STUDIES ON THE BIOLOGY OF *SPHAEROSPORA* SP.

(MYXOZOA: MYXOSPOREA) FROM FARMED ATLANTIC

SALMON, *SALMO SALAR* L. IN SCOTLAND

A thesis presented for the degree of

Doctor of Philosophy to the University of Stirling   1994

by

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August, 1994
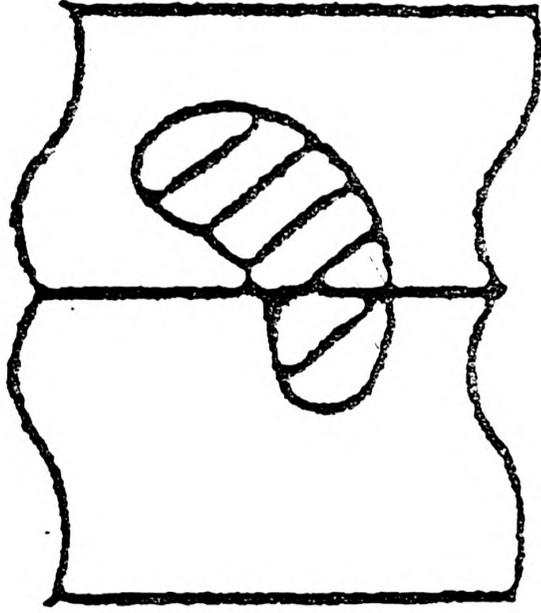
DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

ACKNOWLEDGEMENTS

DEDICATION

To my mother

for her devotion and support

To my father

for the time we had together

To Helen

for all her help, and all her love

ABSTRACT

The life cycle, morphology, development, epidemiology and pathology of a previously unreported myxosporean parasite of Atlantic salmon is described. The parasite infects fish at a number of freshwater smolt-producing hatcheries across Scotland.

A three year epidemiological sampling programme indicated that the parasite had two distinct life cycle phases in the fish. The first, an extrasporogonic stage, was first detected in late June/early July, though a retention experiment showed that infections were pre-patent for two to four weeks prior to their becoming detectable. Extrasporogonic stages rapidly rose in prevalence in a single cohort; this was related to a presumed synchronous release of infective agents from alternate hosts in the rivers supplying farms, perhaps as a result of a temperature stimulus. Extrasporogonic stages were found in the circulating blood, liver sinuses and spleen, but were concentrated in the interstitium of the kidney. Such stages measured $10-60\mu$m in diameter and consisted of a primary cell containing 1-120 secondary cells in its cytoplasm. Secondary cells could contain one, or two, tertiary cells. Ultrastructural observations noted that secondary and tertiary cells were situated in vacuoles in the cytoplasm of primary and secondary cells respectively. Primary cell nuclei showed marked developmental changes in appearance, from early released stages with few secondary cells through to more advanced ones containing many secondary cells. Tertiary cells appeared to be formed by two means; endogenous cleavage of the secondary cell, and the engulfment of one secondary cell by another. The extrasporogonic stages were responsible for morbidity, mortality and histopathological changes. The role of the extrasporogonic stage as a stressor, rendering fish more susceptible to secondary invaders and lowering tolerance to environmental factors and additional stresses was discussed. The host response

resulted in the engulfment and/or the attachment and destruction of some extrasporogonic stages by leucocytes. Extrasporogonic stages were last detected in early September in cohorts.

Sporogonic stages first became detectable in the kidney tubules in late August. The earliest stage comprised an enveloping pseudoplasmodial cell containing two sporoblast cells. This appeared homologous to the secondary cells containing two tertiary cells formed in, and released by, extrasporogonic stages. These stages appeared to reach the kidney tubules via two routes; penetrating between adjacent cells of the basal lamina of the kidney tubule, and by the disruption of the glomerular capillaries followed by entrance to the tubule via the Bowman's space. Glomerular means of entry was more common and caused histopathological changes. Although some tubules were completely occluded by sporogonic stages, they caused little pathology. The two sporoblast cells of each pseudoplasmodium subsequently divided until twelve were present; the twelve then differentiated to form two spores. Sporogenesis was described from both light and electron microscopical observations.

The parasite was identified as a member of the genus *Sphaerospora* by virtue of its spore morphology and dimensions, and presence of an extrasporogonic stage, but showed close affinities with *Leptotheca*. Spores were considerably more broad and deep when immature. Experimental transmissions via the IP-injection of kidney suspensions containing extrasporogonic stages resulted in infections in naive Atlantic salmon and brown trout but not in rainbow trout. Spores and sporogonic stages were not transmissible by IP-injection or orally. The *Sphaerospora* sp. from Atlantic salmon was compared with related species in the literature, especially PKX and *S. truttae*. Despite a close similarity to *S. truttae*, the parasite was not assigned to this species due to a lack of information on *S. truttae* ultrastructure and extrasporogonic stages, and the presence

of intracellular sporogonic development in *S. truttae*.

Studies of infection intensity showed distinct variations between individual farms. A number of aspects of the epidemiology of the salmon *Sphaerospora* pointed to a limited potential for proliferation for individual extrasporogonic stages. Studies of salmon cohorts with different infection histories indicated that previously infected fish were not susceptible in their second year. Fish in their first year, and year old fish not previously exposed, were susceptible. Mature spores could be found in fish held on freshwater away from any source of reinfection 18 months after they had first been detected. However spores were lost from the kidney tubules of fish within three months of their transfer to sea-cages as smolts.

A study of the habitats at and around infected farms for actinosporean life cycle stages in alternate hosts resulted in the discovery of five species of actinosporean of four distinct genera. All species were detected from the oligochaete population of a settlement pond at one farm. Two were identified to species level, *Synactinomyxon longicauda* and *Triactinomyxon mrazeki*, but the remainder appeared to be new species of the genera *Aurantiactinomyxon*, *Raabeia* and *Synactinomyxon*. Polar filaments of *Aurantiactinomyxon*, *Raabeia*, *Synactinomyxon* sp. *1* and *Synactinomyxon longicauda* everted in response to mucus from Atlantic salmon, brown trout and bream. In the case of *S. longicauda*, the sporoplasm of spores hatched and was motile. The spore release patterns of worms infected with *Aurantiactinomyxon* sp. were studied and showed that peak release occurred during the night. The ultrastructure of *Aurantiactinomyxon* was described, polar capsule formation being particularly unusual.

