanded interconnected sacs; the sacculus (containing the sagitta), the utriculus (containing the astericus), and the lagena (containing the lapillus). The sagitta is usually the largest of the three otoliths and is most often used for age determination.

Each otolith is fixed over a region of sensory epithelium (the macula) by an otolithic membrane, into which sensory cilia project. According to Dunkelberger *et al.* (1980), the otolithic membrane consists of two different zones: a structured gelatinous zone, which usually exhibits a reticulated or honeycomb architecture, covers the sensory region of the macula while a non-structured, sub-cupular

Figure 1.1

Diagram of the inner ear of Atlantic salmon (*Salmo salar* L.).

Key:

ac = anterior canal

pc = posterior canal

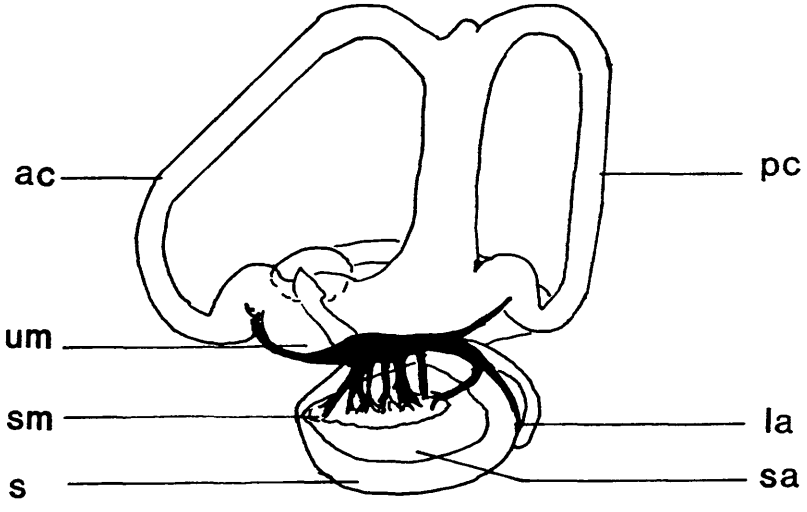
um = utricular maculae

sm = saccula maculae

s = sacculus

sa = sagitta

la = lagena


ac
pc
um
sm
la
s
sa

zone consisting of fibres in very loose networks covers sensory and nonsensory regions of the macula. The gelatinous zone extends from the otolith surface to the tips of the sensory hairs and probably functions primarily in mechanoreception.

The otic sacs are believed to serve several functions (see Lowenstein, 1971; Hawkins, 1986). The semicircular canals and utriculus are usually concerned with the maintenance of equilibrium. The sacculus and lagena usually function as receptors of gravity and sound (Lowenstein, 1971). The otoliths add mass to the gelatinous layer of the three otic sacs, increasing their sensitivity to gravitational and other linear acceleratory forces (Hawkins, 1986; Ross & Pote, 1984).

1.4 STRUCTURE AND COMPOSITION OF THE OTOLITH

Otoliths are chiefly composed of crystallised calcium carbonate (in the form of aragonite) bound by a 'collagenous', fibrous protein, termed otolin (Degens *et al.*, 1969; Dunkelberger *et al.*, 1980). Otolith growth occurs through the deposition of new material over its surface. Ultrastructural analysis has revealed that each increment is made up of a mineral-rich continuous or incremental zone and a smaller matrix-rich, mineral-deficient discontinuous zone (terminology of Tanaka *et al.*, 1981) (Panella, 1980; Dunkelberger *et al.*, 1980). This apparent interruption of crystal growth leads to a characteristic bipartite structure seen as narrow dark (discontinuous) zones and broad translucent (incremental) zones under the light

microscope (Figure 1.2). Scanning electron microscope studies have revealed that the crystals making up the incremental zone are acicular and branched crystallites oriented radially from the core to the perimeter of the otolith (Liew, 1974; Dunkelberger *et al.*, 1980; Watabe *et al.*, 1982; Morales-Nin, 1987). These crystals are laid down in a 100-200 Å-thick matrix fibres. In the discontinuous zone matrix fibres join together to form concentric layers of fibres 300-700 Å wide (Watabe *et al.*, 1981; Morales-Nin, 1987).

1.5 EARLY ONTOGENY OF OTOLITH FORMATION

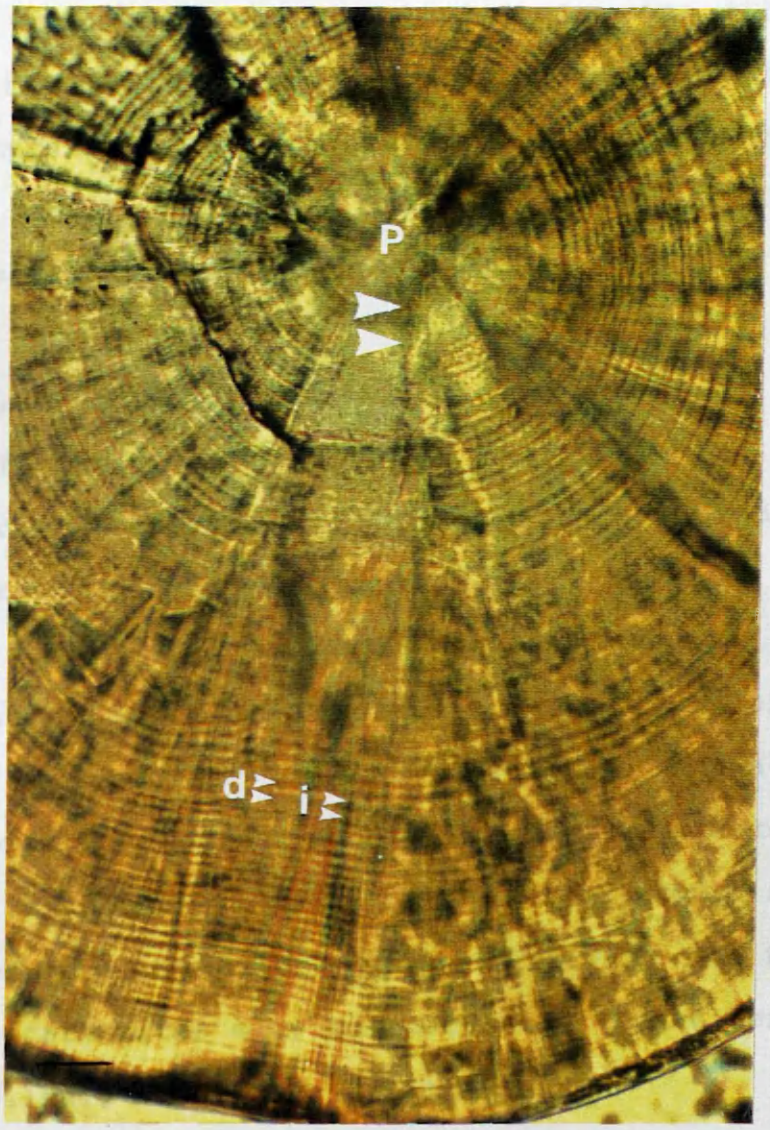
If the pattern of otolith formation in fishes is analogous to that suggested for other vertebrates, the otolith develops from one or more partially calcified primordia exocytosed by cells in the inner ear (Mann *et al.*, 1983). Otoliths are often formed prior to hatching and can be the first calcified tissue to develop (Brother *et al.*, 1976; Radtke & Dean, 1982; Geffen, 1983). The formation of otoliths has been described for larval *Fundulus heteroclitus* (Radtke & Dean, 1982). The process begins with the formation of crystals within a core of organic matrix. The crystals grow to the edge of this core matrix and spherical primordia are common in the calcified core. Spherical primordia have also been reported in other species, for example *Tilapia mossambica* (Tanaka *et al.*, 1981) and *Salmo salar* (Geffen, 1983). In salmonids, several spherical primordia are formed and these fuse together long before hatching. Calcification in *Fundulus heteroclitus*

Figure 1.2

Light micrograph of the sagitta of a three-spined stickleback, *Gasterosteus aculeatus*, showing increments.

P = primordium, arrows point to hatching ring (top) and yolk-absorption/first-feeding ring (bottom) (further details given in Chapter 2).
Light zones are the calcified, incremental zones (i), whilst dark zones are the discontinuous zone (d).

Scale bar = 20 μm


P
d
i

continues with the addition of new crystals which extend beyond the original boundary in an interlocking fashion. This initial crystallisation results in the formation of a nucleus or primordium. Increments begin to be formed 4-5 days later, and in *Fundulus heteroclitus* 6 increments are present prior to hatching. However in some other species increment deposition may not begin until after hatching, for example as in herring larvae, *Clupea harengus* (Geffen, 1982a) and juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Neilson & Geen, 1982).

1.6 OTOLITH MINERALISATION

The formation of otoliths involves the net inward transport of calcium and hydrogen carbonate ions to the mineralising surface (Figure 1.3). However ion fluxes through the system are bidirectional (Mugiya & Uchimura, 1989) and under some conditions dissolution of the outer otolith surface may occur. Little is known about the physiological process of otolith mineralisation, although current evidence suggests that it bears many similarities to other mineralisation systems involving the formation of calcium carbonates e.g. Mollusc shell (Dunkelberger *et al.*, 1980; Wilbur, 1980; Mann, 1983).

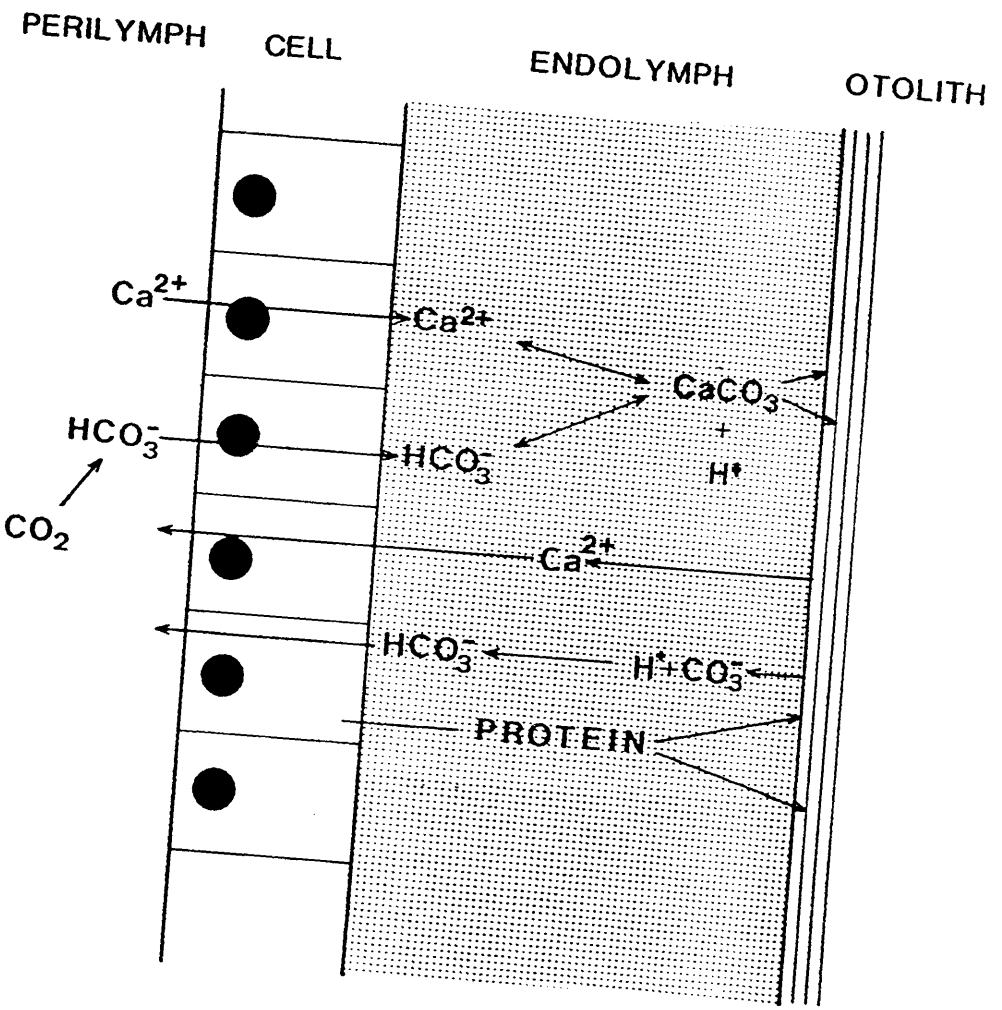
Wilbur (1980) proposed that mineralisation systems had three commmon properties;

1. All systems involve the transport of ions and provide supersaturated concentrations of ions (i.e. which exceed the solubility product) at the mineralising surface. This makes possible the

Figure 1.3

Schematic diagram of the movement of ions to and from the otolith surface.

Protein refers to proteinaceous matrix deposited onto otoliths. Ca^{2+} = calcium ions, HCO_3^- = hydrogen carbonate ions, $CaCO_3$ = calcium carbonate.


PERILYMPH
CELL
ENDOLYMPH
OTOLITH
Ca^{2+}
Ca^{2+}
$CaCO_3$
+
H^+
HCO_3^-
HCO_3^-
CO_2
Ca^{2+}
HCO_3^-
$H^+ + CO_3^-$
PROTEIN

formation of crystal nuclei and the growth of crystals.

2. A sufficiently alkaline pH must be maintained so that, once begun, mineralisation can continue.

3. Crystal formation is often intimately associated with organic material.

The observation that otolith increments are composed of a mineral rich and a mineral deficient zone suggests that there is a variation in one or more of the above properties. Consequently the periodic deposition of mineral rich zones may (i) be related to a diurnal physico-chemical limitation to crystallisation (involving either a lowering of the calcium and hydrogen carbonate ion concentration at the otolith surface or a decline in endolymph pH), or (ii) involve the organic matrix. An analogy with mineralisation of mollusc shell suggests either that organic matrix, deposited in the mineral deficient layer (discontinuous zone), acts as a barrier to crystallisation or that soluble organic matrix, containing compounds that can inhibit crystal growth, is secreted on to the mineral deficient layer (Wheeler *et al.*, 1981; Wilbur & Saleuddin, 1983).

It is likely that the mechanisms determining periodic mineralisation are under endocrine control (Campana & Neilson, 1985; Mugiya, 1987) either directly or indirectly via metabolic influences (Geffen, 1983; Mosegaard *et al.*, 1988). The mechanisms controlling periodic calcification may also be influenced indirectly by environmental factors that influence hormonal cycles and metabolic processes (Campana & Neilson, 1985). Our present understanding of the

process and regulation of otolith formation is too fragmentary to identify the actual mechanisms involved and this has led to much speculation.

7. THE STUDY SPECIES

The present study looks at increment formation in two British freshwater teleosts; the parr of Atlantic salmon, *Salmo salar* and the three-spined stickleback, *Gasterosteus aculeatus*. The two species used in this project were chosen because they are fast growing, have predictably variable growth patterns and because there is a considerable literature on their ecology and physiology.

a. Atlantic salmon parr, *Salmo salar*.

Atlantic salmon exhibit much variability in the timing of life-history events. During the first six months of life sibling populations of juvenile Atlantic salmon diverge into those fish that will migrate to sea as smolts the following spring (S1), and those that will delay migration for a further year (S2). These subpopulations become recognisable on the basis of size, as the length-frequency distribution of the population changes from being unimodal to bimodal by late autumn (Thorpe, 1987; Figure 1.4; Figure 1.5). The upper mode (UMG) consists of the fish that will smolt aged 1+, while their siblings smolting at 2+ form the lower mode (LMG). The rapid segregation by size occurs as a result of the LMG fish losing appetite and virtually ceasing growth from late summer onwards, whereas the UMG fish maintain and even increase their appetite and growth

Figure 1.4

Monthly length frequency distributions in a sibling Atlantic salmon (*Salmo salar*) population (taken from Thorpe, 1987; Environmental regulation of growth patterns in juvenile Atlantic salmon; In: Age and Growth of Fish).

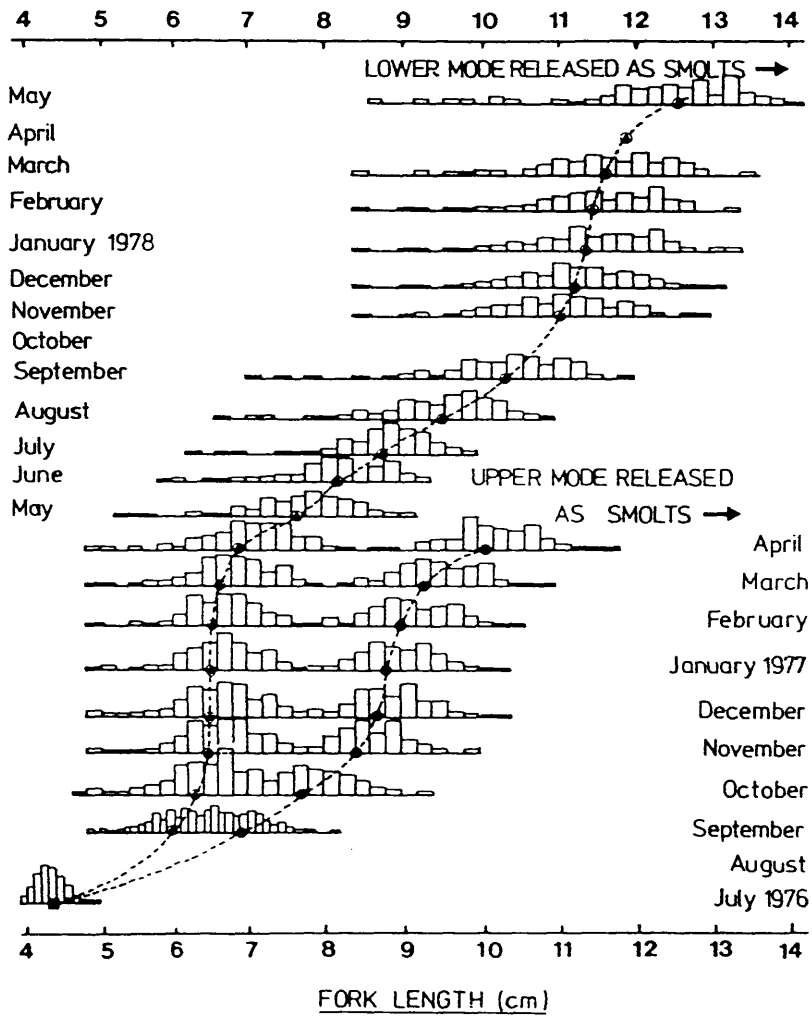

4 5 6 7 8 9 10 11 12 13 14
LOWER MODE RELEASED AS SMOLTS →
May
April
March
February
January 1978
December
November
October
September
August
July
June
May
UPPER MODE RELEASED
AS SMOLTS →
April
March
February
January 1977
December
November
October
September
August
July 1976
4 5 6 7 8 9 10 11 12 13 14
FORK LENGTH (cm)

Figure 1.5

'O+' Atlantic salmon parr (*Salmo salar*), following the bimodal split, showing the difference in size between S1 (destined to smolt at end of first year) and S2 (destined to smolt in second year) parr.

Figure 1.6

An adult male three-spined stickleback (*Gasterosteus aculeatus*).

Scale bar for both figures = 2 cm.

(Metcalfe *et al.*, 1986; 1988). This dramatic divergence in growth rates of the two types of fish occurs even when they are maintained under the same laboratory conditions with food supplied to excess. In the present study this phenomenon is used to investigate increment periodicity and accretion rate in siblings growing at different rates.

b. Three-spined stickleback, *Gasterosteus aculeatus* (Figure 1.6).

The three-spined stickleback has a short life span and most Scottish populations are annual (Ukegbu, 1986). Sticklebacks breed from April to July and fish begin to show signs of developing gonads between August and October in their first year. Little is known about the growth rates of fish in the first and second month after hatching, because to date no method of aging young fry has been discovered. Studies of the growth of fish > 15 mm total length suggest that the most rapid increase in length occurs in July and August (when the fish are 1-2 months old) and that fish virtually cease growing during the winter months. In Scottish populations at least, adults die at around 12 - 16 months old (Chellappa & Huntingford, 1989). (for a review of the ecology of this species see Wootton, 1984).

1.8 OBJECTIVES OF THE PRESENT STUDY

The primary objective of the present study is to improve current understanding about the process of formation of increments in fish otoliths and the way in which

environmental and physiological factors control increment periodicity and size.

Specifically the thesis considers the following questions:

1. Does otolith accretion show daily rhythmicity in the parr of Atlantic salmon, *Salmo salar* and the three-spined stickleback, *Gasterosteus aculeatus*? (Chapter 2)

2. How are otoliths formed and in particular what is the role of the organic matrix? (Chapter 3)

3. What intrinsic and extrinsic factors (photoperiod, temperature and patterns of food availability) influence the periodicity of otolith accretion? (Chapter 4)

4. How is daily accretion related to somatic growth and basal metabolism? (Chapter 5)

Chapter 2

PERIODICITY OF OTOLITH INCREMENT FORMATION

I. INTRODUCTION

Daily increments have a considerable potential for the exact determination of life-history events and the growth of early life-history stages in fish. However daily increments are not usually found in all life-history stages (Pannella, 1971; 1980) and significant deviations from a daily periodicity of increment formation have been reported in some species with slow growing larval phases. For example daily increments do not appear to be formed in many populations of larval herring (Geffen, 1982a; McGurk, 1984; Nichols pers comm.), in the larval stages of several species of flatfish (Geffen, 1982a; Campana, 1984; Alhossaini & Pitcher, 1988), or in the embryonic stages of Atlantic salmon (Geffen, 1983). The rhythm of deposition may also be interrupted by physiological stress (Pannella, 1980; Campana, 1983a; Morales-Nin, 1987) or cease under certain environmental conditions unfavourable to somatic growth (Taubert & Coble, 1977). The presence of prominent sub-daily increments (Campana & Neilson, 1985) may make it difficult to discern the fundamental rhythmicity of increment formation. In addition increments may be too small (i.e. when growth is slow) to resolve under the light microscope (Campana, 1984a; Jones & Brothers, 1987; Morales-Nin & Ralston, 1990). Consequently for each new species and life-history stage studied it is necessary to determine whether increment deposition is daily, how long

it persists without interruption and whether sub-daily increments are present.

Increment periodicity has been assessed by a variety of indirect and direct approaches, the reliability of which have been reviewed (Gjosaeter *et al.*, 1984; Geffen, 1987). Indirect approaches include sequential sampling of a population to compare changes in modal size with that predicted from otolith increment and size data (Methot & Kramer, 1979; Wright *et al.*, 1990) and by examining the degree of synchronisation in percentage completion of the outermost increment within a group of fish experiencing the same conditions (Tanaka *et al.*, 1981; Geffen, 1982b). However these methods are based on assumptions which may not be reliable (Geffen, 1987), such as that samples for size-frequency data are not representative of the population concerned. Consequently a direct approach to validation is most desirable. Direct validation procedures include the sequential killing of reared fish of known age in the laboratory (Brothers *et al.*, 1976; Neilson & Geen, 1982), or the introduction of datable marks on to the otoliths. Dated marks can be applied to fish either in the laboratory or in the wild. Marks may be chemically or intrinsically induced. Chemical marking techniques commonly employ oxytetracycline hydrochloride (Campana & Neilson, 1982) or other fluorescent compounds, for example calcein (Wilson *et al.*, 1987) and alizarin complex (Tsukamoto *et al.* 1988) which are incorporated into calcified tissues within a day of administration. Alternatively intrinsic marks may be used such as hatch checks, or artificially

induced checks caused by stress (Pannella, 1980; Campana, 1983a; Ralston & Miyamoto, 1983) or abnormally short photoperiod and temperature fluctuations (Mosegaard *et al.*, 1986) on known dates.

The conditions under which validation studies are conducted are very important, since factors such as feeding level, feeding periodicity, container size and temperature affect the growth rate of fishes and may also influence increment periodicity (Geffen, 1982a; Neilson & Geen, 1982; Radtke & Dean, 1982; McGurk, 1984; Alhossaini & Pitcher, 1988). In some laboratory studies, an increment periodicity of less than one per day has been related to the inability to maintain the high larval growth rates observed in the wild (Alhossaini & Pitcher, 1988). Rearing conditions in the laboratory often produce unnatural stresses leading to the formation of sub-daily increments and interruptions or checks in increment growth (Pannella, 1980; Morales-Nin, 1987). Further, there tends to be less distinction between incremental and discontinuous zones in fish reared under constant temperature and light levels than in fish reared in field enclosures (Geffen, 1982a; Campana & Neilson, 1982). For these reasons, validation studies should ideally be carried under both laboratory and field conditions (Gjosaeter, 1982; Geffen, 1982a; Campana & Neilson, 1985). Field enclosures or ponds, have been employed in several studies (Liew, 1974; Geffen, 1982a; Simoneaux & Warlen, 1987), although releasing marked fish in the wild, as has been carried out recently for Japanese red sea bream (Tsukamoto *et al.*, 1989), is more desirable. However

because of the large numbers of fry that need to be marked to collect sufficient returns, releasing fish is generally not feasible (Gjosaeter, 1982).

The aim of the present study is to determine the periodicity of increment deposition in the sagitta otoliths of Atlantic salmon parr, maintained in laboratory tanks under natural photoperiod and temperature and of three-spined stickleback, held under both laboratory and field conditions.

Specifically it will ask the following questions:

1. Is increment deposition daily?
2. Do somatic growth rates influence increment periodicity?
3. Are increments formed throughout the life-history (stage)?

2.2 GENERAL METHODS

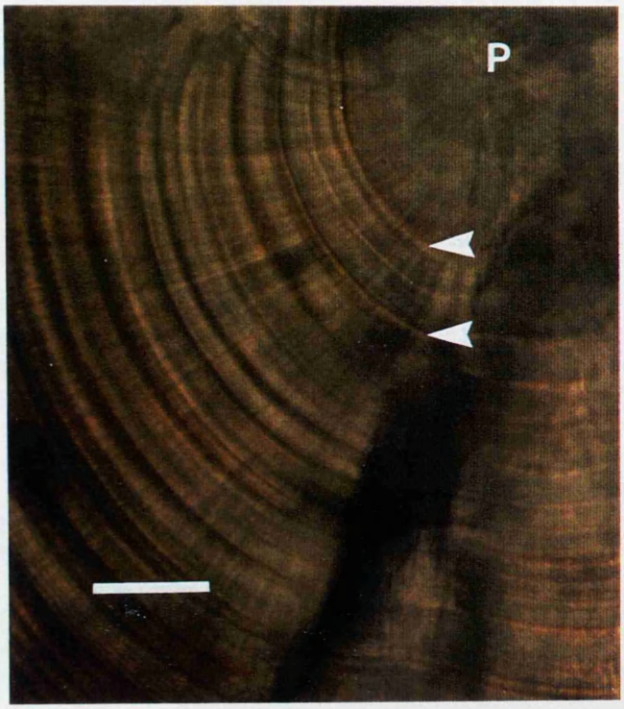
2.2.1 OTOLITH MARKING

The day of first feeding is associated with a change in the opacity and discreteness of otolith increments in both salmon parr (Figure 2.1) and three-spined sticklebacks (see Figure 2.9). This change in otolith microstructure was used as a natural mark from which the number of increments following first feeding could be determined.

Figure 2.1

Yolk absorption and first feeding rings (arrows) of Atlantic salmon parr (Salmo salar).

Scale bar = 25 µm.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Several methods of artificially inducing a mark on the otoliths of both salmon parr and juvenile sticklebacks were tested. These included subjecting fish to rapid temperature changes of $\pm 4^{o}C$, 24h immersion in a tank containing 4% seawater and oxytetracycline (4 $mg.l^{-1}$), feeding with chironomid larvae that had been fed on oxytetracycline labelled food, and a 6L:6D photoperiod, often together with heating of the environmental water to $3^{o}C$ above ambient temperature during the light phases, as suggested by Mosegaard *et al.* (1986).

The types of marks induced by these methods are shown in Figures 2.2 - 2.4. In both species the 6L:6D photoperiod method proved to give the most reliable mark. The 6L:6D photoperiod mark was easier to distinguish from natural checks than other forms of induced mark. It also proved to be a more successful method for inducing a mark on otoliths than methods involving tetracycline (percentage success in inducing marks was 90-100% with the 6L:6D method, compared with 0-60% using tetracycline).

2.2.2 OTOLITH REMOVAL AND PREPARATION

The sagittae of Atlantic salmon parr were exposed by an oblique cut through the dorsal surface of the head and removed with watchmakers forceps.

The otoliths of three-spined stickleback fry (<10 mm TL; the distance from the tip of the snout to end of the caudal fin) were usually removed within 2 hours of death, as the otoliths of freshly killed fry were easier to locate than those of preserved animals. When necessary, fry were preserved in either 75% iso-propanol or 95% ethanol. Fry were placed in a drop of water on a slide and the sagitta otoliths were gently teased out with fine mounted entomological pins. In cases where a sagitta did not come

Examples of sagitta marking methods

Figure 2.2

Oxytetracycline mark in stickleback sagitta: sagitta removed two days after 24 h immersion in oxytetracycline chloride 4mg.l^{-1}; a) under transmitted light, b) under UV light.

Scale bar = 10 µm.

Figure 2.3

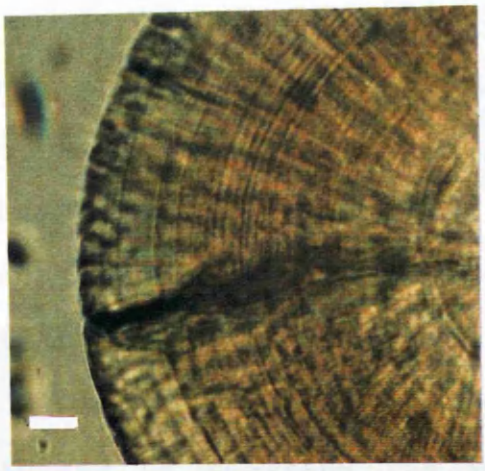
Temperature induced mark in a salmon parr sagitta (arrows): parr subjected to a 4°C temperature rise within 30 minutes.

Scale bar = 10 µm

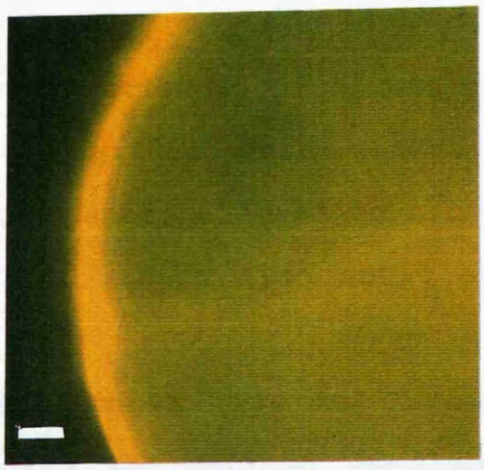
Figure 2.4

3 day 6L:6D (temperature raised 3 °C during light phases) induced mark (M) in a stickleback sagitta.

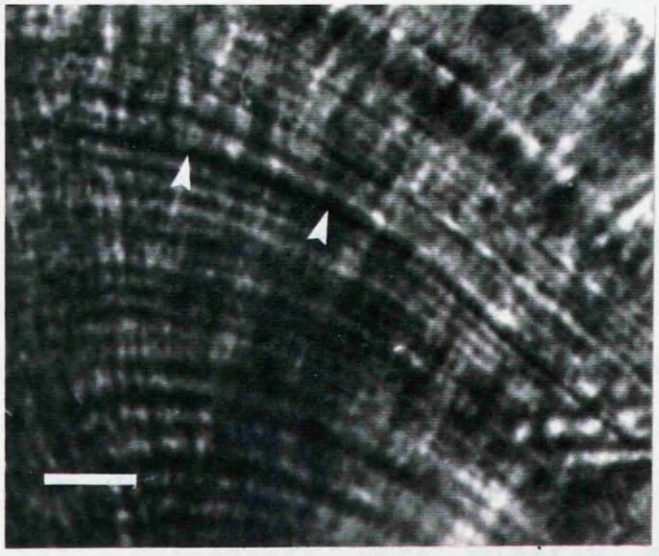
Scale bar = 10µm.

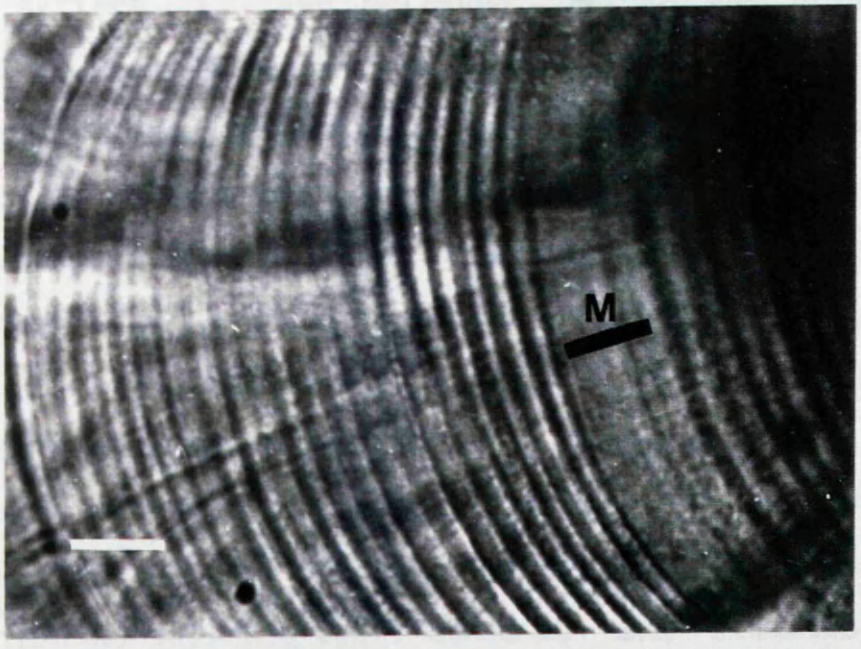


a



b





free, a weak sodium hypochlorite solution (0.01 M) was used to dissolve remaining tissue. Sagittae were washed in water, gently blotted with filter paper, allowed to air dry and then mounted in immersion oil, which also acted as a clearing agent.

The otoliths of juvenile sticklebacks (>10 mm TL) were exposed by removing the operculum and cutting up through the basioccipital pad. All three otoliths from both sides were removed, although the astericus and lagena were rarely used because of the problems in resolving their very fine increments.

Owing to the problems of resolving increments in thick otoliths, the sagittae of salmon parr and of juvenile sticklebacks (>15 mm TL) were ground. Sagittae were fixed to a glass slide with superglue (Bostick). The sagittal surface was then ground with 600 Å carborundum powder to a plane where all increments were clearly visible. The sagitta surface was then polished down to the primordium using 25 µm diamond paste on a Kent 3 lapping wheel (Engis Ltd.; loaned from DAFS Freshwater Fisheries Laboratory). The sagittae were washed with distilled water, dried and left to clear in immersion oil for 30 days prior to examination.

2.2.3 SCANNING ELECTRON MICROSCOPY

To ensure resolution of small winter increments, 5 sagittae from each validation experiment in which salmon (maintained during winter - see p20) and sticklebacks were subject to low temperatures (maintained during winter outside and at 5oC in the laboratory - see p25),

were examined in detail using scanning electron microscopy.

The preparation of sagittae for scanning electron microscopy were based on methods reviewed by Campana & Neilson (1985). One of the pair of sagittae was embedded in epoxy resin (Araldite) allowed to polymerise at 60oC for two days and then ground as for light microscopy. The surface of the sagitta was further polished with a graded series of diamond pastes (6, 1, 0.25 μm). The sagitta was then etched with ethylene diamine tetraacetic acid (E.D.T.A.) at pH 7.5 for 3-5 minutes and either rinsed in 95% ethanol and allowed to air dry or dried using critical-point dehydration. The sagittae were mounted on aluminium stubs with double-sided sellotape and silver paint and coated with a 150 Å layer of palladium-gold before examining under the SEM (Phillips 500) at 15 kV.

2.2.4 COUNTING

Initial counts were made under x 400 magnification, using a hand tally and later compared with counts made from a video monitor, using a Panasonic video camera attached to an Olympus microscope at x 400 or x 1000 magnification. Critical illumination and high video contrast were used to enhance the image. Counts were made along two axes, primordium to rostral and primordium to post-rostrum (terminology of Messieh, 1972). Increments in both sagittae were counted three times and counts on the same otolith were not made consecutively. Counts from both sagitta pairs were averaged for all counts, since estimated numbers of increments did not differ significantly between paired sagittae (ANOVA $F_{1,19} = 0.92$; $P>0.99$). Only counts which differed by 5% or less were used for validation; 93% of all sagittae examined fell into this category. Sagitta radius

was measured using an eyepiece graticule (calibrated with a micrometer slide) and eyepiece units were converted to micrometres.

2.2.5 OBSERVER RELIABILITY

1. Intra-observer reliability was tested against an automated method of quantifying increments, using image analysis to display the differences in increment zone density as a 3-dimensional graph (y-mod, semper 5), although with limited success being able only to distinguish up to 14 increments. Intra-observer reliability was also tested in a double blind experiment using sagittae from fish of known age (Table 2.1).
2. Inter-observer reliability. 4 readers, all with some experience, as experience can have a significant effect on counting accuracy (Brothers, 1987) were given a set region of sagitta formed over 74 days to count (Table 2.1).

2.3 VALIDATION EXPERIMENTS ON ATLANTIC SALMON PARR UNDER CYCLIC LIGHT-DARK CYCLES AND TEMPERATURE

2.3.1 METHODS

Fish were reared from first feeding in 2 m radial flow tanks (Thorpe & Wankowski, 1979) at the DAFS Almondbank Smolt Rearing Station, under ambient photoperiods and water temperatures (daily means from 17^{o}C in summer to 0^{o}C in winter). EWOS Ltd. pelleted food was dispensed automatically at 20-30 minute intervals throughout the day.

Table 2.1

Reliability in increment counting

Data presented as mean ± S.E. for five increment counts per reader. Two sets of results are presented for reader 1 (the author) based on counts from sagittae of known age salmon (a.) and a stickleback (b.). All other results are for other readers counting increments in the stickleback sagitta (as in b.). Deviation from known age determined using a D. test.

Observer	Mean count	Age of fish$_1$	D-test	
			t	P
1 a.	215.8 ± 3.25	211	1.48	≙0.99
1 b.	72.4 ± 0.51	74	3.13	≙0.90
2	64.0 ± 0.97	74	10.31	NS
3	59.2 ± 0.58	74	18.62	NS
4	55.6 ± 1.02	74	18.04	NS

1 = age in days following first feeding ring.

Under hatchery conditions Atlantic salmon usually remain as parr for one or two years and so validation experiments to test the one-increment-per-day hypothesis were carried out on both 0+ and 1+ fish. The difference in the growth history of S1 and S2 parr (see Chapter 1) provided a means of testing whether increment deposition was independent of growth rate.

Validation experiments were caried out in 2 m radial flow tanks, under the following treatments.

For 0+ parr

Over a 285 day period following first feeding, samples of 20-30 parr were killed at 30-50 day intervals and their fork lengths and weights were measured.

For 0+ - 1+ S2 parr

After inducing a mark on their otoliths in late February of their first year (age 0+), samples were taken every 50-80 days through to September of their second year.

2.3.2 RESULTS

Increment width, as determined from light microscope observations, ranged from 1.72 - ~0.80 μm in the otoliths of S2 parr. The maximum and minimum increment width corresponded to the times of maximum (17°C) and minimum (0°C) water temperatures, respectively. Light microscope measurements of minimum increment width were confirmed from scanning electron microscope observations (minimum increment width = 0.82 μm) (Table 2.2a).

In both experiments the number of increments was in close agreement with the number of days following marking.

Differences in the growth rate of O+ group parr, as shown by changes in length-frequency distributions of samples collected in the O+ age group experiment (Figure 2.5), were found to have no effect on otolith increment periodicity (285 day sample S1 $\bar{x}$ = 273; S2 $\bar{x}$ = 285; paired t-test; t_{16} = 2.13; P<0.95). The slope of the linear regression of increment counts (based on all data sets) was not significantly different from 1.0 in either S1 or S2 O+ group parr (b = 0.952; r^2 = 0.992; P <0.001; N = 102; Figure 2.6), as predicted from the one increment per day hypothesis. Similarly the slope of the linear regression for O-1+ parr (b = 0.96; r^2 = 0.971; P<0.001; N = 80; Figure 2.7) did not differ significantly from 1.0. In both experiments the regressions explained over 92 % of the variance and 95 % confidence limits of known age ranged from $\pm$ 2.16 days for fish sampled 46 days from first feeding to $\pm$ 8.66 days for fish sampled 285 days after first feeding.

2.3.3 DISCUSSION

The present study has demonstrated a daily periodicity of increment deposition in the sagitta of hatchery-reared Atlantic salmon throughout the parr stage. Further, this daily rhythmicity was found to be independent of growth rate as daily increments occurred in both the fast growing S1 and slow growing S2 parr. Confirmation of a growth-independent deposition rate has also been shown for individual fish of known growth history (see Chapter 5). A

Table 2.2a

Comparison of i) median increment counts and ii) median increment widths from S2 salmon parr sagittae, determined using either a light microscope or a scanning electron microscope (SEM).

Data presented as median, range and number of sagittae (N). Significant differences between counts derived from light microscopy and sem are compared using a Mann-Whitney U-test.

i) COUNT

	MEDIAN	RANGE	N	U-TEST
LIGHT MICROSCOPE	272	240 - 281	12	$U_{12,5} = 12.5$
SEM	278	275 - 282	5	$P > 0.90$

ii) INCREMENT WIDTH

	MEDIAN	RANGE	N	U-TEST
JULY 17°C				
LIGHT MICROSCOPE:	1.47	1.37 - 1.72	5	$U_{5,5} = 10$
SEM :	1.55	1.38 - 1.82	5	$P > 0.90$
JANUARY 0°C				
LIGHT MICROSCOPE:	0.86	0.80 - 0.95	5	$U_{5,5} = 11$
SEM	0.88	0.82 - 0.92	5	$P > 0.90$

Figure 2.5

Changes in the fork length frequency distribution of Atlantic salmon parr in the '0+' group validation experiment, showing the bimodal separation into the Upper and Lower Modal Groups.

Histograms plotted as percentages of each modal group.

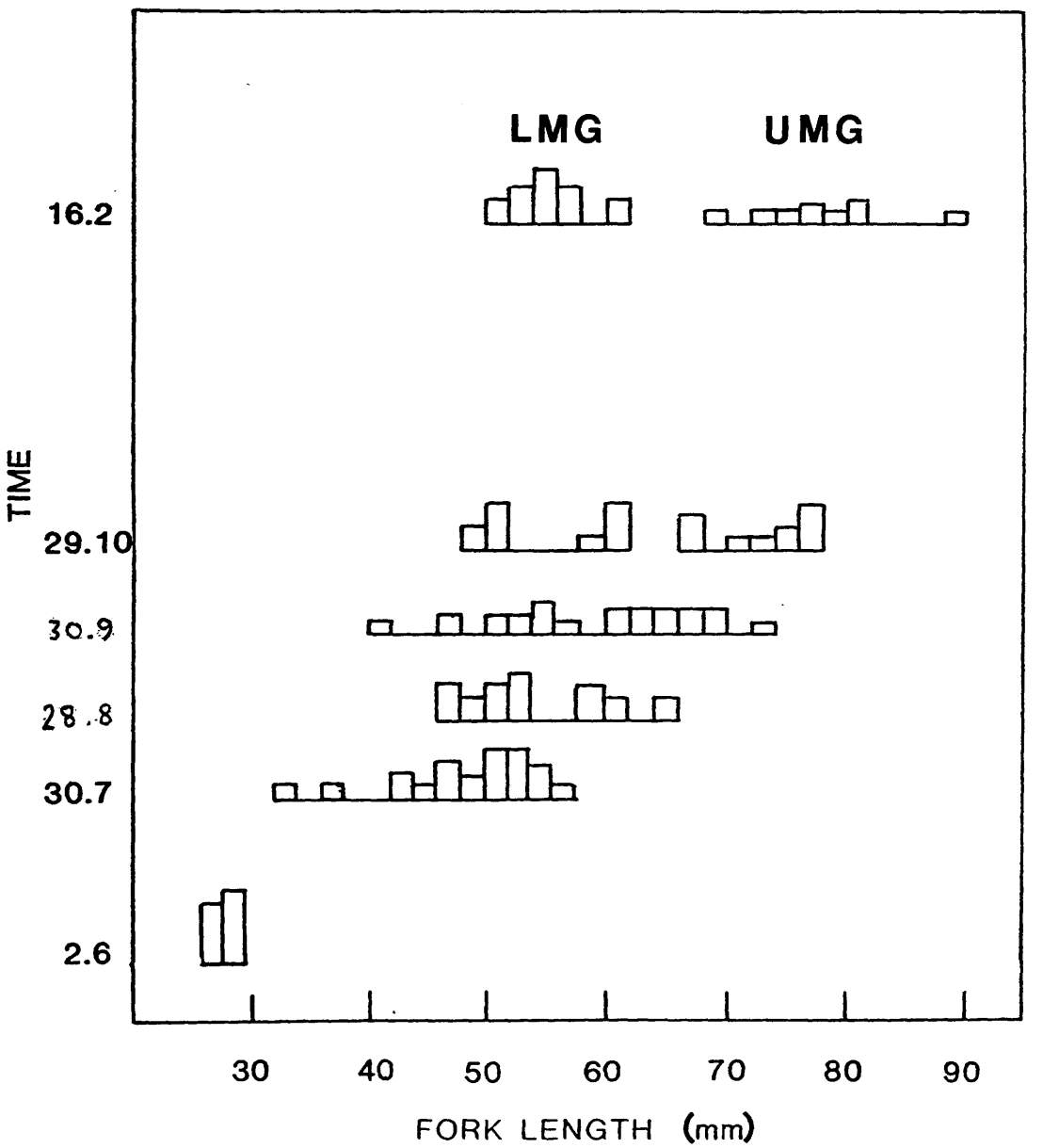

LMG
UMG
16.2
29.10
30.9
28.8
30.7
2.6
TIME
30
40
50
60
70
80
90
FORK LENGTH (mm)

Figure 2.6

Relation between the number of sagitta increments and known age in 0+ salmon parr (*Salmo salar*) maintained under natural photoperiod and temperature.

Data presented as mean and 95% confidence limits (bars) for increment counts from >5 individuals. The regression line for all data sets, $y = 3.51 + 0.952x$ ($r^2 = 0.992$; $P<0.001$; N = 102 sagitta pairs).

where two points at a given time are shown ● = S2 parr and ■= S1 parr.

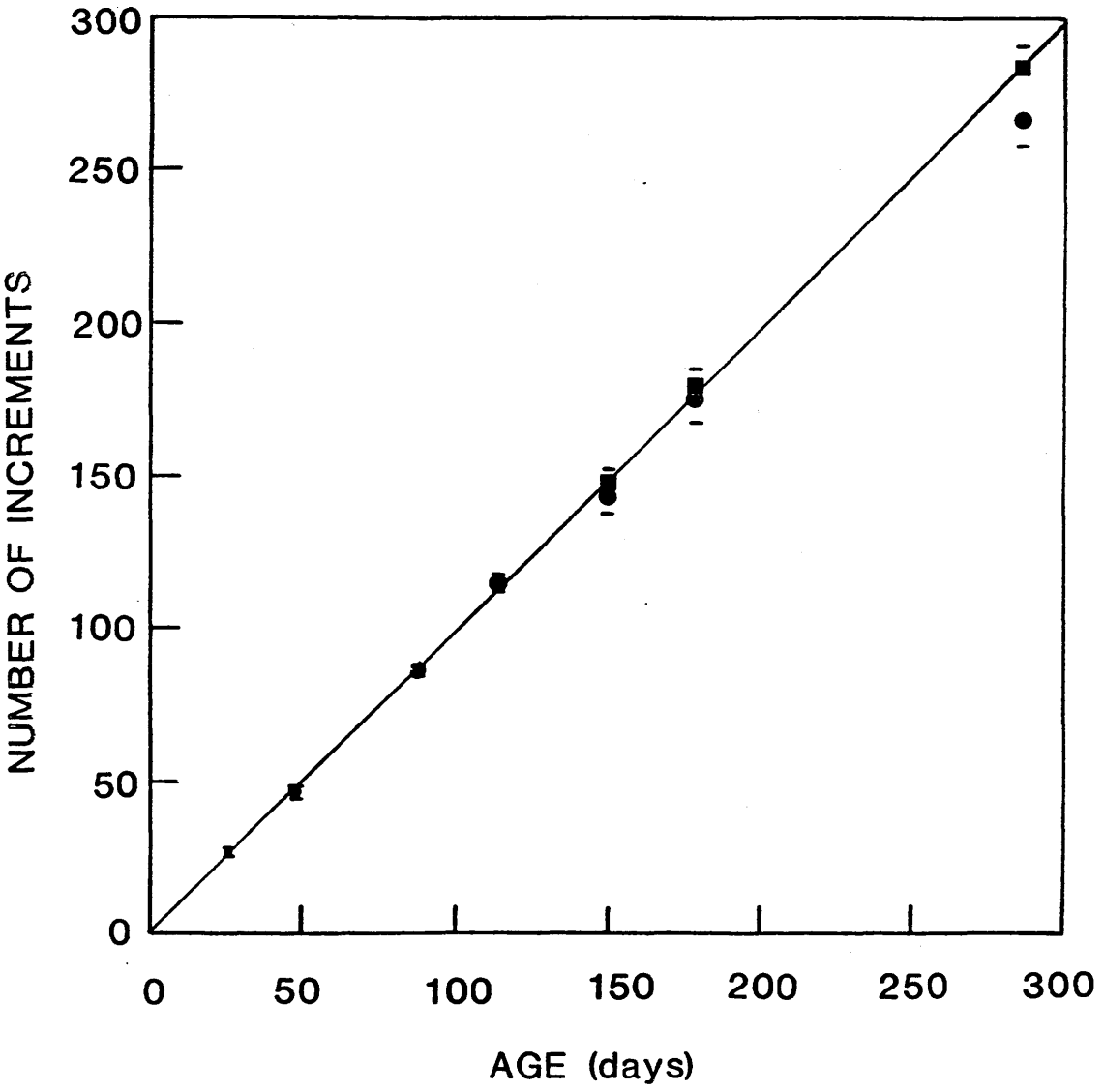
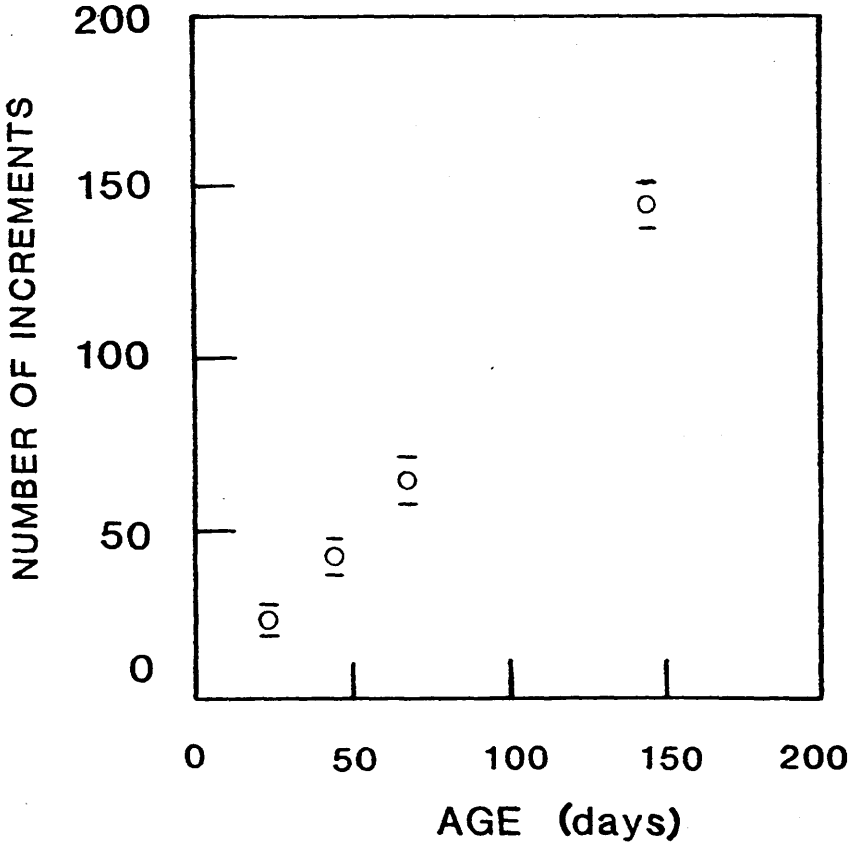

300
250
200
150
100
50
0
NUMBER OF INCREMENTS
0
50
100
150
200
250
300
AGE (days)

Figure 2.7

Relation between the number of sagitta increments and known age in 0+ - 1+ S2 salmon parr (*Salmo salar)* maintained under natural photoperiod and temperature.

Data presented as mean and 95% confidence limits (bars) for increment counts from >5 individuals. The regression for all data sets, $y = 0.455 + 0.96x$ ($r = 0.971$; $P<0.001$; $N = 80$).


NUMBER OF INCREMENTS
200
150
100
50
0
0
50
100
150
200
AGE (days)

daily periodicity of increment formation has also been reported for post-hatch embryonic (alevin) (Mosegaard *et al.*, 1986), but not for pre-hatch embryonic stages of Atlantic salmon (Geffen, 1983). Since hatchery-reared parr experience the same photoperiod and temperature regimes as parr living in the natural habitat, otolith increments would be expected to be produced daily in the otoliths of wild parr. Consequently sagitta increments may well provide an accurate means of determining the time of first feeding and age following first feeding in Atlantic salmon parr.

2.4 VALIDATION EXPERIMENTS ON THREE-SPINED STICKLEBACKS UNDER CYCLIC LIGHT-DARK CYCLES AND TEMPERATURE

2.4.1 METHODS

Validation was carried out both on reared fry of known age and on wild caught fish after inducing a mark on their otoliths (see below).

Adult sticklebacks were collected from the Endrick river, near Drymen, Dunbartonshire, Scotland. Males were allowed to establish nests in aquaria and to mate with receptive females. Eggs were collected within four hours of spawning and transferred to an incubation tank with a continuous flow of water maintained at 90-95% oxygen, 15^{o}C $\pm$ 0.5. Fry hatched after between 11-12 days of incubation and were transferred to 10L aquaria (maximum stocking density 4 fry/L) and fed on Liquifry 1 & 2, Artemia, *Daphnia* nauplii, and chopped Tubifex, according to stage of development.

Once they had reached an age of at least 60 days, juveniles were transferred to 60L tanks (10 - 20 /tank), with a gravel/sand substrate, shelters and plants. The water was filtered continuously (using charcoal filtration) and maintained at a constant temperature ($\pm 0.5^{o}C$) and under natural photoperiod. To allow foraging to continue throughout the day, *Tubifex* worms were scattered throughout the tanks twice daily and *Daphnia* were added occasionally as a supplement.

Laboratory validation of reared fry: Samples of fry (N=5-10) reared in the laboratory in May, 1987 were killed (by an overdose of benzocaine) daily for the first five days and then at 17, 27, 43, 75, 130 and 300 days after hatching. Daylength was adjusted in accordance with ambient photoperiod and temperature was maintained at $15 \pm 0.5^{o}C$ from hatching to September and $10 \pm 0.5^{o}C$ from October onwards.

Laboratory validation of marked fry: Wild caught juveniles were collected in July and November and maintained under similar conditions after marking.

Validation experiments were made, after inducing a mark on the fishes' otoliths, at two starting months, July (when fish were between one and three months old) and November (when fish were between five and seven months old). In July/August, 3 groups of 15 fish were maintained for 33 days and in November/December 2 groups of 15 fish were maintained for 28 and a third group was maintained for 56

days (November - January), before killing. Daylength was adjusted in accordance with ambient photoperiod and temperature was maintained at 15 $\pm$ 0.5^{o}C in July, 10 $\pm$ 0.5^{o}C in October and 5^{o}C, 8L:16D in December.

Field validation of marked wild caught juveniles: Validation was carried out on wild caught fish under the following two treatments.

In one a 2000L tank was used (between November 1987 and March 1988) and in the other a 2000L pond (established in March 1988, at the University Field Station, Rowardennan, Stirlingshire). Both enclosures were provided with pond weed and gravel substrate and the pond was supplied with a continuous flow of water. Chironomid larvae and *Daphnia* were added to the tank during winter months. Once established the pond required no additional food supply.

Juveniles were caught from the Endrick River and transferred to the outdoor tank in December. A mark was induced on the otoliths with a 6 day 6L:6D photoperiod and then samples of 20 fish were collected in January and March.

Adults were introduced into the pond prior to the breeding season and their offspring removed and marked in late July using a combination of short photoperiod and changing temperature and then returned to the pond. Groups of 10 - 20 fish were then removed and killed at intervals of between 4 and 6 weeks to minimise disturbance to the pond community up to December 1988.

2.4.2 RESULTS

Formation of otoliths: Two pairs of otoliths, the sagittae and asterici were present in stickleback embryos from 2 to 3 days prior to hatching. At hatching the sagitta otoliths were spherical, with a radius of 30-40 μm. Observations on the otolith microstructure of embryos, yolk-sac larvae and free-swimming fry revealed that changes in deposition pattern and increment structure, seen under both the light microscope (Figure 2.8) and the scanning electron microscope (Figure 2.9), accompanied hatching and first feeding (following the absorption of the yolk-sac). Two to three faint rings were present at hatching and a dense zone was prominent in all larvae at yolk-sac absorption.

Increment resolution: The contrast between incremental and discontinuous zones in increments was lower in laboratory reared fish than in pond-reared specimens, as has commonly been reported in other validation studies (Gjosaeter, 1982). This made reading and examination of the smaller increments difficult.

Changes in otolith radius of fish of known age, maintained at temperatures above 10^{o}C suggested that otoliths practically ceased growing in fish of more than 80 days old (Figure 2.10). As a consequence it was not possible to determine if increments were still deposited at a daily rate after that time using light microscopy. However increments could still be resolved during the phase of slow winter growth by means of ultrastructural observations of

Figure 2.8

Primordium and post-hatching rings of a 2 day hatched three-spined stickleback (*Gasterosteus aculeatus*), as seen under transmitted light.

Pa denotes accessory primordium

P denotes primordium, I denotes post-hatching rings.

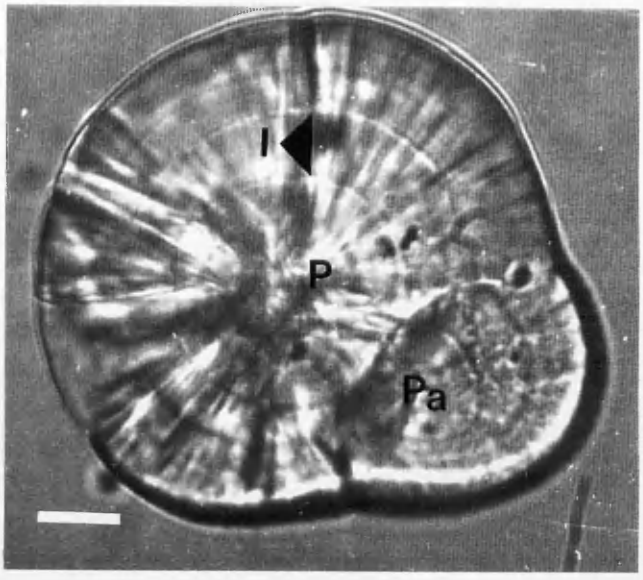
Scale bar = 10 um.

Figure 2.9

Scanning electron micrograph of the primordium (P), hatching (H) and first feeding (F) rings of a 100 day post hatched three-spined stickleback (*Gasterosteus aculeatus*).

Arrows indicate daily increments.

Scale bar = 10 um.


I
P
Pa

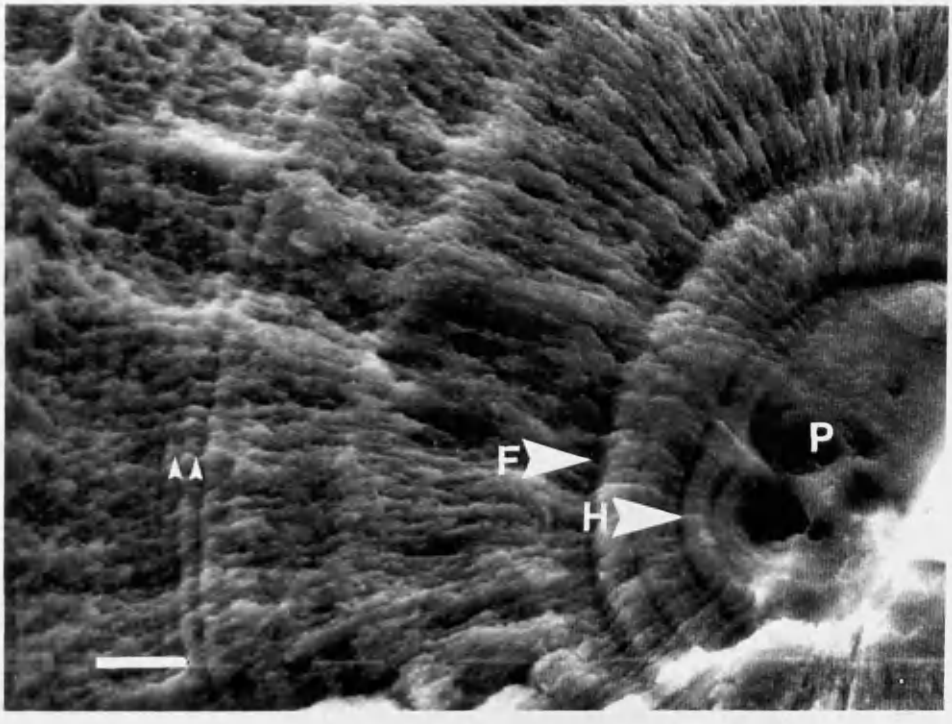
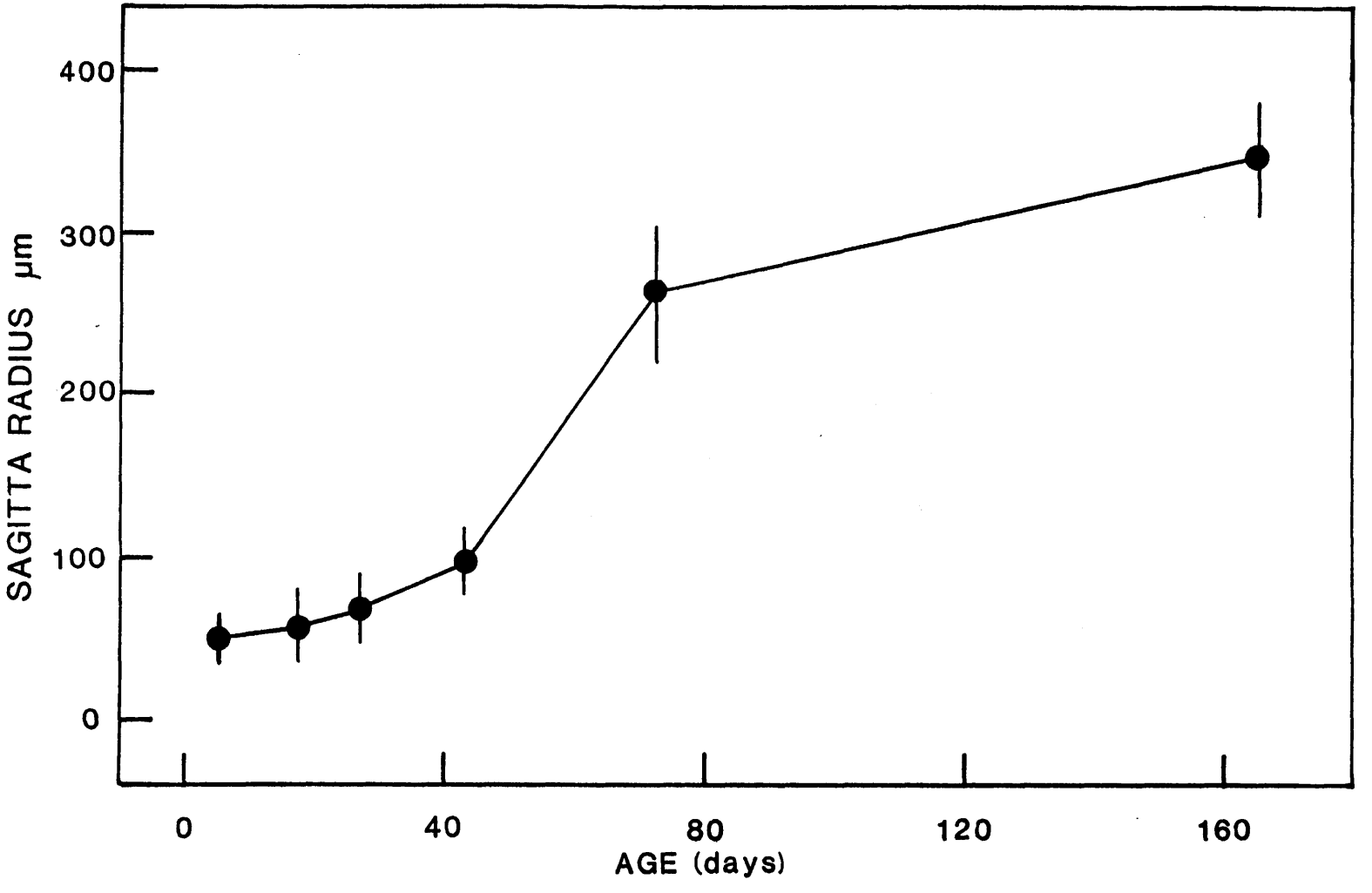

F
H
P

Figure 2.10

The relation between age and sagitta radius for three-spined sticklebacks (*Gasterosteus aculeatus*).

Data presented as mean and range for 5 data sets. Line fitted by eye to mean data sets.


SAGITTA RADIUS µm
400
300
200
100
0
0
40
80
120
160
AGE (days)

the sagitta surface. Increment width ranged from a median of 3.6 μm, in summer to 0.44 μm in winter (Figure 2.11).

Validation; reared fry: No effect of container size was found, the number of increments in juvenile fish held under laboratory conditions was not significantly different from the number of days following hatching in fish reared up to 130 days old ($b = 0.997$; $P<0.001$; $N= 59$; $SD\ b = 0.0086$; Figure 2.12), this is as predicted from the one increment per day hypothesis. The increments in fish reared to 300 days after hatching were difficult to see even using the SEM, which may account for the range in increment counts (276-292) and the high standard error ($\bar{x}$ count = 284 ± 12; $t = 1.33$; $P < 0.95$).

Marked wild caught fish; In marked fish maintained in both the laboratory and the field, the number of increments following marking did not differ significantly from that predicted by the one increment per day hypothesis (Table 2.2). However the increments of fish (< 180 days old) held at 5°C were narrow ($\bar{x}$ = 0.70 ± 0.12 μm; cf. 1.72 μm at 10°C) and difficult to resolve under the light microscope; this was reflected in a high coefficient of variance between counts. The increments of fish housed in the field enclosure during December to March were also too small to resolve reliably with the light microscope and so only counts from sagittae examined with SEM were used. In these fish the number of increments from the induced mark in these fish was not significantly different from that

Figure 2.11

Scanning electron micrograph of the increments deposited by a three-spined stickleback (*Gasterosteus aculeatus*) maintained in an outdoor enclosure during February.

Arrows indicate increments (0.5 - 0.7 µm).

Scale bar = 1 µm.

Figure 2. 12

Regression between the number of sagitta increments and known age in three-spined sticklebacks (*Gasterosteus aculeatus*) from the Endrick River reared under laboratory conditions.

Data presented as mean and 95% confidence limits (bars) for increment counts from >5 individuals. The linear regression line, $y = -0.2756 + 0.9973x$ ($r^2 = 0.998$; $P < 0.001$; $N = 59$ sagitta pairs).

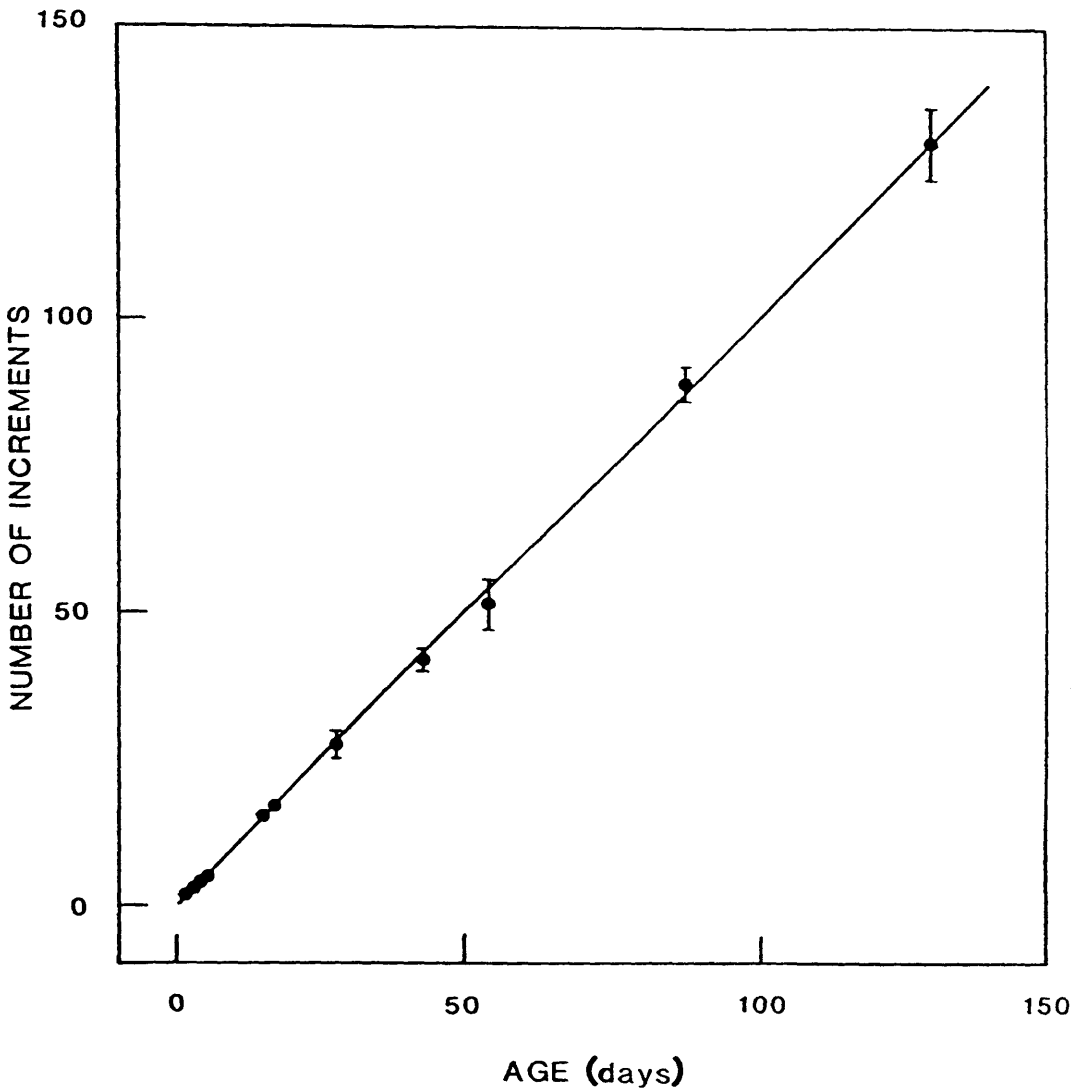

150
100
50
0
NUMBER OF INCREMENTS
0
50
100
150
AGE (days)

Table 2.2

Mean sagitta increment counts for marked juvenile three-spined sticklebacks held under laboratory conditions and in a field enclosure.

All juveniles were collected from the River Endrick, near Drymen, Dunbartonshire. Table shows month in which experiment was begun, age (as estimated from increment counts) of fish when experiment was begun, temperature regime ($\pm$ 0.5 ^{o}C), time interval (in days) following marking, mean increment count following mark, and significance level as determined from a D - test (values for t are given). a and b refer to one group of fish subject to a change in temperature regime from 10 - 5 ^{o}C. The increments produced under the 5 ^{o}C regime were clearly distinct from those produced under 10 ^{o}C as they were much thinner. Consequently the number of increments produced during the 10^{o}C (a) and 5^{o}C (b) regimes were counted separately.

Laboratory reared fish

starting date	age	T°C	interval	count				P
				mean	SE	N	t	(≐)
July	120-140	15	35	34.2	0.47	15	1.7	0.95
July	120-140	15	35	34.6	0.60	15	0.7	0.99
July	120-140	15	35	34.5	0.55	10	0.7	0.99
November	180-205	10	28	26.9	0.63	15	1.7	0.95
November	180-205	10	28	26.4	0.89	12	1.8	0.95
November	180-205a	10	23	22.4	0.80	14	0.9	0.99
December	203-228b	5	23	20.4	0.72	14	3.6	0.90

Field enclosure reared fish

starting date	age	T°C	interval	count				P
				mean	SE	N	t	(<)
December	~200	2-7	83	78	3.6	20	1.39	0.95
August	~ 50	11-20	15	14	0.6	15	0.67	0.99
August	~ 50	6-20	153	148	3.2	18	1.56	0.95

predicted from the one increment per day hypothesis at (X^2 = 1. 55; P<0.90).

Checks: Increment checks were observed in both laboratory (see Figure 2.4 for example) and wild caught fish. In wild caught fish marked for use in validation studies, no checks were seen in the first 50 days (as estimated from daily increments) from hatching but after that age checks were often present and the mean frequency of checks increased with increasing age (Figure 2.13). In the laboratory, transferring fish from one tank to another often (in 13/20 fish examined) resulted in a check being formed. However the frequency of checks in laboratory reared fish was too great to be accounted for by transfers alone.

2.4.3 DISCUSSION

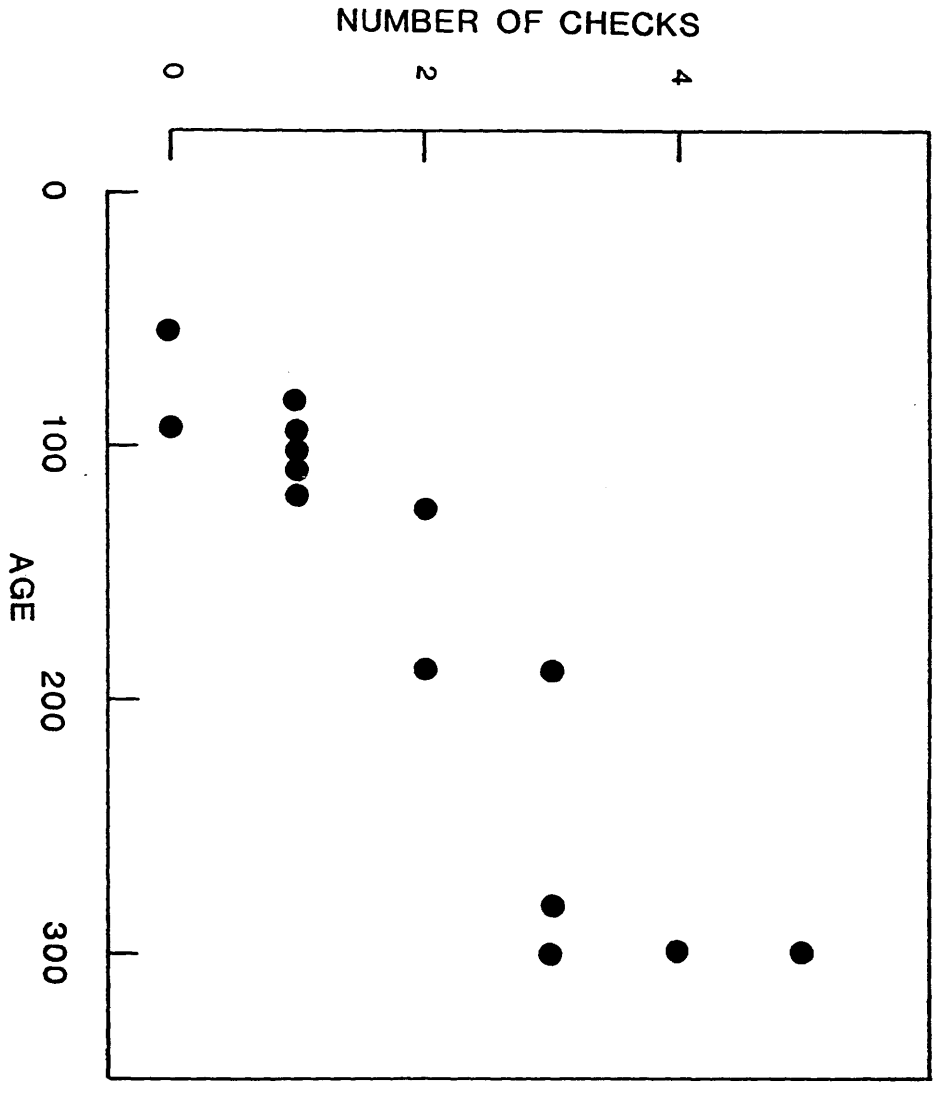
This study has demonstrated that sagitta increments are deposited daily after hatching in sticklebacks and continue to be deposited at a daily rate up to adulthood.

The present study has also demonstrated the problem of resolving narrow increments. On the basis of light microscope observations alone, it would appear that increment deposition in sticklebacks may virtually cease when somatic growth declines in winter. A complete cessation in increment formation has been reported in the green sunfish, *Lepomis macrochir*, subject to temperatures lower than 10°C (Taubert & Coble, 1977) and also in *Oncorhynchus nerka* held under 5°C (Marshall & Parker, 1982). Both of these studies were based on light microscope

Figure 2.13

Relation between number of checks on sagitta and age (as determined from daily increments) in three-spined sticklebacks, *Gasterosteus aculeatus*, collected from the River Endrick.

Points represent number of checks observed in a given sagitta.


NUMBER OF CHECKS
0
2
4
AGE
0
100
200
300

observations. However several studies have shown that narrow increments cannot always be detected using light microscopy (Campana *et al.*, 1987; Jones & Brothers, 1987; Morales-Nin & Ralston, 1990). Similarly the use of scanning electron microscopy in the current study has shown that during the winter months, narrow increments, that are too small to resolve with the light microscope, are deposited.

It is evident from the laboratory validation experiments that temperature is just one of a number of factors that influence increment width (also see Chapter 5). Studies of growth in wild populations, using length-frequency analysis, suggest that the sticklebacks virtually cease growing during winter (Ukegbu, 1986; Chellappa & Huntingford, 1988). This also appears to be the case in fish, 150-180 days old, held under warm (10^{o}C) laboratory conditions.

Checks, or discontinuities, are characteristic of most otolith growth sequences, where they may record periods of perturbation or stress to the fish (Pannella, 1980). In laboratory reared fish, catching and transfering fish out of water to another tank was found to be one cause of check formation. Campana (1983b) has shown that holding young coho salmon, *Oncorhynchus kisutch*, out of water for 20 seconds leads to a temporary cessation in the uptake of calcium from water and results in a reduced calcium deposition on to the otolith, causing a check to be formed. The cause of checks in wild fish is not known, although many factors, such as river spates, could result in some form of physiological stress. Since checks may represent

growth interruptions of unknown duration, their presence may prove to be a significant problem in the accurate interpretation of a growth sequence (Campana & Neilson, 1985). The occurrence of several checks in the sagitta of older fish, found in the present study could explain why increment counts were low ($\bar{x}$ = 284) relative to that predicted from the one increment day hypothesis in fish reared for 300 days.

2.5 SUMMARY

This study has shown that:

1. Sagitta increments are deposited daily in Atlantic salmon parr and in the three-spined stickleback.
2. Increment resolution and check formation may be a problem to ageing sticklebacks older than 150 - 180 days.

Chapter 3

THE PROCESS OF OTOLITH FORMATION

3.1 INTRODUCTION

Teleost otoliths are composed of calcium carbonate in the form of aragonite crystals and an organic proteinaceous matrix, termed otolin, characterised by a high abundance of acidic amino acids (Degens *et al.*, 1969). Little is known about the processes involved in otolith formation, although studies of the composition and structure of otoliths (Dunkelberger *et al.*, 1980; Watabe *et al.*, 1982; Mann *et al.*, 1983; Ross & Pote, 1984; Morales-Nin, 1987) and the surrounding otolithic membrane and sacculus (Dale, 1976; Dunkelberger *et al.*, 1980) have led to the development of several theories.

In general, as with the formation of other mineralised structures in which calcium carbonate is formed, otolith mineralisation must involve the attainment of a supersaturated state (i.e. exceed the solubility product), the nucleation of the mineral and a means of controlling crystal orientation and growth (Crenshaw, 1982).

The endolymph of freshwater fish contains a lower concentration of total calcium (bound and ionic) than that found in the plasma (Mugiya & Takahashi, 1985). Maintenance of the endolymph calcium concentration may involve active transport through the saccular wall, as inhibitor experiments on isolated sacculae have provided evidence for a calmodulin mediated Ca-ATPase pump mechanism (Mugiya, 1986). Whilst there has been no published estimate for the

solubility product of calcium carbonate in teleost endolymph, an estimate of apparent solubility product (Misogianes & Chasteen, 1979) using the calcium and total carbon dioxide concentrations reported for rainbow trout (*Oncorhynchus mykiss*) endolymph (Mugiya & Takahashi, 1985), suggests that the ion concentration in the endolymph is lower than that needed for calcification to occur (Appendix 1). Consequently an elevation of calcium and carbonate ion concentration at the otolith surface would be expected. The maintenance of high calcium carbonate concentrations at the crystallising surface of the otolith may involve calcified spherules, as spherical structures, believed to contain calcium, have been found in close contact with the otolith surface in Cod, *Gadus morhua* (Dale, 1976) and *Sarotherodon niloticus* (Saito quoted in Mugiya, 1987).

The pH of the endolymph is considerably higher than that of the plasma, thus favouring crystallisation (Mugiya & Takahashi, 1985). Indirect evidence from inhibitor experiments using acetalozamide suggest that the enzyme carbonic anhydrase may play a role in otolith mineralisation (Mugiya, 1977; Mugiya *et al.*, 1979; Mugiya & Takahashi, 1985).

In common with most mineralised tissues, the otolith is surrounded by an extracellular organic matrix, the sub-cupular meshwork (Dunkelberger *et al.*, 1980). Organic matrix is also incorporated into the otolith, and serves to separate individual crystals and bind the crystal and crystal layers into a unified structure (Dunkelberger *et al.*, 1980; Pannella, 1980; Watabe *et al.*, 1982; Morales-

Nin, 1987). The organic matrix may be involved in nucleation as initial otolith calcification in *Fundulus heteroclitus* has been shown to occur in a core matrix (Radkte & Dean, 1982). The organic matrix of otoliths may also provide a template for crystal growth and may determine crystal orientation (Dean *et al.*, 1983; Mann *et al.*, 1983; Ross & Pote, 1984), as has been indicated in other mineralising systems (Wilbur & Saleuddin, 1983). However there is little experimental evidence on this point.

As otoliths are formed within the otic sacs the precursors of the extracellular matrix must be produced by cells in the otic sacs, although these have not been identified (Mugiya, 1987). In TEM sections, vesicular structures have been observed on the apical surface of the saccula epithelium (Dale, 1976; Dunkelberger *et al.*, 1980; Popper & Hoxter, 1981) and may correspond to basophilic vesicles observed by Gauldie & Nelson (1988). However the histochemical composition of these structures has not been investigated and so their relevance to matrix formation is not known.

Studies of mineralisation in molluscs have shown that calcifying matrix is rich in sulphated glycoproteins and proteoglycans (Wada, 1980; Crenshaw, 1982; Hascall *et al.*, 1982). In vertebrates, extracellular matrices found in mineralised tissue are usually composed of a proteoglycans ground substance and type I collagen fibres, that are adhered to cells by means of a fibrous glycoprotein, fibronectin (Darnell *et al.*, 1986). Little is known about

the biochemical composition of the matrix found in the subcupular meshwork and otoliths, although PAS and alcian blue staining material have been identified in the endolymph, suggesting the presence of mucosubstances (e.g. glycoproteins and proteoglycans) (Mugiya, 1966).

Extracellular matrix contains both water soluble and insoluble components (Crenshaw, 1982). A calcium-binding protein has been extracted from the soluble matrix of mollusc shell and shown to be capable of inhibiting and regulating the rate of crystal formation *in vitro* (Wheeler *et al.*, 1981; Sikes & Wheeler, 1982; Krampitz, 1982; Wilbur & Saledullin, 1983). Similar calcium binding proteins have also been demonstrated in the calcified matrices of vertebrate tissues, such as egg shell (Krampitz, 1982). However no attempt has been made to determine if such a protein is also present in otoliths.

The aims of this study was to advance current understanding about the process of otolith formation. Specifically, the study was designed to investigate:

1. The structure of the sacculus epithelium, subcupular meshwork and otoliths (using histological techniques and scanning electron microscopy).
2. The occurrence and composition of spherules in the sacculus (using histochemical techniques and scanning electron microscopy).
3. The composition, formation and role of organic matrix in mineralisation (using histochemical techniques and scanning electron microscopy).

4. The calcium binding properties of soluble matrix extracted from the otolith.

3.2 STRUCTURE AND HISTOCHEMISTRY OF SACCULUS AND OTOLITHS

3.2.1 METHODS

Sampling and fixation: Atlantic salmon parr (*Salmo salar*) were obtained from the DAFS Smolt Rearing Station, Almondbank. Fish were decapitated and their labyrinths (otic sacs and semicircular canals) were immediately fixed *in situ* for histological or ultrastructural (scanning electron microscopy) examination. Tissue for histochemical studies was quench-frozen, after removal, for cryosectioning. The quenching medium was isopentane cooled to gelation-point (-165^{o}C) in liquid nitrogen.

Histology: After removal from the skull, labyrinths were transferred to fresh fixative (10% neutral buffered formal saline). Initially labyrinths were embedded in paraffin wax, after dehydration using a graded alcohol series. However, as found with ultrastructural studies (Dale, 1976; Dunkelberger *et al.*, 1980), sectioning of labyrinths proved very difficult owing to the combination of the thin fragile tissue layer and the hard mineralised otolith. Decalcification of fixed material, prior to embedding, was not suitable as it resulted in the loss of matrix fibres associated with the otolith. For these reasons, subsequent labyrinths were double embedded using 1% celloidin in

methyl benzoate and paraffin wax in accordance with standard techniques (Drury & Wallington, 1977) and tissue blocks were decalcified, prior to sectioning, using 3% ethylene diamine tetracetic acid (E.D.T.A.). 6 µm sections were cut on a Leitz rotary microtome. After dewaxing in Histoclear sections were brought to water and stained with haemalum and eosin or Mallory's trichrome stain.

Whilst double embedding provided a better means of preparing labyrinths, a relatively high degree of tissue distortion and shrinkage, due to processing and sectioning of this fragile tissue, was unavoidable.

Tissue sections were examined under transmitted light, using a Leitz Orthoplan microscope and photographs were taken with Kodacolor 100 ASA film, using a blue filter and even field illumination. Unfortunately owing to the density of the tissues, correct exposures for the tissue led to overexposure of the background and consequently resulted in most photographs having a yellow/green background hue.

Cryosectioned tissue: 8 µm thick sections of whole labyrinths, whole sacculae and isolated sagittae were sectioned with a cryostat (Bright Starlet) at -29^{o}C. Sections were collected on to coverslips and allowed to air dry, before dehydrating up to 70% ethanol and staining with haemalum and eosin.

Scanning electron microscopy: Preparation of whole sacculae and otoliths was similar to that used by Dale (1976). In an attempt to maintain the structure of calcified tissues a

[1] Following dehydration the sacculus was gently teased open, using watchmakers forceps and a scalpel, to reveal the inner sacculus surface and otoliths.

solution of 0.1 M phosphate buffer (Na_2HPO_4/NaH_2PO_4) adjusted to pH 7.4, 0.2% $CaCl_2$, 7% sucrose was used in all fixatives and rinses. Partial decalcification of tissue, to identify matrix organisation, was achieved by the use of 0.1 M sodium cacodylate buffer, 0.1% $CaCl_2$, 7% sucrose. The primary fixative of 2.5% glutaraldehyde/1.5% paraformaldehyde and secondary fixative of 1% osmium tetraoxide were made up in these buffer solutions. The procedure was as follows:

1. Primary fixation 2 - 3 hours.
2. Rinse in basic solution (2 changes): 30 minutes each.
3. Secondary fixation: 1 hour.
4. Rinse in basic solution (2 changes): 10 minutes each.
5. Post fix in uranyl acetate: 30 minutes, followed by rinse (used only for preparations of matrix)
6. Dehydration in graded acetone series (30, 50, 70, 90, 2 x 100%, dry absolute acetone): 10 minutes each.