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Chapter 1
General introduction

1   GENERAL INTRODUCTION

1.1   General introduction

Myxosporeans are generally regarded as parasites of cold-blooded aquatic vertebrates (Mitchell, 1977).   The vast majority of the more than 1200 species descriptions to date have been from teleost fish in both the freshwater and marine environments (Lom, 1987; Cone, 1992), although a number of reports exist of species of the Class parasitic in invertebrates (Kudo, 1920; Overstreet, 1976).

Individual myxosporean species usually appear to show a degree of host and tissue specificity, but as a Class have been identified from virtually all fish species and fish tissues examined.

In wild populations of fish, epizootics appear to be uncommon, or are at least rarely reported (Mitchell, 1977) since presumably in nature there exists the normal interactive balance exhibited by most host-parasite systems.   However, a number of marine myxosporeans may cause significant problems in wild fisheries (Alvarez-Pellitero & Sitja-Bobodilla, 1992).   Where artificially created intensive culture regimes have been developed, or are developing, myxosporeans are frequently and increasingly being encountered as significant pathogens (Dykova & Lom, 1988b; Lom & Dykova, 1992d; El-Matbouli, Fischer-Scherl & Hoffmann, 1992b; Dykova, 1992; Körting, 1992; Alvarez-Pellitero & Sitja-Bobadilla, 1992; 1993).

1.1.1   Higher taxonomy

The myxosporean spore is formed by several cells of three functionally and morphologically differentiated types.   Trophozoite stages also exceed the simple unicellular level exhibited by most protistan groups (Lom, 1987).   The term

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'pluricellular' was coined to describe this level of cellular organisation and distinguish it from the multicellularity of metazoans (Grassé, 1970; Grassé and Lavette, 1978).

Myxosporeans have been studied for over a century. Shulman (1966; English translation, 1990) gave an account of the history of their study and classification. More recently, comprehensive reviews and articles have been produced by Mitchell (1977), Lom (1987), Lom & Dykova (1992d) and El-Matbouli *et al*. (1992b).

Grassé (1970) proposed that pluricellularity justified the removal of the Myxosporidia, along with the related Actinomyxidia, into a new taxon, the Myxozoa. However, it was not until the classification of the Protozoa as a whole was revised by Levine, Corliss, Cox, Devoux, Grain, Honigberg, Leedale, Loeblich, Lom, Lynn, Merinfield, Page, Poljansky, Sprague, Vavra & Wallace in 1980 that the Myxozoa became accepted as one of seven separate phyla within the protistans as follows:-

<u>**Kingdom**</u>	**Protista**
<u>**Sub-Kingdom**</u>	**Protozoa**
<u>**Phylum**</u>	**Myxozoa Grassé, 1970**
<u>**Classes**</u>	**1. Myxosporea Bütschli, 1881**
	2. Actinosporea Noble, 1980

The current definition of the phylum Myxozoa is:-

> "spores of multicellular origin, with one or more polar capsules and sporoplasms; with 1, 2 or 3 (rarely more) valves; all species parasitic"
>
> (Levine *et al*., 1980)

Interestingly, there exists a limited amount of evidence supporting the assumption that myxosporeans may have a metazoan evolutionary ancestry. According to Lom (1969a) and Lom & dePutorac (1965) there are distinct similarities in structure and development between the myxosporean polar capsule and the nematocyst of coelenterate cnidarians. Ultrastructurally, double septate junctions and aggregates of transmembrane particles have been shown in freeze fracture plates of myxosporean spores by Desportes-Livage & Nicolas (1990), which are also found in the cnidarian epithelial membrane. However, in general, the cnidarian link has still to be explored in detail (Lom & Dykova, 1992a).

1.1.2. Taxonomy of the class

The class Myxosporea was defined by Lom & Dykova (1992d) as:-

> "Parasites characterised by spores composed of several cells transfigured into 1 to 7 spore shell valves, 1 to 2 amoeboid infective germs (sporoplasms) and 2 to 7 nematocyst-like polar capsules. The latter contain an extrudable filament with an anchoring function. Pluricellularity and morphological and functional specialisation of cells also found in the trophozoite stages... During the life cycle, some cells (the generative ones) are enclosed within the others (somatic cells). Parasites of organ cavities [coelozoic] and tissues [histozoic] of fish... amphibians, reptiles and invertebrates."

The taxonomy of the class Myxosporea was revised by Lom & Noble (1984). These authors expressed concern with respect to the almost exclusive, but necessary,

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reliance on spore morphology to differentiate taxa (see chapter 3). This meant that their classification was designed as a logical improvement upon existing schemes rather than a phylogenetically based system. It was therefore emphasised that further, perhaps radical revision of taxonomy might become necessary in the future once features such as vegetative stages, presporogonic [extrasporogonic] stages and intermediate [alternate] hosts became more clearly understood.

At present the classification of taxa (fig.1.1) remains based on the morphology and morphometrics of the spore, in terms of the number, nature and relative positions of the suture, polar capsules and valves, and the absence or presence of valvular projections. Spores often possess mucous envelopes which swell on their release into water. These probably enhance floating ability and thus aid dispersion (Lom and Vavra, 1963). Polar filament coil number and angle of their coils are useful taxonomic features (Lom, 1969b). Filaments can be caused to evert by the use of concentrated urea (Lom, 1964).

Most myxosporean species develop in either histozoic or coelozic locations within the host. Some groups, such as *Myxobilatus* and *Hoferellus* may have histozoic intracellular or intercellular developmental stages in, for example, the tubular epithelium of the kidney which are followed by a coelozoic sporogonic phase in the tubule lumen of the same organ (Molnar, 1988a).

1.1.3 Life history of myxosporeans

Another set of advances in our understanding of the life cycle of myxosporeans has completely overturned the established views of the scientific community and further illustrated the unpredictable nature of research on this group of organisms.

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fig.1.1   Classification within the Myxosporea

Class **MYXOSPOREA**

1. Order Bivalvulida

    sub order Sphaeromyxina
        family Sphaeromyxidae
            genus *Sphaeromyxa*
    sub order Variisporina
        family Myxidiidae
            genera *Myxidium, Zschokkela, Coccomyxa*
        family Ortholineidae
            genera *Ortholinea, Neomyxobolus*
        family Sinuolineidae
            genera *Sinuolinea, Davisia, Myxoproteus, Bipteria, Shulmania*
        family Fabesporidae
            genus *Fabespora*
        family Ceratomyxidae
            genera *Leptotheca, Ceratomyxa*
        family Sphaerosporidae
            genera *Sphaerospora, Hoferellus, Wardia, Palliatus, Myxobilatus*
        family Chloromyxidae
            genera *Chloromyxum, Caudomyxum, Agarella*
        family Auerbachiidae
            genera *Auerbachia, Globospora*
        family Alatosporidae
            genera *Alatospora, Pseudoalatospora*
        family Parvicapsulidae
            genera *Parvicapsula, Neoparvicapsula*
    sub order Platysporina
        family Myxobolidae
            genera *Myxobolus, Henneguya, Thelohanellus, Unicauda, Dicauda,*
                Phlogospora,Neohenneguya, Trigonosporus

2. Order Multivalvulida

        family Trilosporidae
            genera *Trilospora, Unicapsula*
        family Kudoidae
            genus *Kudoa*
        family Pentacapsulidae
            genus *Pentacapsula*
        family Hexacapsulidae
            genus *Hexacapsula*

Until recently the fish to fish transmission of myxosporeans remained a mystery. However, Markiw & Wolf (1983) reported that rather than being direct, as had always been assumed, the life cycle of *Myxobolus cerebralis*, the causative agent of salmonid whirling disease, involved a second spore forming stage in an oligochaete worm. The spores formed in, and released by the worm were of the myxozoan class Actinosporea, and were infective for salmonids. The myxosporeans and actinosporeans had previously been classified as separate classes in the phylum Myxozoa. Initially these studies proved to be extremely controversial with some workers being unable to reproduce such results (Hamilton & Canning, 1987), and it was not until the last five years that the first experimental verification of the two host life cycle was reported (El-Matbouli & Hoffmann, 1989). This aspect of myxosporean biology remains an emerging science with few species' life cycles completed, many questions unanswered and many uncertainties as to its applicability to all species (Lom & Dykova, 1992a).

In the last fifteen years another set of research has established the existence of extrasporogonic proliferative cycles in some myxosporean genera such as *Sphaerospora* and *Chloromyxum*, mainly those parasitic in cyprinids in Europe (Lom, Dykova & Pavlaskova, 1983; Lom, Pavlaskova & Dykova, 1985; Baska & Molnar, 1988). There are few complete descriptions of such stages to date, and many aspects of their identity, biology and ultrastructure remain to be elucidated. Although the first of these vegetative stages to be found was reported from common carp by Csaba in 1976 their definite role in the life cycle of *Sphaerospora renicola* was not established for a number of years (Molnar, 1984; 1988b). However, even within genera in which extrasporogonic stages have been found, it is not known whether all species have such stages or not, and few extrasporogonic stages have been positively identified to species level.

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These life cycle advances, many of which are even now regarded by some workers as controversial, may revolutionise our understanding of the biology and taxonomy of this group of organisms, and by increasing our knowledge of the host-parasite relationship help develop new strategies for control.

1.1.4   Background to the present study

A protozoan parasite has been detected in recent years in the kidneys of Atlantic salmon from a number of Scottish smolt producing hatcheries with increasing frequency. At some sites, managers considered this to be a significant pathogen.   A preliminary investigation into the parasite's taxonomic position suggested that it could belong to the myxosporean genus *Sphaerospora* (Branson, 1987).   Kidney infecting species of myxosporeans in UK salmonids have been very rarely reported (Kennedy, 1974; Wootten & Smith, 1980) other than those of the important and enigmatic pathogen PKX, the causative agent of proliferative kidney disease (PKD) of salmonids.

The aim of the present study was therefore to investigate the biology of the parasite by a range of techniques and approaches.   These would include detailed morphological and ultrastructural studies of the parasite's development, combined with a long term sampling programme in order to detect and describe all stages of the parasite in the salmon host and establish their epidemiology and pathogenic potential; experimental infections to determine the parasite's host range; and studies of farms harbouring infections to try to find and study potential oligochaete alternate hosts and/or actinosporean alternate life cycle stages.

Chapter 2
Life cycle in the fish

2   LIFE CYCLE IN THE FISH

2.1   INTRODUCTION

2.1.1   The taxonomic position of *Sphaerospora* spp

Myxosporean parasites of the genus *Sphaerospora* (Thélohan, 1892) have received particular attention in recent years due to the fact that in a variety of fish species, especially those cultured under intensive or semi-intensive conditions, a number of *Sphaerospora* spp have been held responsible for significant mortalities and are therefore of economic consequence.

Since the revised classification of the class Myxosporea (Lom & Noble, 1984), the genus *Sphaerospora* has been further redefined (Lom *et al.*, 1985b) to include:

"myxosporeans having spherical or subspherical, anteriorly pointed spores. The two shell valves adhere together along a straight meridional suture. The thickened borders of the valves may be markedly elevated anteriorly. The two anteriorly located polar capsules are situated in a level parallel to the sutural plane and open apically, close to the suture line. The surface of the shell valves may bear ridges or short, blunt or pointed projections. There are two uninucleate sporoplasms.

The vegetative stages of the sporogonic cycle are pseudoplasmodia formed by uninucleate primary cells containing one to twelve sporogonic cells, and are, according to species, mono- or disporous. They live mostly in the lumen of the urinary tract with prevailing affinity to the renal tubules, in which they may occur also intracellularly. Some species live in the gill and skin tissues. Their hosts are freshwater and marine fish.

In some species, vegetative reproduction may also proceed in the blood and tissues of other organs, including the swimbladder and the eye. In these repetitive cycles, secondary cells reproduce within the primary cells up to a certain number. This number and the morphology of the stages is different in various organs and, also, in different hosts."

Lom *et al.*(1985b) suggested that if these repetitive, vegetative cycles from the

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blood and other organs, now known as extrasporogonic cycles, were only found in a restricted range of *Sphaerospora* spp the genus might have to be split. However they considered it possible that all species had such stages.

The higher taxonomic affinities of the genus remain as outlined by Lom & Noble, (1984):

Class	MYXOSPOREA	Bütschli, 1881
Order	BIVALVULIDA	Shulman, 1959
sub-Order	VARIISPORINA	Lom & Noble, 1984
Family	Sphaerosporidae	Davis, 1917
Genus	*Sphaerospora*	Thélohan, 1892

The type species for the genus was emended to *Sphaerospora elegans* (Thélohan, 1892) by Lom, Dykova, Pavlaskova & Grupcheva (1983).