Tissue was then transferred to a plastic cell with a pipette and covered in dry acetone. The tissue in the cell was then dehydrated in a critical point drier (SAMDRI-780). [1] The dried tissue was mounted on to aluminium stubs with double sided sellotape and silver paste and coated with a 150 Å layer of palladium gold in a sputter coater (POLARON 5000) before being examined with a Phillips 500 scanning electron microscope at 12.5 kV.

The ultrastructure of otolith increments was also investigated, from ground sagitta prepared for increment counts (Chapter 2).

Staining reactivity: Double embedded and cryostat sections (with and without diatase) were stained with periodic acid Schiff, alcian blue 8GX (pH 1.0 and 2.5), toluidene blue pH 2.0 (Drury & Wallington, 1977) and bromophenol blue (for double embedded sections; Pearse, 1986) or Ninhydrin-Schiff (for croystat sections; Yasuma & Itchikawa, 1952 in Pearse 1985), to demonstrate carbohydrates, mucosubstances (sulphated and acidic), metachromasia and proteins. Light green or alcian blue (pH 2.5; for distinction of acid [alcian staining] and neutral [PAS staining] glycoproteins) was used as a counterstain for PAS sections.

Carbonic anhydrase localisation: Carbonic anhydrase activity was localised in cryostat sectioned tissue using the method of Rosen (1970; in Pearse, 1985). 10^{-5} M acetazolamide (which inhibits carbonic anhydrase activity) was used in the control incubation medium.

Fibronectin: Reactivity for fibronectin was determined in acetic acid fixed tissue, according to the following schedule. Tissue was fixed in 1% acetic acid in 95% ethanol for 4-5 hours, washed and dehydrated in 95% ethanol (10 minutes) and absolute alcohol (2 changes 10 minutes each). Following dehydration, tissue was double embedded and 6 µm thick sections were cut.

Sections were dewaxed in histoclear, rehydrated and washed in distilled water and phosphate buffered saline (PBS) for 15 minutes. Sections were incubated for 45 minutes (at room temperature) in rabbit anti-(human)-fibronectin, made up to

a concentration of 1:60 with PBS. Sections were washed for 5 minutes in PBS after incubation. The sections were then incubated in fluorescein isothiocyanate isomer (FITC; a fluorescent probe) labelled anti-rabbit IgG antiserum at a concentration of 1:200. Sections were then rinsed and washed in PBS before mounting in glycerol and PBS (9:1) pH 9. The tissue was examined under UV light (incident lighting; 350 - 550 nm), using a Leitz Orthoplan fluorescent microscope, with no sub-stage illumination; photographs were taken with Kodak Ektachrome 400 ASA film.

Localisation of calcium using *in vitro* incubation in oxytetracycline: Whole sacculi were removed immediately after death and incubated in a teleost saline (Mugiya, 1986) containing 4 $mg.l^{-1}$ oxytetracycline chloride, fluorescent calcium chelator (Koenigs *et al.*, 1986), for 20 minutes. Sacculi were then rinsed three times in fresh saline and quench-frozen to $-29^{o}C$ and sectioned as above. Sections were stained with haemotoxylin and eosin and examined under UV light (incident lighting; 350 - 550 nm), with a Leitz Orthoplan microscope.

3.2.2 RESULTS

General structure of sacculus: Under low power (x100) the sacculus consisted of an outer wall of connective tissue, ranging between 40 µm and 100 µm and an inner epithelial layer (Figures 3.1). The epithelial layer consisted of a multi-layered sensory region (the macula), containing hair

Figure 3.1

Light micrograph of the cross-sectioned sacculus of Atlantic salmon parr.

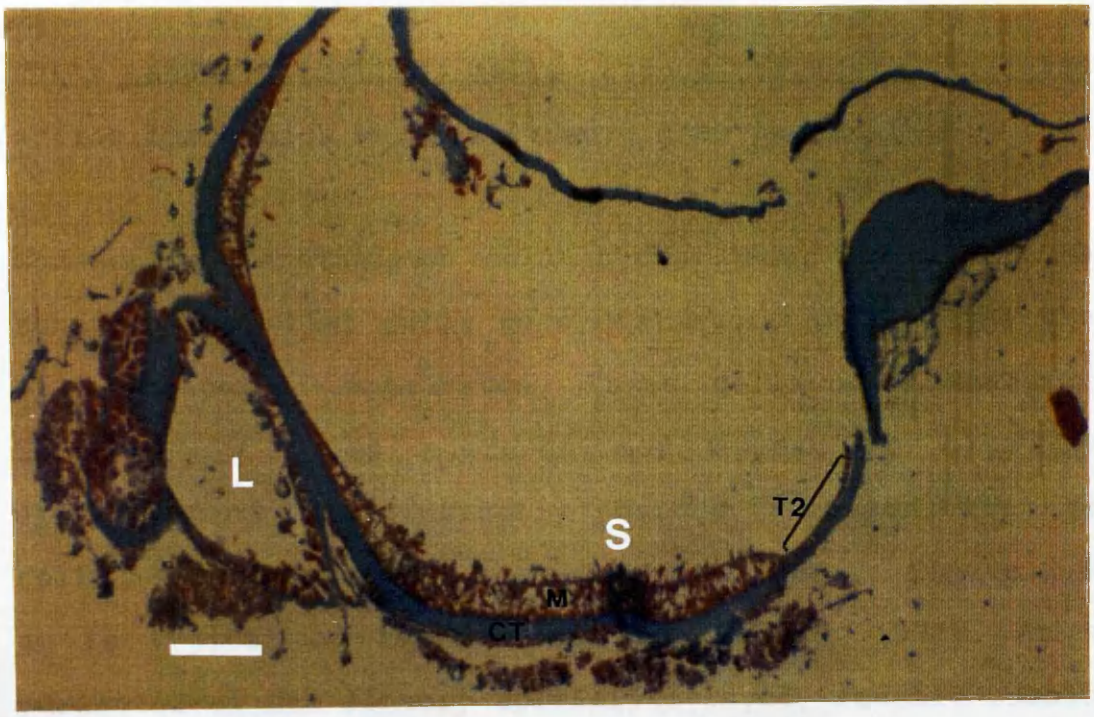
S denotes sacculus, L denotes lagena. M denotes macula
Mallory's triple stain. T2 denotes type T2 cells
Scale bar = 100 µm.

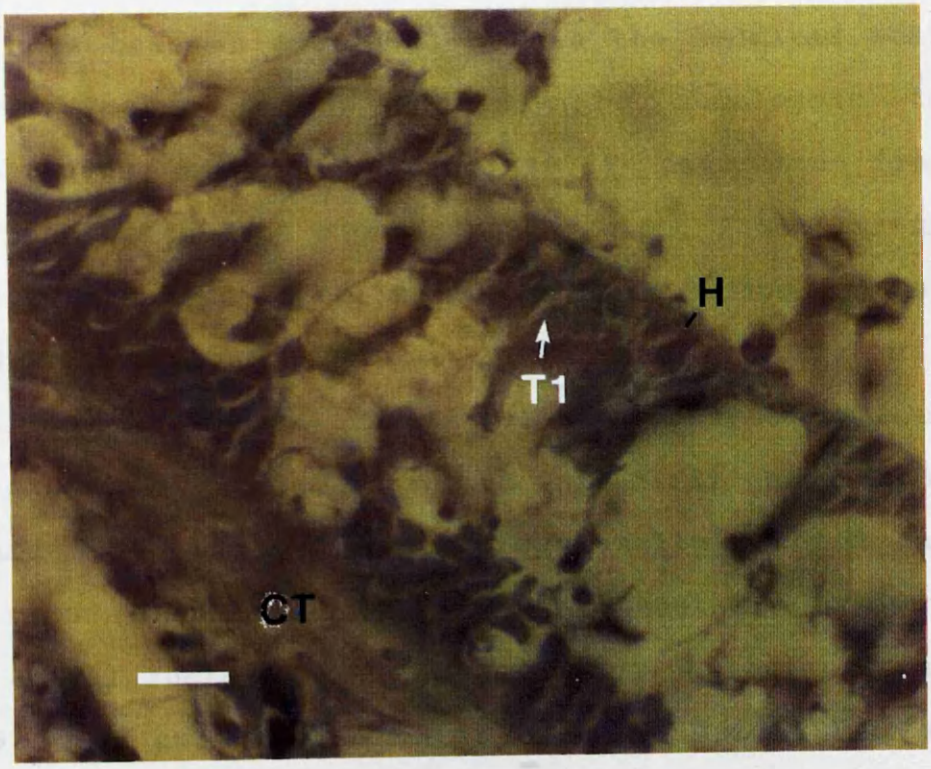
Figure 3.2

Light micrograph of a cross-section through the sacculus sensory epithelium (macula) of Atlantic salmon parr, showing a supporting (type I; T1) cell.

H denotes hair cells
CT denotes connective tissue

Bromophenol blue stain.
Scale bar = 10 µm.


L
S
T2
M
CT


H
T1
CT

cells and supporting cells and a thinner non-sensory epithelium, which was rarely more than one cell thick.

Non-sensory epithelium cell types: Four types of non-sensory epithelial cells were distinguishable on the basis of histological appearance. These cells were designated Types I - IV as most do not appear to have been distinguished histologically before. Between the hair cells, supporting cells were present (Type I; Figure 3.2), as is typical of teleost maculae (Popper, 1977). At the perimeter of the sensory epithelium layer, roughly cuboidal shaped cells (Type II; Figure 3.3) ~ 30 µm in height, with relatively large basal nuclei, were found. Many of these were heavily granulated and a fibrous-looking secretion was seen on the apical surface of some cells. This secretion may have been an amorphous gel in the hydrated state (N. Spurway pers. comm.) and may be homologous to the 'membranous material' Dale (1976) described from TEM sections of the sub-cupular zone region. A similar type of cell to the type II cells, but with an irregular shape, 13 - 23 µm in height, was seen at the base of cristae in the lapillus (Figure 3.4). These cells gave way to smaller (~15 µm) cuboidal or columnar shaped epithelial cells (Type III; Figure 3.5), with a basophilic, basally located nucleus. Vesicles (<1 µm in diameter) were generally evident near the apical surface of these cells and vesicular structures (0.5 - 1 µm) were evident on the apical surface of epithelia. At the outer perimeter of the sacculus epithelium, corresponding to the region in closest contact

Figure 3.1a

Schematic representation of the sacculus, showing arrangement of different non-sensory epithelia cells (T1 - T4), hair cells (HC), sub-cupular meshwork (SCM), connective tissue (CT) and sagitta.

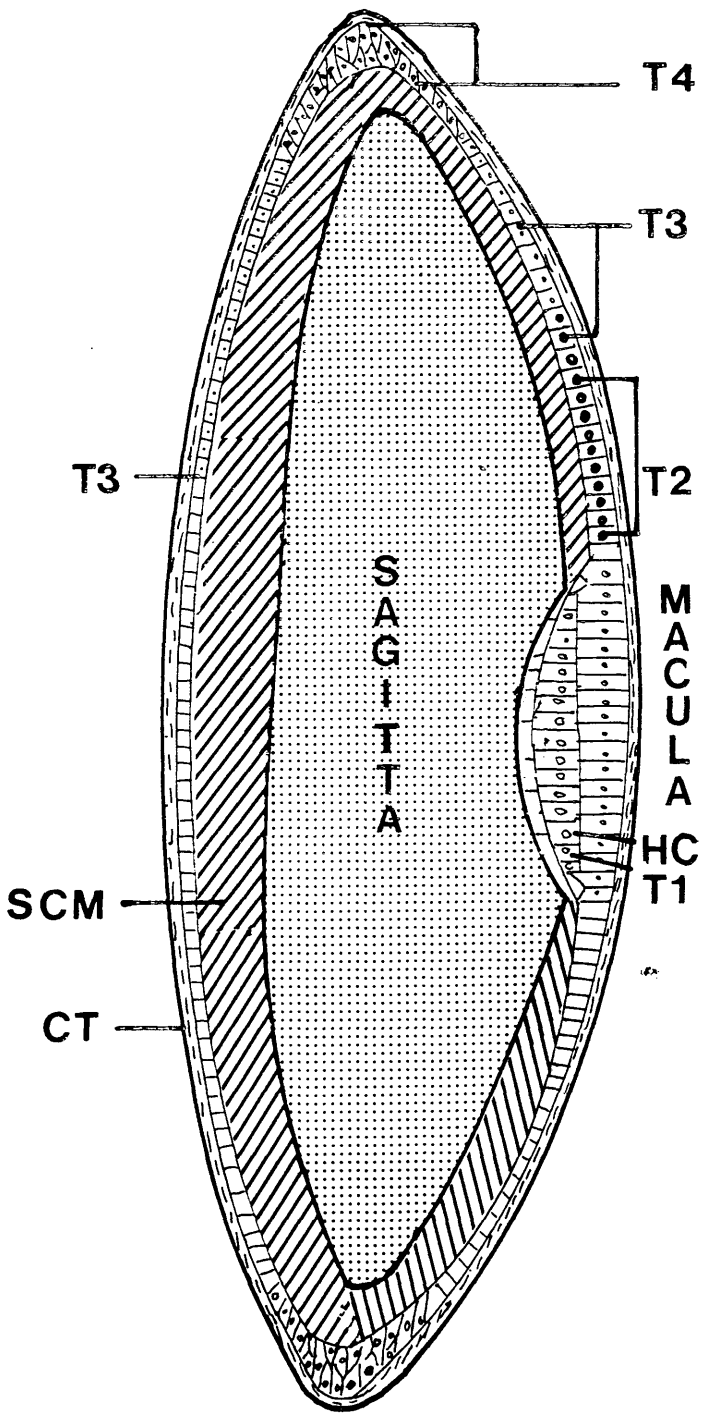


Figure 3.3

Light micrograph of a cross-section through the sacculus of Atlantic salmon parr, showing a type II cell.

S denotes fibrous secretion, C denotes connective tissue layer.
Bromophenol blue stain.
Scale bar = 25 µm.

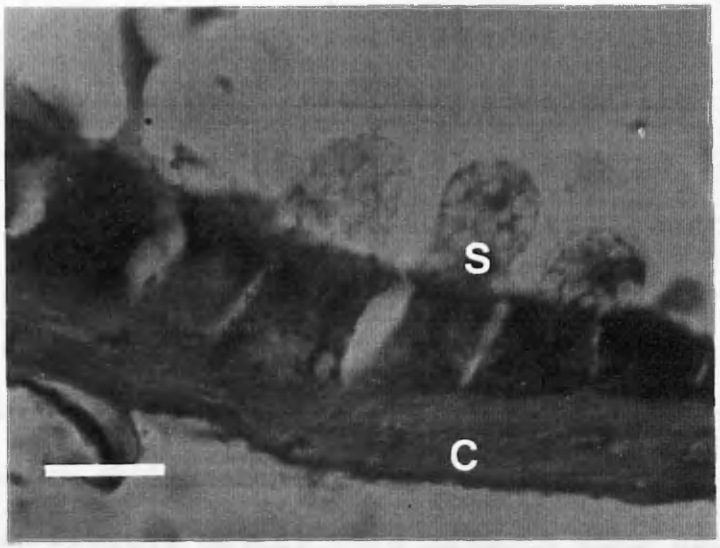
Figure 3.4

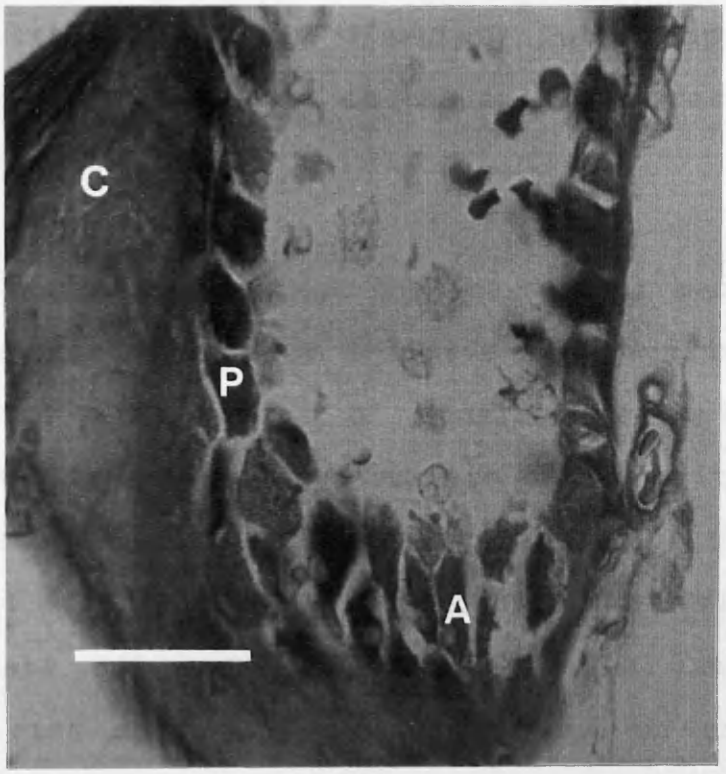
Light micrograph of a cross-section through the lapillus cristae of Atlantic salmon parr, showing cells similar to type II sacculus cells.

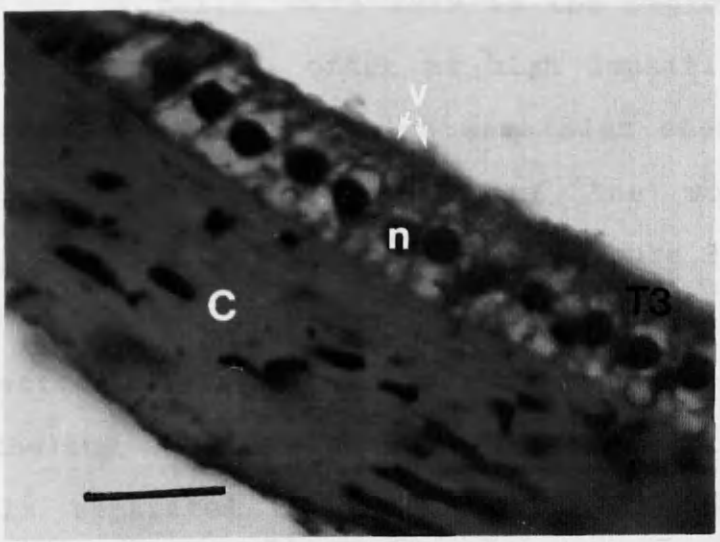
C denotes connective tissue layer, P denotes PAS positive cells and A denotes alcian blue positive cells.
PAS/Alcian blue (pH 2.5) stain.
Scale bar = 25µm.

Figure 3.5

Light micrograph of a cross-section through the sacculus of Atlantic salmon parr, showing a type III cell(T3).
C denotes connective tissue layer; n denotes nucleus
Haemalum and eosin stain. v denotes vesicles
Scale bar = 20 µm.


S
C


C
P
A


V
n
C
T3

to the sagitta surface, strongly basophilic irregular shaped cells (Type IV; Figure 3.6), 12-16 µm in height, with a very large nucleus (~70-80% of cell section) were found. Type IV cells always occurred in a multiple layer, unlike other cells outside the sensory epithelium. On the apical surface of these cells large densities (1600-2200 mm^{-2}) of small (0.5-2 µm) spherical structures within an extracellular fibrous matrix were seen in some sections.

Larger spherules (4-12 µm diameter), which appeared to have a core, were also found on the apical surface of non-sensory epithelia.

Sub-cupular meshwork: Although the sub-cupular meshwork was lost in most sections, some did have the remains of this layer together with pieces of decalcified otolith. The layer extended from the apical surface of the epithelium to the surface of the otolith (Figure 3.7). The layer consisted of fibrous-looking amorphous material in the hydrated state and matrix fibres. Spherical structures, similar in size and shape to those found on the apical surface of type IV cells, were seen in the region closest (~ 50 µm) to the otolith, often at high densities (1000-2000 mm^{-2}) (Figure 3.8). Spherules were also seen in much lower densities in other regions of the sub-cupular meshwork as were small (<1 µm) particles (Figure 3.9).

Scanning electron microscopy: The apical surface of the sensory epithelium consisted of the surfaces of the apical sensory cells separated by the apical surfaces of the

Figure 3.6

Light micrograph of a cross-section through the sacculus of Atlantic salmon parr, showing type IV cells.

s = spherule structures, m = alcian positive matrix
Haemalum - eosin and alcian blue (pH 1.0) stain.
Scale bar = 20 µm.

Figure 3.7

Light micrograph of a cross-section through the sub cupular meshwork (SCM) and sensory epithelium (E) of Atlantic salmon parr.

C = cartilage, CM = matrix with calcified staining properties, O = position of otolith in intact specimen.

Haemalum and eosin stain
Scale bar = 10 µm.

Figure 3.8

Light micrograph of a cross-section through the sub-cupular meshwork (SCM) near the otolith surface.

O = position of otolith in intact sacculus, L = tissue layer, s = spherules similar to those found on type IV cells.

Haemalum-eosin and alcian blue (pH 1.0) stain

Scale bar = 25μm.

Figure 3.9

Light micrograph of a cross-section through the sub-cupular meshwork (SCM).

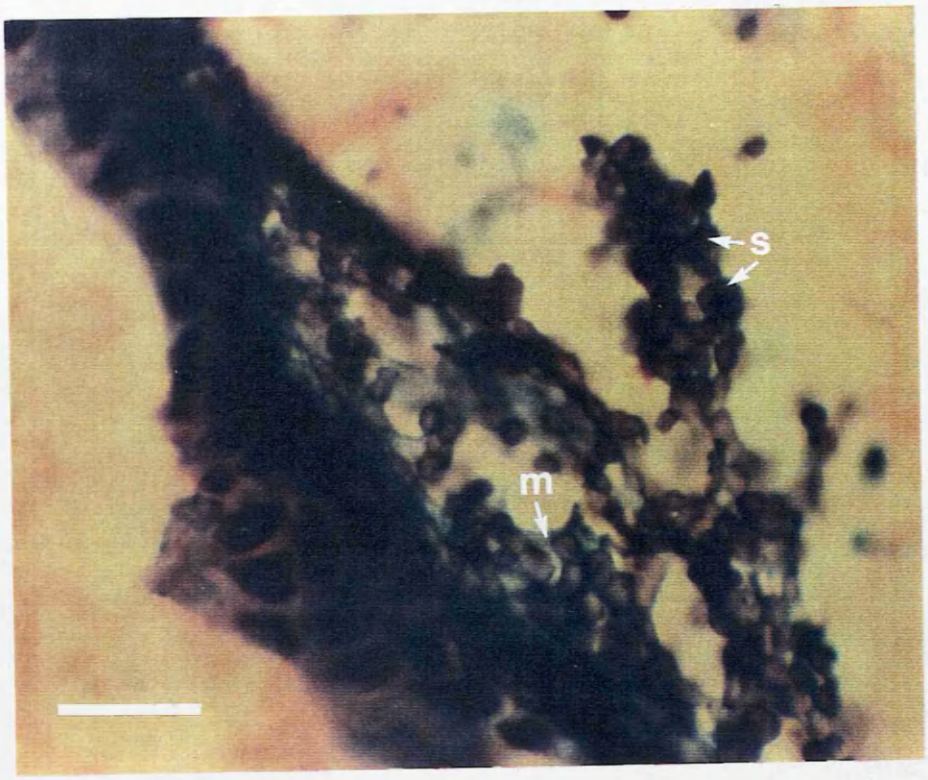
s = spherules similar to those found on type IV cells.

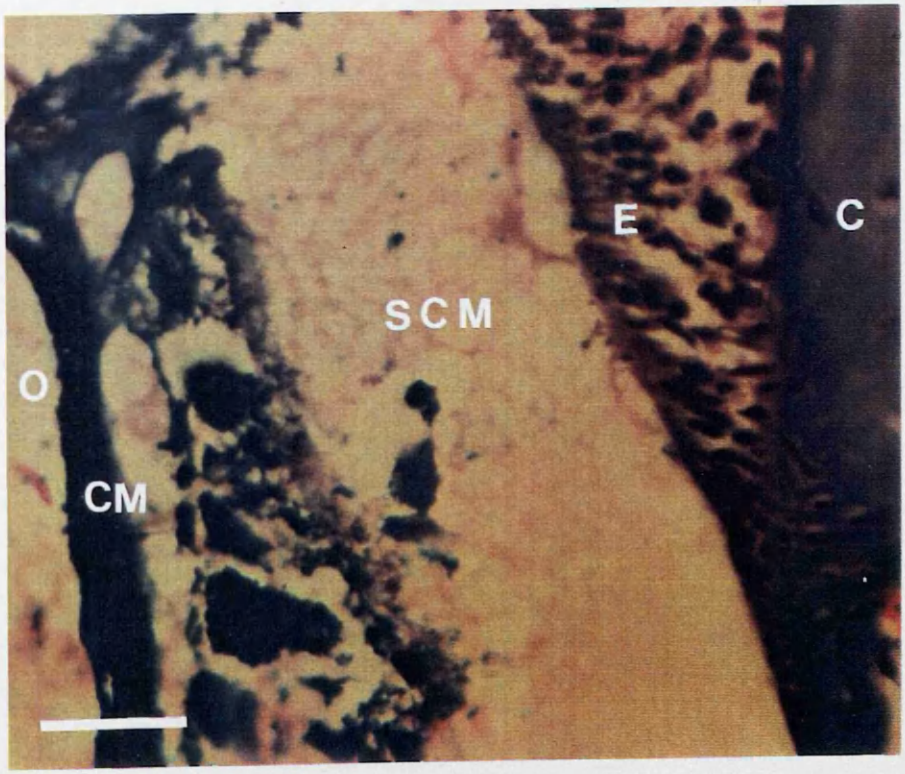
g = protein staining granules

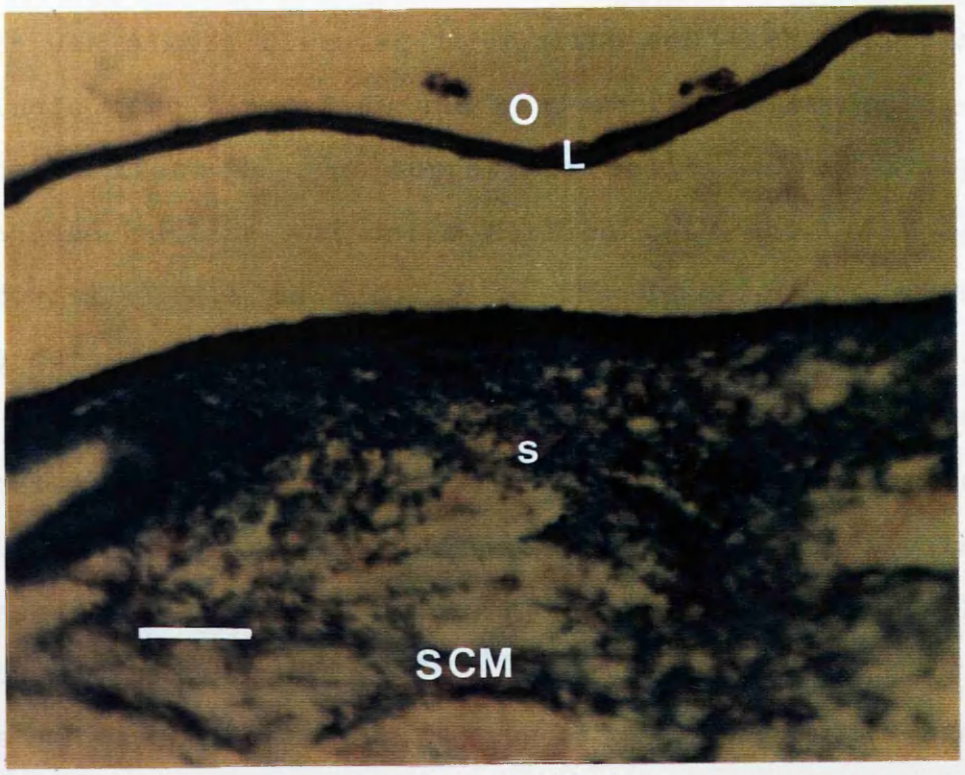
f = matrix fibres

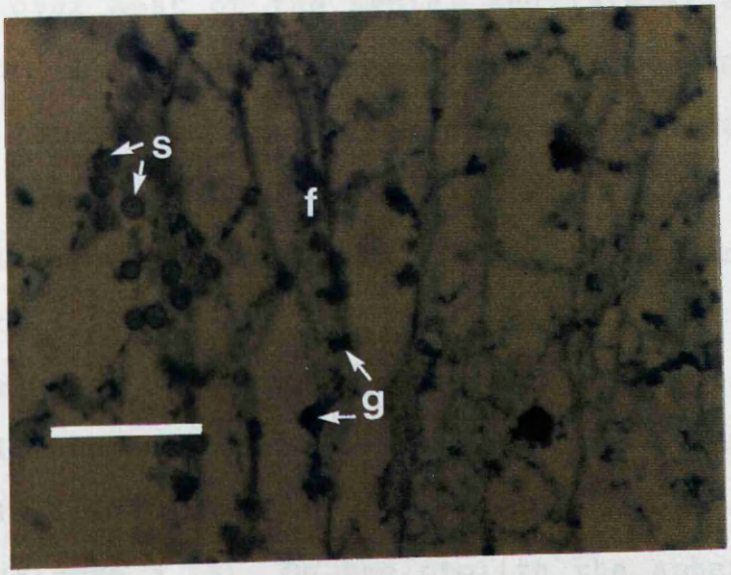
Bromophenol blue stain

Scale bar = 25μm.


s
m


E
C
SCM
O
CM


O
L
s
SCM


s
f
g

supporting cells (Figure 3.10). The apical surface of hair-cells was almost circular in outline and each cell carried a single long kinocilium and many shorter stereocilia. The apical surface of the supporting cells had an irregular elongated outline and cells carried only short, knob-like microvilli. This is similar to the descriptions given in more detailed studies by Dale (1976) and Popper (1977).

In the non-sensory region a fibrous secretion was evident on the apical surface of some cells, and the cells appeared to be erupting (Figure 3.11). The secretion was similar in appearance to the fibrous material of the subcupular meshwork (see below).

The subcupular meshwork, lying between the macula and the sagitta, usually remained attached to the otolith after having been removed from the sacculus (Figure 3. 12). It was therefore possible to examine the medial surface of the meshwork. This examination revealed a reticular structure consisting of coarse and fine inter-connected fibres or trabeculae over most of the medial and lateral surface of the otolith. The coarse fibres formed large (0.25 µm), hexagonal, honeycomb-like meshes crossed by finer fibres. Around the perimeter of the otolith thicker (~ 3.2 µm) more cylindrical shaped fibres with a nodular appearance were seen (Figure 3.13).

Small spherules (0.5-2 µm) were seen on the medial surface of the otolithic membrane and on the surface of the otolith (Figure 3.14). The spherules had a rough, reticular surface structure (Figure 3.15). On the otolith the spherules were enmeshed in a veil-like fibrous material; this bound them

Figure 3.10

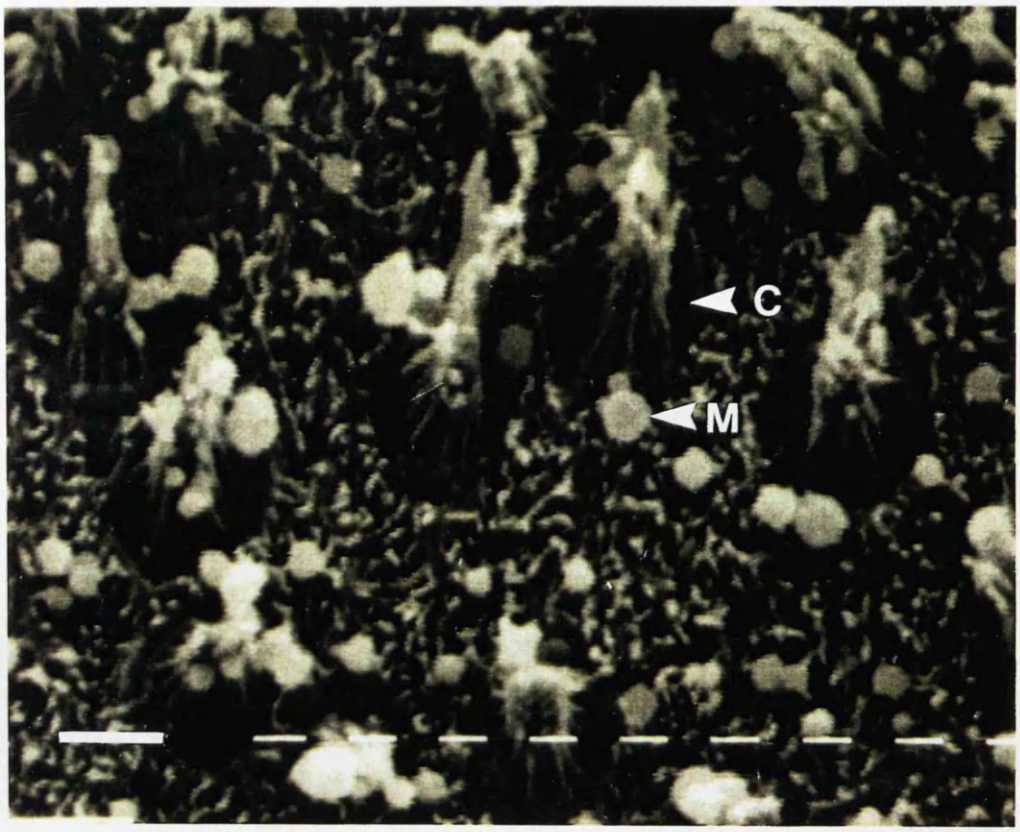
Scanning electron micrograph of the apical surface of the sacculus sensory epithelium, from an Atlantic salmon parr.

Sodium cacodylate buffer was used in fixation (see text).
C = stereocilia of a hair cell
M = knob-like microvilli of a supporting cell
Scale bar = 5 µm.

Figure 3.11

Scanning electron micrograph of the apical surface of non-sensory sacculus epithelium showing a cell with a fibrous secretion (M).

Sodium cacodylate buffer was used in fixation (see text).
C denotes cell which appears to be erupting.
Scale bar = 2µm.


C
M

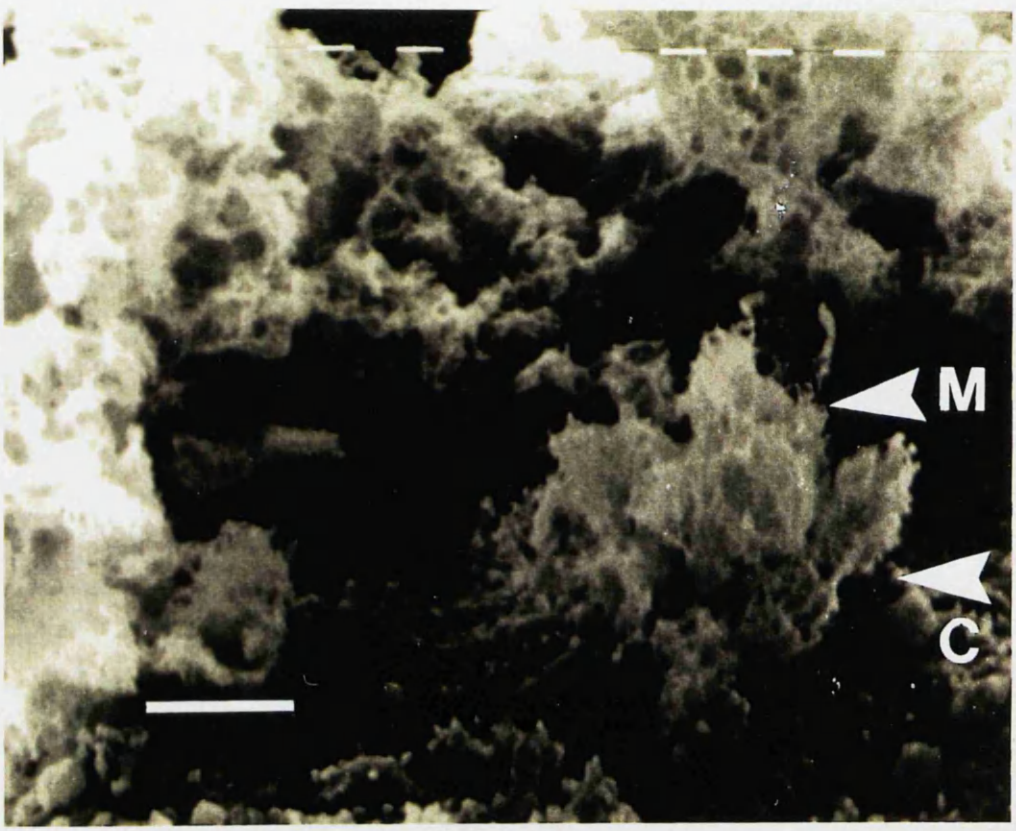

M
C

Figure 3.12

Scanning electron micrograph of the subcupular meshwork (SCM) remaining on surface of the sagitta.

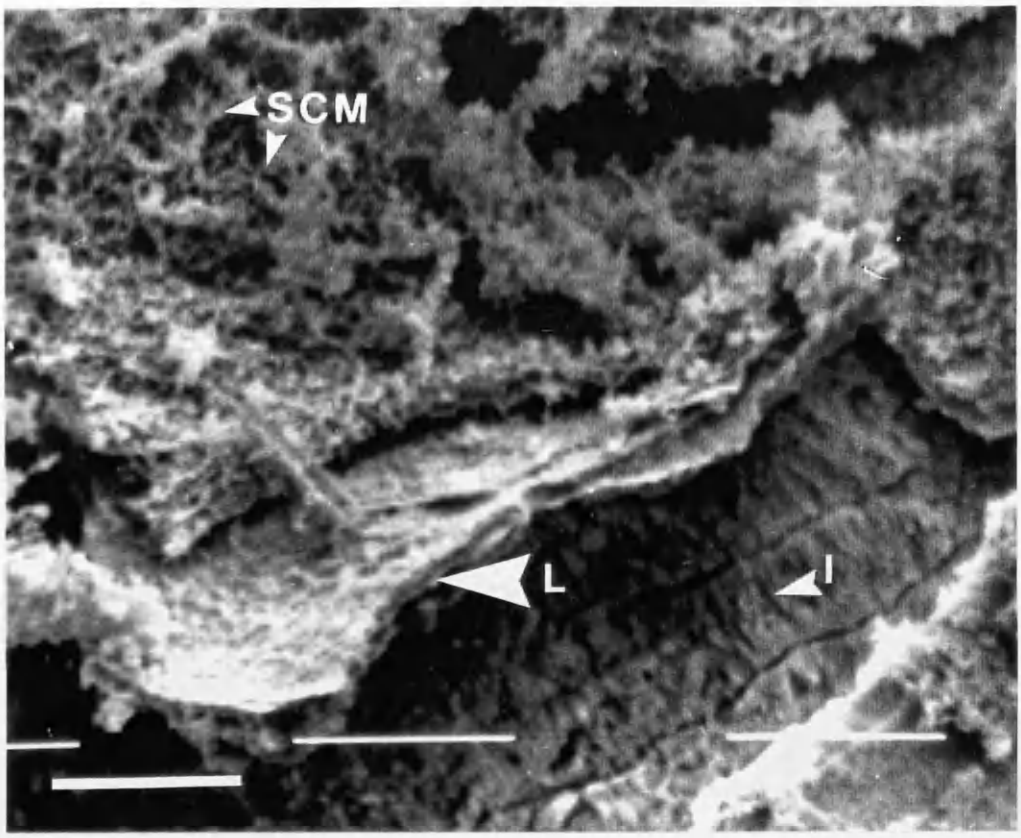
Sodium cacodylate buffer was used in fixation (see text).
L = a dense layer covering most of the sagitta, I = increment seen on the lateral surface of the exposed sagitta.

Scale bar = 2μm

Figure 3.13

Scanning electron micrograph of the coarse fibres of subcupular meshwork (SCM) close to the sagitta surface.

Phosphate buffer was used in fixation.
N = nodule-like structures on fibres, f = finer interconnecting fibers.
Scale bar = 10μm.


SCM
L
I

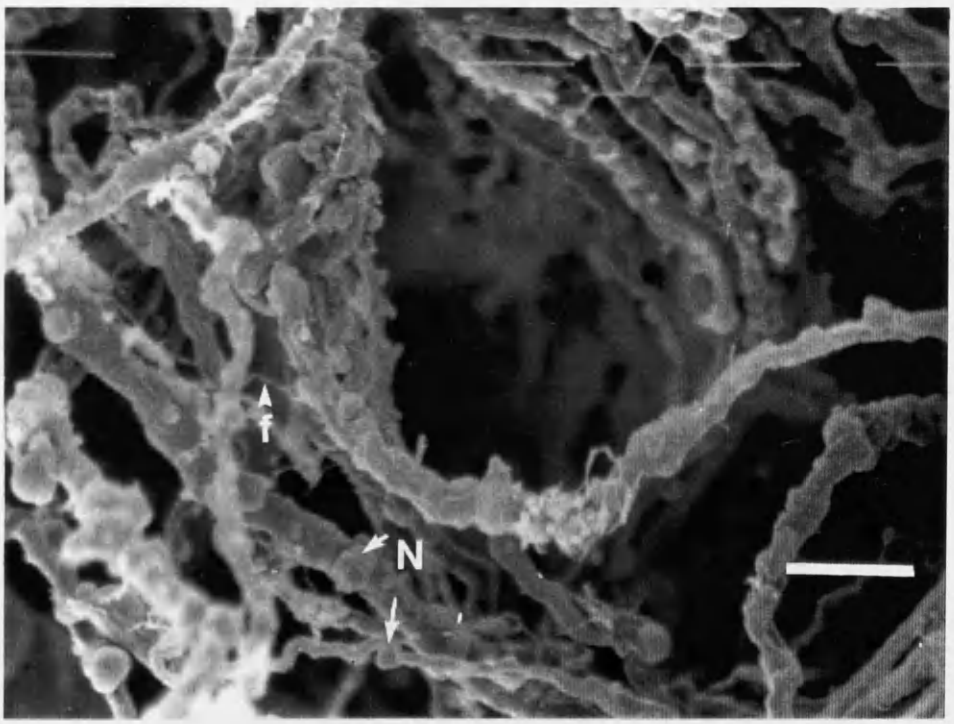

f
N

Figure 3.14

Scanning electron micrograph of spherules on the sagitta surface, showing different stages of embedding into matrix covering the sagitta.

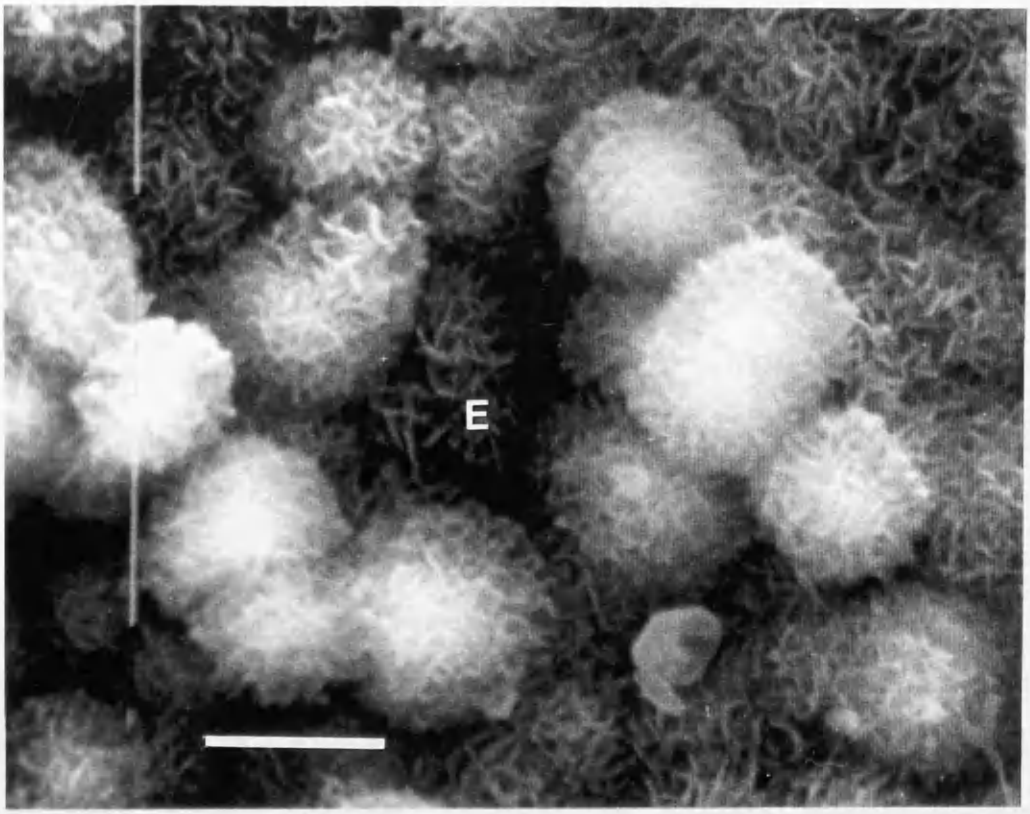
Phosphate buffer was used in fixation (see text).
E denotes embedded spherule.

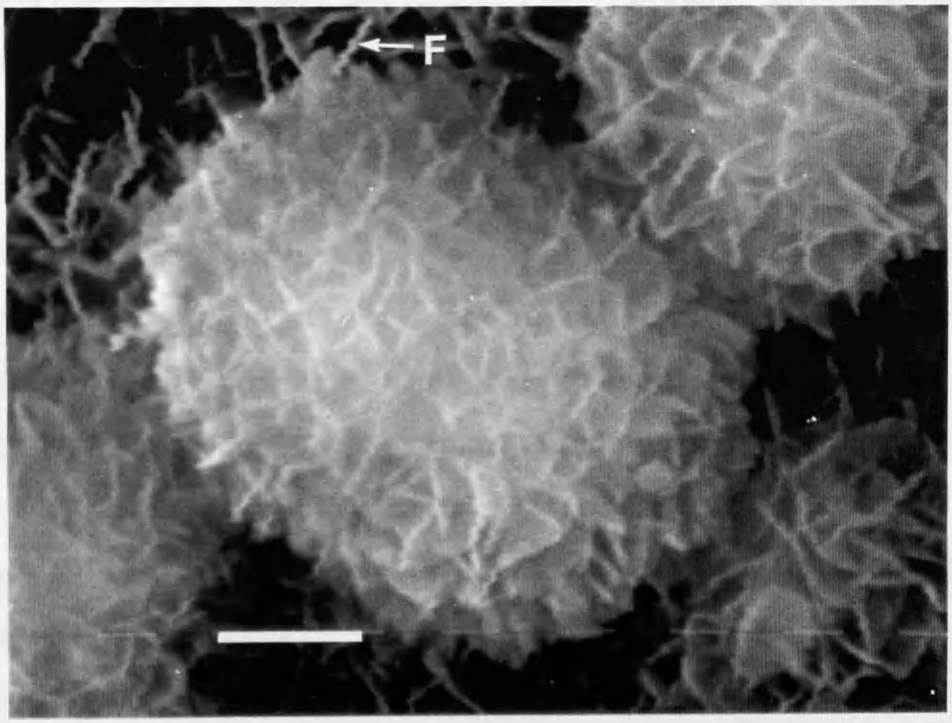
Scale bar = 5 μm.

Figure 3.15

Scanning electron micrograph of spherules on the sagitta surface, showing detailed structure of spherule surface.

F denotes one of the many matrix fibres which appear to attach the spherules together and which occur throughout the spherule.
Phosphate buffer was used in fixation (see text).
Scale bar =0·5μm.


E


F

together and to the tissue layer, and many spherules appeared to be partially or completely embedded in this material. Remains of a thin, (0.5 µm), tissue layer covering at least part of the spherules and otolith was seen in both calcified and decalcified preparations (Figure 3.16). In places the layer of spherules and fibrous material was disrupted and the surface of the otolith could be seen underneath (Figure 3.17). However the fibrous material of the spherule layer was continuous with fibres on the bare otolith surface.

The medial side of the sagitta was convex and in the region that faces the sensory epithelium was traversed by a relatively large furrow, termed the *sulcus acusticus* zone (Figure 3. 18). A pattern of concentric increments or lamellae were seen on both the lateral surface and the ground medio-lateral surface of the sagitta. The increments were arranged radially from the centre out to the edges (cf. Figure 2.11). The smallest increments (0.8 - 4.2 µm) consisted of a crystallised layer, the incremental zone and a heavily etched layer, the discontinuous zone. Detailed observations indicated that the discontinuous zones formed sutures between the crystallised layer, which had the effect of interrupting crystal growth (Figure 3.19). Matrix fibres were continuous between the calcified and discontinuous layer, although they tended to be thicker in the latter. Individual crystallised layers were composed of branched aggregates of crystals that individually measured about 0.25 µm in diameter arranged with their longest axes (c-axes) orientated towards the primordium

Figure 3. 16

Scanning electron micrograph of spherules (S) on the sagitta surface (o), showing attachment to a layer (L) covering the sagitta, homologous to the layer in Figure 3.12.

L denotes thin matrix layer
S denotes spherule

Phosphate buffer was used in fixation (see text).

Scale bar = 2 µm.

Figure 3.17

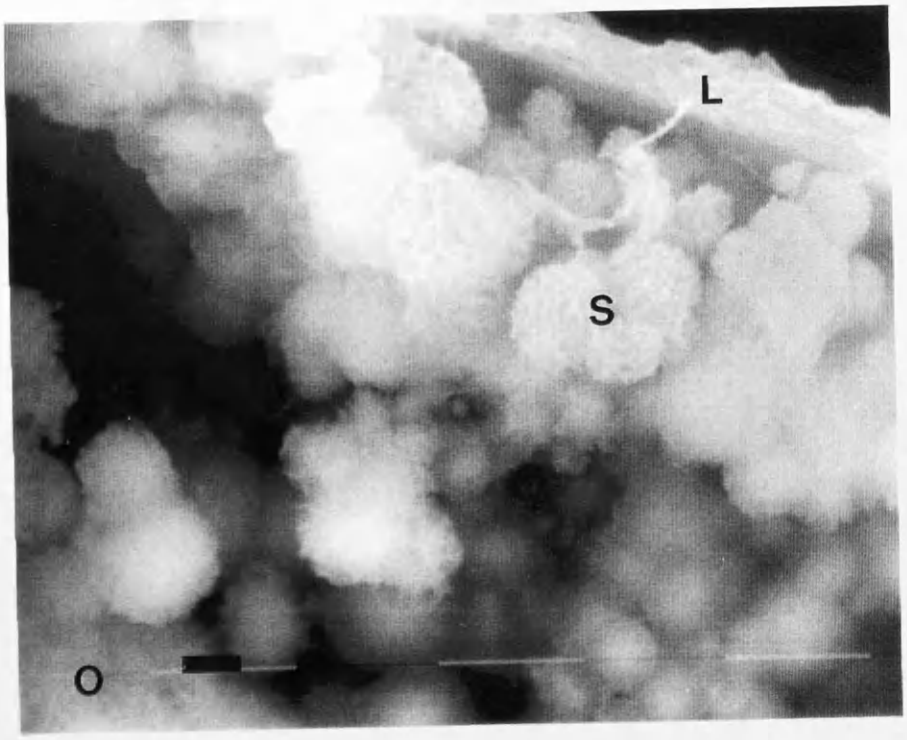
Scanning electron micrograph of spherules on the sagitta surface, showing an exposed region of sagitta (o).

Phosphate buffer was used in fixation (see text).

M denotes the matrix that is continuous between otolith surface and spherule layer.

Phosphate buffer was used in fixation (see text).

Scale bar = 5µm.


L
S
O

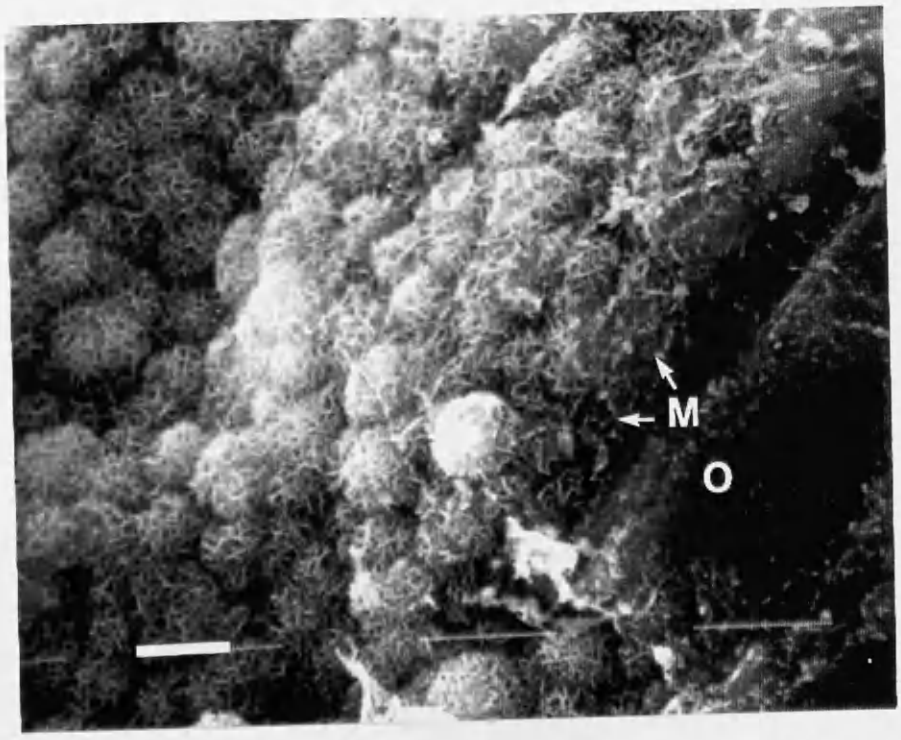

M
O

Figure 3.18

Scanning electron micrograph of the medial surface of a sagitta, showing the sulcus acusticus (SA) and remnants of gelatinous layer (G).

Sodium cacodylate buffer was used in fixation (see text). Scale bar = 100μm.

SA
G

Figure 3.19

Scanning electron micrograph of sagitta increments on the lateral surface, showing the distribution of matrix fibres over the incremental (I) and discontinuous zone (D).

Sodium cacodylate buffer was used in fixation (see text).
Critical-point dehydrated specimen.
Horizontal scale bar = 1 μm.

Figure 3.20

Scanning electron micrograph of a sagitta increments from a ground and EDTA etched medial section. (Ethanol dried).

D = heavily etched discontinuous zone, S = dense border of crystallised zone, C = crystals of crystallised zone, c = crystals that have grown across discontinuous zone.
Scale bar = 1μm.

D
D
I

C
C
S
D

(Figure 3.20). Some crystals bridged the heavily etched discontinuous zone. The front of each crystallised layer was denser than the rest of the layer, and may have been overlain either by crystals or matrix.

Vesicles and extracellular secretion: Basophilic vesicles were seen close to the apical surface and vesicular structures (1-2 μm; Figure 3. 21) were found on the apical surface of supporting cells of the sensory epithelium (Type I) and the apical surface of Type III cells within the transitional epithelia. Vesicles in Type I and Type III cells showed a positive reaction to PAS and Bromophenol blue stains indicating the presence of carbohydrate and protein (Table 3.1). In PAS - alcian blue stained sections Type II cells showed an intense reactivity to PAS or alcian blue or a combination of the two. The fibrous secretion (or amorphous gel) observed on the apical surface of Type II cells showed positive reactions to protein stains and, in PAS/alcian blue (pH 2.5) stained sections, reacted predominantly with either PAS or alcian blue. A lack of reactivity to alcian blue in PAS/alcian blue stained tissue indicates neutral mucosubstances (neutral glycoproteins), whereas reactivity to alcian blue indicates acidic mucosubstances (Pearse, 1985). This suggests that there is a change in the relative composition of neutral and acidic mucosubstances in the secretion produced by the type II cells. The secretion of type II cells was also found to exhibit metachromasia with toluidene blue indicating the presence of glycosaminosglycans (possibly

Figure 3.21

Light micrographs of basophilic vesicular structures (v) found on the apical surface of sensory epithelium.
k = Kinocilia, h = haircell, t1 = type I cell. Double embedded section.

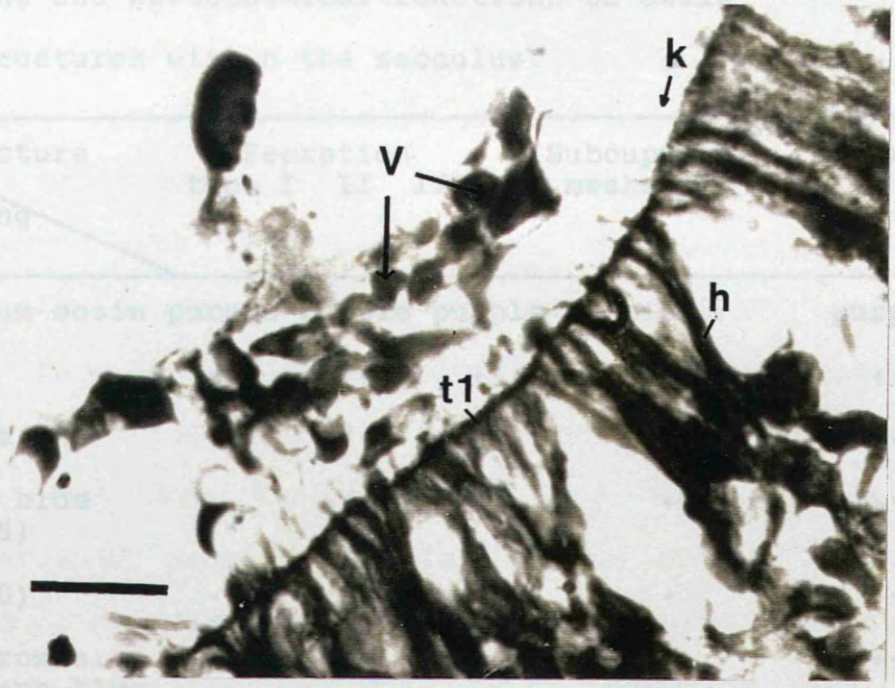
Scale bar = 20 μm.

Figure 3.22

Light micrograph of fibronectin positive material on apical surface of of sensory epithelium and type II (T2) cells, in the sacculus of Atlantic salmon parr. Light micrograph (inset) of a control (not incubated in anti-fibronectin) is given below.

Photograph taken under UV light (excitation wavelength = 350 - 525 nm).

Scale bar = 20μm.


k
V
h
t1

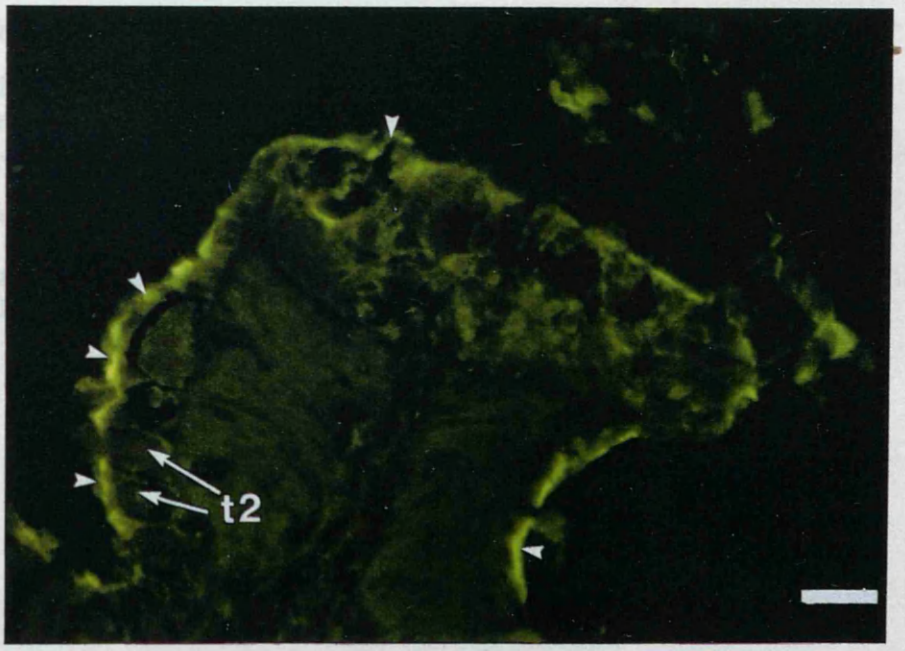

t2

Table 3.1

Staining and histochemical reactions of cells and structures within the sacculus.

Structure / Staining method	Secretion type I	Secretion type II	Secretion type III	Subcupular meshwork	Spherule
Haemalum-eosin	purple	purple	purple	pink	purple[1]
PAS	+	+/++	+	+	++[1]
diatase PAS	+	+/++	+	+	++[1]
Alcian blue (pH 2.5)	-	+/++	-	+	++[1]
(pH 1.0)	-	++	-	-	++[1]
Metachromasia: toluidene blue (pH 4.1)	-	+/++	-	+	++[1]
Bromophenol blue	++	+	++	+	+[1]
Nin-hydrin Schiff	++	+	++	+	+[1]
Tetracycline	-	-	-	+	++
Carbonic anhydrase				-/+	+[1]

Reactivity is denoted by +/++ (weak/strong)

[1]denotes staining oforganic matrix in association with spherule

proteoglycans) and stained blue with alcian blue at pH 1.0, indicating sulphated glycoprotein.

The staining reactivity of matrix in the sub-cupular meshwork varied regionally. In the apical region around type IV cells and close to the otolith surface, the matrix associated with spherules showed a strongly positive reaction to alcian blue and was metachromatic, indicating the presence of acidic glycoproteins and glycosaminosglycans (possibly proteoglycans). In other regions the matrix showed weak reactions to PAS and alcian blue. The aggregates of organic material on the thread-like fibres reacted to protein stains. The central region of the matrix also stained faintly pink with haemalum and eosin suggesting that it contained collagen and the region close to the otolith stained intensely blue, indicating that the matrix was calcified (Drury & Wallington, 1977).

Fibronectin: A positive reaction to anti-fibronectin was found throughout the sacculus, at the interface between the subcupular meshwork and the apical surface of the epithelium (Figure 3. 22). Reactivity was most intense in the fibrous secretion of the type II cells.

Spherules: Spherules were found to incorporate oxytetracycline, indicating calcium accretion (Figure 3.23). Organic material was also present on the surface of these spherules and sites of carbonic anhydrase activity were localised within this organic material (Figure 3.24). Material at the centre of these spherules reacted to alcian

Spherules (cryosectioned)

Figure 3.23

Light micrograph of a group of spherules (s) on the apical surface of epithelium after incubation with $.4mgl^{-1}$ oxytetracycline.

Photographed under UV light (excitation wavelength = 350 - 525 nm). All yellow fluorescent material has incorporated tetracycline.

Scale bar = 5µm

Figure 3.24

Light micrograph showing localisation of carbonic anhydrase activity on surface organic material.

Arrows indicate areas of carbonic activity as shown by cobalt sulphite precipitation.

Scale bar = 10µm.

Figure 3.25

Light micrograph of a group of spherules showing metachromatic core (C) material.

Toluidene blue stain

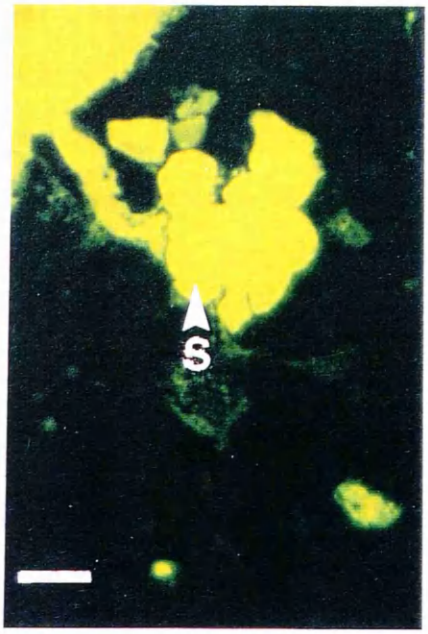
Scale bar = 10µm.

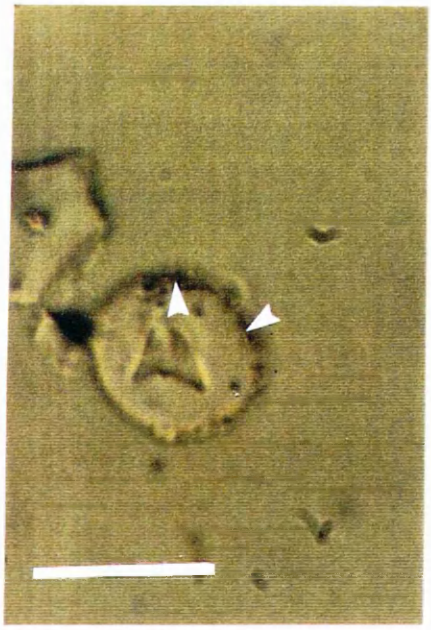
Figure 3.26

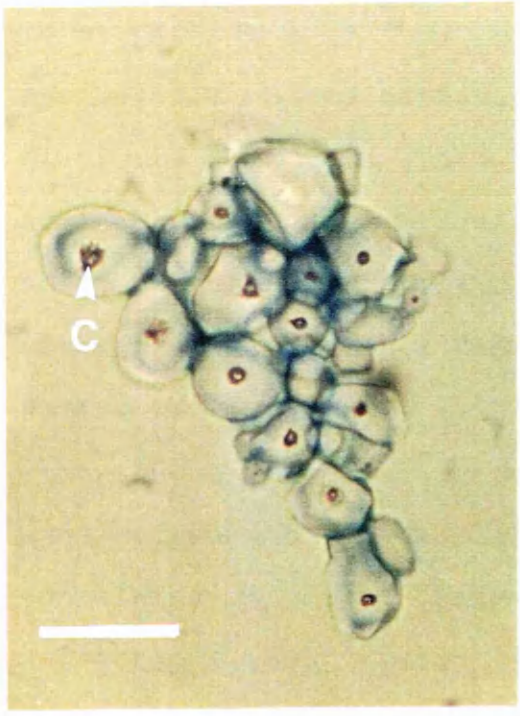
Light micrograph of a group of spherules showing PAS stained material between and within the spherules.

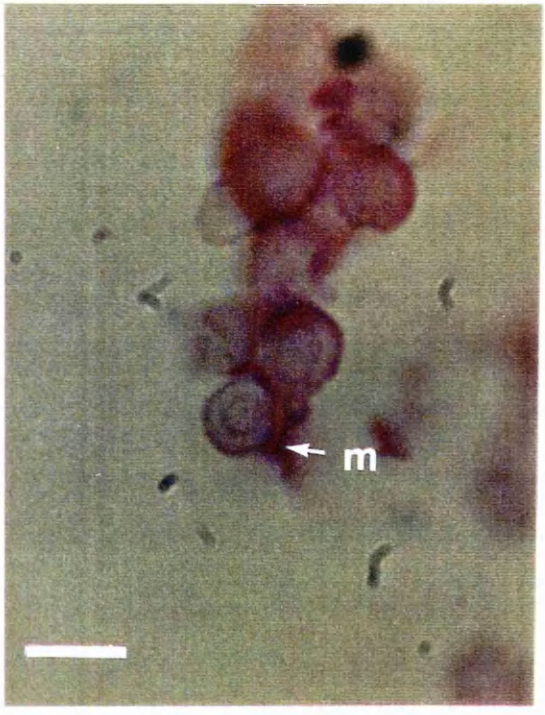
m denotes matrix on surface of spherule

Scale bar = 10µm.


S




C


m

blue (pH 1.0) (Figure 3.26) and PAS (Figure 3.27) suggesting that calcium accretion occurred around an organic core containing sulphated glycoprotein and glycosaminosglycans (possibly proteoglycans).

3.3 CALCIUM BINDING PROPERTIES OF SOLUBLE MATRIX

3.3.1 METHODS

Soluble matrix was obtained from sagittae of freshly killed; 1+ & 3+ and 4+ Atlantic salmon. A minimum of 20 mg of otoliths was required to yield sufficient matrix (~ 12 ug matrix) for each experiment. All experiments were replicated twice. Otoliths were cleared of connecting tissue in 1% sodium hydroxide, rinsed in water and ground to powder in a 1.5 ml eppendorf tube with a porcelain pestle. 0.1 ml of distilled water was added for each 20 mg of ground otolith. The slurry was decalcified by a gradual addition of 0.1 M HCl at 20°C with the pH maintained at 3 to 5, as used for oyster shell (Wheeler et al., 1981). After the mineral had dissolved (a minimum decalcification period of four hours was used), the resulting solution was centrifuged twice at 13,000g for ten minutes to sediment undissolved otolith and insoluble matrix. The supernatant containing soluble matrix was then dialysed against distilled water. The protein content of the extract was determined using a Lowry assay (Lowry et al., 1951). The reactivity of the extract to carbohydrate (PAS) and protein (Ninhydrin) stains was tested on chromatography paper.

The effect of salmon soluble matrix on the rate of precipitation of CaCO3 was determined by recording pH following either the rapid addition of 3 ml of 20 mM $CaCl_2$ to a solution containing 3 ml of 20 mM $NaHCO_3^-$ and 0.3 ml of H_2O or redialysed matrix protein or the addition of 0.3 ml of H_2O or redialysed matrix protein to a solution containing 3 ml of 20 mM $NaHCO_3^-$ and 3 ml of 20 mM $CaCl_2$ (following Wheeler et al., 1981). The overall reaction for the precipitation is;

$$Ca^{2+} + HCO_3^- \longrightarrow CaCO_3 + H^+$$

The decrease in pH was followed using a pH meter and the reaction medium was stirred continuously. The initial pH of the hydrogen carbonate solution was adjusted to 8.7 + 0.1, giving precipitation with 0.3 ml of water added at 20°C in less than 6 minutes (precipitation is observed between pH 8.0 - 7.4).

3.3.2 RESULTS

The soluble matrix extracted from otoliths reacted positively to PAS and Ninhydrin staining, indicating the presence of carbohydrate and protein.

Recordings of a series of precipitation experiments are shown in Figure 3.27 and 3.28. As reported by Wheeler et al. (1981), the precipitation was marked by an almost instantaneous downward shift in pH when CaCl2 was added to the hydrogen carbonate solution. After this sudden shift, the pH was relatively stable until nucleation, and consequent precipitation, began (at a pH of 8.1), following

Figure 3.27

Decrease in pH with time associated with calcium carbonate precipitation, when a) 0.3ml soluble matrix containing 36 µg of protein, or b) 0.3 ml H_2O [control], is added to the reaction medium at time 0 (=3.0 ml of 3.0 mM $CaCl_2$ and 3.0ml 3.0 mM $NaHCO_3$).

Data for a) = mean and range for three experiments, data for b) = mean and standard error for five control experiments.

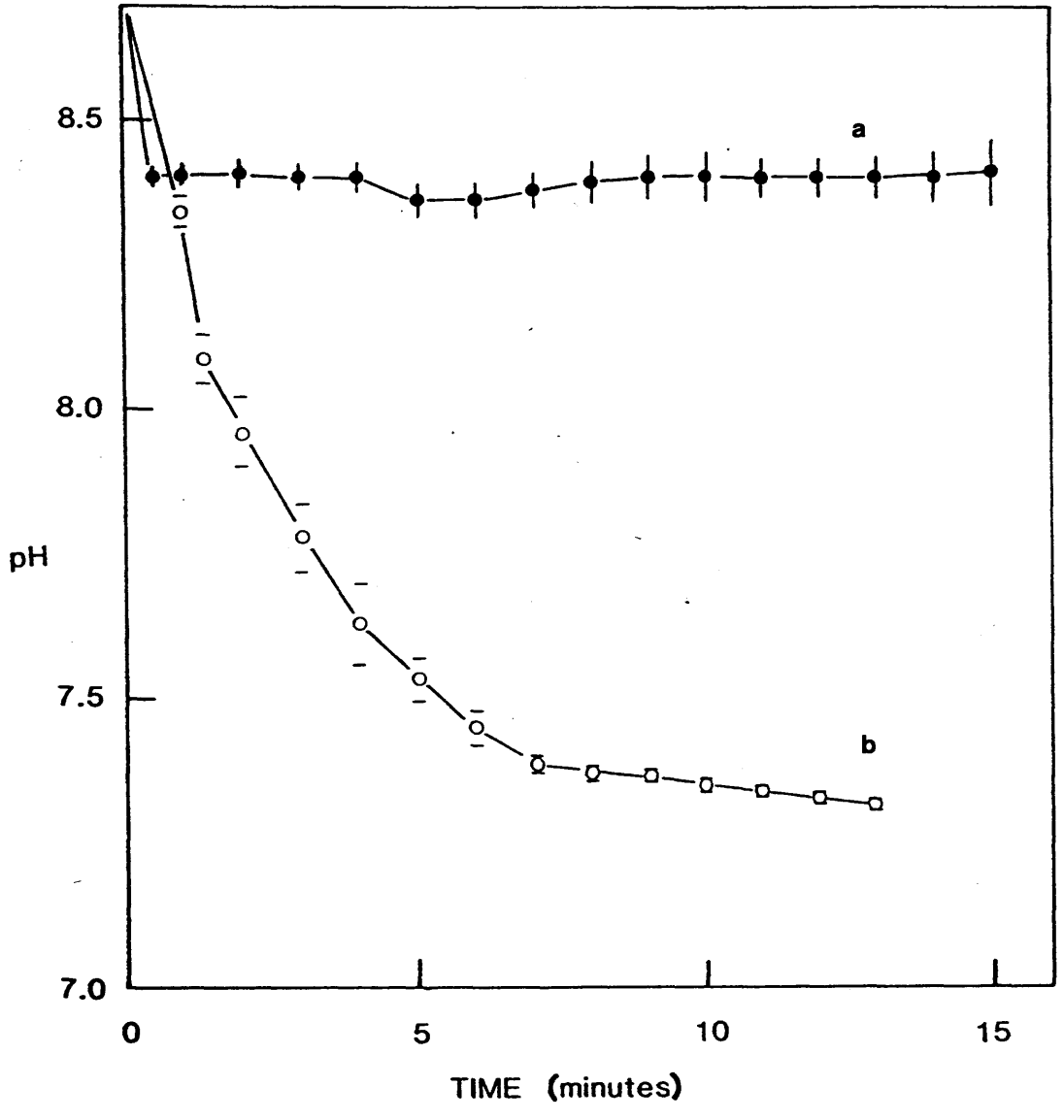
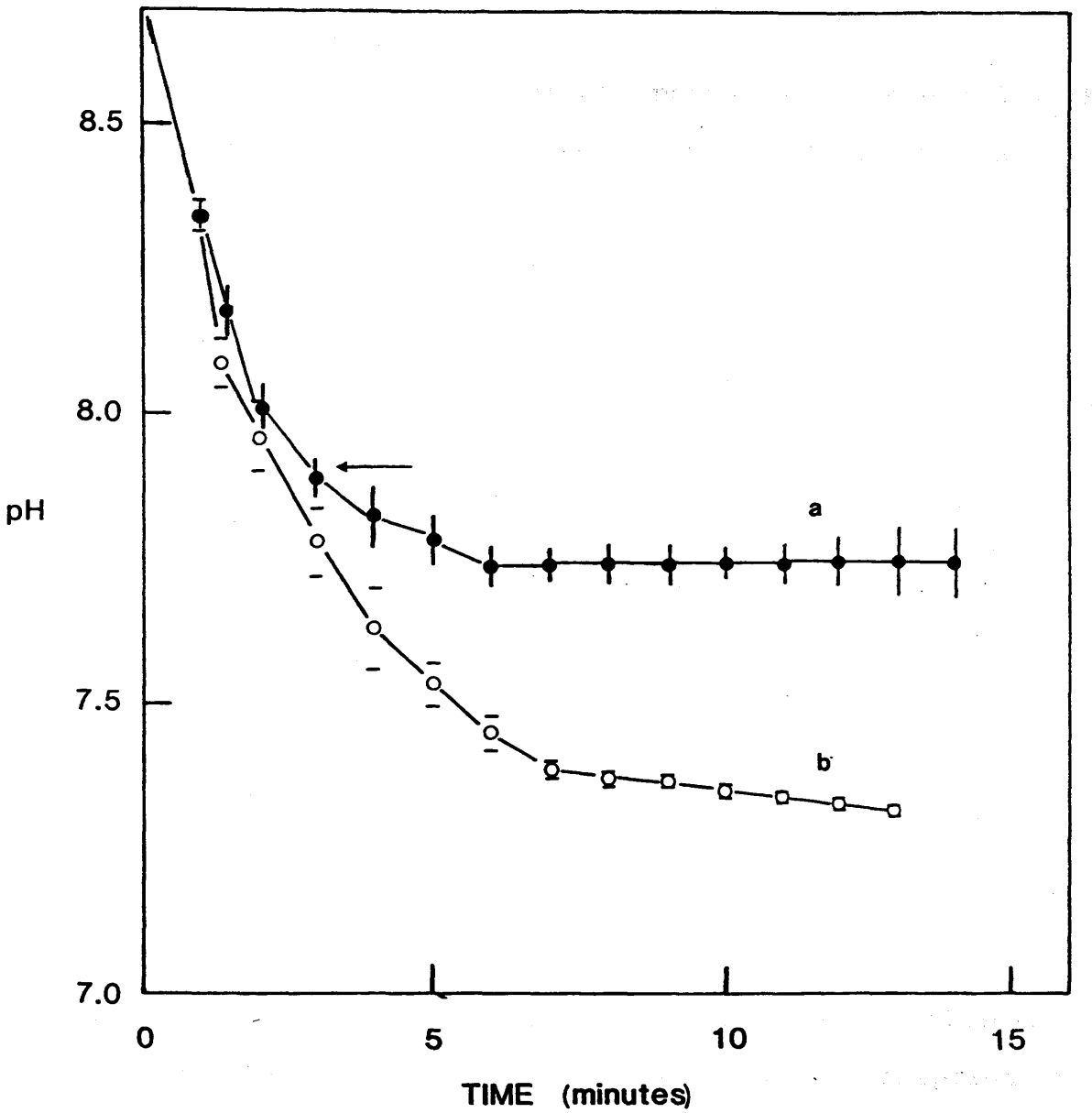

8.5
8.0
7.5
7.0
pH
a
b
0
5
10
15
TIME (minutes)

Figure 3.28

Decrease in pH with time associated with calcium carbonate precipitation, when a) 0.3 ml soluble matrix containing 36 µg of protein, or b) 0.3 ml H_2O [control], is added to the reaction medium after precipitation has begun at pH 7.9 (=3.0 ml of 3.0 mM $CaCl_2$ and 3.0 ml 3.0 mM $NaHCO_3$). Arrow denotes pH at which matrix or water was added.

Data for a) = mean and range for three experiments, data for b) = mean and standard error for five control experiments.


8.5
8.0
7.5
7.0
pH
a
b
0
5
10
15
TIME (minutes)

which it again declined in the controls and a precipitate was observed.

The duration of the stable period was increased markedly over the control by the addition of soluble matrix at > 6.8 $\mu g\ ml^{-1}$ (a total of 2.2 μg). In addition once nucleation had occurred, the rate of net precipitation or crystal growth, as shown by the decrease in pH ($\Delta pH/\Delta t$), was less for reactions with soluble matrix than for controls. At 12 $\mu g\ ml^{-1}$ (a total of 3.6 μg of protein; Figure 3.27), the decrease in pH was delayed for hours, suggesting inhibition of nucleation. Precipitation, following nucleation, could also be delayed by the addition of a similar concentration of soluble matrix (at pH 7.9; Figure 3.28).

3.4 DISCUSSION

3.4.1 STRUCTURE OF THE SACCULUS

On the basis of observations made in this chapter it is possible to provide a more detailed description of the teleost sacculus than has previously been reported (Dale, 1976; Dunkelberger, 1980). Most significantly, the present study has indicated that there are differences in non-sensory epithelia cell types, one of which (type II cells; Figures 3.3, 3.11) may be involved in the formation of the sub-cupular meshwork. Scanning electron microscope observations also indicates the presence of a dense fibrous layer, not previously described, covering both a spherule layer and the otolith. A schematic diagram of part of the

sacculus, illustrating the arrangement of cells and other structures observed, is given in Figure 3.29.

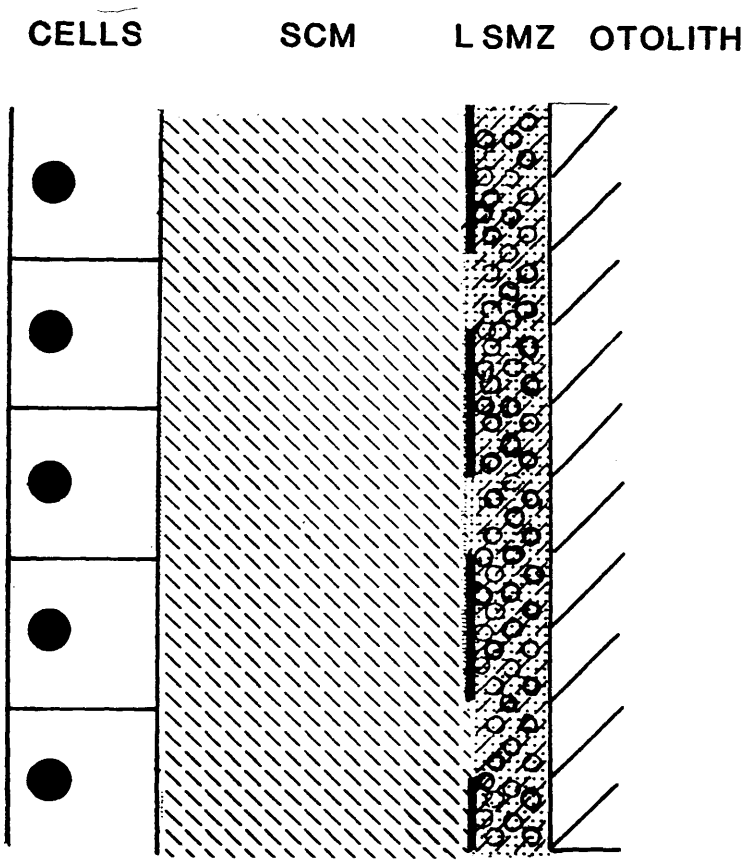
3.4.2 THE ROLE OF CALCOSPHERULES IN ION TRANSPORT

The presence of spherules in the subcupular zone and on the otolith surface agrees with Dale's (1976) observations on cod, *Gadus morhua*, otoliths. These spherules are similar to the spherules reported in the otolith primordium of *Tilapia nitoloca* otoliths (Tanaka *et al.*, 1981) and the core matrix of larval *Fundulus heteroclitus* and in many salmonid larvae (Radtke & Dean, 1982). The uptake of oxytetracycline by these spherules, demonstrated in the current study, confirms Dale's suggestion that the spherules contain calcium. However the spherules reported here differ from those observed by Dale in that they varied in size and were generally much smaller (2 μ cf. 10 μ seen in cod). In addition to the sites in which Dale examined, these calcospherules were also found on the apical surface of type IV cells, the region adjacent to the most active calcifying site on the otolith (Mugiya, 1977; Dunkelberger *et al.*, 1980).

It is evident from the arrangement of aragonite crystals seen in sections of the otolith that calcium carbonate is not deposited in the otoliths in the form of spherules. Indeed observations from the present study confirm that these calcospherules form a layer in close contact with the otolith, and are not actually incorporated on to the surface. This finding is contrary to that suggested by Dale (1976) who believed that the different stages of embedding

Figure 3.29

Schematic representation of section through sacculus, showing possible arrangement of epithelial cells, sub-cupular meshwork (SCM), dense matrix layer (L), spherule-matrix zone (SMZ) and otolith.


CELLS
SCM
L SMZ
OTOLITH

of the spherules in the surface of the otoliths provided evidence to suggest that the spherules were the building units of the otoliths.

The presence of large numbers of spherules on both the otolith surface and the apical surface of cells, suggests that these structures may be a means of storing and transporting calcium carbonate to the otolith. Further, the low solubility product of the endolymph (Mugiya & Takahashi, 1985; Appendix 1) would suggest that ion concentration at the otolith surface is in some way elevated. Consequently it would seem reasonable to infer that spherules may have a role in maintaining the high ion concentration at the otolith surface.

The presence of calcified bodies close to a mineralising surface, such as the spherules found in this study, is unusual (cf. Wilbur, 1980) but has been observed in other mineralising systems. For example calcified spherulites have been reported on the regenerating shell of many molluscs (Saleuddin, 1983). An ionic maintenance role has been demonstrated for intracellular calcospherules found in the mantle epithelium of Lamellibranch molluscs (Istin & Mansoni, 1973). In this case ion concentration in the extrapallial fluid was maintained by the dissolution of calcospherules in the region close to the calcifying shell. The dissolution of these calcospherules was catalysed by carbonic anhydrase (Istin & Girard, 1970) located on the their surface (Roinel *et al.*, 1973). The localisation of carbonic anhydrase on the surface of large calcospherules,

in the teleost sacculus suggests that this enzyme association may have a similar role.

3.4.3 THE ROLE OF ORGANIC MATRIX IN OTOLITH MINERALISATION

Composition of extracellular matrix: The present study has demonstrated that, as in most mineralisation systems, the extracellular matrix of the subcupular meshwork is 'collagenous' and contains sulphated acid glycoproteins and glycosaminoglycans (possibly in the form of proteoglycans). Further, fibronectin was found to be involved in the adhesion of the extracellular protein fibres, comprising the subcupular meshwork, to both the epithelium and the otolith.

Production of extracellular matrix: The fibrous or amorphous proteinaceous material secreted by the type II cells was similar both structurally and histochemically to the organic matrix of the sub-cupular meshwork, suggesting that these cells are involved in the production of matrix precursors. Further the apical region between type II cells and their secretion exhibited a marked reactivity to fibronectin, which provides binding sites for extracellular proteins. The functional significance of differences in the staining reactivity of this secretion deserves further investigation.

Nature of vesicular structures: Gauldie & Nelson (1988) reported basophilic vesicles in and on the apical surface of the macula of orange roughy, *Hoplostethus atlanticus*.

The present study has identified such structures on the supporting (type I) cells and in other non-sensory epithelia. All these cells may secrete products that have some role in matrix production as their histochemical reactivity corresponds to substances found in the endolymph (Mugiya, 1966) and organic matrix.

Matrix role in nucleation: The organic matrix found in the sacculus may have a role in nucleation, as has been indicated in most other mineralising systems (Wilbur & Saleuddin, 1983) and from the initial otolith calcification found in a core matrix of *Fundulus heteroclitus* (Radtke & Dean, 1982). In the present study, matrix, containing glycosaminoglycans and sulphated glycoprotein, was found in association with calcospherules. The formation of calcospherules also appeared to involve these substances, as the organic material at their centre, had a similar staining reactivity. Interestingly the staining reactivity of the organic material found in these calcospherules is also similar to that of components of the molluscan extrapallial fluid in which crystallisation is known to occur (Wada, 1980; Crenshaw, 1982).

The role of matrix in incremental growth: Observations on the otolith ultrastructure using scanning electron microscopy agreed with reports for other teleosts including *Fundulus heteroclitus* (Dunkelberger *et al.*, 1980; Watabe *et al.*, 1982), *Tilapia mossambica* (Watabe *et al.*, 1982), *Dicentrarchus labrax* (Morales-Nin, 1987), and *Gadus morhua*

(Radtke, 1989). The arrangement of increment layers in the otolith suggests that accretion occurs predominantly around the medial-lateral margin of the otolith, as has also been indicated from autoradiography (Mugiya, 1974). Increments were composed of a multiple crystalline layer (= incremental zone), and an organic matrix layer (= discontinuous zone or interlamellar layer).