Although there are reports of *Sphaerospora* infections of aquatic amphibians and reptiles (Desser, Lom & Dykova, 1986), the vast majority of species have been reported from teleost fish. One report exists of *Sphaerospora* from an elasmobranch (Arthur & Lom, 1985).

Arthur & Lom (1985) listed 36 species of *Sphaerospora* that conformed to the generic definition (table 2.1). Another 14 species have since been assigned (table 2.2). The majority of species described to date, 39, are from freshwater fish, with one brackish and ten marine reports.

As in other genera of Myxosporea, new species of *Sphaerospora* have been described on the basis of very small variations in spore morphology, or because they

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table 2.1   Species assigned to the genus *Sphaerospora* as reported by Arthur & Lom, (1985)

	Species	Authority	Habitat
1.	*Sphaerospora amurensis*	Akhmerov, 1960	FW
2.	*Sphaerospora angulata*	Fujita, 1912	FW
3.	*Sphaerospora araii*	Arthur & Lom, 1985	Marine
4.	*Sphaerospora brevis*	Polyansky, 1955	Marine
5.	*Sphaerospora branchialis*	Razmashkin & Skripchenko, 1967	FW
6.	*Sphaerospora carassi*	Kudo, 1920	FW
7.	*Sphaerospora chinensis*	Lom, Dykova, Pavlaskova & Grupcheva, 1983	FW
8.	*Sphaerospora cristata*	Shulman, 1962	FW
9.	*Sphaerospora dogieli*	Shulman, 1962	FW
10.	*Sphaerospora elegans*	Thélohan, 1892	FW/Marine
11.	*Sphaerospora galinae*	Evlanov, 1981	FW
12.	*Sphaerospora gasterostei*	Schuurmans-Stekhoven, 1920	FW
13.	*Sphaerospora gobionis*	Lom, Pavlaskova & Dykova, 1985	FW
14.	*Sphaerospora hupehensis*	Lee & Nie, 1965	FW
15.	*Sphaerospora hypophthalmichthydis*	Chen & Hsieh, 1984	FW
16.	*Sphaerospora krogiusi*	Lom & Noble, 1984	FW
17.	*Sphaerospora markewitschi*	Donets, 1962	FW
18.	*Sphaerospora masovica*	Cohn, 1902	FW
19.	*Sphaerospora minima*	Kashovsky, 1974	FW
20.	*Sphaerospora minuta*	Konovalov, 1967	FW
21.	*Sphaerospora molnari*	Lom, Dykova, Pavlaskova & Grupcheva, 1983	FW
22.	*Sphaerospora notropis*	Fantham, Porter & Richardson, 1939	FW
23.	*Sphaerospora pectinacea*	Bocharova & Donets, 1974	FW
24.	*Sphaerospora periophthalmi*	Fantham & Porter, 1943	Marine
25.	*Sphaerospora perlata*	Gurley, 1894	FW
26.	*Sphaerospora petruschewskii*	Shulman, 1962	FW
27.	*Sphaerospora poljanskii*	Kulemina, 1969	FW
28.	*Sphaerospora reichnowi*	Jacob, 1953	FW
29.	*Sphaerospora renalis*	Bond, 1938	Marine
30.	*Sphaerospora renicola*	Dykova & Lom, 1982	FW
31.	*Sphaerospora rostrata*	Thélohan, 1895	Marine
32.	*Sphaerospora rota*	Zaika, 1962	FW
33.	*Sphaerospora sapae*	Donets, 1962	FW
34.	*Sphaerospora schulmani*	Allamuratov, 1966	FW
35.	*Sphaerospora sphaerica*	Dogiel, 1948	Marine
36.	*Sphaerospora tincae*	Plehn, 1925	FW

table 2.2   New species of *Sphaerospora* assigned since Arthur & Lom, (1985)

	Species	Host	Authors	
1.	*Sphaerospora colomani*	*Acipenser ruthenus*	Baska, 1990	FW
2.	*Sphaerospora dicentrarchi*	*Dicentrarchus labrax*	Sitja-Bobadilla & Alvarez-Pellitero, 1992	Marine
3.	*Sphaerospora diminuta*	*Lepomis gibbosus*	Li & Desser, 1985	FW
4.	*Sphaerospora epinephali*	*Epinephalus malabaricus*	Supammataya, Fischer-Scherl, Hoffmann & Boonyaratpalin, 1991	Brackish
5.	*Sphaerospora gobionis*	*Gobio gobio*	Lom, Pavlaskova & Dykova, 1985	FW
6.	*Sphaerospora hankai*	*Ictalurus nebulosus*	Lom, Desser & Dykova, 1989	FW
7.	*Sphaerospora ictaluri*	*Ictalurus punctatus*	Hedrick, McDowell & Groth, 1990	FW
8.	*Sphaerospora inequalis*	*Clarias lazera*	Landsberg, 1986	FW
9.	*Sphaerospora ohlmacheri*	*Rana catesbeiana*	Desser, Lom & Dykova, 1986	FW
10.	*Sphaerospora paulini*	*Semotilus atromaculatus*	Lom, Desser & Dykova, 1989	FW
11.	*Sphaerospora scardinii*	*Scardinius erythrophtalmus*	El-Matbouli & Hoffmann, 1992	FW
12.	*Sphaerospora testicularis*	*Dicentrarchus labrax*	Sitja-Bobadilla & Alvarez-Pellitero, 1992	Marine
13.	*Sphaerospora truttae*	*Salmo trutta*	Fiscer-Scherl, El-Matbouli & Hoffmann, 1986	FW
14.	*Sphaerospora sp.*	*Sparus aurata*	Sitja-Bobadilla, Franco-Sierra & Alvarez-Pellitero, 1992	Marine

have been found in a new host species, new site, or geographic location. It has been proposed on a number of occasions that members of the genus *Sphaerospora* have a very restricted host specificity, with many species described from only a single fish species (Arthur & Lom, 1985; El-Matbouli & Hoffmann, 1992).