A similar pattern is also seen in molluscan nacre, which results from the alternate accretion of organic matrix and calcium carbonate crystals. The matrix overlying the crystals may limit crystal development (Wilbur & Saleuddin, 1983). Support for this limitation concept has come from evidence of sclerotization of the interlamellar layer by phenoloxidase in mollusc nacre (Gordon & Carriker, 1980). Another type of matrix material, condensing from the extrapallial fluid on to the interlamellar layer, is believed to provide nucleation sites for the subsequent crystalline layer (Beverlander & Nakahara, 1969). Such a regulation of crystal growth is consistent with the variations in crystal and matrix density seen in the present study and in previous studies (Dunkelberger *et al.*, 1980; Watabe *et al.*, 1982; Morales-Nin, 1987; Radtke, 1989). However it remains possible that the matrix layer may be deposited after the crystal layer has virtually ceased growing due to low ion concentration or some other form of inhibition.

3.4.4 THE ROLE OF SOLUBLE MATRIX

The present study has demonstrated that there is a glycoprotein component of the soluble matrix that can inhibit crystal nucleation *in vitro*, even after crystallisation has begun. These observations indicate that this glycoprotein may regulate mineralisation. Calcium binding proteins have been demonstrated in other calcium carbonate mineralised tissues and in molluscan extrapallial fluid (Wilbur & Saleuddin, 1983). In oysters the effects of soluble matrix on nucleation and inhibition are not simply due to removal of free calcium (Sikes & Wheeler, 1982), so the effect of these proteins is not limited by their calcium binding capacity. Inhibition by soluble matrix may be brought about by negatively charged groups of the calcium binding protein reacting with calcium ions of the lattice of the upper crystal surface, so preventing further addition to the lattice (Crenshaw & Ristedt, 1976; Weiner & Traub, 1981).

Iwata (1975) found that the typical pore pattern of mollusc nacre was only seen when demineralisation was carried out with agents that fixed the soluble matrix, suggesting that soluble matrix was deposited in the pores of the interlamellar matrix. Interestingly SEM investigations of the otolith, in which cetylpyridinium chloride, was added to the fixative (which fixes glycoproteins), have found small nodules in the interlamellar matrix that are not found when cetylpyridinium chloride is not used (Watabe *et al.*, 1982;

Morales-Nin, 1987). Clearly the role of soluble matrix in otolith calcification deserves further attention.

3.4.5 PROCESS OF OTOLITH FORMATION

On the basis of observations made in this chapter and evidence from previous studies of otolith formation and shell formation in molluscs it is possible to postulate a series of mechanisms to explain the formation of incremental and discontinuous zones in teleost otoliths. These mechanisms are summarised in Figure 3.30.

The present study has shown that a calcospherule-matrix layer is continuous with the otolith and possibly with the subcupular meshwork. These observations agree with Dunkelberger *et al.* (1980) TEM observations from *Fundulus* sacculi. Consequently it is likely that the insoluble matrix of the sub-cupular meshwork is incorporated into the otolith and that crystals grow out from the otolith surface. As crystallisation does not occur throughout the subcupular meshwork, an elevated ion concentration at the otolith surface and a modification of, or addition to, the matrix at the otolith would be expected.

On the otolith surface insoluble matrix would provide nucleation sites for calcification and a template for crystal growth. Soluble matrix may also be secreted into the surrounding endolymph and become incorporated within the intercrystalline matrix to control the rate of crystal growth.

The formation of the interlamellar matrix may involve a modification of the insoluble matrix, i.e. sclerotization,

FIGURE 3.30

Diagram illustrating the possible mechanism of increment formation in four stages.

1. Glycoprotein-glycoaminosglycan material (n) is deposited or is condensed out of the endolymph on to the otolith and provides sites for crystal growth.
2. Aragonite crystals (c) grow out from the perimeter of the otolith surface. During this stage soluble matrix is secreted by epithelial cells into the endolymph to regulate the rate of crystal growth.
3. Crystal growth is periodically inhibited by an increased production of soluble matrix that is incorporated into matrix and may condense on the growing crystal surface and is further limited by an increased deposition of matrix (di).
4. Glycoprotein material condenses out on to the interlamellar layer, providing new sites for crystal nucleation.

OTOLITH
1
2
3
4
c
dp
n

providing a physical interruption to crystal growth, and the production of relatively high concentrations of soluble matrix, that would inhibit crystallisation. A nucleating matrix component may later condense out of the endolymph, as in the interlamellar matrix of the nacreous layer of molluscs (Beverlander & Nakahara, 1969), providing a new nucleation front for the subsequent crystal layer.

In this way a cyclical peak in the secretion of both soluble matrix and insoluble matrix could account for the observed incremental pattern. The observation that calcospherules, enmeshed in matrix, occur on both the apical surface of epithelial cells and in close proximity to the otolith, may be of significance to ion transport, and deserves further investigation.

3.5 SUMMARY

The present study has found:

1. Four histologically distinct types of non-sensory epithelia are present in the sacculus.
2. Calcospherules are present on the surface of the otolith and apical surface of certain epithelial cells.
3. Calcified matrix has a sulphated glycoprotein, glycoaminosglycans component.
4. A calcium-binding protein is present in the soluble matrix.

Chapter 4

THE CYCLICITY OF OTOLITH FORMATION AND ITS CONTROL

4.1 INTRODUCTION

Clearly for otolith increments to be of use in ageing, the processes involved in their regulation either must be synchronised to cyclical environmental events, or must possess an endogenous circadian rhythm (Geffen, 1987). In addition, increment formation must be independent of somatic growth. Synchronisation of increment formation with environmental factors has been reported for a number of species, but the mechanisms involved differ between species and life-history stages. For example light-dark cycles appear to be necessary for daily increment formation in larval *Lepomis macrochir* (Taubert & Coble, 1977) and *Fundulus heteroclitus* (Radtke & Dean, 1982), whilst temperature fluctuations have been reported to be the major factor influencing increment formation in temperate stream fishes (Brothers, 1978; 1981). Feeding frequency has also been reported to influence increment formation in some species; *Oncorhynchus tshawytscha* (Neilson & Geen, 1982), *Pleuronectes platessa* (Alhossaini & Pitcher, 1988), but not in others; *Lepomis macrochir* (Taubert & Coble, 1977), *Oncorhynchus nerka* (Marshall & Parker, 1982), *Platichthys stellatus* (Campana, 1983a). In juveniles of some species, for example *Platichthys stellatus* (Campana & Neilson, 1982), increment deposition has been shown to continue at a daily rate under constant light, suggesting an endogenous

regulation of increment formation. Campana & Neilson (1985) proposed that the endogenous circadian rhythm was entrained to photoperiod, but could be masked by sub-daily temperature cycles or feeding patterns. However few studies have attempted to control for the three main environmental factors whose cyclic variation may influence increment formation. Consequently there is little unambiguous experimental evidence of an endogenously regulated cycle of increment formation. One aim of the present study is therefore to investigate increment deposition in the absence of cyclic variations in light, temperature and food availability.

In addition, although a variety of exogenous influences on increment periodicity have been identified (see Campana & Neilson, 1985), little is know about exactly how these affect the deposition of the two layers comprising the increment. In goldfish, *Carassius auratus,* the order of formation of the two increment zones is dependent on photoperiod, as a reversal of the light-dark cycle induced a reversal in the order of the two zones (Tanaka *et al.*, 1981). Support for this finding also comes from a study of otolith calcification of goldfish, using ^{45}Ca (Mugiya *et al.*, 1981). In rainbow trout diurnal rhythmicity of otolith formation, associated with photoperiod, has been demonstrated for both calcification (Mugiya, 1984; 1987) and organic matrix formation, the two cycles being in antiphase (Mugiya, 1987). Since the decline in otolith calcification coincided with a diurnal decline in plasma calcium concentration, Mugiya (1984) suggested that the

cycle of otolith calcification was related to the supply of calcium ions to the endolymph. Further he found a seasonal reversal in the rhythm of otolith calcification associated with a reversal in the plasma calcium cycle. Thus in rainbow trout, although there is a cycle of otolith calcification associated with photoperiod, the rhythm is apparently controlled by the factors that regulate the calcium concentration of the plasma.

The aims of the work described in this chapter are therefore to investigate the regulation of increment formation in Atlantic salmon parr by examining the following:

1. Does periodic deposition continue in the absence of cyclic changes in light levels, temperature and food availability?
2. How extrinsic rhythms affect:
   a) the time of formation of the discontinuous zone;
   b) the rhythm of otolith calcification; and
   c) the secretion of organic matrix precursors?
3. How is increment formation related to plasma calcium concentration?

4.2 ROLE OF ENVIRONMENTAL CYCLES IN INCREMENT FORMATION

4.2.1 MATERIALS AND METHODS

The importance of diurnal light-dark transitions, temperature variations and feeding frequency in the regulation of increment formation were investigated in a

series of experiments in which these parameters were varied systematically.

0-age S1 (destined to smolt at 1 year old) parr Atlantic salmon parr, reared at the DAFS Smolt Rearing Station, Almondbank, were moved to an experimental tank with recirculating water and allowed to acclimatise for at least four days prior to experimentation.

Subjects were maintained in circular 60L tanks with a recirculating power filtration giving a tangential flow of 6.5 L/minute. They were provided with shelter and fed with chironomid larvae from an automatic feeder.

A mark was induced on the otoliths of parr using a 3 day 6L:6D photoperiod. Duplicate groups of 15 parr were maintained under the following experimental treatments before sampling:

1. The natural control group was held under a 12L:12D photoperiod (daylight light intensity = 0.22 μmol $m^{-2}s^{-1}$), varying temperature (ranging between 8 and 11^{o}C) and were fed approximately 2% of wet body weight once per day at about 1300h. Note all food was consumed within an hour.

2. The constant conditions group was held under constant light (0.22 μmol $m^{-2}s^{-1}$) and constant temperature (10 $\pm$ 0.5^{o}C) and were fed automatically at intervals throughout the day, receiving approximately 2% of wet body weight per day.

3. The cyclic temperature group was held under cyclically varying temperature, (ranging between 8 and 11^{o}C in the first run and 13-16^{o}C in the second run) while in constant light (0.22 μmol $m^{-2}s^{-1}$), and were fed automatically at

intervals throughout the day, receiving approximately 2% of wet body weight per day.

4. The cyclic feed group was fed approximately 2% of wet body weight once around 1300 h each day and held under constant light (0.22 µmol $m^{-2}s^{-1}$) and constant temperature (10 ± 0.5°C). Note all food was consumed within an hour.

5. The photoperiod group was held on a 12L:12D photoperiod (daylight light intensity = 0.22 µmol $m^{-2}s^{-1}$), while at constant temperature (10 ± 0.5°C) and were fed automatically at intervals throughout the day, receiving approximately 2% of wet body weight per day.

6. The short photoperiod group was held under a 6L:6D photoperiod (daylight light intensity = 0.22 µmol $m^{-2}s^{-1}$), constant temperature (10 ± 0.5°C) and were fed automatically at intervals throughout the day, receiving approximately 2% of wet body weight per day.

7. The sub-daily temperature cycle group was subject to a 2°C (10-8-10°C) temperature variation for 4 hours in the daylight hours, a 12L:12D photoperiod (daylight light intensity = 0.22 µmol $m^{-2}s^{-1}$) and were fed automatically at intervals throughout the day, receiving approximately 2% of wet body weight per day.

After 28 days the fished were killed and their sagittae were removed, prepared and increments counted as described in Chapter 2. Data for replicates were pooled for analysis as there were no significant differences within treatments (ANOVA P>0.95).

4.2.2 RESULTS

Analysis of variance showed that constant light, constant temperature and single or multiple feeding regimes had no

significant effect on the periodicity of increment deposition, increments continuing to be produced daily in parr maintained under experimental conditions 1-5 and 7 (Table 4.1). Increment width did not vary significantly between these treatments (ANOVA, $F_{6,165} = 0.16$; $P>0.90$), although increments of fish held under constant temperature were less distinct than those subject to a diurnally varying temperature. Fish subjected to a 6L:6D photoperiod regime, however, produced on average two indistinct increments per day, as in the case of otolith marks. Each of these sub-daily increments was approximately half the thickness of increments produced under a 12L:12D photoperiod for a given temperature (mean increment thickness in natural control group = 2.3 μm; mean increment thickness in 6L:6D group = 1.05 μm).

4.3 INFLUENCE OF PHOTOPERIOD ON ZONE FORMATION

4.3.1 METHODS

Changes in completion of the outermost increment: The state of completion to the nearest quartile of the outermost incremental zone was estimated by comparing the width of the outermost incremental zone with that of the adjacent increment at intervals over a 27 hour period (12L:12D; 0700-1900h). Groups of 3 fish were sampled every three hours, and their otoliths were removed and carefully ground down to the primordium (see Chapter 2). The sagittae were photographed at x1000 magnification and the negatives were

Table 4.1

Mean number of sagitta increments produced by Atlantic salmon parr held under experimental conditions, during 28 day experiments.

Data presented as means ($\bar{x}$), standard errors (SE) and deviation from expected time interval as determined from D - test (values for t statistic and P are given.

NUMBER OF INCREMENTS DEPOSITED WITHIN A 28 DAY TREATMENT

TREATMENT	NUMBER OF INCREMENTS					INCREMENT WIDTH	
	$\bar{x}$	SE	N	t	P ($\simeq$)	$\bar{x}$	SE
1. constant conditions	27.4	0.26	27	2.3	0.90	2.18	0.21
2. varying conditions	27.1	0.36	30	2.5	0.90	2.33	0.32
3. varying temperature	27.9	0.47	28	0.2	0.99	2.40	0.36
4. single feed	27.0	0.34	30	2.9	0.90	2.22	0.39
5. 12L:12D photoperiod	27.6	0.54	28	0.6	0.99	2.29	0.23
6. 6L:6D photoperiod	52.6	1.56	15	2.2	0.90	1.05	0.41
7. sub-daily temperature	26.6	0.77	28	1.82	0.95	2.31	0.30

projected on to a screen from which measurements were taken.

Calcium uptake into sagitta increments: Groups of between 30 and 50 Atlantic salmon parr were moved to an experimental tank (Figure 4.1) with recirculating water and fed to excess on chironomid larvae every 4 to 6 hours prior to and throughout the experimental period. All feeding times during the experimental period were recorded. ^{45}Ca (Amersham), was introduced to the experimental tank, to give a final activity of approximately 37 $KBql^{-1}$.

In initial experiments, sampling was initiated two to three hours after the addition of ^{45}Ca, in order to determine whether calcium was deposited continuously on otoliths. This methodology, as used by Mugiya and coworkers (1981) to investigate *in vivo* otolith calcification in goldfish, does not allow for isotopic equilibration of labelled and non-labelled calcium between either the plasma and environmental water or the free calcium pool (Simkiss, 1974). A comparison of sagitta activity/unit area from fish maintained in labelled water for three days with that of sagitta activity from fish subsequently transferred to non-labelled water for nine and fifteen days demonstrated that calcium stores in the body can significantly contribute to otolith calcification (Figure 4.2). Mugiya's technique therefore ignores any contribution to otolith calcification from calcium reserves within the fish. Consequently in order to estimate calcium fluxes in otoliths, fish were pre-labelled for two days, by which time plasma calcium

Figure 4.1

Diagram of the experimental tank used for ^{45}Ca experiments.

Key:

P = power filtration pump

v = vibrator pump (for aeration)

s = shelter

t = trap; a net hidden under a shelter. The net is pulled up over the shelter, allowing for the removal of fish with minimal disturbance to other fish or stress to the trapped fish.

Scale bar = 20 cm.

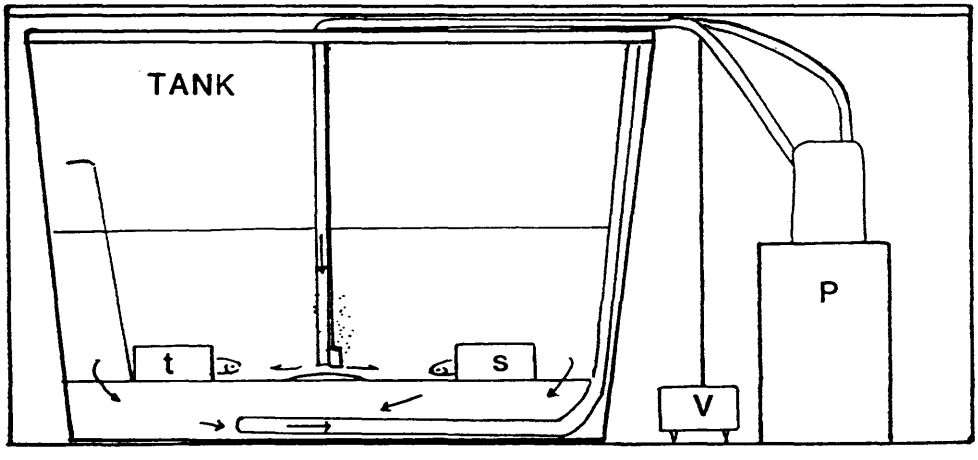
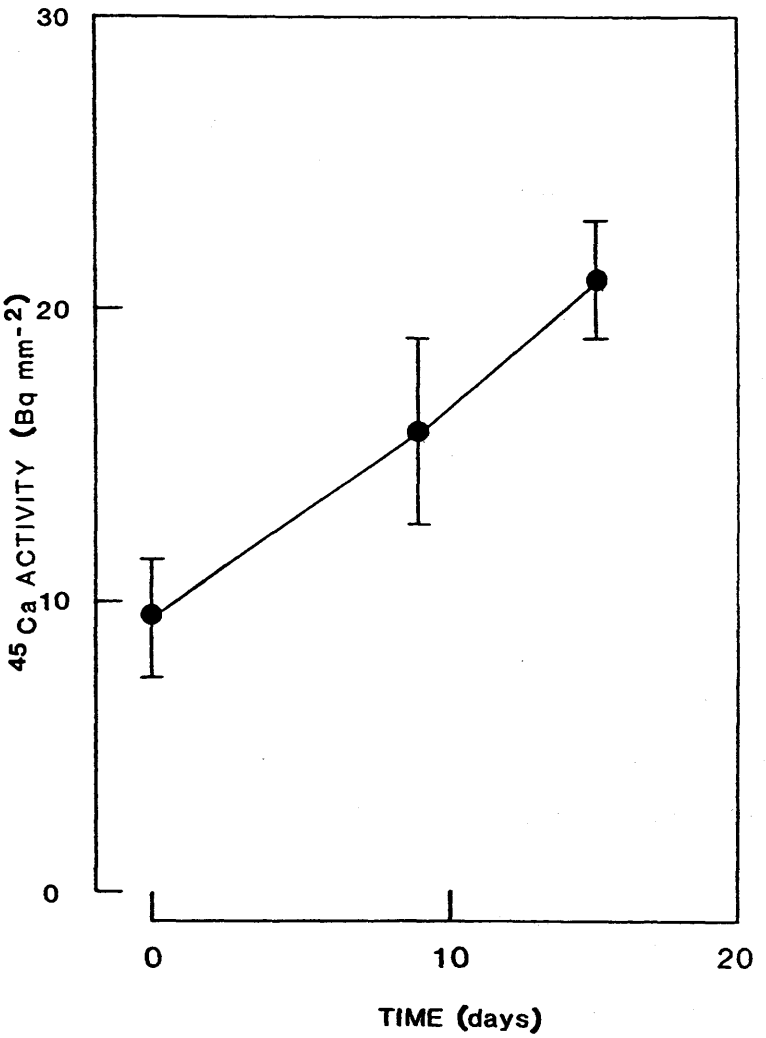

TANK
t
s
P
V

Figure 4.2

Changes in mean activity of calcium-45 deposited on to salmon parr (*Salmo salar*) sagittae through time, following transference to non-labelled water.

Data presented as mean ^{45}Ca activity per mm^{-2} of sagitta surface for 6 individuals (bars represent 95% confidence limits).


30
20
10
0
^{45}Ca ACTIVITY (Bq mm^{-2})
0
10
20
TIME (days)

activities had stabilised (Figure 4.3), indicating equilibration between the labile calcium stores and radioactive calcium.

Experiments were carried out at ambient photoperiod and constant temperature under the following regimes:

1. No equilibration. Day-time introduction, 16L:8D (L = 0600 - 2200h; August/September), 15°C +0.5°C. Isotope was introduced at 1000h and groups of five fish were taken at 1200h and every three hours for 27 hours thereafter.

2. No equilibration. Night-time introduction, 13L:11D (L = 0800 - 2100h; March), 15°C +0.5°C. Isotope was introduced at 2400h, and groups of 10 fish were sacrificed at every 2 hours following the introduction of the radioisotope until four hours after dawn.

3. Equilibrated. 12L:12D (L = 0700 - 1900h; March), 6°C. Isotope was introduced at 1000h two days prior to sampling. Groups of five fish were killed at intervals of between 2 and 4 hours (sampling being more intensive during the period of apparent cessation of calcium apposition) over a 48 hour period.

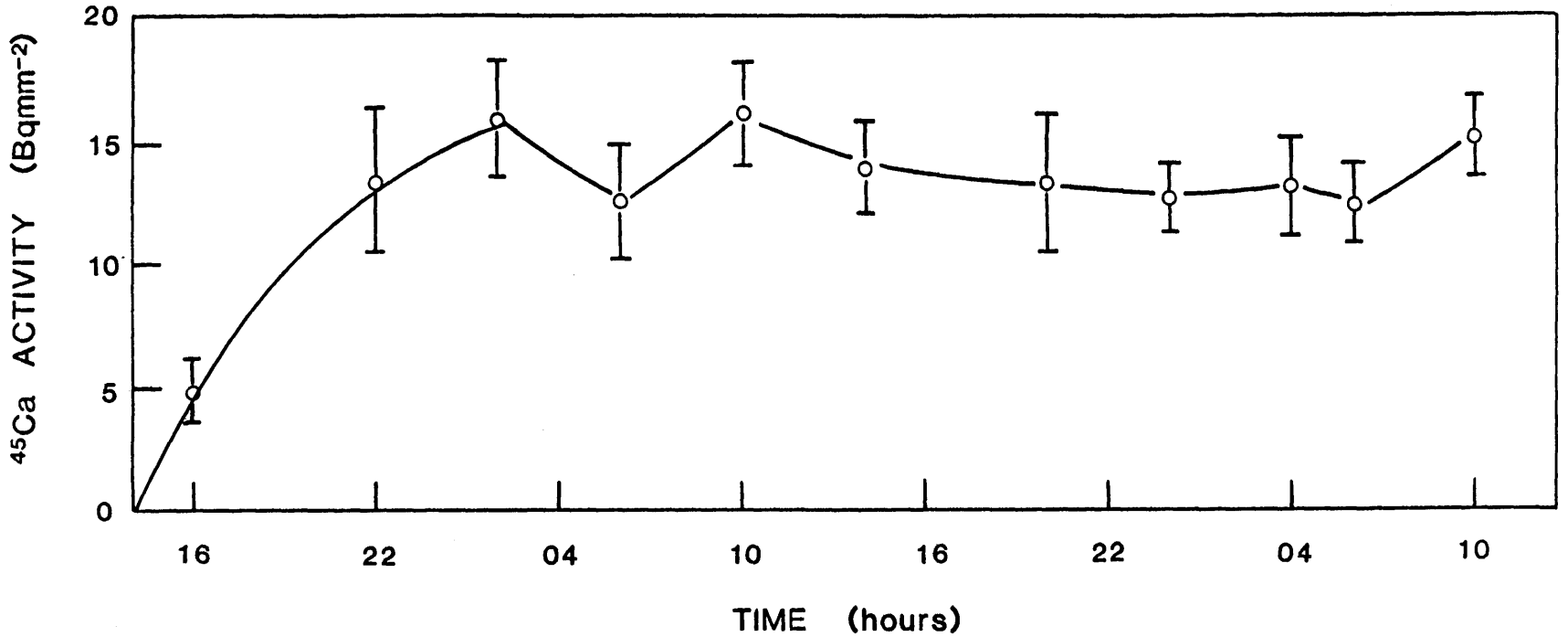
4. Equilibrated. 8L:16D (L = 0900 - 1700h; January/February), 4°C. Isotope was introduced at 1000h two days prior to sampling. Groups of five fish were killed at intervals of between 2 and 4 hours (sampling being more intensive during the period of apparent cessation of calcium apposition) over a 48 hour period.

All treatments were replicated. Treatments 1, 2 and 3 were carried out on immature 1+ parr (range in mean wet weight < 3 g) and treatment 4 was carried out on O+ parr. However

Figure 4.3

Mean activity of calcium-45 within the blood of salmon parr (*Salmo salar*) through time, in the 15 day period following introduction of the isotope to the environmnetal water.

Data presented as mean ^{45}Ca activity per µl for 6 individuals (bars represent 95% confidence limits).


20
15
10
5
0
^{45}Ca ACTIVITY (Bqmm^{-2})
16
22
04
10
16
22
04
10
TIME (hours)

only the results for fast growing S1 parr, (destined to smolt in their first year; see Thorpe, 1987) (mean wet weight = 6.7 g) are presented as the otoliths of S2 parr did not incorporate sufficient isotope for reliable measurements of uptake. A narrow size range of fish was used in each experiment, although the mean size of fish differed between experiments.

At each sample time fish were gently removed, one at a time, in a trap and killed by a blow to the head. Blood was extracted from the caudal artery by heparinised capillary tubes. Duplicate water samples were taken in conjunction with fish sample times to determine the level of isotope remaining in the water. After blood collection the head was severed and the sacculi were removed and washed briefly (<2s) in 0.78% NaCl, and dried to a constant weight. In the first experiment the dried sagittae were weighed to within one mg and the activity expressed per total sagitta weight. However as total weight may not reflect otolith surface area, the surface area of saggitae was estimated, as suggested for scale uptake measurements (Smagula & Adelman, 1982), in subsequent experiments. Surface area was measured using a digitising table and computer software package (Cherry software program), from traces made using a camera lucida. Sagitta activity was expressed as $Bqmm^{-2}$. Sagittae were dissolved in 5 µl 60% perchloric acid and 10µl hydrogen peroxide at $70^{O}C$ for 2 hours. Blood samples were dissolved in an equal volume of 60% perchloric acid and twice the volume of hydrogen peroxide at $70^{O}C$ for 2 hours as in the method of Mahin & Lofberg (1966). Radioactivity

was determined using a Phillips P7300 scintillation counter with Ecoscint (National Diagnostics) scintillation fluid.

Significant differences in sagitta activity within experiments were determined using analysis of variance (ANOVA). Significant differences between sample means within experiments were determined using Duncan's multiple range test (Torries, 1960).

Matrix production: Seven groups of five fish were killed at intervals over a 27 hour period (12L:12D; 1900-0700 h) and their sacculi were removed whole and fixed in neutral buffered formal saline (pH 7.5). Histological sections were prepared using the methods given in Chapter 3. The activity of two cell types (I & II - see Chapter 3) found to have secretory products with a similar staining reactivity to organic matrix was investigated. Observations indicated that the secretory activity of type II cells could be classified into five states of activity (Figure 4.4). Similarly the activity of type I cells was classified into five states of activity (Table 4.2), based on the density of PAS staining vesicles in a fixed area of epithelium. The state of secretory activity for both cell types was determined for three sections from each sacculus and a median and range activity score determined for each sample time. A total median activity score was determined for all samples for a given cell type and significant deviations from the total median were determined using the Kruskal-Wallis test.

Figure 4.4

Diagrams of changes in appearance of type II saccula cells associated with secretory activity.

1. = Faint granulation - very weak reactivity to PAS.
2. = sparse granulation - PAS positive.
3. = PAS +ve granulation throughout cytoplasm, although no reactive cytoplasm ~ 50% of cell section.
4. = intense PAS +ve granulation, cytoplasm can hardly be seen, and some PAS +ve secretory material may be seen on surface.
5. = cell appears to be entirely PAS +ve, a thick layer of PAS +ve material covers the surface of the cell.

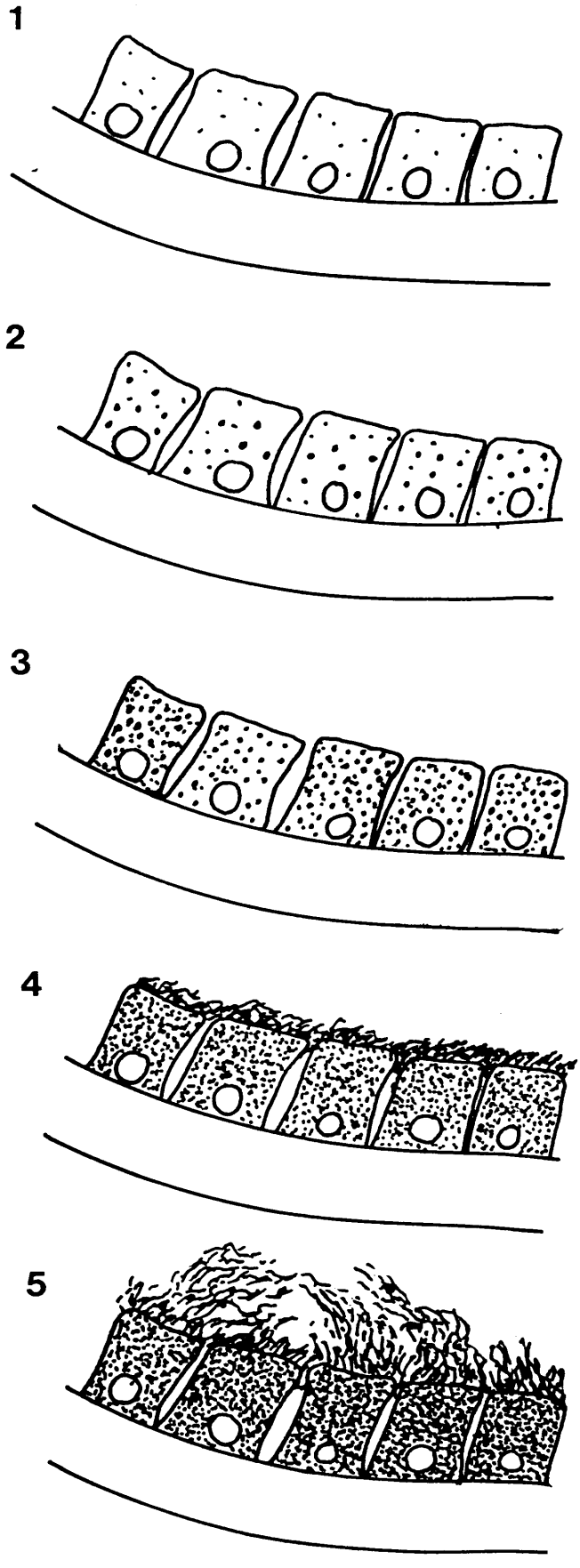

1
2
3
4
5

Table 4.2

Stages of secretory activity in type I cells of the sacculus, as indicated by the mean density of PAS positive vesicles at the apical region.

Stage	Number of PAS vesicles in 0.05 mm^2 apical region of cells
1	0 - 4
2	5 - 9
3	10 - 14
4	15 - 19
5	20 +

4.3.2 RESULTS

Pattern of increment completion: The pattern of increment completion is shown in Figure 4.5. Only the incremental zone was evident in fish sampled between 0700 (dawn) and 0200 h. The discontinuous zone was formed between these times and appeared to be completed by 0700.

Calcium uptake into otoliths: When ^{45}calcium was introduced at 1000h radioactivity was detected in the sagittae as early as three hours following the introduction of the isotope to the environmental water (Figure 4.6). However when the isotope was introduced at 2400 h (regime 2), after 3 hours of darkness, no significant activity was detected in sagittae collected during the 6 hours of darkness (ANOVA $F_{1,28} = 2.3$; $P>0.95$; Duncans multiple range test **N.S.**), even though ^{45}Ca was taken up from the water, as evident from the increase in activity of blood samples (Figure 4.7). Following the dark/light transition a significant increase in mean sagitta activity (Duncans multiple range test $P>0.05$) was observed. This difference between day-time and night-time incorporation would suggest that there is no net calcium accumulation in the later part of the night but that following dawn there is a net inward flux on to the otolith, which exceeds any outward flux, resulting in a detectable net accumulation of calcium.

Regimes 3 and 4 (equilibration)

In both 12L:12D (regime 3; Figure 4.8) and 8L:16D (regime 4; Figure 4.9) photoperiod regimes mean sagitta activity increased throughout the daylight hours and in the early hours of darkness (ANOVA $P<0.001$) but significantly

Figure 4.5

Relationship between state of completion of the outermost incremental zone and time in 1+ salmon parr (*Salmo salar*).

The state of completion was estimated by comparing the width of the outermost calcium zone with that of the previous increment's calcium zone. Owing to the problems of determining width in thin increments widths were estimated to the nearest quartile.
Data is based on observations from 4-6 individuals per sample time.

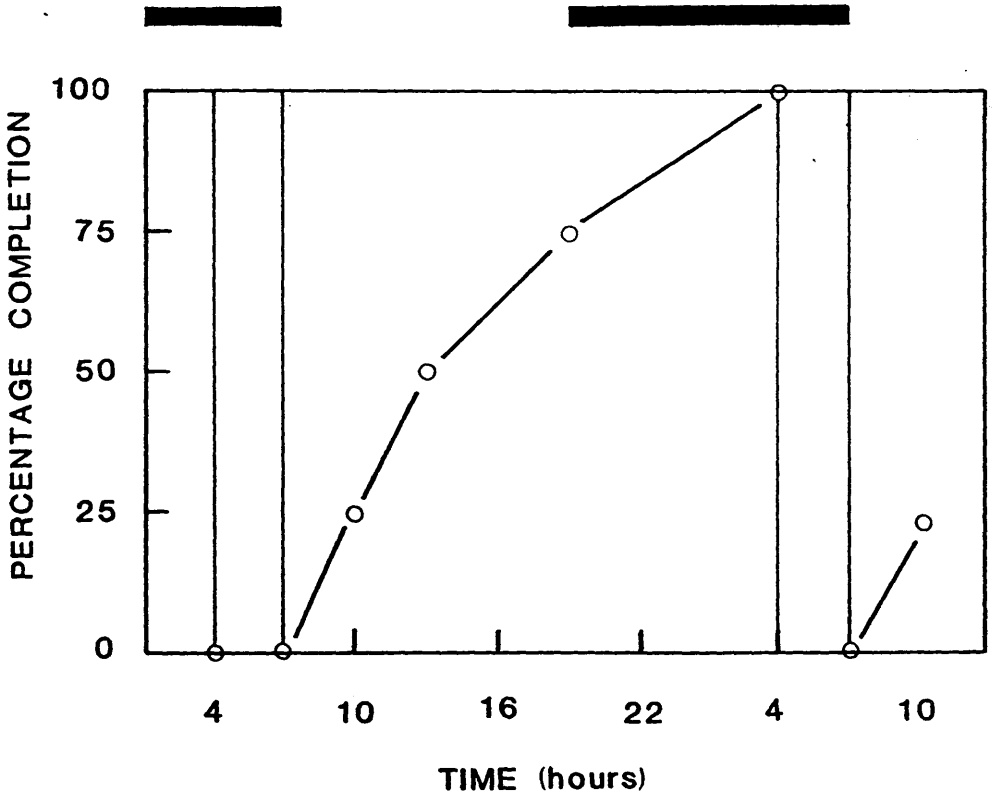

PERCENTAGE COMPLETION
100
75
50
25
0
4
10
16
22
4
10
TIME (hours)

Figure 4.6

Relation between mean activity of calcium-45 deposited on to salmon parr (*Salmo salar*) sagittae and time following introduction, in parr maintained at 16L:8D, 15^{O}C (isotope introduced at 1000h).

Data presented as mean ^{45}Ca activity per mg^{2} of total sagitta weight for 5 individuals (bars represent 95% confidence limits). Horizontal bars represent dark periods.

☆ denotes significant decline and increase in isotope activity between consecutive night-time samples (Duncan multiple range test P>0.05).

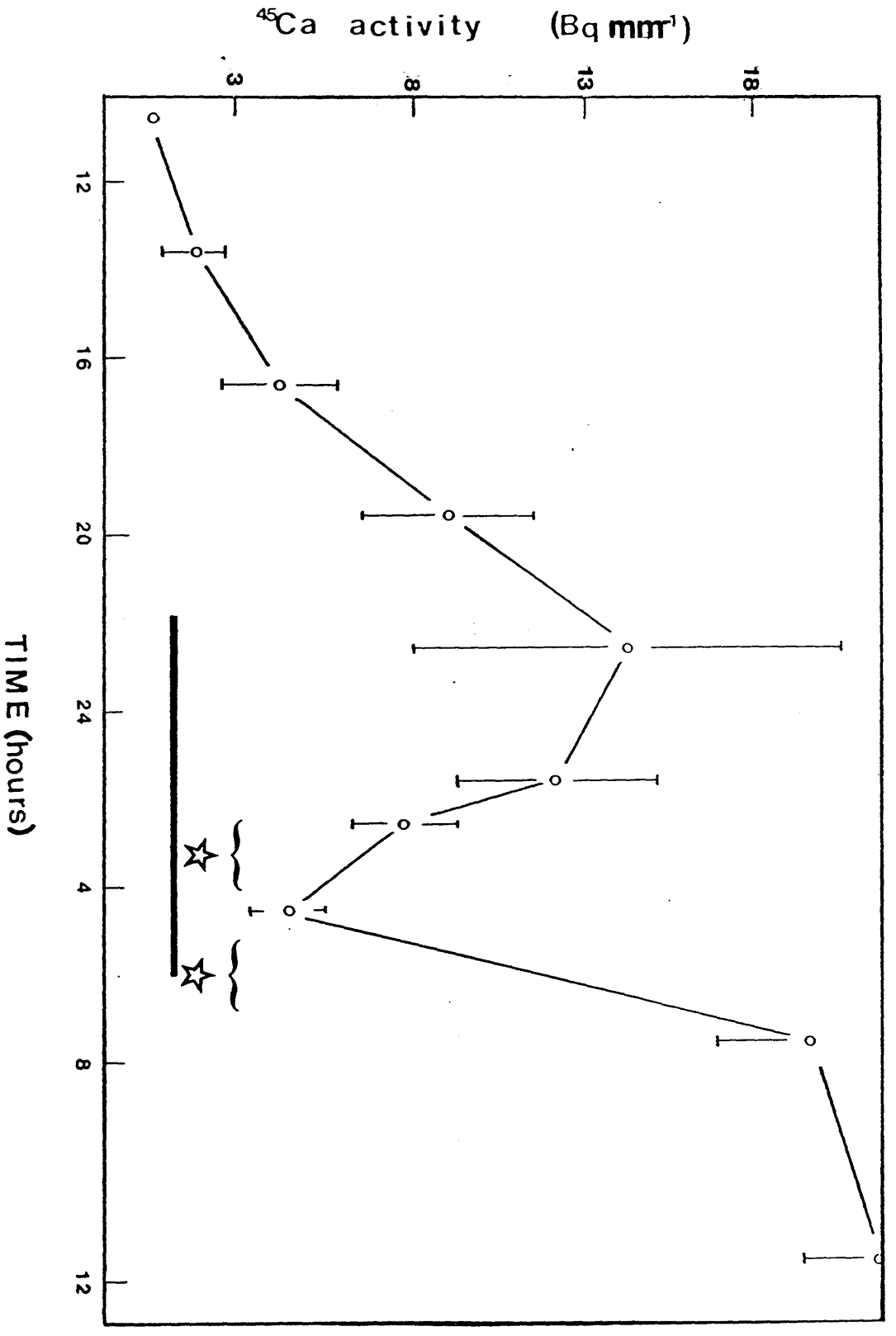

^{45}Ca activity (Bq mm^{-1})
3
8
13
18
TIME (hours)
12
16
20
24
4
8
12

Figure 4.7

The relationship between the incorporation of calcium-45 into the blood (●) and sagittae (o) of salmon parr (*Salmo salar*) and time following introduction at night.

Data presented as mean ^{45}Ca activity per mm^2 of total sagitta for 5-10 individuals and mean activity per µl of blood (bars represent 95% confidence limits). Horizontal bars represent dark periods.

☆represents significant change in sagitta activity between consecutive samples (Duncan multiple range test $P>0.05$).

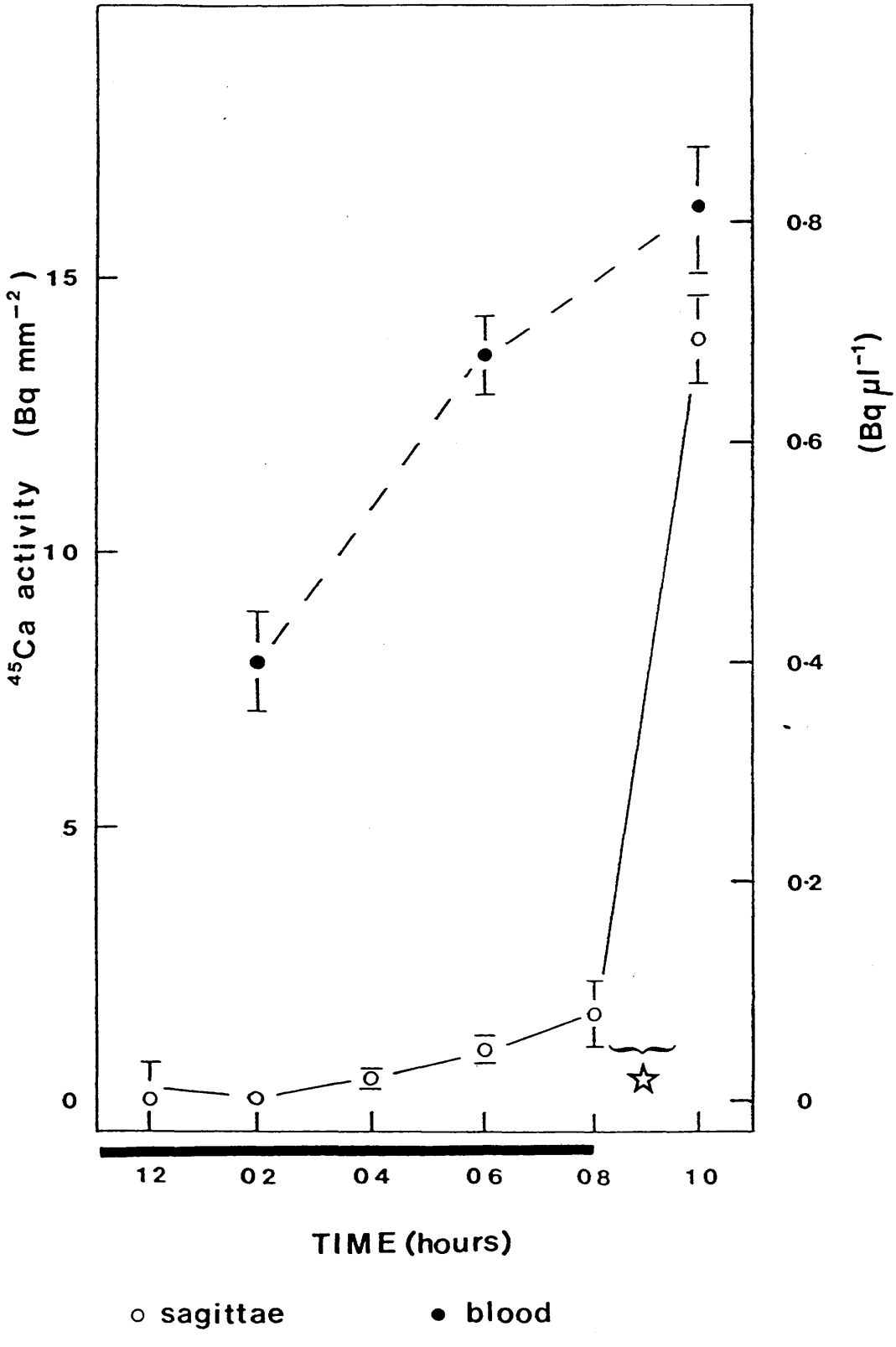

^{45}Ca activity (Bq mm^{-2})
15
10
5
0
0·8
0·6
0·4
0·2
0
(Bq µl^{-1})
12
02
04
06
08
10
TIME (hours)
○ sagittae
● blood

Figure 4.8

Relation between mean activity of calcium-45 deposited on to sagittae and time, in salmon parr (*Salmo salar*) maintained at 12L:12D, 6^{o}C (isotope introduced two days prior to sampling).

Data presented as mean ^{45}Ca activity per mm^2 of total sagitta for 5 individuals (bars represent 95% confidence limits).

Horizontal bars represent dark periods.

★denotes significant decrease and following increase in isotope activity between consecutive samples (Duncan multiple range test P>0.05).