This degree of specificity has been confirmed in studies of extrasporogonic blood stages of *Sphaerospora* spp. from related fish species such as cyprinids, where despite having almost indistinguishable spore forms in the kidney of their respective hosts, their extrasporogonic stages appear different both morphologically and in terms of development (Lom *et al.*, 1985b; Baska & Molnar, 1988; Kepr & Trsova, 1989).

Confusion has also existed in terms of generic identity. A number of *Sphaerospora* spp. were previously assigned to *Leptotheca* (*S. brevis, S. krogiusi, S. perlata*) or to *Podospora, Myxoproteus* or *Chloromyxum*; conversely a large number of species previously known as *Sphaerospora* have been transferred to other genera such as *Mitraspora, Myxobolus* and *Ortholinea* (Arthur & Lom, 1985). Lom & Noble (1984) expressed their concern at the almost exclusive, but necessary, reliance on spore morphology and morphometrics to differentiate myxosporean taxa and indicated that major taxonomic revision might become necessary once non-spore related features became more clearly understood.

2.1.2 Morphology

Sporogonic stages

Sporogony in *Sphaerospora* spp has been well described at the light microscope level. Dykova & Lom (1982) outlined sporogonic development in *S. renicola* from common carp, as did Lom *et al.*, (1983a). Desser *et al.*, (1986), Feist, Chilmonzyk &

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Pike (1991) and El-Matbouli & Hoffmann (1992) described the sporogonic stages of *Sphaerospora ohlmacheri* from bullfrog tadpoles, *Sphaerospora elegans* from stickleback and *Sphaerospora scardinii* from rudd respectively. At the electron microscope level, the ultrastructure of development has been described for *Sphaerospora* sp. in goldfish (Hamilton, 1980), *Sphaerospora renicola* in common carp (Lom, Dykova & Lhotakova, 1982), *Sphaerospora angulata* and *Sphaerospora carassii* in common carp (Desser, Molnar & Horvath, 1983), *Sphaerospora tincae* and *Sphaerospora galinae* from tench (Lom, Körting & Dykova, 1985), *Sphaerospora ictaluri* from channel catfish (Hedrick, McDowell & Groff, 1990), *Sphaerospora epinephali* from grouper (Suppamattaya, Fischer-Scherl, Hoffmann & Boonyaratpalin, 1990; 1991; 1993), *Sphaerospora elegans* from stickleback (Feist *et al.* 1991), *Sphaerospora dicentrarchi* from Mediterranean sea bass (Sitja-Bobadilla & Alvarez-Pellitero, 1992b), and *Sphaerospora* sp. from gilt head sea bream (Sitja-Bobadilla, Franco-Sierra & Alvarez-Pellitero, 1992).

Myxosporeans of the genus *Sphaerospora* differ markedly from many other genera, as spores are not formed in macroscopic cyst-like trophozoites or plasmodia, but singularly or in pairs in small, monosporous or disporous pseudoplasmodia. Large polysporic trophozoites of genera such as *Myxobolus* contain large numbers of vegetative nuclei and generative cells. Within the trophozoite, many pansporoblasts are produced, often by the envelopment of one generative cell by another (Lom and Dykova, 1992d). The inner cell of each pansporoblast then proceeds to divide and differentiate within the outer one, to produce two spores in the case of disporous species, or one spore in monosporous species. Thus a polysporic plasmodium contains many pansporoblasts and a large number of spores. However Lom *et al.* (1982) studied the ultrastructure of sporogonic stages of *Sphaerospora renicola* from carp, and compared sporogenesis with

that of genera producing spores in large plasmodia. They demonstrated that the earliest stage of *S. renicola* in the kidney tubule, which is a single enveloping pericyte surrounding an inner sporoblastic cell could be considered homologous to the individual pansporoblast units in other genera. Since the enveloping cell of the *Sphaerospora* sporogonic stages contains just one (vegetative) nucleus, they cannot be classed as true plasmodia. The term "pseud plasmodium" was thus established, to describe the individual "trophozoites" of *Sphaerospora* spp. Disporous and monosporous pseudoplasmodia are therefore broadly equivalent to pansporoblasts inside true polysporic plasmodia, with the pseudoplasmodial cell itself homologous to the pericyte cell of the pansporoblast.

Mono- or disporous development has been considered a species characteristic (Lom *et al.*, 1985b). Some species of *Sphaerospora*, like *S. molnari* (Lom *et al.*, 1983b) are monosporous but most are disporous, such as *S. elegans* (Feist *et al.*, 1991). Very few species have both monosporous and disporous pseudoplasmodia, for example, *S. epinephali* (Supamattaya *et al.*, 1991) and *S. scardinii* (El-Matbouli & Hoffmann, 1992).

The sporogonic stages of most *Sphaerospora* spp are coelozoic in the lumena of the kidney tubules. However, a few species have sporogonic stages in other organs (table 2.3).

Extrasporogonic stages

Molnar (1979a) noted that within populations of common carp, *Cyprinus carpio*, with gill sphaerosporosis, individual fish were either completely free of infection or carried massive numbers of sporogonic pseudoplasmodia. If only one

table 2.3   *Sphaerospora* spp. producing spores in non-urinary system locations

Site	Species	Host
Gill filaments	*S. branchialis*	*Cyprinus carpio*
	S. carassii	*Carassius carassius*
	S. chinensis	*Cyprinus carpio*
Gall bladder	*S. masovica*	*Abramis brama*
	S. pectinacea	*Perca fluviatilis*
	S. periophthalmi	*Periophthalmamus koelreuteri*
Gill/skin/blood	*S. molnari*	*Cyprinus carpio*
Dermis	*S. sp.*	*Sparus aurata*
Head kidney	*S. tincae*	*Tinca tinca*
Intestine	*S. hypophthalmichthydis*	*Hypophthalmichthys molitrix*
Testes	*S. testicularis*	*Dicentrarchus labrax*
Histozoic (numerous organs)	*S. dicentrarchi*	*Dicentrarchus labrax*

pseudoplasmodium in an infected fish was produced by each infective agent (believed then to be a "mud aged" spore) then such a finding would suppose either a single massive invasion of each infected fish, or a very long term exposure to countless minor invasions. Molnar therefore proposed that the severity of sphaerosporosis in infected fish was a consequence of an intensive vegetative multiplication of some kind following the intake of only a few spores which resulted in an ensuing massive infection of the gills.