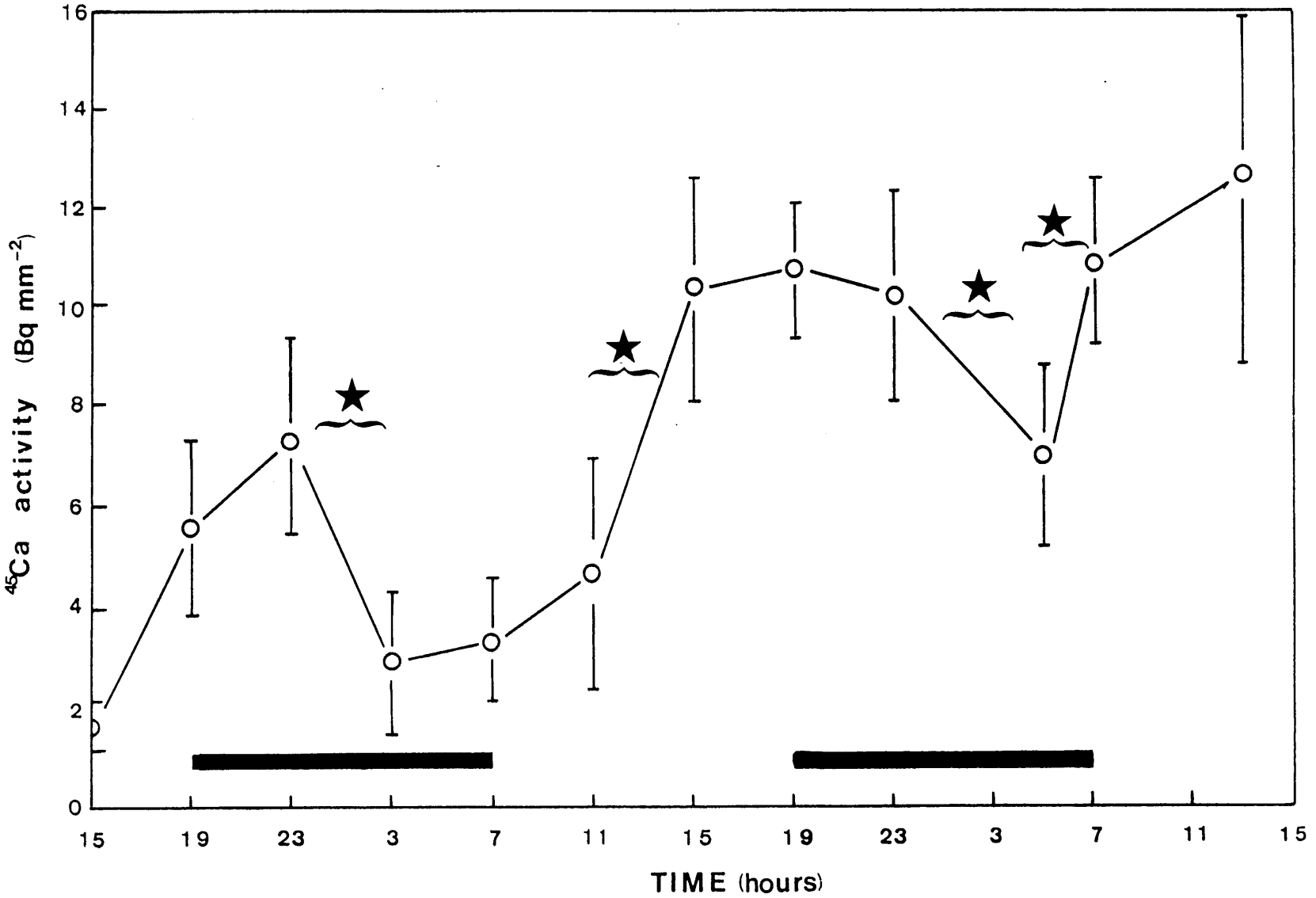
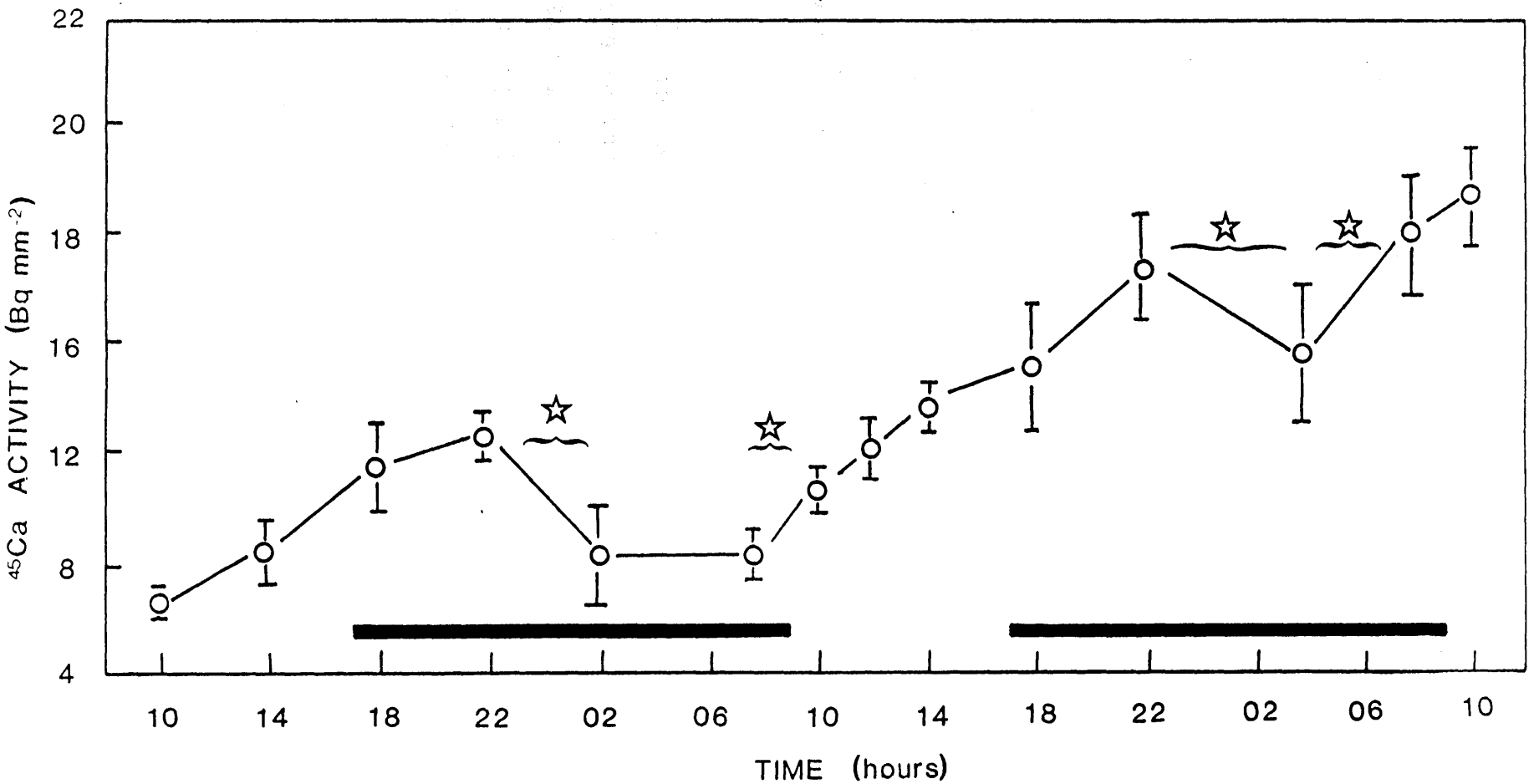

16
14
12
10
8
6
4
2
0
^{45}Ca activity (Bq mm^{-2})
15
19
23
3
7
11
15
19
23
3
7
11
15
TIME (hours)

Figure 4.9

Relation between mean activity of calcium-45 deposited on to sagittae and time, in S1 salmon parr (*Salmo salar*) maintained at 8L:16D, 4°C. (isotope was introduced two days prior to sampling to allow for equilibration between labelled and unlabelled calcium reserves).

Data presented as mean ^{45}Ca activity per mm^2 of total sagitta for 5 individuals (bars represent 95% confidence limits). Horizontal bars represent dark periods.

☆ denotes significant decrease and following increase in isotope activity between consecutive samples (Duncan multiple range test $P>0.05$).


22
20
18
16
12
8
4
^{45}Ca ACTIVITY (Bq mm^{-2})
10
14
18
22
02
06
10
14
18
22
02
06
10
TIME (hours)

decreased 3-5 hours prior to the dark-light transition (Duncan multiple range test; $P>0.05$). These findings suggest that there is a positive net flux of calcium on to the otolith during daylight hours and early evening but that at night-time there is a negative net flux, leading to a decline in mean activity. Nevertheless within a day the net accumulation of calcium on to the otolith is significantly greater then net losses occurring at night-time (mean net daily accumulation; 12L:12D; day 1: 2300h $\bar{x}$ = 7.39, day 2: 2300h $\bar{x}$ = 10.6; 8L:16D; day 1: 2200h $\bar{x}$ = 12.4, day 2: 2200h $\bar{x}$ = 17.4; Duncan multiple range test $P> 0.05$), as would be expected from the formation of daily increments.

Matrix production: The secretory activity of both type I and II cells varied cyclically with a significant peak in activity occurring in sacculi collected at 0200 and 0500h, 5 hours prior to dawn (Figure 4. 10; Kruskal-Wallis test; $P> 0.01$). At this time a layer of PAS positive matrix was observed close to the apical surface of the type II cells, and appeared to be continuous with it.

4.4 THE ROLE OF PLASMA CALCIUM IN OTOLITH CALCIFICATION

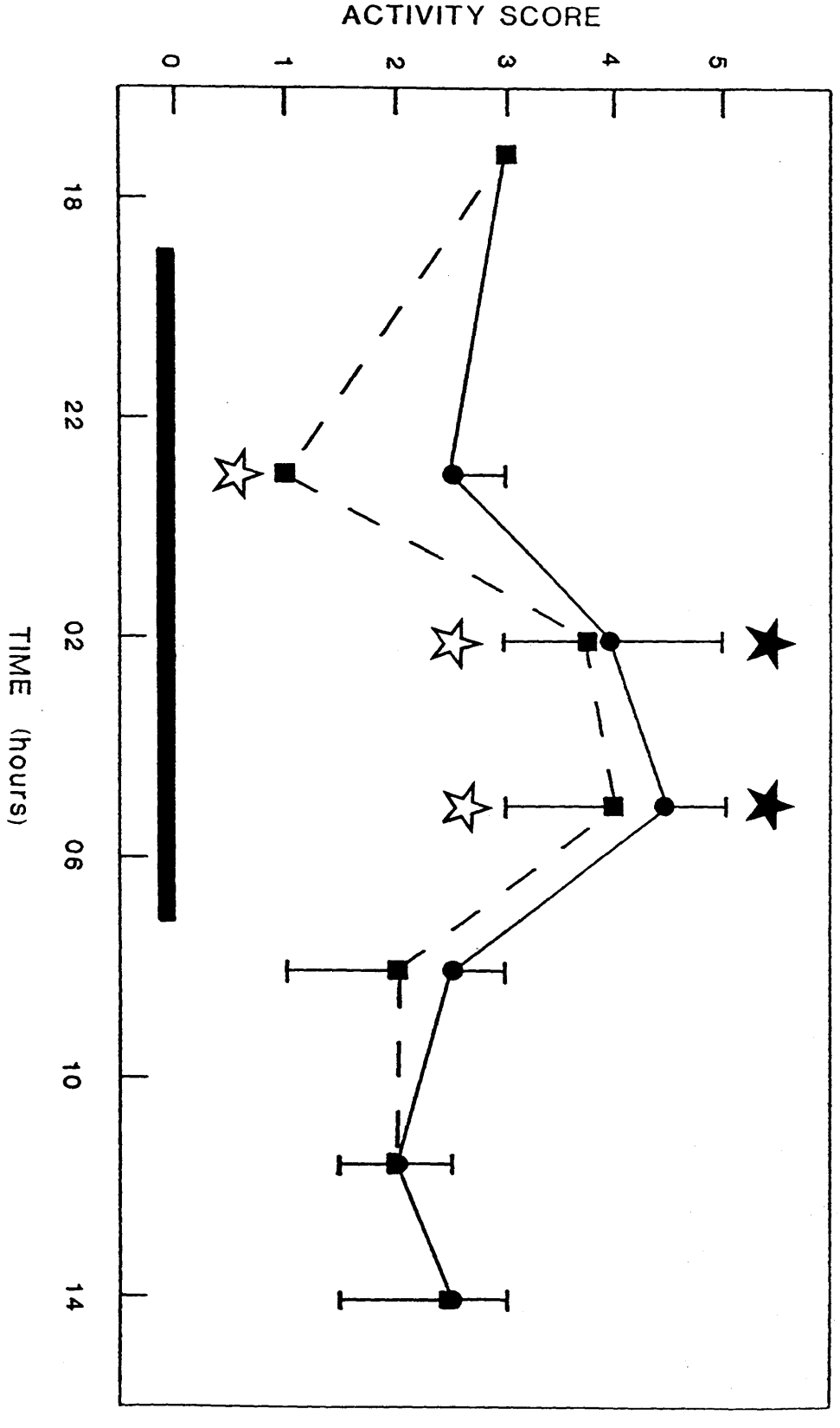
4.4.1 METHODS

Diel variation in plasma calcium concentration: To determine if plasma total calcium concentration varied diurnally, blood samples were taken from a group of 1+ parr maintained in a 2m radial flow tank under 12L:12D

Figure 4.10

Variation in the secretory activity of type I (■) and type II (●) saccula cells of Atlantic salmon parr during a 27 hour period.

Data presented as median activity score (see text for calculation) and range for each of the two cell types. Significant deviations from the total median activity score of each cell type are denoted by * P>0.01 (Kruskal-Wallis test).


ACTIVITY SCORE
0
1
2
3
4
5
TIME (hours)
18
22
02
06
10
14

photoperiod and temperature 10-12°C (September, 1988) at the DAFS Smolt Rearing Station, Almondbank.

Samples of 1+ S2 fish (wet weight 9 - 24 g) were taken at intervals of 3 or 6 hours (in part of the second 24 hours) over a forty-eight hour period. Fish were removed, one at a time, by gently netting and then killed prior to extracting blood from the caudal artery using heparinised capillary tubes. Water samples were taken at each sampling period, to check for variations in water calcium concentration. After centrifugation, plasma and haematocrit volume were determined by converting capillary length to volume using a pre-determined calibration factor. The plasma was stored at -18°C for 24 hours until analysed.

In a second experiment fish were held under laboratory conditions similar to those experienced by fish in radioisotope experiments, to determine if there were differences in plasma calcium fluctuations between hatchery and laboratory reared fish. Groups of 5 fish were sampled every eight hours over a twenty-four hour period (12L:12D). As ionic calcium is the only form of plasma calcium available to the endolymph both total calcium (i.e. calcium that is bound, predominantly by protein, and ionic 'free' calcium) and non-protein bound calcium were determined. The plasma from each fish was divided into two aliquots and one was deproteinised with 0.1% of the volume of perchloric acid to determine protein free calcium concentration. Deproteinised plasma samples were then centrifuged and the supernatant removed for analysis.

Lanthanum chloride (1% in total volume) was added to plasma samples before analysis and total calcium was determined using an atomic absorption spectrophotometer (Philips PU8200) with an acetylene/air flame. The length and weight of each specimen was recorded.

Influence of experimentally induced hypocalcemia on plasma calcium and otolith increment formation: In order to determine if a depression in plasma calcium could influence otolith calcification, ^{45}calcium activity was determined prior to and following experimentally inducing hypocalcemia in fish maintained in ^{45}Ca labelled water. Hypocalcemia was achieved by adding calcium-free water (deionised water containing a natural concentration of Na, Cl, HCO_3 ions) to labelled water. The effect of hypocalcemia on plasma calcium levels was determined in fish subjected to the same treatment but maintained in non-labelled water. Further the effect of hypocalcemia on increment deposition was investigated using SEM techniques (Chapter 2) on sagittae removed from five fish, fifteen days after inducing hypocalcemia. All experiments were carried out in the day-time (16L:8D) and hypocalcemia was induced at 1100 h (6 hours after dawn). The isotope was introduced two days before hypocalcemia was induced to allow for isotopic equilibration. Groups of five fish were killed at 2 hourly intervals prior to and following the introduction of the calcium free water and at 24 hours to determine if calcification had resumed.

4.4.2 RESULTS

Total calcium concentration in the plasma ranged from 2.5-3.3 mM, with a mean of 2.88 $\pm$ 0.06 mM. A significant negative deviation from the mean plasma calcium concentration occurred at 0400h on the first day (paired t-test $t_8 = 4.45$; $P < 0.01$; Figure 4.11) but not the second day. Significant positive deviations occurred at 1000h (paired t-test $t_9 = 4.11$; $P < 0.01$) the first day and 0700h on the second day (paired t-test $t_8 = 2.47$; $P < 0.05$). The large variation in plasma calcium concentration between individuals at a given time may have masked individual cyclicity.

In laboratory maintained parr, protein-free calcium was found to vary in relation to total calcium concentration (Table 4.3), representing approximately 71% of the total calcium of the plasma.

Influence of experimentally induced hypocalcemia on plasma calcium and otolith increment formation: Dilution experiments indicated that a reduction in the environmental water calcium concentration from 184 µmol to 111 µmol (39.7 %) at 1100h, was sufficient to induce a short-term (~6 hour) 17.8% reduction in protein-free plasma calcium concentration of salmon parr (Table 4.4). The specific activity of calcium in the plasma increased following induced hypocalcemia, possibly as a result of resorption from calcium reserves, that had taken up ^{45}calcium. Scanning electron microscope observations of the sagittae

Figure 4.11

Variations in total calcium concentration in the plasma of 1+ salmon parr (*Salmo salar*) over a 48 hour period.

Data presented as mean deviation from total mean ($\bar{x}$ = 2.88 $\pm$ 0.06 mM) together with 95% confidence limits, of measurements from 8 - 10 individuals. Horizontal lines represent dark periods. Significant deviations from total mean are represented by * $P<0.05$ ** $P<0.01$ paired t-test.

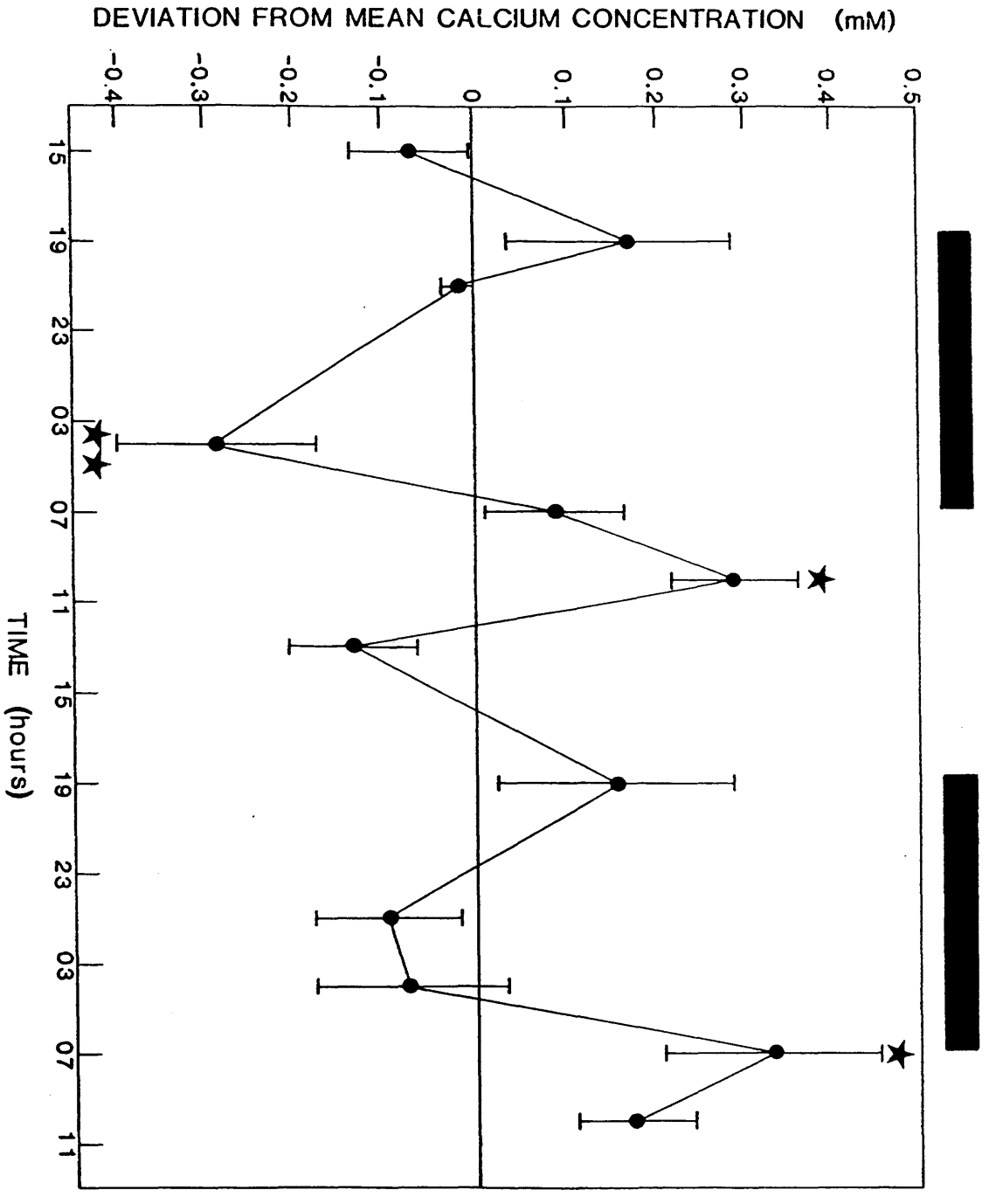

DEVIATION FROM MEAN CALCIUM CONCENTRATION (mM)
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
15
19
23
03
07
11
15
19
23
03
07
11
TIME (hours)

Table 4.3

Variations in total and protein-free calcium concentration of the plasma of Atlantic salmon parr, held under 12L:12D (2000 - 0800h).

Data presented as mean, standard error of the mean (S.E.) and range in percentage of total calcium that protein-free calcium comprises for three sample times. No significant differences between the percentage of total calcium that was comprised by protein-free calcium was found using a Mann-Whitney U-test ($P>0.50$).

	1200h	2000h	0400
total	2.73 ± 0.72	2.86 ± 0.84	2.62 ± 0.83
protein-free	1.76 ± 0.42	2.12 ± 0.70	1.86 ± 0.46
% total	63 - 74	66 - 76	64 - 74

Table 4.4

Changes in specific activity of calcium in the plasma associated with induced hypocalcemia.

Mean ($\pm$ S.E.) protein-free calcium concentration of the plasma, specific activity of the protein-free calcium concentration of the plasma, and specific activity of the water before and after inducing hypocalcemia are presented. Specific activities were estimated using the mean calcium concentrations of plasma and water found in the non-labelled experiment and the mean activities ($Bq\mu l^{-1}$) of plasma found in the ^{45}Ca labelled experiment.

	Ca^{2+} conc^{n}		specific activity	
	plasma	water	plasma	water
	(mM)	(μM)	($BqmM^{-1}$)	($Bq\mu M^{-1}$)
BEFORE	2.14 ± 0.06	184	25.29 ± 4.1	18.89
AFTER	1.76 ± 0.12	111	24.03 ± 3.9	20.46

from parr subject to hypocalcemia, indicated that a matrix layer was deposited on the day of treatment (Figure 4.12). The introduction of calcium-free water induced a change from a net inward to a net outward flux of calcium from the otolith for over four hours (Figure 4.13). However this trend had reversed within 24 hours of the introduction of calcium-free water as the mean activity of the sagittae had increased significantly from that prior to the introduction (Duncan multiple range test $P>0.05$).

4.5 DISCUSSION

4.5.1 ROLE OF ENVIRONMENTAL CYCLES IN INCREMENT FORMATION

The results of the environmental manipulation experiments demonstrate that the daily periodicity of increment formation persists in the absence of variation in daily light-dark, temperature cycles and cyclic food supply. Consequently increment deposition in the parr stage is probably controlled by an endogenous circadian rhythm. As daily increment periodicity has also been reported for post-hatch embryonic (alevin) (Moosegaard *et al.*, 1986), but not for pre-hatch embryonic stages of Atlantic salmon (Geffen, 1983), it would appear that regulation of increment deposition in the parr stage is initiated close to hatching.

The continuation of diel increment formation in constant light or darkness, at constant temperatures and with food available across the day, has been reported for juvenile

Figure 4.12

Scanning electron micrograph of ground sagitta showing matrix check produced on day of hypocalcemia experiment.

Sagitta was critically point dehydrated after grinding. Scale bar = 2μ.

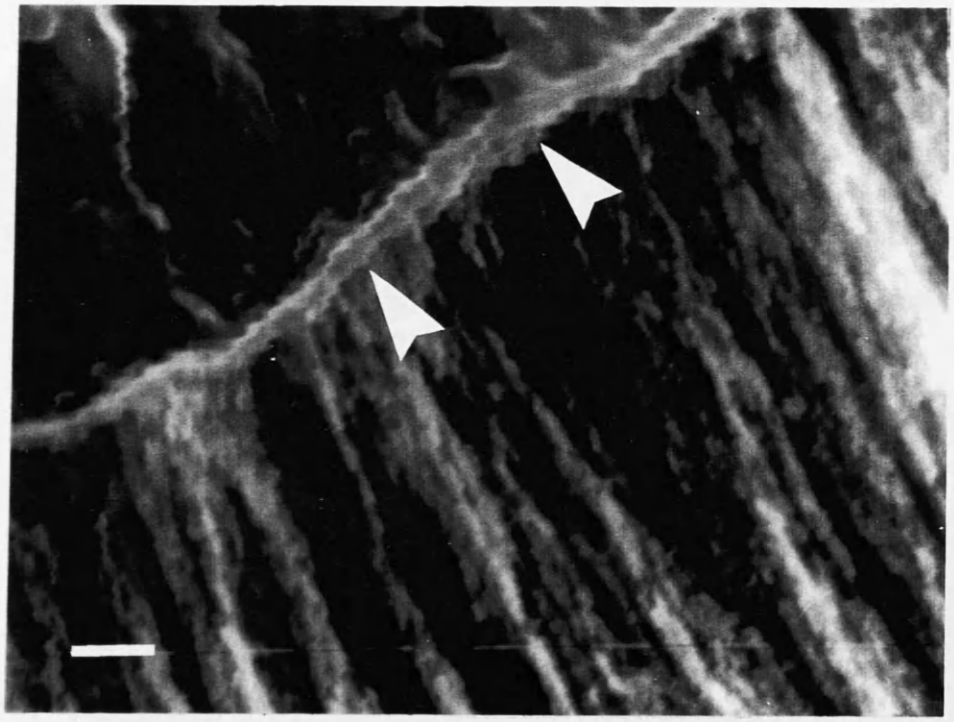
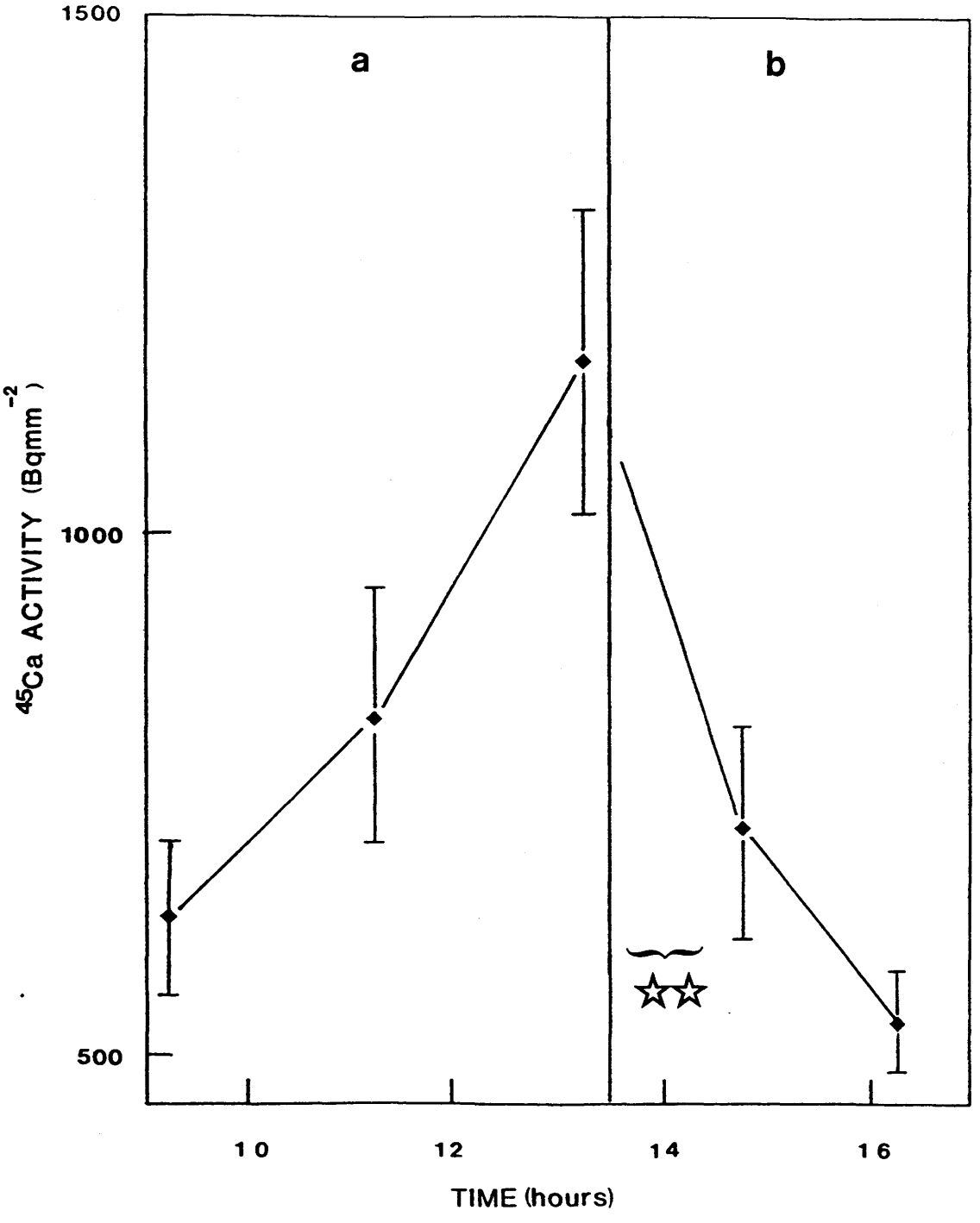


Figure 4.13

Relation between mean activity of calcium-45 deposited on to sagittae and time, in salmon parr (*Salmo salar*) prior to (a) and following (b) the introduction of calcium-free water.

Data presented as mean ^{45}Ca activity per mm^2 of total sagitta for 5 individuals (bars represent 95% confidence limits).

☆denotes significant decline in isotope activity between consecutive samples (Duncan multiple range test $P>0.05$).


1500
1000
500
a
b
^{45}Ca ACTIVITY (Bqmm^{-2})
10
12
14
16
TIME (hours)

stages of several species, including the starry flounder, *Platichthyes stellatus* (Campana & Neilson, 1982). However in other species food availability (Neilson & Geen, 1982) or temperature fluctuations (Brothers, 1978), appear to influence the rate of increment formation. Further a 24h light-dark cycle has been shown to be necessary for daily increment formation in the larval stages of some species (Taubert & Coble, 1977; Radtke & Dean, 1982), although as Campana & Neilson suggest (1985) this dependence may be age mediated, as this influence has been found in the larval but not the juvenile stages of plainfin midshipman, *Poricthys notatus* (Campana, 1984b).

Whilst patterns of food availability did not influence increment periodicity in the salmon used in this experiment, no account was taken of possible differences in feeding motivation within a day. Recent evidence suggests that the feeding intensity of hatchery reared parr varies diurnally, with a peak in feeding activity following dawn (Rawlings & Talbot, pers.comm.). Consequently although food was available throughout the day, food intake may still have varied cyclically. Pannella (1980) suggested that increment periodicity may be related to the number of peaks in feeding activity and Neilson & Geen (1982) found that Chinook salmon, produced one increment per day when fed once per day but produced significantly more than one increment per day when fed four times per day. However on the basis of this hypothesis daily increment deposition would be expected to cease if fish stopped feeding. Parr destined to smolt in their second year (S2) are known to

just consume a maintenance ration by around October (Higgins & Talbot, 1985; Metcalfe *et al.*, 1986). However they continue to deposit increments daily (Chapter 2). Other studies have also shown that starved fish often continue depositing increments at a daily rate (Taubert & Coble, 1977; Marshall & Parker, 1982; Campana, 1983a). Thus there appears to be little evidence to support Pannella's hypothesis of a relationship between increment periodicity and peaks in feeding activity.

Otolith growth is sensitive to temperature in a number of species (Brothers, 1978; 1981; Mosegaard *et al.*, 1986; 1988) and temperature fluctuations can induce prominent sub-daily increments in some species (Neilson & Geen, 1982). However in the present experiments although constant temperature resulted in relatively poor contrast between the continuous and discontinuous zones in the increments of salmon parr, daily increments continued to be formed. This indicates that increment formation is not dependent on a diel temperature fluctuation. This finding is contrary to Brother's (1978) suggestion that increment periodicity is mediated by diurnal variations in water temperature in temperate stream fishes. Confusion between temperature-induced sub-daily increments and daily increments is unlikely to be a problem in analysis of otoliths in wild Atlantic salmon parr because the water temperature in their stream habitat falls at night, coinciding with the formation of the discontinuous zone. Further, even if minor changes ($\pm 2^{o}C$) in stream temperature do occur during the day-time, the sub-daily temperature experiment of the

present study suggests that such changes are unlikely to induce the formation of sub-daily increments.

Whilst the present study demonstrated that a 24h light-dark cycle was not required for the maintenance of a daily increment periodicity, an abnormally short photoperiod of 6L:6D induced two faint increments within a day. This suggests that the processes regulating increment deposition are entrained by light-dark transitions; such transitions therefore act as zeitgebers to the endogenous rhythm regulating increment deposition in Atlantic salmon parr. Tanaka *et al.* (1981) also demonstrated an influential role for photoperiod by showing that a reversal of the light-dark cycle could reverse the order of formation of the discontinous and incremental zones in goldfish. Further differences in calcium-45 isotope incorporation rates into the otoliths of goldfish (Mugiya *et al.*, 1981) and rainbow trout (Mugiya, 1984) have been found to vary in relation to the light-dark cycle.

In conclusion, the present study strongly suggests that increment deposition is regulated by a circadian rhythm synchronised to light/dark cycles within a 24h period. Campana & Neilson (1985) suggested that such a photoperiod-mediated mechanism may be typical in teleost otolith formation. However this investigation is one of the few studies to test this hypothesis.

4.5.2 DIURNAL VARIATION IN OTOLITH CALCIFICATION AND MATRIX PRODUCTION

Formation of a discontinuous zone in the salmon otolith coincided with the peak secretory activity of the matrix precursor producing cells and with a time of no net calcium accumulation on to the otolith. These results agree with previous reports of a diurnal variation in otolith calcification (Tanaka *et al.*, 1981; Mugiya *et al.*, 1981; Mugiya 1984; 1987; Watabe *et al.*, 1982) and demonstrates that the formation of a discontinuous zone is associated with an increased production of matrix precursors, as has been suggested (Watabe *et al.*, 1982; Mugiya, 1987; Morales-Nin, 1987). In rainbow trout there is a seasonal inversion in the net flux of calcium to the otolith, the lowest net flux occurring at 1600 h June and at 0400h (Mugiya, 1984) or 2200h (Mugiya, 1987) in December. In contrast in Atlantic salmon parr, the changes in the net flux of calcium to the otolith remained in phase with the light/dark cycle in all experiments, indicating that the factors controlling otolith calcification are entrained to photoperiod. This synchronisation between the return to a net accumulation of calcium on to the otolith and the dark/light transition would explain why a 6L:6D photoperiod regime induced the production of approximately 2 increments per day in salmon parr otoliths. Further, such an effect of light/dark transitions on otolith calcification and matrix production would account for the fact that a reversal of the light-dark cycle led to a reversal in the order of

formation of the discontinuous and continuous incremental zones in goldfish (Tanaka *et al.*, 1981).

4.5.3 THE INFLUENCE OF PLASMA CALCIUM CONCENTRATION

Whilst total and protein-free calcium in the plasma of salmon parr declined around the time of no net accumulation of calcium on the otolith (and again at around 1300h), there was no evidence of a regular diurnal fluctuation in calcium concentration as has been reported for goldfish (Mugiya *et al.*, 1981) and rainbow trout (Mugiya, 1984). However any individual cyclic variations in plasma calcium may have been masked by the high variation in calcium concentration at each sampling point. Further, an experimentally induced depression of plasma calcium, of an order (17.8 %) that may occur naturally within the animal, was found to correspond with a net loss of calcium from the otolith.

The coincidence between changes in net calcium accumulation on to otoliths and plasma calcium (Mugiya *et al.*, 1981; Mugiya, 1984) has been taken as evidence for a plasma-calcium-mediated regulation of otolith calcification. Mugiya's (1986) evidence for a calmodulin-mediated calcium transport process in isolated sacculi, provided a possible mechanism by which calcium transport to the endolymph could be sensitive to extrinsic calcium concentration. The results of the hypocalcemia experiment is consistent with this theory, as the hypocalcemia-induced net loss of calcium from the otolith could cause a lowering of the ion concentration at the otolith surface. However

testing this hypothesis requires information on how endolymph calcium concentration is related to plasma calcium concentration, and on the effect of such changes on ion concentration at the otolith surface. Furthermore whilst a depression in the calcium concentration at the otolith surface may have occurred and affected the rate of calcium accumulation, this alone could not explain the formation of a matrix rich zone when hypocalcemia was induced or under natural conditions at night. Indeed, Mugiya's theory ignores the possible role of organic matrix in regulating otolith formation and assumes that extrinsic calcium would only influence calcium transport to the otolith.

Other mechanisms could also account for the observed association between plasma calcium concentrations and otolith calcification. As otolith calcification is limited by the number of nucleation sites provided by the organic matrix (Crenshaw, 1982; Mann *et al.*, 1983) and the soluble matrix appears to have the capability of regulating calcification (Chapter 3), the influence of extrinsic calcium may be indirect, affecting the secretion of matrix precursors. Extrinsic calcium may act directly on the sacculus, by stimulus-secretion coupling, or indirectly by influencing hormones that control matrix production. The release of many hormones has been shown to be influenced by extrinsic calcium concentration (Grau & Helms, 1989). Calcium transport to the otolith may also be under hormonal control, as has been found in Gastropod shell formation (Dogterom *et al.*, 1979; Dogterom & Doderer, 1981), and

hormone regulation may be responsible for the diurnal fluctuation in plasma concentration reported in some species (Mugiya *et al.*, 1981; Mugiya, 1984).

4.6 SUMMARY

The present study has demonstrated that:

1. Increment formation is endogenously controlled in Atlantic salmon parr

2. Increment formation is entrained to light-dark cycles in salmon parr as;

a. discontinuous zone formation occurs late at night, coinciding with a net loss of calcium from the otolith, and ceases at dawn.

b. calcium accumulation on to otoliths declines at night and resumes at dawn,

c. matrix production is highest at the time when the discontinuous zone is formed.

3. The influence of light-dark cycles is mediated either by extrinsic calcium concentration or by a common hormonal influence that influences both plasma calcium concentration and matrix production.

Chapter 5

THE INFLUENCE OF SOMATIC GROWTH AND METABOLIC RATE ON OTOLITH ACCRETION

5.1 INTRODUCTION

Daily otolith increments can potentially provide an accurate means of studying the early growth history of fishes (see review by Campana & Neilson, 1985). Experiments in which both somatic and otolith growth have been studied simultaneously have often shown a positive correlation between growth rate of the whole fish and otolith increment width (Volk *et al.*, 1984; Neilson & Geen, 1985). Such relationships have been used to back-calculate growth rates from otolith radii (Methot, 1981; Radtke & Scherer, 1982), providing a quicker and direct alternative for the construction of an age-length curve than the collection of age-at-length data (Barkman & Bengtson, 1987).

However, some species of fish fail to produce increments on a daily basis (Geffen, 1982a), and those that do may show continuous growth of otoliths even during periods of starvation or negative growth (Brothers, 1981; Marshall & Parker, 1982). This suggests that there is no fixed relationship between otolith and somatic growth. Indeed, recent studies have shown that there may be an uncoupling of the two growth processes such that individuals in populations experiencing slow somatic growth develop

disproportionately large otoliths (Mosegaard *et al.*, 1988; Reznick *et al.*, 1989; Secor & Dean, 1989).

Two theories have been proposed for the control of otolith accretion. Mosegaard & Titus (1987) suggested that, whereas somatic growth rates peak at intermediate temperatures, otolith growth rates are governed by a metabolic process, that is an increasing function of temperature. More recently Secor & Dean (1989) have suggested that sagitta accretion rate is determined by the interaction between two components: the daily periodicity of increment formation (that continues even under periods of no somatic growth) and an amplitude component that varies with somatic growth rate.

In this chapter, data on otolith radius and size are used to derive otolith radius/length relationships for the three-spined stickleback, *Gasterosteus aculeatus,* and Atlantic salmon parr, *Salmo salar* L.. Further, the influence of somatic growth and metabolic rate on otolith accretion is investigated in Atlantic salmon parr.

Atlantic salmon parr provide an ideal subject for investigating the relationship between otolith accretion and somatic growth because individuals within a sibling population adopt different developmental patterns, regardless of externally-applied constraints on growth (Thorpe, 1987). During the first six months of life sibling populations of juvenile Atlantic salmon diverge into those fish that will migrate to sea as smolts the following spring, and those that will delay migration for a further year. These subpopulations become recognisable on the basis

of size, for the length-frequency distribution of the population changes from being unimodal to bimodal by late autumn (Thorpe, 1977). The Upper Modal Group (UMG) consists of those fish that will smolt aged 1+, while their siblings smolting at 2+ form the Lower Modal Group (LMG). The rapid segregation by size occurs as a result of the LMG fish losing appetite and virtually ceasing growth from late summer onwards (Metcalfe *et al.*, 1986), whereas the UMG fish maintain and even increase their appetite and growth (Metcalfe *et al.*, 1988). This dramatic divergence in growth rates of the two types of fish occurs even when they are maintained under the same laboratory conditions with food supplied to excess.

The two modal groups thus provide an opportunity to compare somatic-otolith growth relationships in individual fish growing at markedly different rates under the same environmental conditions.

The possible role of metabolic rate in determining otolith accretion (Mosegaard & Titus, 1987; Mosegaard *et al.*, 1988) was also investigated by examining the influence of temperature and food intake, which affect metabolism, on otolith accretion and by comparing increment width with oxygen consumption rates.

The aims of this chapter are summarised below:

1. to construct an otolith radius/length relationship for three-spined sticklebacks;

2. to compare otolith accretion and somatic growth in individual UMG and LMG salmon parr;

3. to investigate the influence of temperature and food intake on otolith calcification, as determined from ^{45}Ca uptake rates; and
4. to compare otolith accretion and oxygen consumption in individual UMG and LMG salmon parr.

5.2 SAGITTA-SOMATIC GROWTH RELATIONSHIPS IN THREE-SPINED STICKLEBACKS

5.2.1 MATERIALS AND METHODS

Larval sagitta radius: The sagitta rostral (terminology of Messieh, 1972) radius was measured, from a microscope and video monitor enhanced image at x1000 magnification, of otoliths from laboratory reared larvae descended from River Endrick parents. Total larval length was measured to the nearest 0.1 mm.

Juvenile sagitta radius: The sagitta rostral radius of wild caught sticklebacks from the River Kelvin (Ukegbu collection; see Ukegbu, 1986) of known length, was measured using a calibrated eyepiece graticule at x 400 magnification.

5.2.2 RESULTS

Sagitta radius (SR) was linearly correlated with total length in 5 - 8 mm TL larvae (r^2 = 0.856; N = 27; P<0.01; Figure 5.1), according to the regression:

$$S.R.\ (\mu m) = -5.98 + 7.68TL(mm)$$

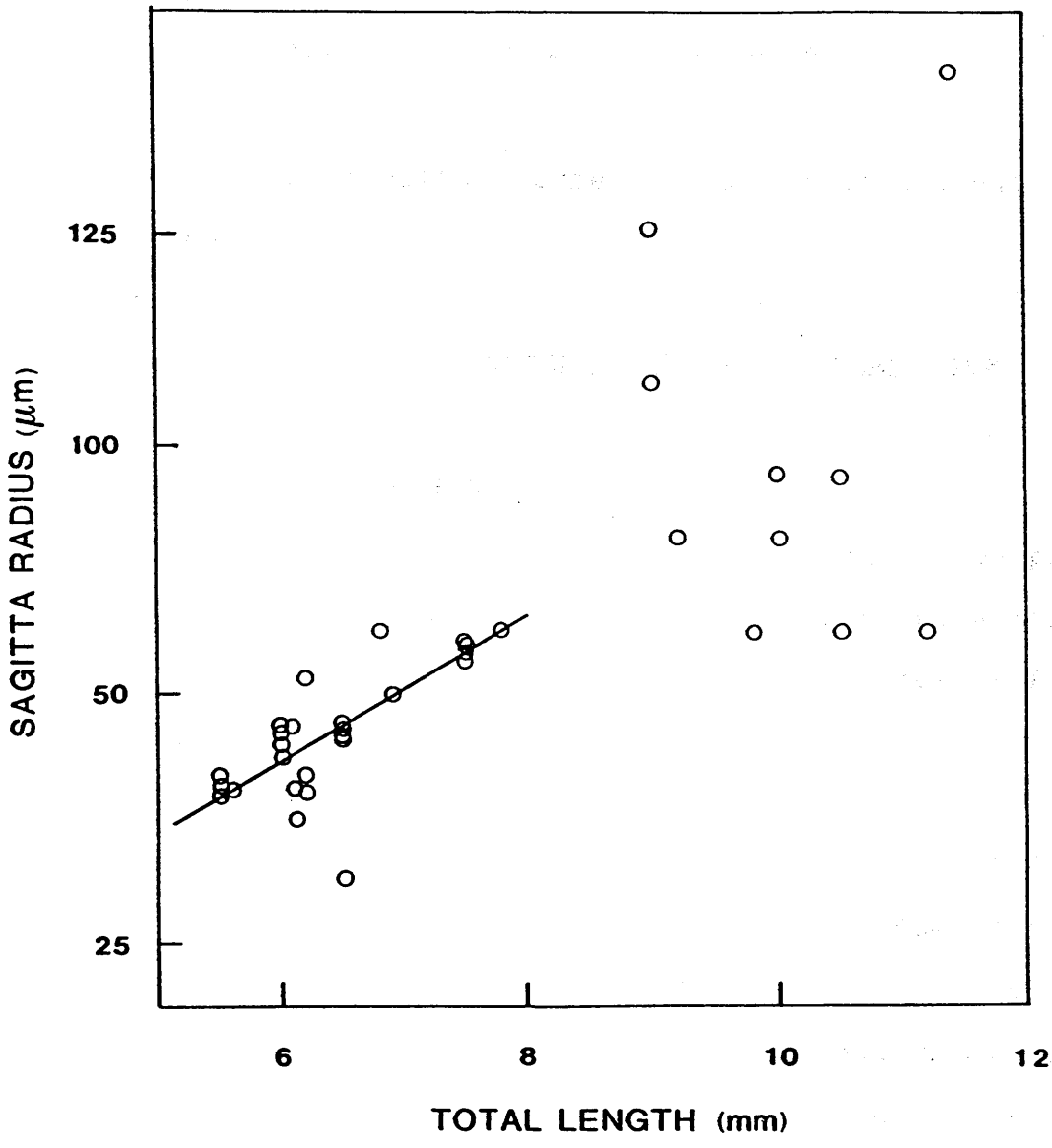
Figure 5.1

The relationship between total length and sagitta radius for larval laboratory reared three-spined sticklebacks, from the River Endrick.

Points plotted are individual data sets. Equation linking sagitta radius (y) to total length (x), in individuals less than 8 mm TL;

$$y = -5.98 + 7.864x \ (r^2 = 0.856;\ P<0.001).$$

where sagitta radius is in um and length is in mm. No relationship was apparent in individuals in the range 9 - 12 mm TL.


SAGITTA RADIUS (μm)
125
100
50
25
6
8
10
12
TOTAL LENGTH (mm)

but varied markedly in the size range 9 - 12 mm TL. Sagitta radius of wild caught juveniles was highly correlated with fish total length (TL) in mm (Figure 5.2) according to the formula:

$$SR(mm) = 0.130 + 3.266 \times 10^{-3} TL$$

$$(r^2 = 0.836;\ N = 40;\ P<0.001)$$

indicating that sagitta accretion is proportional to somatic growth.

5.3 OTOLITH-SOMATIC GROWTH IN ATLANTIC SALMON PARR

5.3.1 MATERIALS AND METHODS

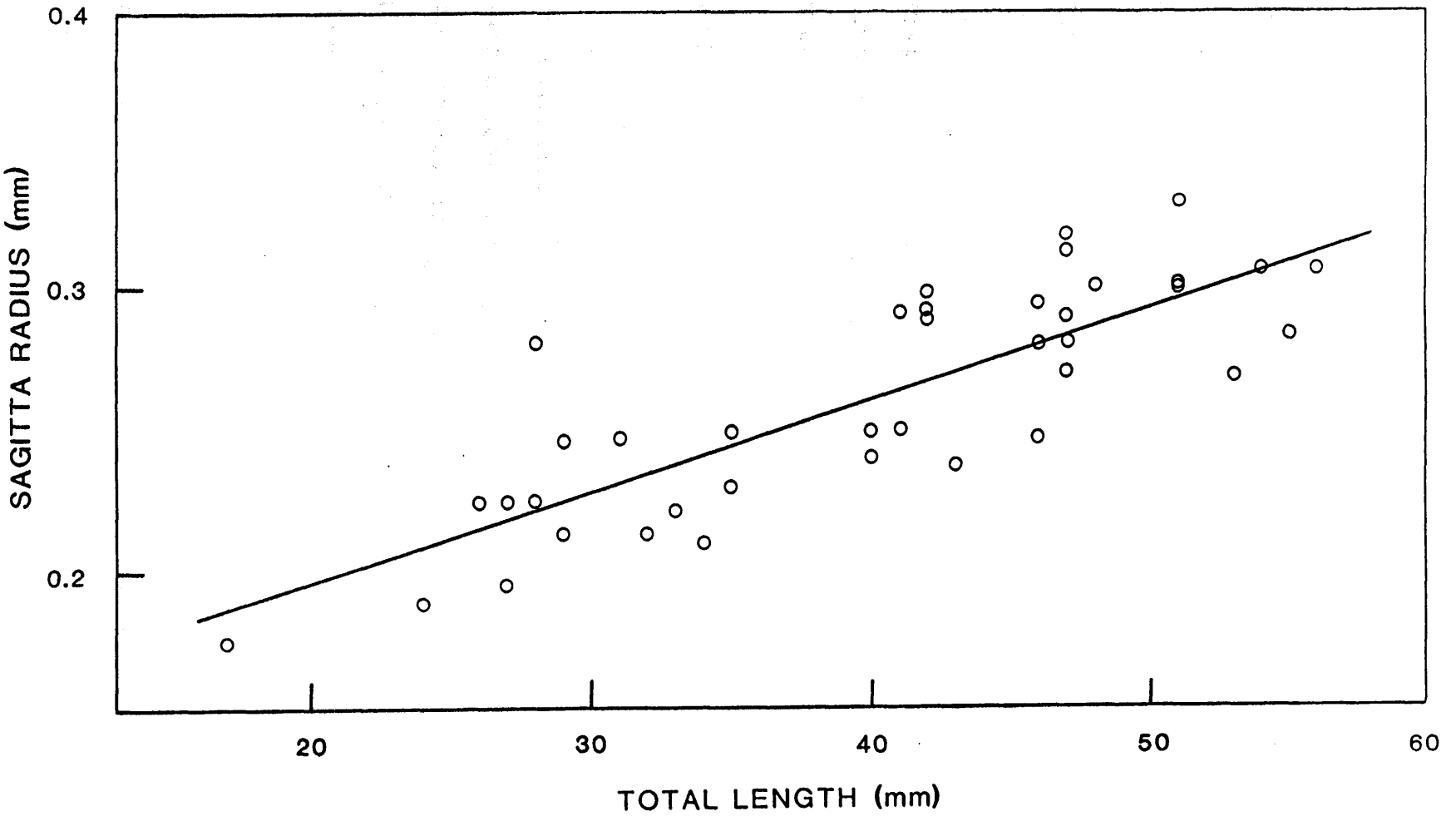
The data were obtained from sibling populations of 0+ parr derived from sea-run parents collected in the River Almond, Perthshire. In the first experiment fish were reared from first feeding in a 2 m radial flow tank (Thorpe & Wankowski, 1979). In the second, fish were reared from first-feeding (29th April 1988) in a single 1 m radial flow tank, firstly at the University Field Station, Rowardennan and after 18 October at the DAFS Smolt Rearing Station, Almondbank. At both sites the fish experienced ambient photoperiods and water temperatures, and were fed to excess on EWOS pelleted food dispensed automatically throughout the day.

A group of salmon parr were marked and monitored by Dr. N. B. Metcalfe for this study. On 9 June a number of fish were marked as individuals (by means of unique combinations of alcian blue dye-spots injected subcutaneously; Kelly, 1967) and their fork-lengths measured to the nearest mm.

Figure 5.2

The relationship between total length and sagitta radius for wild caught three-spined sticklebacks, from the River Kelvin.

Points plotted are individual data sets. Equation linking sagitta radius (y) to total length (x), both measured in mm, $y = 0.130 + 3.266 \times 10^{-3}x$ ($r^2 = 0.836$; $P < 0.001$).


0.4
0.3
0.2
SAGITTA RADIUS (mm)
20
30
40
50
60
TOTAL LENGTH (mm)

The fish were re-marked and re-measured at approximately monthly intervals until they had clearly separated into the two smolting groups, recognisable by means of the bimodal length-frequency distribution (Thorpe, 1977). A sample of 13 Lower and 11 Upper Modal Group fish were then killed for otolith analysis.

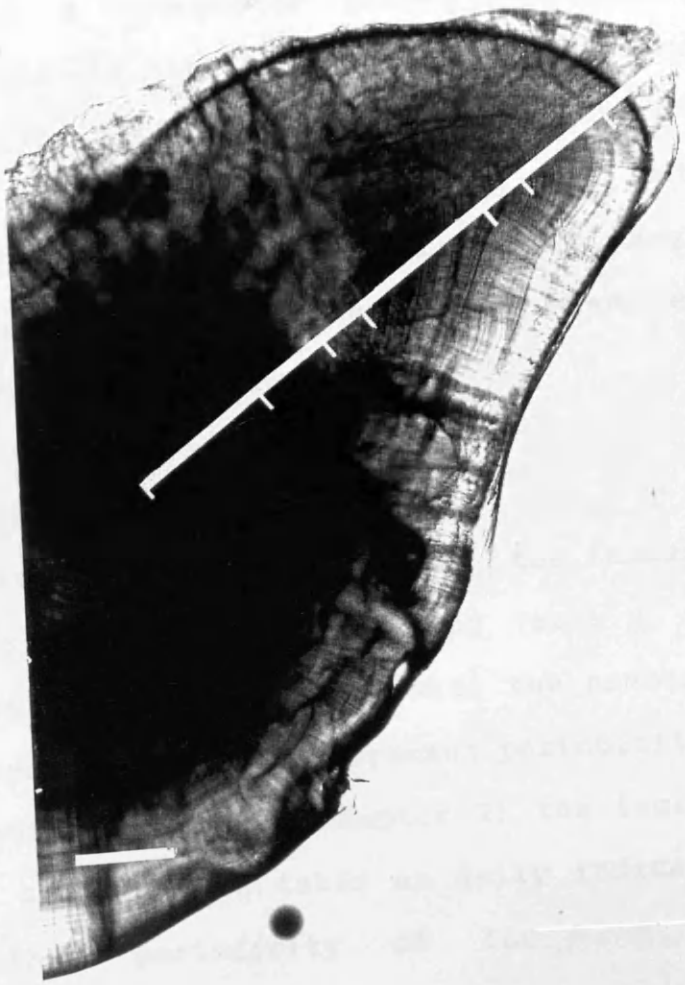
Otoliths were exposed by an oblique cut through the dorsal surface of the head. The left sagitta was fixed to a glass slide with superglue (Bostick) and ground on the sagittal surface to a plane where all increments were clearly visible, using a graded series of diamond pastes (6, 1 and 0.25 μ) on a Kent 3 lapping and polishing wheel (Engis Ltd). The sagittae were then washed and dried, and left to clear in immersion oil for 30 days prior to examination.

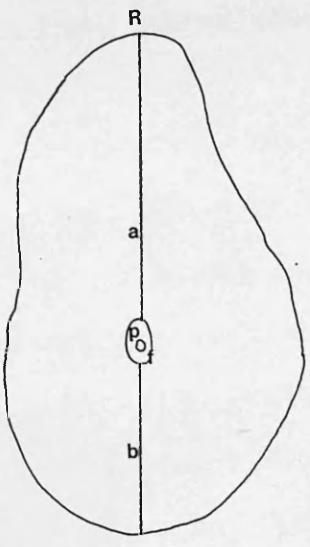
A light microscope fitted with a camera lucida was used to produce images of the rostral region of each sagitta (terminology of Messieh, 1972) at magnification of x330. Under natural photoperiods the sagittal increment produced by salmon parr at first feeding is clearly distinguishable as a change in increment clarity (Chapter 2). A comparison of rostral radius, post-rostral radius and otolith weight in groups of salmon parr up to 250 days old, indicated that the former was more closely related to otolith radius (r^2 = 0.925; $P<0.001$; N = 102), than was post-rostral radius (r^2 = 0.822; $P<0.01$; N = 102). Consequently measurements were made along the rostral radius (see Fig. 5.3). By counting increments from the first feeding point it was therefore possible to locate the increment produced on each of the seven dates that fork-lengths were measured. The position

Figure 5.3

The rostral region of the sagitta of a 211 days-old salmon parr, showing the relative size of the sagitta each day that fork length was measured.

inset: Schematic diagram of salmon parr sagitta showing rostrum (R), post-rostrum (PR), primordium (p), first feeding ring (f), and rostral (a) and post-rostral (b) radius.




R
a
p
o
f
b
PR

of these seven increments was then traced along a fixed radius through the rostral peak and the otolith radius on each of the seven dates obtained by referring to a scale drawn from a micrometer slide. The accuracy of these measurements was tested by replicating the procedure for 10 otoliths; the replicated results were within a mean of 96% (range 94-98%) of the original. Dr. N.B. Metcalfe did not provide the results of the fish fork length data until after the otolith radii had been measured, to prevent subjective bias.

5.3.2 RESULTS

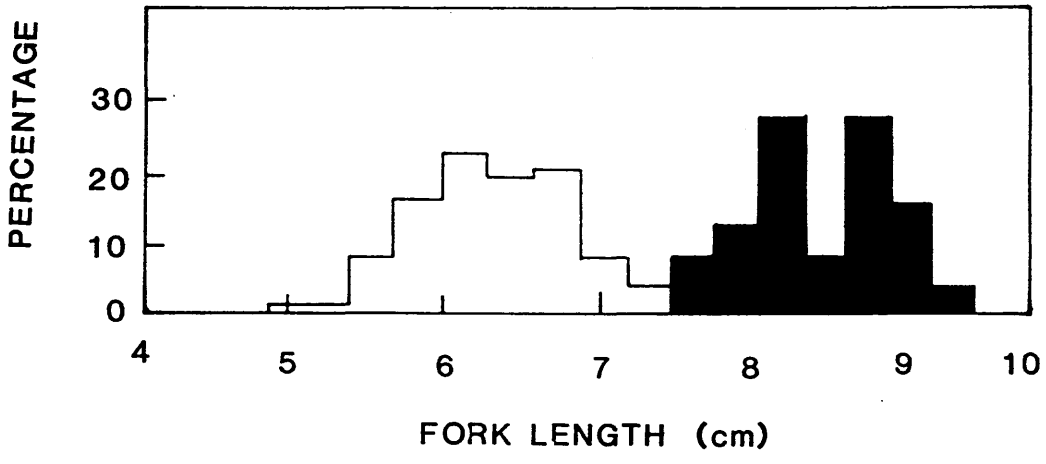
Periodicity: At the time of death, the number of increments following the first feeding ring (mean $\pm$ s.e. = 215.8 $\pm$ 3.25) was in close agreement with the number of days since first feeding (211). As increment periodicity remains daily throughout this period (Chapter 2) the increments observed in this study can be taken as daily indicators of otolith size; this periodicity of increment deposition is maintained regardless of the growth and developmental pattern of the salmon parr.

Development of bimodality: By the time of the final sampling date the sibling population of parr had clearly diverged into the two developmental groups, as indicated by the bimodal length-frequency distribution (Fig. 5.4). The sample taken for otolith analysis were fish that could be reliably assigned to one or other modal group on the basis of size. Thus for the Lower Modal Group: mean length on 8

Figure 5.4

The fork length frequency distribution of Atlantic salmon parr on 8 November 1988, showing the bimodal separation into the Upper (black) and Lower (white) Modal Groups.

Histograms plotted as percentages of each modal group. (Data provided by Dr. N.B. Metcalfe).


PERCENTAGE
30
20
10
0
4
5
6
7
8
9
10
FORK LENGTH (cm)

November was 63.5 mm (range = 57-68); while for the Upper Modal Group was 85.3 mm (range = 81-93).

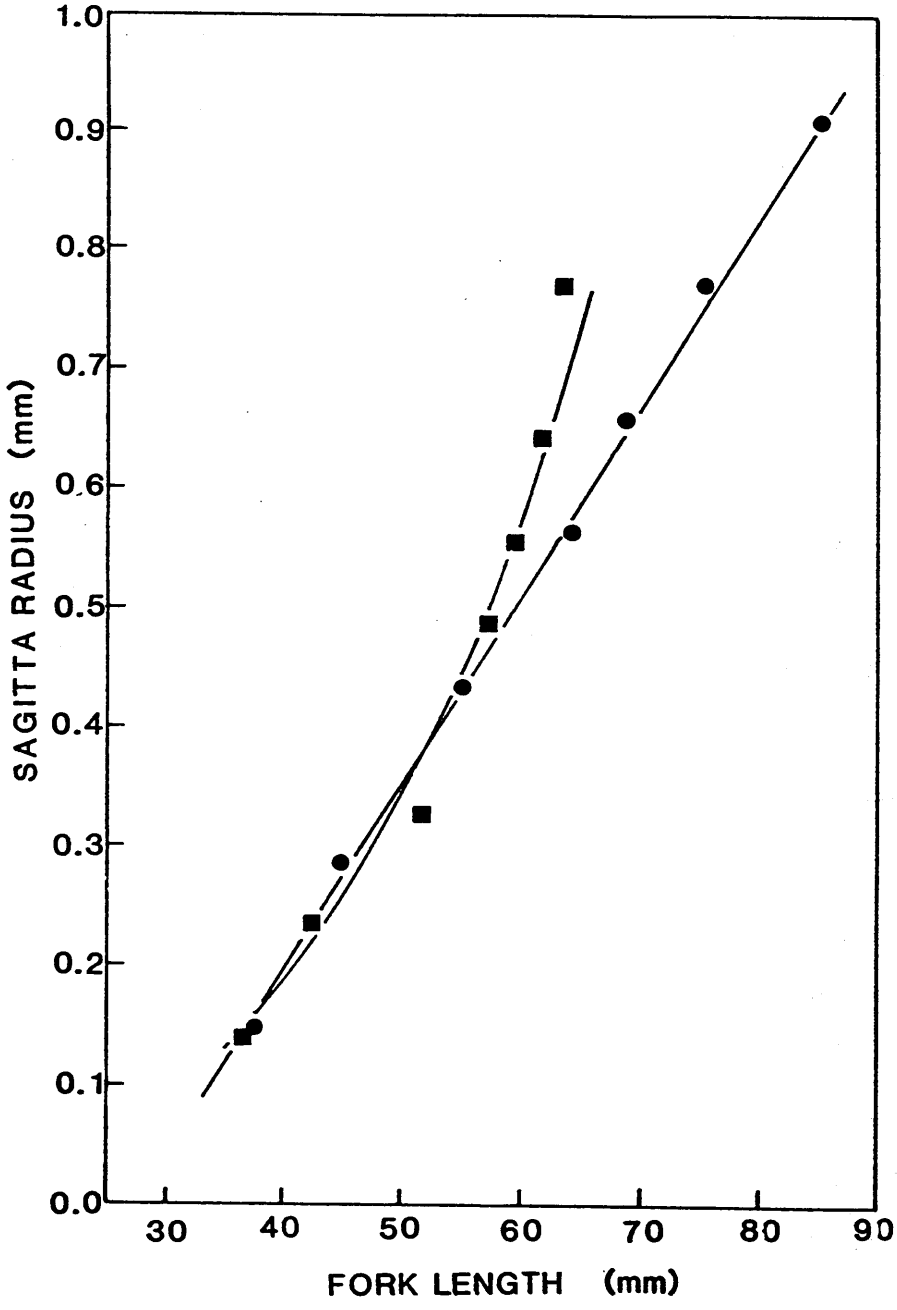
Relationship between sagitta and somatic growth: In Upper Modal Group fish (i.e. salmon destined to smolt aged 1+) there was a highly significant linear correlation between fish fork length and sagitta radius (r^2 = 0.93; P<0.001; Fig. 5.5). Fork length explained 89.9% of the variance in sagitta radius, and otolith growth varied in proportion to somatic growth. However, this was not true in fish entering the Lower Modal Group (destined to smolt aged 2+). The somatic growth of these fish declined rapidly after August, yet the sagittae continued to grow at their previous rate. Thus in LMG fish the specific growth rate (Ricker, 1979) measured in terms of fork length over the six weeks prior to 11 November dropped to 0.064 $\pm$ 0.012% per day, whereas that of otolith radius remained at 0.467 $\pm$ 0.050% per day (corresponding figures for UMG fish over the same period: fork length = 0.304 $\pm$ 0.017 per day, otolith radius = 0.423 $\pm$ 0.049% per day). This resulted in a curvilinear relationship between fork length and sagitta radius in Lower Modal Group fish (Fig. 5.5). Note that a study of sagitta radius and age in groups of parr killed at intervals throughout a 285 day period from first feeding indicated that sagitta and somatic growth declined dramatically in both UMG and LMG parr following October (Wright & Rowe unpubl. data).

The regression of otolith size against age remained linear in fish from both modal groups until November, despite the

Figure 5.5

The relationship between fork length and sagitta radius for Upper (●) and Lower (■) Modal Group salmon parr.

Points plotted are means [n = 13 (LMG) or 11 (UMG)] at each day of monitoring. Equations linking sagitta radius (y) to fork length (x), both measured in mm: for UMG fish, $y = 0.01554x - 0.420$ ($r^2 = 0.899$, $P<0.001$); for LMG, $y = 2.5927 \times 10^{-6} (x^3) + 0.020$ ($r^2 = 0.870$, $P < 0.001$).


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SAGITTA RADIUS (mm)
30
40
50
60
70
80
90
FORK LENGTH (mm)

virtual cessation of somatic growth in Lower Modal Group parr (Fig. 5.6). However, the two smolting groups differed in the rate of otolith growth (Fig. 5.6; covariance analysis comparing regression slopes: $F_{1,127} = 10.10$, $P<0.01$), which is perhaps merely a reflection of the fact that it is the faster growing parr that then adopt the developmental pathway of smolting at 1+.

The relationship between otolith and somatic growth was initially similar for the two groups of fish, such that the fork length-sagitta radius equation obtained from UMG fish (see legend to Fig. 5.5) gave accurate predictions of sagitta radius on the basis of fork length for LMG fish until some 2.5 months after first-feeding (Fig 5.7). After this point they deviated markedly from the predicted relationship, first having small otoliths and subsequently disproportionately large otoliths for their size. In LMG fish there thus appears to be a distinct change in the relative growth rates of soma and otoliths some months after first feeding. The timing of this uncoupling can be estimated from Fig. 5.5 as the point when the two lines diverge. This occurred when the otolith radii of LMG fish averaged approximately 0.4 mm, corresponding to an age of 99 days (= 6 August, from the regression predicting age (days) from sagitta radius (mm) for LMG fish: age = 204.92(radius) ± 17.16).

Figure 5.6

The relationship between age and sagitta radius for Upper and Lower Modal Group salmon parr.

Data presented as in Figure 5.5 with means $\pm$ S.E.; regression lines exclude the last sampling point as sagitta accretion deviates from linear after this time. For UMG(●) fish, $y + 0.00541x - 0.055$ ($r^2 = 0.884$, $P<0.001$); for LMG(■) $y = 0.00447x - 0.042$ ($r^2 = 0.916$, $P<0.001$).

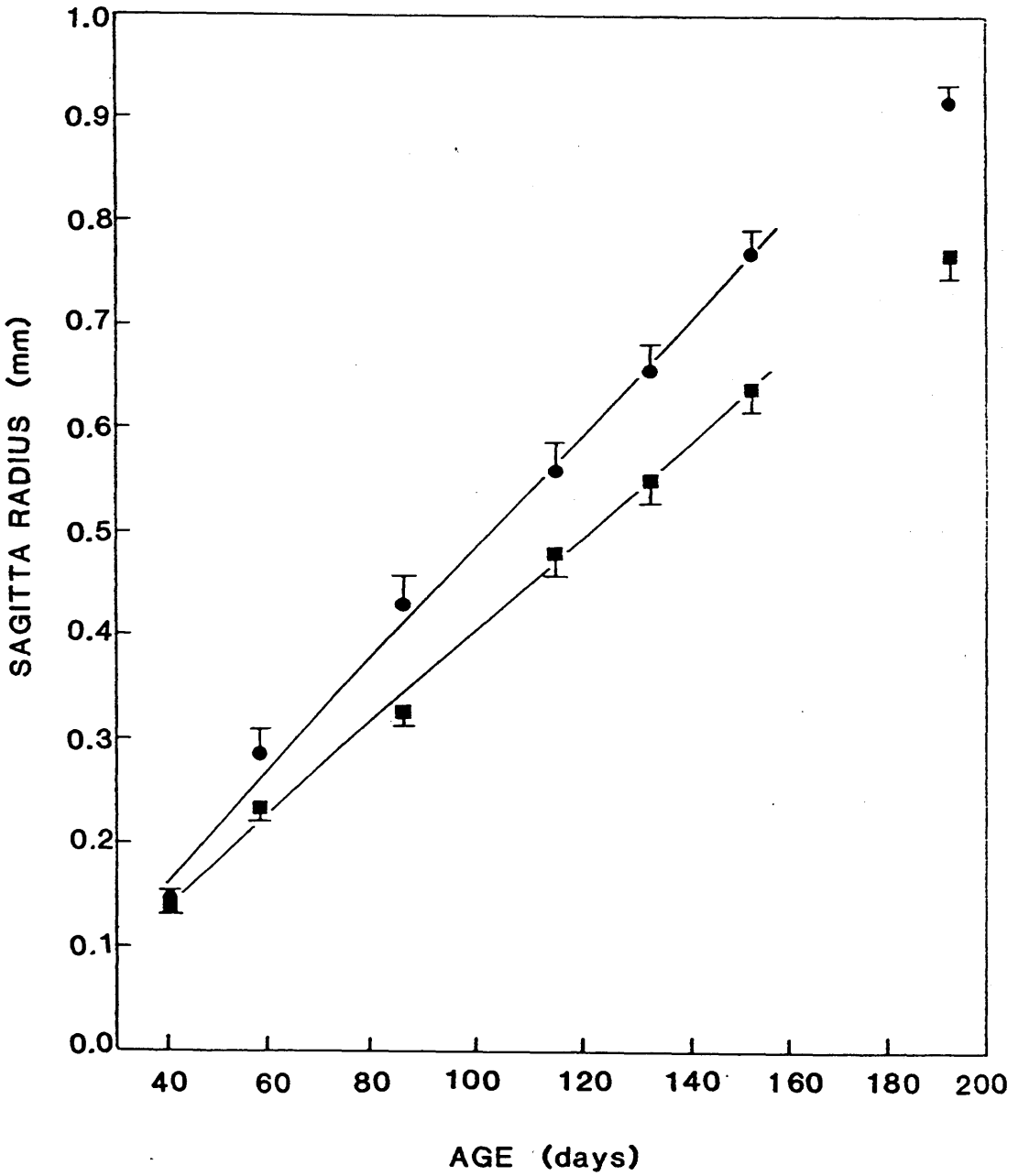
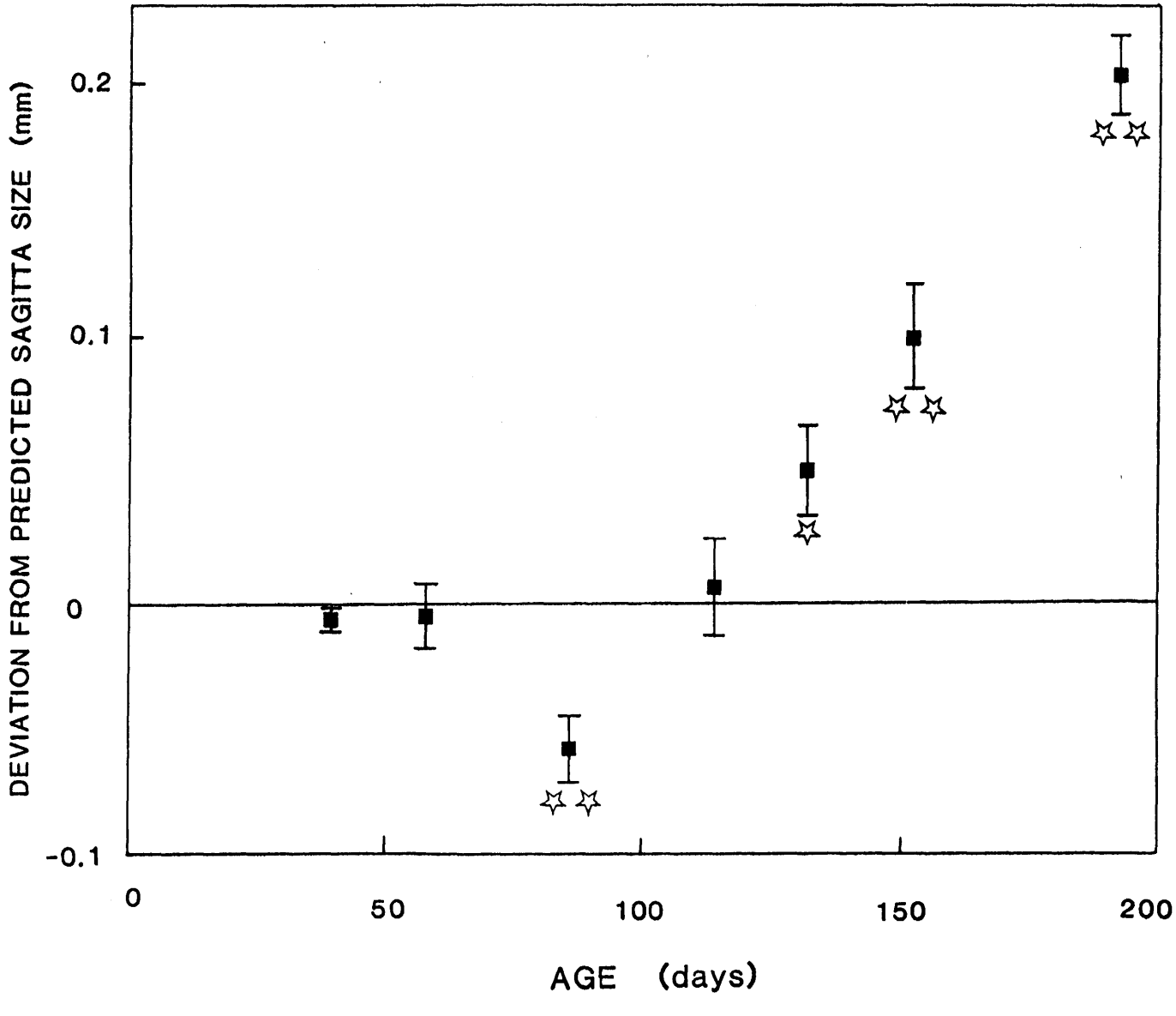

SAGITTA RADIUS (mm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
40
60
80
100
120
140
160
180
200
AGE (days)

Figure 5.7

The mean deviation (mm, $\pm$S.E.) of the sagittas of Lower modal Group salmon parr from the size predicted for fish of that length; predictions based on the UMG fork length-sagitta radius relationship from Figure 5.5. Asterisks indicate significant deviations from the predicted sagitta sizes (paired t-tests; *$P<0.05$, **$P<0.001$).


DEVIATION FROM PREDICTED SAGITTA SIZE (mm)
0.2
0.1
0
-0.1
0
50
100
150
200
AGE (days)

5.4 INFLUENCE OF TEMPERATURE AND FOOD INTAKE ON OTOLITH CALCIFICATION RATE

5.4.1 MATERIALS AND METHODS

Temperature: Four experiments were carried out under 12L:12D, at 4, 6, 10 and 15^{o}C using the methods described in Experiment 3, Chapter 4. Groups of ten fish were allowed to equilibrate for two days in water containing 37KBql^{-1} ^{45}Ca and food was withheld 24 hours prior to and during the experiment. Five fish were removed at 1000h and the other five fish were removed at 1600h. The difference in mean ^{45}calcium activity of sagittae was determined using the methods described in Chapter 4.

Influence of food intake: Two groups of 20 UMG parr (weight range = 4-6 g) were moved to individual tanks with a recirculating filtered water supply and fed either to excess (>10 chironomid larvae/fish/ day) or a low ration (1-2 chironomid larvae/fish/day) for seven days. Temperature was maintained at 10^{o}C $\pm$ 0.5^{o}C. Each ration group of parr were then transferred to a test tank containing 37KBql^{-1} ^{45}Ca and allowed to equilibrate for two days. Respective ration levels were maintained during the experimental period. Following equilibration 10 fish were killed at 1000h and the other 10 fish were killed at 1600h. Gut contents were examined to determine stomach fullness. Differences in mean ^{45}Ca activity between sample times were

then compared for the two groups, using the methods described in Chapter 4.

5.4.2 RESULTS

Temperature: Difference in mean sagitta ^{45}calcium activity between sample times increased in proportion to temperature (Figure 5.8), over the studied range.

Food intake: Food intake was higher in parr fed on high rations, which had between 4 and 7 (mean = 5) partly digested chironomid larvae in their stomachs, than in parr fed on low rations, which had between 0 and 2 (mean = 1). The net increase in mean ^{45}Ca activity of sagittae from parr fed on high rations was also greater than that for parr fed on low rations, suggesting a greater net accumulation of calcium (Table 5.1).

5.5 OXYGEN CONSUMPTION AND INCREMENT WIDTH

5.5.1 MATERIALS AND METHODS

A single-cell recirculating flow respirometer was constructed for measurement of resting rate oxygen consumption (Figure 5.9). The respirometer had a sealable 3L tank and the water it contained was filtered and aerated continuously except when measurements were taken. The test cell was covered with a polystyrene sheet to reduce disturbance to fish. A Rank polagraphic oxygen electrode (loaned from DAFS Scottish Crop Research Institute) was fitted with an inlet and outlet head, as used by Higgins (1985), to enable water from the cell to flow over the

Figure 5.8

Relation between changes in mean calcium-45 accumulation 3-6 hours after the light-dark transition and temperature in Atlantic salmon parr.

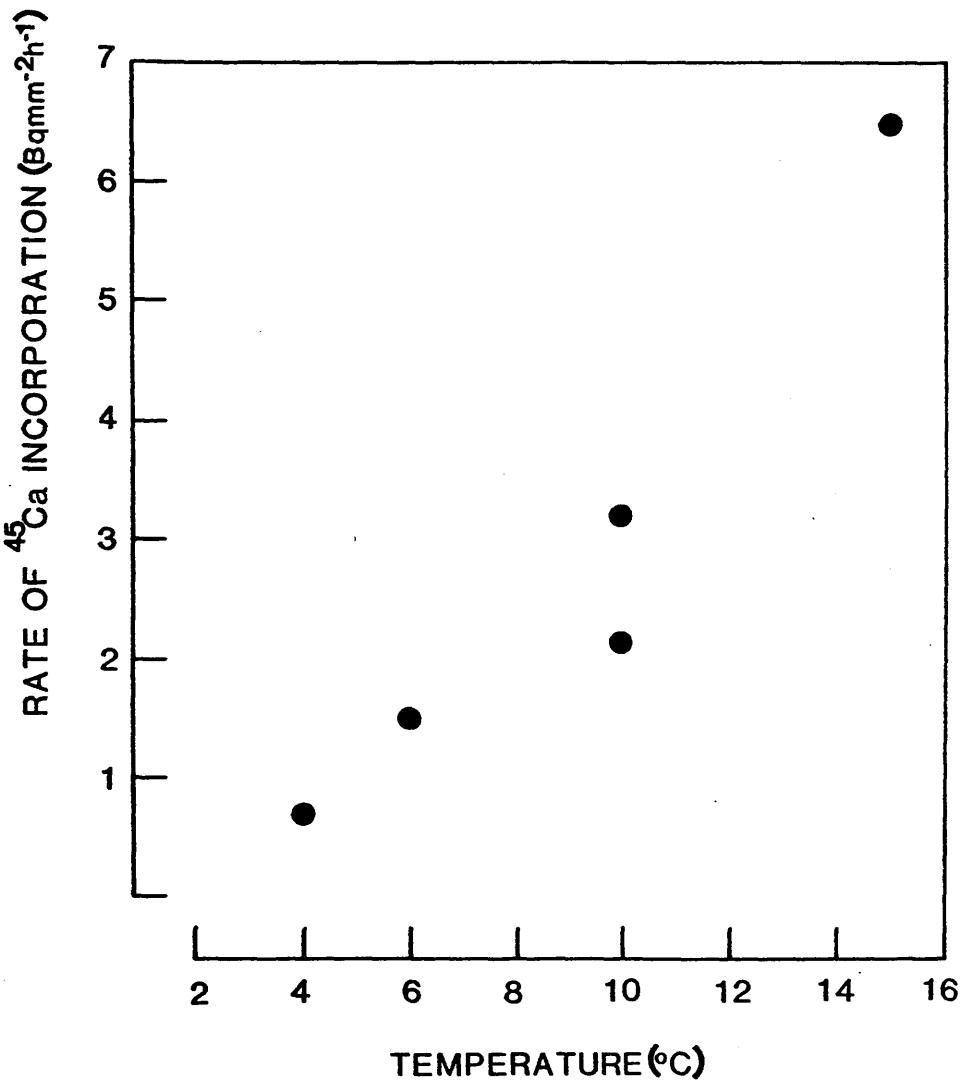

RATE OF ^{45}Ca INCORPORATION ($Bqmm^{-2}h^{-1}$)
7
6
5
4
3
2
1
2
4
6
8
10
12
14
16
TEMPERATURE (°C)

Table 5.1

Relation between changes in mean calcium-45 accumulation 3-6 hours after the light-dark transition and food ration in Atlantic salmon parr.

Data presented as mean of 5 sagitta pairs at 3 and 6 hours, together with the difference between means for fish fed on high (>5 larvae in stomach) and low rations (0-2 larvae in stomach).

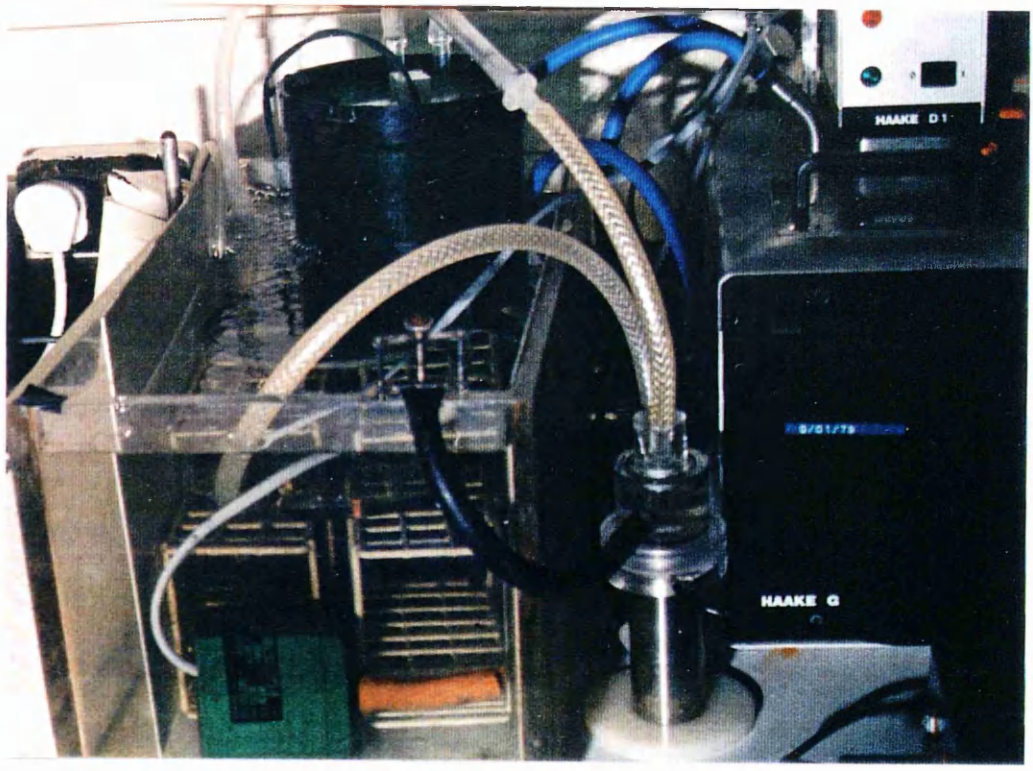
RATION \ TIME	MEAN SAGITTA ACTIVITY ($Bqmm^{-2}$) 1000	1500	difference
HIGH	1.048 ± 0.086	1.644 ± 0.065	0.1192
LOW	0.766 ± 0.040	1.241 ± 0.116	0.0950

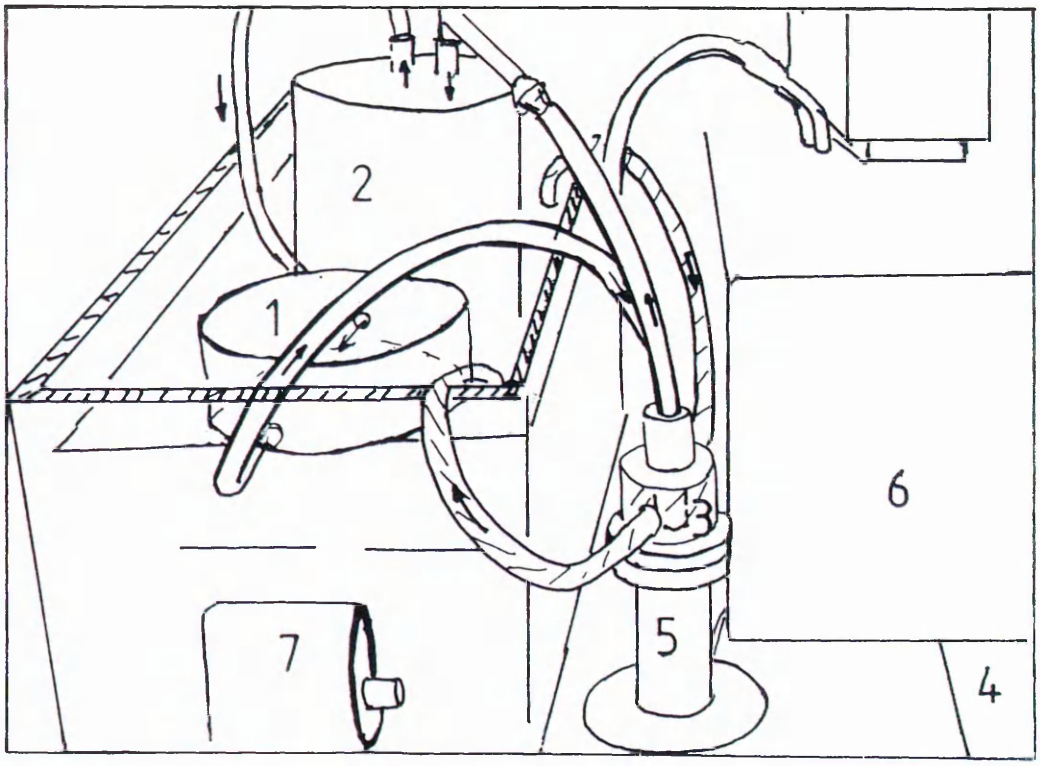
Figure 5.9

Single cell respirometer constructed for measurements of oxygen consumption in Atlantic salmon parr.