Later, whilst studying *Sphaerospora angulata* infections of *Cyprinus carpio*, Molnar (1980a) suggested that pre-sporoblastic stages of *S. angulata* existed and, noting a distinct similarity histologically, first linked the parasite with the "unidentified extracellular protozoan" of Csaba (1976) from the blood of common carp. In the same year, Molnar (1980a) suggested that the parasite also showed a correlation, in terms of coincident infection, with swimbladder disease of carp, known as swimbladder inflammation or SBI. The aetiological agent of SBI had remained unidentified despite its serious economic effects in the carp farms of central and eastern Europe

Dykova & Lom (1982) studied *Sphaerospora* sp. from carp kidneys. Although identical to that described by Molnar (1980a) as *S. angulata*, this species differed markedly from the original description of the parasite and thus they established it as a new species, *S. renicola*. They too linked carp kidney sphaerosporosis with the proliferative, so-called C-blood stages of Csaba (1976).

Körting (1982) and Kovacs-Gayer, Csaba, Bekesi, Bucsek & Szakolczai (1982) also linked C-blood stages with *S. renicola* sporogonic stages, but identified a morphological similarity between *S. renicola* sporogonic stages and protozoan parasites in the swimbladder associated with SBI. Lom *et al.* (1983a) concluded that the C-

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protozoan blood stage was a myxosporean by virtue of its ultrastructure, especially with respect to its having the characteristic myxosporean enveloped cell condition. Stages consisted of a primary cell containing one to eight secondary cells, each of which could contain a single tertiary cell. Subsequent rupturing of the primary cell would liberate secondary cells to act themselves individually as primary cells, and thus act as a proliferative mechanism. C-stages were seen penetrating between the epithelial cells of the kidney tubules to enter the lumen, and were ultrastructurally very similar to *S. renicola* sporogonic stages. However, despite being able to transmit such C-stages to infection-free carp by intra-peritoneal (IP) injection, experimentally infected fish never developed sporogonic stages in the kidney tubules.

Csaba, Kovacs-Gayer, Bekesi, Bucsek, Szakolczai & Molnar (1984) studied the swimbladder organism in detail. They named it the K-protozoan, and established its affinities with myxosporeans, especially *S. renicola* lumenal stages, as well as postulating it as the aetiological agent responsible for SBI. These stages were broadly similar to C-stages, but up to 46 secondary cells were found in primary cells. The end product within primary cells in the swimbladder were secondary cells each containing two tertiary cells. It was these, referred to as "triple formations" which were reminiscent of the earliest tubular stages of *S. renicola*. Independently, Körting, Hoffman, Neukivch & Fuhrmann (1984) reached the same conclusions studying carp from German farms.

Molnar (1984) transmitted K-stages from infected fish swimbladders into naive controls and subsequently obtained renal stages of *S. renicola*. He also transmitted C-stages from the blood of infected carp into experimental fish, but failed to obtain *Sphaerospora* infections in the kidney. Molnar & Kovacs-Gayer (1986a) showed similar results. That C-blood protozoan stages were lifecycle stages of *S. renicola* remained in

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doubt until Molnar (1988b) was successful in obtaining sporogonic stages of *S. renicola* as a result of the injection of C-blood stages.

In the life cycle of *S. renicola* there are thus two morphologically distinct extrasporogonic stages, one in the blood and one in the swimbladder. However Odening (1989) described K-stages of *S. renicola* from the brain, spleen, liver and pancreas of infected fish prior to their appearance in the swimbladder.

Therefore, it seems that the function of extrasporogonic stages is to act as a proliferative mechanism, a very large number of spores being produced as a result of an infection by few initially invasive stages.

Lom *et al.* (1985b) have since demonstrated morphologically different C-stages in the blood of a range of freshwater fish species, as have Baska & Molnar (1988). The latter authors found large forms, akin to *S. renicola* K-stages in the circulating blood of asp and white bream. They proposed that the whole extrasporogonic cycle occured in the blood in such species, whilst in carp some stages had a predilection for the swimbladder. Kepr & Trsova (1989), in abstract form, noted the presence of extrasporogonic stages in a further eight fish species in Czechoslovakia with little further detail. Extrasporogonic stages therefore seem to be a feature of many *Sphaerospora* species. Almost always, extrasporogonic stages can be found in the circulating blood, but often they are found in increased number in certain sites within the host (table 2.4). It is also noticeable that few extrasporogonic stages have definitely been identified to species level. This is because, although extrasporogonic stages have often been found concurrently with sporogonic stages in the kidney tubules, most authors have not identified the accompanying spores to species level. Since many fish species have their own, apparently very host specific species of *Sphaerospora*, in a number of cases an

table 2.4   Extrasporogonic stages of *Sphaerospora* spp.

Fish species	Myxosporean species (site)	Extrasporogonic site	Authors
Cyprinus carpio (common carp)	*Sphaerospora renicola* (kidney tubule lumen)	Blood (C-stages) Swimbladder (K-stages)	Csaba, (1976); Lom, Dykova & Pavlaskova, (1983); Körting,(1982); Kovacs-Gayer *et al*, (1982)
Gobio gobio (gudgeon)	*Sphaerospora gobionis* (kidney tubule lumen)	Blood	Lom, Pavlaskova & Dykova, (1985); Baska & Molnar, (1988)
Rutilus rutilus (roach)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Lom *et al*, (1985); Baska & Molnar, (1988)
Tinca tinca (tench)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Lom *et al*, (1985)
Blicca bjoerkna (white bream)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Baska & Molnar, (1988)
Scardinius erythrophthalmus (rudd)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Baska & Molnar, (1988); Kepr & Trsova, (1989)
Abramis ballerus (blue bream)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Baska & Molnar, (1988)
Aspius aspius (asp)	*Sphaerospora sp* (kidney tubule lumen)	Blood	Baska & Molnar, (1988)
Alburnus alburnus (bleak)	*Sphaerospora sp* (kidney tubule lumen)	Biood	Baska & Molnar, (1988); Kepr & Trsova, (1989)
Gila bicolor (chub)	*Sphaerospora sp* (kidney tubule lumen)	Blood Kidney interstitium	Hedrick, Kent, Toth & Morrison, (1988)