Respirometer consisted of a 3L tank (**2**) with a sealable lid (by means of an O-ring), containing a recirculating pump and charcoal filter. Water was pumped into a 400ml test cell (**1**), at $2Lmin^{-1}$ and out to a Rank polagraphic oxygen electrode (**3**). The water was then returned to the 3L tank. When measurements were not being taken the 3L tank was left open and the water was aerated to give at least 90% air saturation. The water temperature was maintained at $10^{o}C \pm 0.25\ ^{o}C$ with a cooling unit (**6**) and water jacket, and water at this temperature was circulated around the electrode chamber by means of a pump(**7**).

Measurements from the oxygen meter (**4**) were recorded using a BBC chart recorder at 3 cms^{-1}. **5** = Stirrer.


HAAKE D1
HAAKE G


2
1
6
5
7
4

electrode. The electrode was calibrated to $mg.l^{-1}$ oxygen using a Jencons portable oxygen meter. The volume of the system was determined volumetrically.

10 fish (5 LMG and 5 UMG) were transferred from the DAFS Smolt Rearing Station, Almondbank, to a tank with recirculating filtered water supply at Glasgow, in January (by which time most LMG parr had ceased growing). Fish were individually marked with alcian blue dye and measured (length and weight) before introducing to the tank, at the time of oxygen consumption measurements and at the end of experiments. A mark was induced on their otoliths using a 3 day 6L:6D photoperiod. The water temperature in the tank was maintained at 10 $\pm$ 0.5 ^{o}C and chironomid larvae were supplied twice a day.

Fish were moved to the respirometer test cell (maintained at 10 $\pm$ 0.25 ^{o}C) and allowed to settle for 16 hours, as oxygen consumption is usually elevated for at least 4 hours after introduction to a respirometer cell (Higgins, 1985). Feeding times were so timed to ensure the fish were starved for 34 hours (18 hours in stock tank + 16 hours in test cell) prior to oxygen consumption measurements, thus ensuring complete evacuation of the gut (Higgins & Talbot, 1985). Three one hour measurements were made over a 24h period, from which a daily mean resting rate of oxygen consumption was calculated. Oxygen consumption was expressed as $mg\ l^{-1}\ h^{-1}\ g\ wet\ weight^{-1}$.

All fish were killed after 50 days. Five fish were tested twice within this period and the others were tested only once. Both sagittae were extracted from each fish and

ground and polished as described in Chapter 2. The increments on the days when measurements of oxygen consumption were made were located by backtracking from the day of killing. Increment width along the maximum rostral radius on these days was recorded on acetate sheets from a microscope and video monitor enhanced image at x2500 magnification. Measurements were made using a digitising table and software (Cherry software). The accuracy of these measurements was tested by replicating the procedure for both sagittae 5 times; the replicated results were within a mean of 90 % (range 87-98 %) of the original.

5.5.2 RESULTS

Oxygen consumption was logarithmically correlated with fish weight, the slope being greater in UMG parr than in LMG parr (Table 5.2). The values obtained were similar to those reported by Higgins (1985) using a more sophisticated respirometer. Specific growth rates of LMG (<76 mm FL) parr during the 50 day experimental were practically zero (range = 0.00 - 1.86 $mgday^{-1}$) and ranged from 5.36 to 12.22 $mgday^{-1}$ in UMG parr (>85 mm FL). However increment width was significantly correlated to oxygen consumption in both UMG and LMG parr (combined data; r_s = 0.91; $P<0.01$; Figure 5.10), increment width and oxygen consumption being generally lower in LMG than UMG parr.

Table 5.2

Regression parameters for regression:

$$\text{oxygen consumption} = A \times \text{Weight}^{b} \qquad (mg.l^{-1}h^{-1})$$

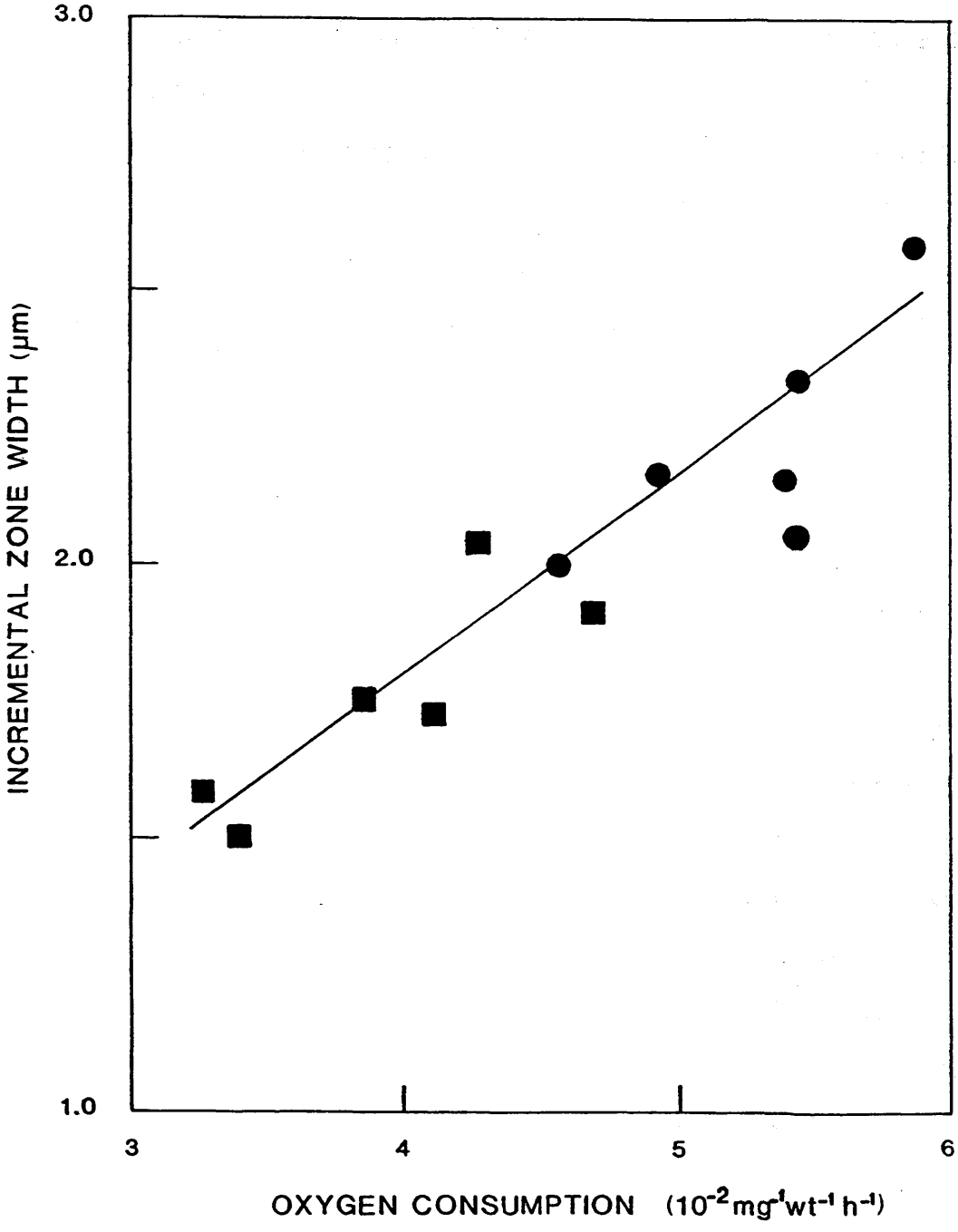
for Atlantic salmon parr (*Salmo salar* L.).

SIZE CLASS	A	b	N	r^2	P
LMG	0.04036	0.975	7	0.89	>0.01
UMG	0.04080	1.138	6	0.73	0.1

Figure 5.10

The relationship between oxygen consumption and sagitta incremental zone width for Upper (●) and Lower (■) Modal Group salmon parr, held at 10°C.

Points plotted are single data sets, based on a mean of three one hour oxygen consumption measurements during the 20 hours an incremental zone was produced. Regression linking oxygen consumption for all data sets,
$y = 0.347 + 36.47x$ ($r^2 = 0.902$; $P<0.001$; or if data sets are ranked Spearmann rank corelation: $r_s = 0.91$; $P<0.01$).


3.0
2.0
1.0
INCREMENTAL ZONE WIDTH (μm)
3
4
5
6
OXYGEN CONSUMPTION (10⁻² mg⁻¹wt⁻¹ h⁻¹)

5.6 DISCUSSION

5.6.1 SAGITTA - SIZE RELATIONSHIPS IN THREE-SPINED STICKLEBACKS

The present study suggests that sagitta radius may provide a means of backtracking somatic growth in the early larval (5-8 mm TL) and juvenile stages of the three-spined stickleback. However further information on the relation between somatic and sagitta growth in individual fish is needed to assess the reliability and precision of back-calculated estimates of growth.

5.6.2 REGULATION OF SAGITTA ACCRETION IN ATLANTIC SALMON PARR

The present study has demonstrated a linear correlation between sagitta radius and fork length in salmon parr destined to smolt at 1+ (i.e. fish entering the UMG) up to 211 days following first feeding. The sagittae of these fish were found to accrete at an almost constant rate throughout the 211 day study. However, a steady increase in otolith radius was also found in LMG fish despite a virtual cessation of somatic growth. This demonstrates that the two growth processes are not inextricably coupled and are positively correlated only when somatic growth is high. Further it should be noted the correlation between sagitta radius and somatic growth in UMG parr is based on a 19 to 40 day interval between samples and so a direct correspondance between increment width and daily somatic growth cannot be assumed. Indeed Bradford & Geen (1987)

have demonstrated from a study of sagitta growth and somatic growth in individual Chinook salmon, that a correlation between somatic growth and increment width only holds when width is averaged over at least 30 day intervals.

'Uncoupling' between otolith and somatic growth has been observed when growth has ceased (Brothers, 1981; Marshall & Parker, 1982) and under conditions, such as hyperoptimal temperatures, that inhibit somatic growth (Mosegaard *et al.* 1988). Metabolic rate has been suggested to have an important influence on increment deposition (Geffen, 1983; Campana & Neilson, 1985). Mosegaard & Titus (1987) developed this suggestion and proposed that otolith growth rates were governed by a metabolic process that was an increasing function of temperature (whereas somatic growth rates peak at intermediate temperatures). This concept was further developed by Mosegaard *et al.* (1988), who formulated a model, using experimental data, that predicted changes in otolith size of Arctic charr (*Salvelinus alpinus*) on the basis of initial weight and temperature. Secor & Dean (1989) have proposed a different type of increment regulation; namely they propose that otolith accretion is determined by the interaction between the daily periodicity of increment formation (that continues even under periods of no somatic growth) and an amplitudinal component that varies in relation to somatic growth rate.

The results of the present study provide little support for the latter hypothesis, since otoliths increased in size

at an almost constant rate despite large changes in the rates of somatic growth. Nor do they correspond entirely with the model of Mosegaard *et al.* (1988), since the observed steady rate of otolith accretion occurred while the fish were exposed to the ambient seasonal temperature cycle (i.e. from approximately 9 ^{o}C in early June to 15 ^{o}C in late July to 7 ^{o}C in November). However, this discrepancy may reflect the simple formulation of the model rather than its underlying concept. For instance, the use of temperature by Mosegaard and co-workers as an index of metabolic rate ignores other factors such as food intake and fish size which also infuence metabolism. Indeed the present study has indicated that when examined independently, calcium accretion was positively related to both temperature and food consumption.

Temperature, food intake and fish size all varied during the period of the individual growth experiments and so using any one as an indicator of metabolic rate would be unreliable. Nonetheless it is likely that metabolic rates in LMG fish are maintained after the point at which somatic growth slows (since water temperatures are still high), and the metabolic model would therefore predict that LMG otoliths would continue to grow beyond this point. This prediction is supported by the demonstration of a positive correlation between increment width and oxygen consumption in both UMG and LMG parr, under resting conditions, even when LMG parr had ceased growing. Thus it would appear that metabolic rate has a major influence on daily increment accretion, although this relationship needs to be

investigated further in active fish. Clearly if metabolic rate does indeed determine the rate of sagitta growth then this suggests a difference in metabolic rate between Upper and Lower Modal Group parr from very early in their development, since sagitta growth was consistently lower in LMG fish (see Fig. 5.7). The present study suggests that oxygen consumption rates are higher in UMG parr once the bimodal split has become clear and this is supported by more extensive data collected in the spring following first feeding by Higgins (1985). Measurements of metabolism and somatic and sagittal growth rates in individuals before the divergence has taken place would be useful to resolve the question of the difference in otolith accretion.

5.6.3 THE TIMING OF THE LIFE-HISTORY 'DECISION' IN SALMON

In terms of life-history strategies the timing of the divergence between somatic and otolith growth rates in Lower Modal Group parr is of interest since it suggests that a physiological change occurs around 100 days after first feeding (Figure 5.6). This change is the earliest and most precise marker yet found of the 'switching-off' of appetite and somatic investment in fish that form the lower modal group, and may correspond with the actual life-history decision. Other lines of evidence also indicate that the decision is made at this time of year. From estimates of short-term growth derived from changes in RNA/DNA ratios of skeletal muscle Villarreal (1983) and Thorpe (1987) predicted that the divergence of the growth groups occurred in late July. Appetite has been found to

fall off by late August (Metcalfe *et al.*, 1986), while experimental manipulations of the environmental conditions suggest that it is the growth opportunities experienced by parr during July and August that influence the developmental pattern they adopt (Thorpe *et al.*, 1989). Furthermore, the decision is unlikely to have been made appreciably before this point, since the proportion of fish entering the LMG can be changed by environmental manipulations made as late as mid July (Pickering *et al.*, 1987).

5.7 SUMMARY

The present study has demonstrated that:

1. Otolith growth is correlated to somatic growth in juvenile three-spined sticklebacks and fast growing Atlantic salmon parr.

2. Otolith growth is not related to somatic growth in S2 parr once somatic growth rate declines.

3. Otolith calcification is influenced by temperature and food intake.

4. Increment accretion is positively related to oxygen consumption, suggesting a relationship between otolith accretion and metabolic rate in both fast growing S1 and slow growing S2 parr.

Chapter 6

GENERAL DISCUSSION

6.1 OBJECTIVES

The results presented in this thesis have dealt with the periodicity of increment formation in the otoliths of Atlantic salmon parr and in the three-spined stickleback (Chapter 2), the mechanism of otolith formation (Chapter 3), the regulation of increment periodicity (Chapter 4) and the influence of somatic growth and metabolic rate on otolith accretion (Chapter 5). The following sections summarise these results and identify areas that require further investigation.

6.2 DAILY INCREMENT FORMATION

Otolith increments are formed daily in 0+ and 1+ salmon parr and so provide a means of age determination throughout this life-history stage. Similarly daily increments have also been demonstrated in the three-spined stickleback up to an age of 300 days. Using daily increments it has been possible to re-examine previous growth estimates providing, for the first time, an accurate means of studying growth in populations of this species (Appendix 2).

6.3 OTOLITH FORMATION

The present study has demonstrated several, previously unknown, aspects of the process of increment formation. The most significant are: the isolation of a calcium-binding protein in the soluble matrix, the localisation of matrix-

producing cells and the localisation of calcospherules both on the otolith and on the apical surface of certain non-sensory epithelial cells (type IV cells). Nevertheless our understanding of the process of otolith formation is still fragmentary. Future studies of otolith formation may benefit from a comparitive approach, as the findings of a number of studies (Dunkelberger *et al.*, 1980; Tanaka *et al.*, 1981; Watabe *et al.*, 1982), including the present one have demonstrated many similarities between otolith mineralisation and other, well investigated, mineralisation systems involving calcium carbonate, most notably the shell of bivalve molluscs (Wilbur, 1980; Crenshaw, 1982).

6.3.1 What are the causes of increment formation?

The formation of mineral rich and mineral deficient layers suggests that mineralisation is periodically limited. Limitation of mineralisation may be related to diurnally varying physico-chemical factors such as a decrease in ion transport or pH. Alternatively it may involve insoluble matrix (acting as a barrier to crystal growth) or the soluble matrix, which contains a calcium binding protein that can inhibit crystal growth. There are many questions that remain to be answered about the influence of all these factors.

6.3.2 Role of ion transport

Ion concentration at the otolith surface must be elevated for mineralisation to occur (Appendix 1), and the presence of large numbers of calcium spherules on the otolith

surface suggests that these structures may be involved in this process. The formation of spherules and their transformation to the aragonite crystal requires investigation. However the presence of an organic core material suggests that the formation of spherules may depend on the secretion of core material, by matrix producing cells. Calcium transport through the sacculus may involve a calcium-calmodulin dependent transport system (Mugiya, 1986), but the presence and location of this system needs to be confirmed. Immunochemical techniques for localisation of calmodulin activity are commercially available (SIGMA chemicals) as are histochemical techniques for localisation of calcium ATPase (Pearse, 1985), although attempts at localisation of this enzyme proved inconclusive in the present study.

6.3.3 Role of endolymph pH

Whilst the influence and regulation of endolymph pH was not investigated in the present study, Mugiya & Takahashi (1985) have shown that the pH of rainbow trout endolymph varies diurnally by 0.4 units. Such a variation may influence the rate of otolith calcification, as pH has a major influence on the solubility product (Appendix 1). Indeed a depression of endolymph pH below the natural range, induced by injection of acetazolamide (Mugiya & Takahashi, 1985) or severe anaerobic stress (Mugiya & Uchimura, 1989), has been found to lead to a dissolution of calcium from the otolith. However the cyclical variation in pH found in rainbow trout does not correspond with the

cycle of otolith calcification reported for this species (Mugiya, 1984; 1987), and so pH is unlikely to have a major role in the periodic mineralisation of otoliths.

6.3.4 Role of insoluble matrix

Both the rate of secretion of insoluble matrix (Chapter 3) and its deposition on to otoliths (Mugiya, 1987; Watabe *et al.*, 1982; Morales-Nin, 1987; Radtke, 1989) are variable, suggesting that calcium transport alone is not responsible for increment formation. The apparent interruption of the calcified layer by a sclerotized interlamellar layer in molluscs (Gordon & Carriker, 1980; Wilbur & Saleuddin, 1983) has been considered a major factor in the incremental growth of shell nacre. Close structural similarities between incremental growth in molluscan nacre and teleost otoliths (Chapter 3; Dunkelberger *et al.*, 1980; Watabe *et al.*, 1982) suggests the pattern of incremental growth may be common to both mineralising systems (Wilbur, 1980).

6.3.5 Role of soluble matrix

Potentially, the calcium-binding protein of soluble matrix may play a major role in regulating and inhibiting mineralisation. However further investigations are needed, particularly as to the regulation and occurrence of this protein in the interlamellar layer.

6.4 REGULATION OF INCREMENT RHYTHMICITY

6.4.1 Relation to other diurnal rhythms

The deposition of daily increments in Atlantic salmon parr under both natural (Chapter 2) and constant environmental regimes (Chapter 4) indicates that otolith formation is controlled by an endogenous circadian rhythm. Further the influence of light/dark cycles on increment zone formation (Chapter 4) suggests that this rhythm is entrained to the light/dark cycle. Entrainment to light-dark cycles suggests the involvement of the pineal-hypophysial complex. Endocrine secretion displays a circadian periodicity in many animals and through the intermediary of metabolic rate, ultimately controls most physiological processes (Simpson, 1978). Endocrinological studies have demonstrated diurnal variations in the levels of several hormones in the plasma of teleosts (Matty, 1985), including thyroxine (T_4) (Eales *et al.*, 1981) a hormone known to influence skeletal growth and calcification in rainbow trout (LaRoche *et al.*, 1966). Many metabolic processes are also known to fluctuate diurnally, including heart rate (Priede, 1978) and oxygen consumption (Ali, 1964). Ali (1964) has demonstrated that the diurnal rhythm of oxygen consumption persists when parr are held under constant darkness, suggesting an endogenous circadian regulation. Ali (1964) also reported that the nadir in oxygen consumption occurred at night. Considering the apparent relationship between oxygen consumption and otolith accretion (Chapter 5) and the reduction in otolith

calcification at night-time (Chapter 4), future investigations may benefit from an examination of the influence of diurnal variations in metabolism on increment formation. Interestingly, work on oxygen consumption rates in salmon embryos (Geffen, 1983) indicated that metabolic rate did not vary in a rhythmical fashion and otolith increments were not formed daily. Mosegaard *et al.* (1986) reported that daily increments were formed in the otoliths of salmon alevins and so it is possible that the onset of daily increment formation may correspond to the ontogeny of a rhythmical fluctuation in metabolic rate at the alevin stage.

Locomotory activity (Varanelli & McCleave, 1974; Richardson & McCleave, 1974; Erikkson, 1978) and feeding patterns (Higgins & Talbot, 1985; Rawlings & Talbot, pers. comm.) of salmon parr also vary diurnally. Feeding intensity has been shown to be entrained to light-dark cycles, increasing in relation to increasing light intensities (Rawlings & Talbot, pers. comm.). Work by Richardson & McCleave (1974) has suggested that the light-dark transition may be the zeitgeber for locomotory activity in Atlantic salmon parr.

As reported by Mosegaard (1986), but in contrast to the results of Taubert & Coble (1977) and Campana (1983a), the present study found that an abnormally short photoperiod could induce the formation of more than one increment per day in both Atlantic salmon parr and sticklebacks. Ali (1964) demonstrated that salmon parr held under a 6L:6D photoperiod, exhibited two cyclical fluctuations in oxygen

consumption, within 30 hours of being subjected to this regime. Studies of locomotor activity have also demonstrated that juvenile salmon co-ordinate their locomotor activity with times of sunrise and sunset, even if the light-dark transitions are shifted forward by 6 hours (Varanelli & McCleave, 1974) and parr may entrain to a 6L:6D regime after 2 - 3 days (Ali, 1964). Thus continuous 6L:6D cycles may confuse the animal and result in sub-daily activity cycles which may disrupt the normal diurnal rhythmicity.

6.4.2 Proximate influences on increment formation

Previous studies have emphasised the possible role of plasma calcium in regulating otolith formation (Mugiya *et al.*, 1981; Mugiya, 1984; 1986; 1987; Campana, 1983b). The effect of induced hypocalcemia found in the present study (Chapter 4) suggests an association between plasma calcium and otolith formation. However this association could arise from hormonal regulation common to both the plasma and sacculus. Such hormonal regulation could influence both ion transport and matrix production in the sacculus. Studies on shell mineralisation in the snail, *Lymnaea stagnalis* (Dogterom *et al.*, 1979; Dogterom & Doderer, 1981) have indicated that a growth hormone may regulate shell formation by controlling both the secretion of calcium binding protein and calcium ion concentration at the mineralising surface.

Even if hormonal regulation does not directly control otolith formation, as might be inferred from studies on

isolated sacculi (Mugiya, 1986), the effect of a decline in plasma calcium may act as a stimulus to matrix cells to increase secretion rather than to decrease ion transport to the otolith. Indeed it should be noted that induced hypocalcemia led to the formation of a matrix rich check, as well as a net calcium efflux from the otolith (Chapter 4).

6.5 OTOLITH GROWTH AND ITS RELATION TO SOMATIC GROWTH

The use of otoliths in backcalculating growth is based on the premise that otolith growth is related to somatic growth (Bagenal & Tesch, 1978). However investigations of otolith and somatic growth (Mosegaard *et al.*, 1988; Reznick *et al.*, 1989), including the present study, have often demonstrated that this assumption is not met. It seems, that unlike skeletal tissue, otolith growth persists at times of slow or no somatic growth (Marshall & Parker, 1982; Campana, 1983a; Mosegaard *et al.*, 1988). Indeed, as seen in Figure 5.6, the growth of otoliths may differ markedly from somatic growth. This, together with the apparent lack of calcium turnover from the otolith (Ichii & Mugiya, 1983; Mugiya & Uchiyama, 1989), suggests that the otolith is a protected structure. The protected nature of otolith accretion may be related to the otolith's function as a part of a sensory system. The otolith growth pattern in Atlantic salmon parr (Chapter 5) suggests that development of the otic sacs is maintained regardless of somatic growth. This maintenance of otic development is probably important to the animals sensory abilities.

Further otic growth does not appear to be maintained much beyond the parr stage, as the otolith mass of a one year old smolt, weighing ~ 12g is nearly as heavy as that of a 3Kg adult (Rowe & Wright unpubl. data).

In teleosts, daily increments have only been demonstrated in otoliths. This may be because incremental growth patterns in other skeletal tissues are lost due to mineral turnover. Glycine uptake into scales has been shown to vary diurnally in some species, indicating diurnal changes in protein deposition, although this variation may merely reflect a temperature induced response (Smith, 1986). ^{45}Ca uptake experiments suggest that calcification of skeletal tissue does not show the diurnal variation (Simkiss, 1974, Ichii & Mugiya, 1983; Appendix 3), that has been demonstrated for otoliths (Mugiya, 1987; Chapter 4). This raises the question of why otoliths grow in an incremental pattern. One expla nation may be related to the apparent stability of the tissue. The periodic deposition of an insoluble matrix layer may be a means of maintaining the integrity of the structure by reducing ion dissolution from the otolith surface. Circumstantial support for this idea has come from experiments that tried to induce ion resorption. Even severe acidosis of the endolymph causes only the dissolution of a single newly formed increment (Mugiya & Uchiyama, 1989). Clearly it is important to the sensory system that otoliths do not readily dissolve, as dissolution could affect the distance between otolith and epithelium. Similarly regulation of otolith accretion is important to maintain proportionality between otolith and

epithelium. This regulation may be brought about by the release of calcium-binding protein and the secretion of the matrix substrate, with one biproduct being daily increments.

6.6 FUTURE STUDIES

6.6.1 Potential use of daily increments for studying growth histories.

The relationship between metabolic rate and increment accretion means that the otolith potentially provides a chronological record of daily metabolism in Atlantic salmon parr. Consequently daily increments may be used to provide information on physical perturbations experienced by the parr, such as acid episodes. Indeed, experimental studies on the influence of acid episodes on growth in Chinook salmon, have demonstrated that sagitta growth rates are reduced when juveniles are exposed to low pH (<4.5) (Geen *et al.*, 1985). If the increments continue to be deposited daily following smolting, as has been suggested by Mosegaard *et al.* (1986) it may be possible to identify the precise age and time at which Atlantic salmon parr move out to sea, as has been shown in Chinook salmon (Neilson *et al.*, 1985).

The occurrence of daily increments in all life-history stages of the three-spined stickleback potentially provides a means of determining how differences in hatching time and in early growth rate effect subsequent development and reproductive success in this species.

6.6.2 *In vitro* culture of sacculi

In vitro culture of sacculi is clearly a desirable technique for studying the effects of extrinsic ion concentrations and hormonal influences on otolith formation. The effects on matrix secretion and deposition and otolith calcification could be studied using radiolabelling techniques and histochemistry. Mugiya (1986) has used radiolabelling and *in vitro* techniques to demonstrate that extrinsic calcium can effect otolith calcification in isolated sacculi of rainbow trout. However he failed to relate the experimental effect of calcium concentration to the natural range in calcium ion concentration found in perilymph, and so the significance of his results is unclear. Attempts in the present study to examine the influence of extrinsic calcium ion concentration using the *in vitro* techniques developed by Mugiya (1986) proved unsatisfactory because of tissue atrophy, possibly as a result of damage during the removal of the very small sacculi.

6.6.3 Significance of plasma calcium to otolith calcification

The significance of plasma calcium concentration to otolith calcification could be examined in large fish, which exhibit daily increments, from direct measurements of calcium ion concentration of the plasma and endolymph of individual fish. It should be noted that the measurement of ionic calcium and protein-free calcium (rather than just total calcium) is important, as ionic calcium is the only

form available for otolith calcification and calcium in mineralising tissues may be largely chelated to low molecular weight carbohydrates (Misoğianes & Chasteen, 1979).

6.7 CONCLUSIONS

In conclusion the work described in this thesis has demonstrated that:

1. Increments are formed daily in the sagitta of Atlantic salmon parr and of the three-spined stickleback.
2. Otolith calcification may be periodically limited by insoluble matrix and a calcium binding protein, and nucleation may involve a matrix containing sulphated glycoprotein andglycoaminosglycans.
3. Calcospherules are present throughout the sacculus and these may be involved in ion storage and transport.
4. The rhythm of increment formation is endogenously controlled and entrained to the light-dark cycle.
5. Induced hypocalcemia influences both plasma calcium concentration and otolith calcification, suggesting a possible direct relationship or a common hormonal regulation.
6. Otolith growth is not always proportional to somatic growth in Atlantic salmon parr and the three-spined stickleback.
7. Increment accretion is positively related to metabolic rate in Atlantic salmon parr.

Appendix 1

Estimation of apparent solubility product for endolymph

The general formula for solubility product cannot be used for body fluids as it is based on a simple solution. Miosgianes & Chasteen (1979) used an apparent solubility product to estimate ion saturation in mollusc extrapallial fluid. The apparent solubility product is defined as:

$$K'_{SP} = M_{Ca}^{2+} \cdot M_{CO3}^{2-}$$

where M is the molal concentration of the two ions.

An apparent solubility product was estimated for rainbow trout endolymph using the formula of Misogianes & Chasteen (1979) and the total calcium and total carbon dioxide concentrations reported by Mugiya & Takahashi (1985). Carbonate concentration was estimated by assuming that the total CO_2 is present as CO_2 and HCO_3. Values of apparent dissociation constant for carbonic acid at pH 8.24 (maximum pH) and 7.85 (minimum pH), 10°C, were calculated using a formula derived for rainbow trout plasma (Boutilier *et al.*, 1984). The value of HCO_3 concentration obtained (23.69 x 10^{-3} mM, pH 8.24; 23.29, pH 7.9) was similar to that reported for the plasma of freshwater teleosts (Heisler, 1984). Using the dissociation constant of HCO_3, a value for apparent solubility product of; $K'_{sp} = 4.14 \times 10^{-7}$ mol^2 kg^{-2} was obtained for a pH of 8.24 and a value of $K'_{sp} = 1.86 \times 10^{-7}$ mol^2 kg^{-2} was obtained for a pH of 7.9. As these values were based on total rather than ionic calcium concentration, these values are likely to overestimate the apparent solubility product. Nevertheless both values are

less than the minimum apparent solubility product required for saturation of calcium carbonate (= 4.5×10^{-7} mol^2/kg^2 $\times 10^{-7}$ mol^2/kg^2; Misogianes & Chasteen, 1979). So it would appear that overall, the endolymph of rainbow trout is undersaturated, and crystals would not be expected to be formed. This would suggest that there are regional differences in ion concentration in the endolymph, with ion concentration being elevated at the otolith surface. Further, the effect of pH on solubility product should be noted as the cyclical change in pH (7.85-8.24) reported by Mugiya & Takahashi (1985) would be expected to siginificantly effect the degree to which calcium concentration at the otolith surface has to be elevated for crystallisation to occur.

Appendix 2

The early growth of the three-spined stickleback, *Gasterosteus aculeatus*.

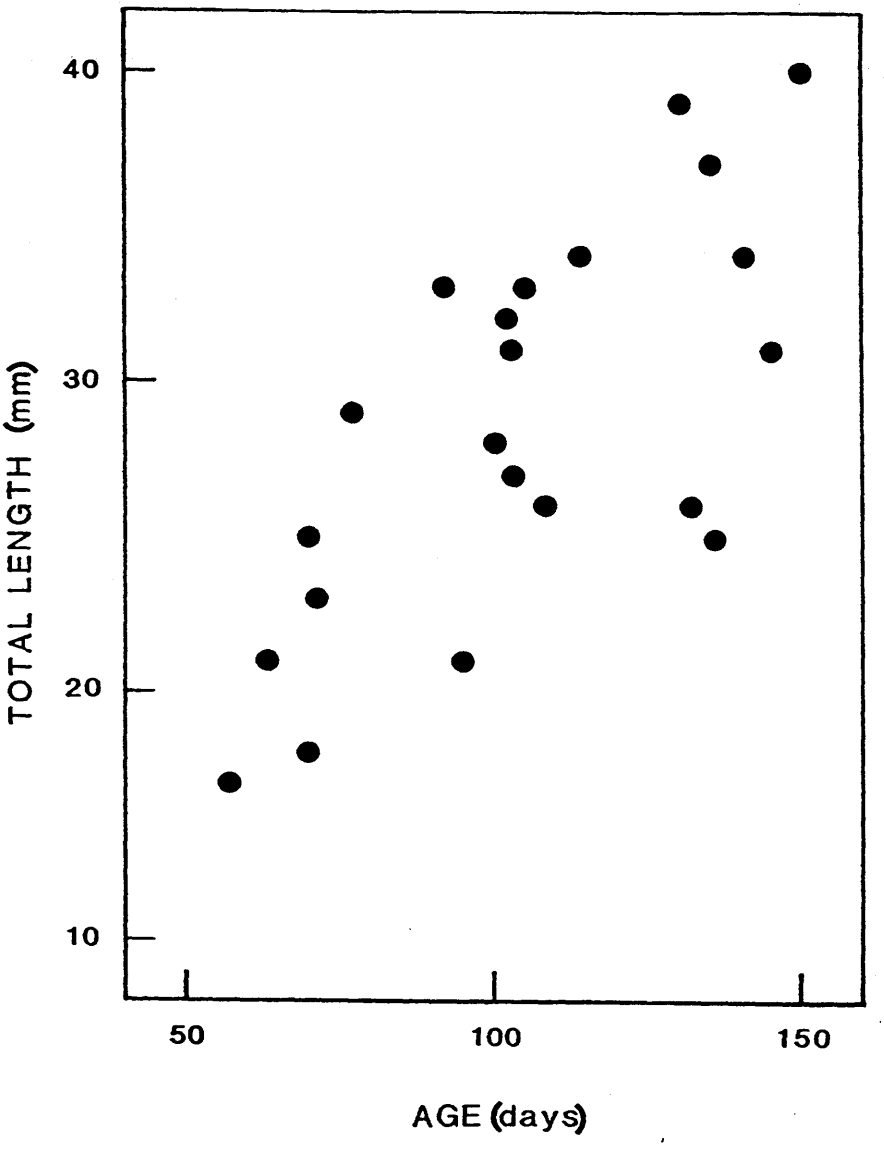
Daily increments have been particularly beneficial to growth studies of species with a protracted breeding season and short-life span as the overlap in modal size groups, arising from differences in hatching dates, make analysis from size-frequency data difficult (Struhsaker & Uchiyama, 1976; Isely & Noble, 1987; Wright *et al.*, 1990). Scottish populations of *Gasterosteus aculeatus* are generally annual (Ukegbu, 1986) and breed between April and July. The growth of sticklebacks has been investigated from length-frequency data and from sub-annual increments in the otoliths (Jones & Hynes, 1950, Mann, 1971; Allen & Wootton, 1981; Ukegbu, 1986). However both methods have their limitations (Wootton, 1984) as there is considerable overlap between size and age categories (Jones & Hynes, 1950) and otolith growth increments cannot always be related to seasonal growth patterns (Ukegbu, 1986). For this reason daily increments were counted in otoliths collected in a previous survey of a stickleback population from the River Kelvin, Glasgow (Ukegbu, 1986) inorder to construct an age-length curve (Figure A2.1).

Increment counts were made for fish collected in four monthly samples, between June and September, 1983, according to procedures described in Chapter 2. The age-length curve derived from these counts indicates that

Figure A2.1

Relationship between total length and age (as determined from daily increments) in three-spined sticklebacks from the River Kelvin, Glasgow.

Points represent individual data sets. Length data and otoliths provided by A. Ukegbu.

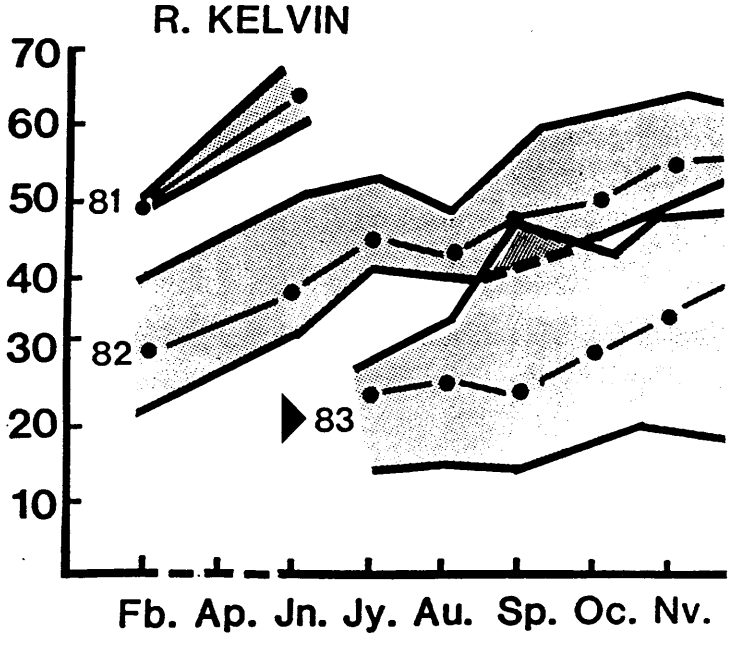

40
30
20
10
TOTAL LENGTH (mm)
50
100
150
AGE (days)

sticklebacks were growing at approximately 0.233 mm/day between the ages of 50 and 150 days old. This contrasts with the relatively slow growth predicted by changes in modal length (Figure A2.2; courtesy of Angela Ukegbu). The difference in growth estimates can be explained by the wide age range (40 - 100 days) of fish collected in monthly length-frequency samples. Consequently length-frequency analysis does not appear to be suitable for studying the early growth of this species.

Figure A2.2

Changes in modal length within monthly samples of three-spined sticklebacks from the River Kelvin.

Figure taken from Ukegbu (1986) and shows modal length and range of 1983 samples from which increment counts were made.


R. KELVIN
70
60
50
40
30
20
10
81
82
83
Fb. Ap. Jn. Jy. Au. Sp. Oc. Nv.

Appendix 3

Comparison of calcium-45 uptake in vertebrae and otoliths

Introduction

To determine if skeletal tissue exhibited a periodic reduction in calcification as seen in otoliths (Chapter 4), samples of vertebrae and otoliths were taken in a replicate of experiment 2 (no equilibration; night-time introduction of isotope: see Chapter 4).

Methods

Mean calcium-45 accumulation on to sagittae and vertebrae was determined from serial samples of salmon parr held under similar conditions to experiment 2 (18L : 6D; 0400 - 2200h, $10^{o}C$). ^{45}Ca was added to the experimental tank at 2400h to give an activity of $37KBql^{-1}$. Samples of 5 fish were removed and killed at four, eight and twelve hours, following the introduction of the isotope. Fish were steamed for 30 seconds and the vertebrae were dissected out, cleaned of connecting tissue and dried to constant weight at $70^{o}C$. Vertebrae were weighed to the nearest mg and ground to a powder in an eppendorf tube, with a small pestle. The ground tissue was allowed to dissolve in perchloric acid (0.1ml/0.1g of tissue) and hydrogen peroxide (0.2ml/0.1g of tissue) for 24h, according to the procedure of Mahin & Lofberg (1966).

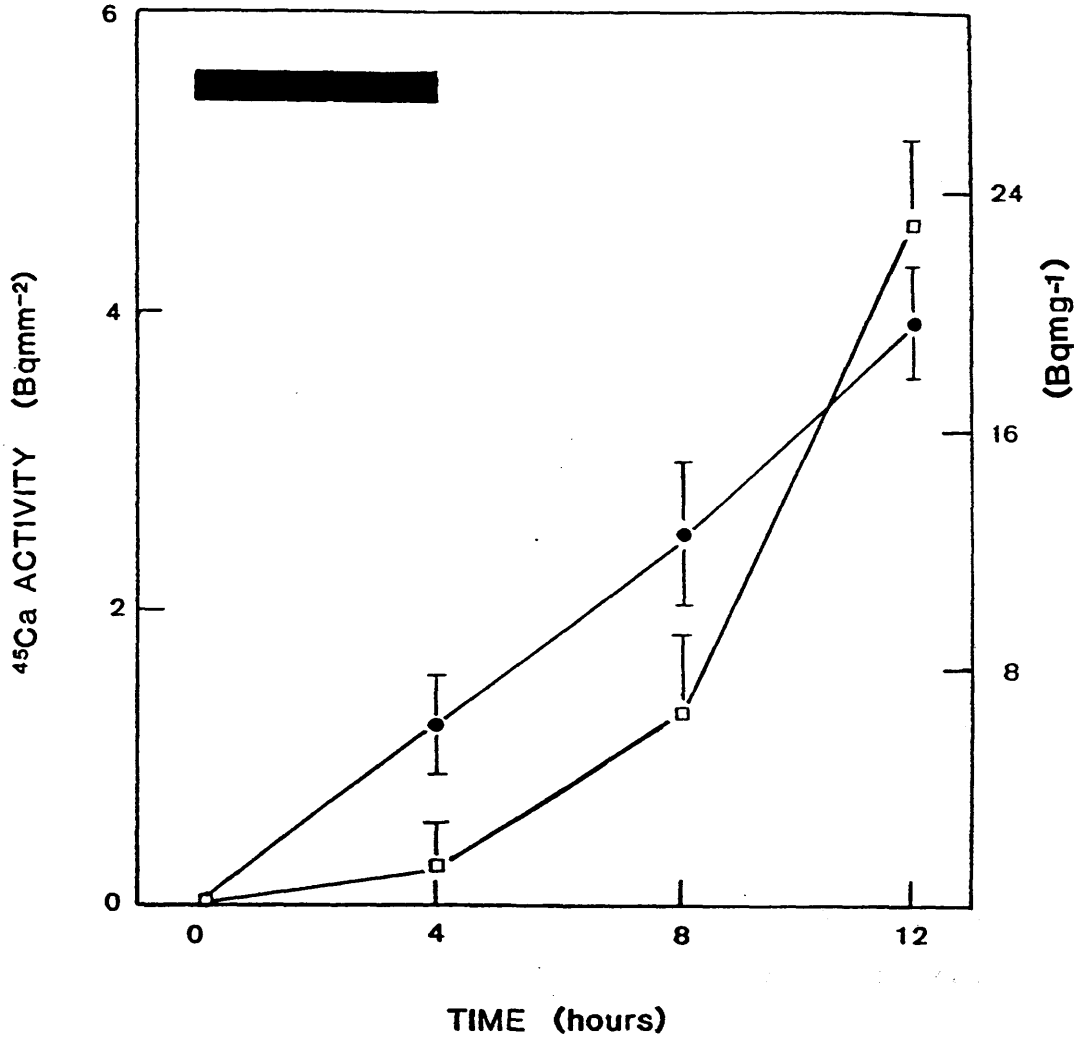
Results and Discussion

Mean sagitta activity did not increase appreciably until following the dark-light transition (Figure A3). The increase in activity during the dark phase may have been due to the relatively early introduction of isotope (introduced at 2 hours after the dark-light transition as opposed to 3 hours as used in earlier replicates). However mean activity of vertebrae increased at an almost linear rate during the experiment. So it would appear that that vertebrae do not exhibit the same pattern of incremental growth that is seen in the otoliths.

Figure A3

Relation between mean activity of calcium-45 deposited on to salmon parr (*Salmo salar*) sagittae (□) and vertebrae (●) and time following introduction, in parr maintained at 18L:6D, $10^{O}C$ (isotope introduced at 1000h).

Data presented as mean ^{45}Ca activity per mm^2 of sagitta (left y-axis) and per mg of ground vertebra (right y-axis) for 5 individuals (bars represent 95% confidence limits). Horizontal bar represents dark period.


6
4
2
0
0
4
8
12
24
16
8
45Ca ACTIVITY (Bqmm-2)
(Bqmg-1)
TIME (hours)

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