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Fish species	Parasite (site in host)	Location	Reference
Gasterosteus aculeatus (three-spined stickleback)	*Sphaerospora sp* *Sphaerospora elegans* *Myxobilatus gasterostei* (kidney tubule lumen)	Blood Kidney interstitium *rete mirabile* of the eye	Hedrick *et al*, (1988); Feist, Chilmonczyk & Pike, (1991); Lom, Pike & Feist, (1991)
Abramis brama	Unknown	Blood	Kepr & Trsova, (1989)
Gymnocephalus cernua	Unknown	Blood	Kepr & Trsova, (1989)
Leuciscus cephalus	Unknown	Blood	Kepr & Trsova, (1989)
Lota lota (burbot)	Unknown	Blood	Dykova & Lom, (1988); Lom, Dykova & Kepr, (1988); Kepr & Trsova, (1989)
Perca fluviatilis (perch)	Unknown	Blood	Kepr & Trsova, (1989)
Ictalurus punctatus (channel catfish)	*Sphaerospora ictaluri* (kidney tubule lumen)	Systemic throughout vascular system	Hedrick, McDowell & Groff, (1990)
Salvelimus fontinalis (brook trout)	Unknown	Kidney interstitium Gill	Cone & Cusack, (1991)
Epinephalus malabaricus (grouper)	*Sphaerospora epinephali* (kidney tubule lumen)	Blood	Supamattaya *et al. (1990; 1993)*

educated guess as to the identity of the extrasporogonic stage can be made. For example only *Sphaerospora cristata* has been described from the burbot, *Lota lota*, and so it is reasonable to assume that the extrasporogonic stages of Kepr & Trsova, (1989) from this fish are those of *S. cristata*. However difficulties arise when extrasporogonic stages are found in species of fish such as roach or tench from which more than one *Sphaerospora* sp. is known (Lom *et al.* 1985b). The increasing evidence of the presence of extrasporogonic stages in many species of *Sphaerospora,* and the likelihood that further study will uncover the presence of such stages in *Sphaerospora* spp. from which only sporogonic stages have as yet been described, prompted Lom *et al.* (1985b) to redefine the genus.

Although the number of reports of extrasporogonic stages of *Sphaerospora* is increasing, there remains a paucity of ultrastructural descriptions of such stages. Lom, Dykova & Pavlaskova (1983a) provided details of the C-stages of *S. renicola* from common carp, whilst Csaba *et al.* (1984), Molnar (1988c), and Dykova, Lom & Körting (1990) described the K-stages of the same parasite. Kent & Hedrick (1986) and Feist & Bucke (1987) studied the ultrastructure of PKX, the extrasporogonic myxosporean causative agent of proliferative kidney disease of salmonid fish, whilst MacMillan, Wilson & Thiyagarajah (1989) did so for the extrasporogonic myxosporean causative of proliferative (hamburger) gill disease, PGD in the USA. Lom, Feist & Pike (1991) studied extrasporogonic stages from the *rete mirabile* of sticklebacks infected with both *Sphaerospora elegans* and *Myxobilatus gasterostei*. Although unable to discriminate for certain which of the two myxosporeans these extrasporogonic stage corresponded to, the authors considered it most likely that it was *S. elegans*. Two accounts of the ultrastructure of the extrasporogonic stages of *Sphaerospora epinephali* were presented

by Supamattaya *et al.* (1991; 1993).

There are a number of reports of possible extrasporogonic stages, from myxosporeans of other genera, indicating that such cycles may be more common, in a range of genera, than is currently evident (Lom and Dykova, 1992d). Although these are reminiscent of the extrasporogonic stages of *Sphaerospora*, in some cases a precise generic identification has not been possible since spores were not found during the studies concerned.

Daniels, Herman & Burke, (1976) described a protozoan in the epithelium of rainbow trout exposed to waters enzootic for whirling disease *Myxobolus cerebralis*. The organism consisted of a primary or outer cell containing up to thirty enclosed cells. Some such cells were seen to be free or released from the enveloping cell. Its ultrastructure can be seen to resemble that of extrasporogonic stages of *Sphaerospora* to a certain extent. This protozoan would appear to be identical to that described by Markiw (1989a) from fish experimentally infected with the invasive actinosporean stage of the *M. cerebralis* life cycle. No evidence that these stages proliferate was found, and it may be that each sporoplasm cell goes on to produce a single *M. cerebralis* trophozoite. Extrasporogonic stages, by definition, are proliferative (Lom, 1987; Lom *et al.*, 1991).

Dykova & Lom (1988a) found possible extrasporogonic stages of *Chloromyxum reticulatum* in the blood of burbot, *Lota lota*. These were similar to the trophozoite stages of the same organism in the liver parenchyma, but their coincidental appearance with extrasporogonic stages of *Sphaerospora cristata* precluded a safe distinction. Since spores of *C. reticulatum* are formed in trophozoites in the gall bladder, the authors were

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able to propose a blood-borne means of transport to the site of infection. This is important from an evolutionary viewpoint, as previously it had been thought that entry was via the intestine. Later, Lom, Dykova & Kepr (1988) linked different blood forms in burbot to infections with *Chloromyxum lenorae* in the kidney tubules and glomeruli. However two of five fish were co-infected with *S. cristata* and one with *Chloromyxum pseudomucronatum* in the urinary bladder.

An interesting non-*Sphaerospora* extrasporogonic proliferation was found in the renal corpuscle cells of pike, *Esox lucius*, infected by *Myxidium lieberkuehni* (Lom, Dykova & Feist, 1989). Infection resulted in the formation of large xenomas in which numerous primary cells were produced, each of which contained a single secondary cell, which in turn contained two tertiary cells. However most stages became degenerate, with very few being released into the kidney tubule lumen to enter sporogony. The majority of sporogonic stages entering the tubule from such infections were from standard intracellular forms in the cells of the tubular epithelium.

Lom & Dykova (1986), Lom (1987) and Lom and Dykova (1992d) referred to extrasporogonic proliferative stages in intra-axonal sites in the central nervous system of, for example *Notropis cornutus* (Ferguson, Lom & Smith, 1985), *Cyprinus carpio*, *Rutilus rutilus*, *Pimephales promelas* (Dykova & Lom, unpublished; quoted by Lom, Feist, Dykova & Kepr, 1989) and those of Stensaas, Stensaas & Sotelo (1967) from *Bufo arenarum*, as extrasporogonic proliferative stages. However Lom *et al.* (1989b) claimed that this was probably not the case and that instead such stages were actually early plasmodia in which sporogony had yet to begin. This fits with the definition of extrasporogonic stages, as they must proliferate in a site other than that at which sporogony occurs.

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Styer, Harrison & Burtle (1991) and Burtle, Harrison & Styer (1991) discussed the similarites between the extrasporogonic stages of *Sphaerospora ictaluri* and the agent of proliferative gill disease, PGD, of channel catfish in the southern United States. This condition was first linked to *Henneguya exilis* infections by McCraren, Landolt, Hoffman & Meyer (1975), but this has been questioned on the basis of epidemiological evidence (Kent, Duhamel, Foott & Hedrick, 1987), and the experimental results of MacMillan *et al.* (1989). *S. ictaluri* extrasporogonic stages are systemic in the vascular system of channel catfish according to Hedrick *et al.* (1990).

The most "enigmatic" myxosporean extrasporogonic stage is that of PKX, the myxosporean agent of proliferative kidney disease of salmonids. Seagrave, Bucke & Alderman, (1980) described the ultrastructure of this parasite from the spleen and kidney interstitium of infected fish. The presence of secondary cells, tertiary cells and "haplosporosomes" led them to relate the organism to the haplosporidian genus *Marteilia*, but also to note its affinities to myxosporeans. Later work has established that PKX is indeed the extrasporogonic proliferative stage of a myxosporean (Kent & Hedrick, 1985a; Feist & Bucke, 1987; Clifton-Hadley & Feist, 1989). For some unknown reason the parasite fails to produce fully mature spores in salmonids, leading to theories that salmonids represent non-natural hosts; such a situation is also presented to explain the high degree of pathogenicity shown by the parasite (Kent & Hedrick, 1985a; Kent & Hedrick, 1985b; Kent & Hedrick, 1986; Feist & Bucke, 1987; Hedrick, Kent, Toth & Morrison, 1988; Clifton-Hadley & Feist, 1989; Bucke, Feist & Clifton-Hadley, 1991). Most authors have proposed that the parasite is likely to be a member of the genus *Sphaerospora*, a hypothesis for which there is some evidence based on the appearance of immature spores in the kidney tubules of recovering fish, and upon the

similarity between the PKX cell and *Sphaerospora* extrasporogonic stages (Hedrick *et al*, 1988; Kent, Whitaker & Margolis, 1993b). However, other kidney-infecting genera could be involved, e.g. *Parvicapsula* (Clifton-Hadley & Feist, 1989) or *Myxobilatus* (MacConnel & Smith, 1990). The fact that the generic identity of the parasite has yet to be established reflects the reliance on spore morphology as the sole criterion for generic and species discrimination in myxosporeans.

2.1.3 Objectives

The objective of this part of the current study was to use light and electron microscopy techniques to study and interpret the morphology and development of all life cycle stages of the parasite under study in Atlantic salmon from a number of Scottish smolt producing hatcheries.

2.2 MATERIALS AND METHODS

2.2.1 Sampling sites

Farm **A** lies on the west coast of Scotland (NN179107) and produces only $S_{1/2}$ Atlantic salmon smolts, that is fish which are transferred to marine rearing facilities in October/November of their first year. Fry are initially maintained on heated bore-hole water from hatching until April when they are put out into tanks served by ambient river water. The farm is sited on a NW flowing, Highland river (Kinglass Water) with small stocks of wild salmonids, and discharges directly into a sea loch (Loch Fyne). Water is often obtained from other sources including an artificial loch (Loch Restal), particularly during the summer when water shortages are frequently a problem.

Farm **B** is situated in the North of Scotland (NH464407) and, although producing mainly S_1 Atlantic salmon smolts for Spring transfer, also rears some $S_{1/2}$s. Fry are maintained on heated river/burn water until put out onto ambient water in March. The farm lies on a relatively slow flowing river (River Beauly) which flows eastward to an estuary (Beauly Firth) and supports a large mixed fish population, including Atlantic salmon, brown and sea trout, eels, minnows and sticklebacks. A burn provides alternative, and warmer, water supplies and is used as much as possible.

Farm **C** is sited on the extreme NW coast of Scotland (NC386546) and produces S_1 and S_2 Atlantic salmon smolts, together with a small number of brown trout, the progeny of stripped returning sea trout. All fish remain on ambient river water throughout their time on site. The farm is supplied by a small river (Amhainnan Stratha-Bhig). A loch (Loch Bad na h-Achlaise) discharges into the river above the farm. The river has very varied flow, which is often much reduced in the summer months and leads to management problems. The river flows NE to a sea loch (Loch Eribol and is a good sea trout and salmon fishery. The farm discharges via a settlement pond to the river. This serves to settle and sediment out fish wastes and uneaten food from the water leaving tanks, and therefore removes many suspended pollutants prior to discharge into the river.

Farm **D** is on the NW coast of Scotland (NG812739) and is a growing-on site, producing S_2 Atlantic salmon smolts from parr transferred at 1+ from other sites. All parr are maintained at ambient temperature until smoltification and sea transfer. The farm is on a westward flowing river (River Kerry) which flows into a sea loch (Loch Kerry).

Sampling procedure

The number of fish required were randomly sampled by netting from the relevant tank. All fish were transported alive to the Institute of Aquaculture in oxygen-aerated river water in plastic bags. If fish were to be examined immediately they were maintained in the laboratory in transportation bags with the water aerated. Where immediate examination was not possible, fish were transferred to 40l plastic flow-through tanks supplied with aerated charcoal filtered mains water at ambient temperature.

2.2.2 Post-mortem procedure

Each fish was subject to a standard post-mortem parasitological examination for the detection of myxosporeans. Fish were sacrificed by means of a sharp blow to the head followed promptly by the severing of the spinal cord behind the head using a scalpel and the destruction of the brain.

Examination of the blood

Blood obtained from the caudal artery or heart was examined by placing a fresh drop on a slide and using a coverslip for the detection of extrasporogonic stages. Blood films were prepared and stained with Giemsa for the same purpose (see below).

The capillaries of the swimbladder and the *rete mirabile* of the eye were examined fresh by means of squash preparations.

Blood smears were produced by placing a small drop of blood from the caudal artery at the bottom centre of an alcohol-cleaned glass slide. The drop was then collected on the underside of a second slide held in contact with the first at 45° to the horizontal and dragged across the first to produce a smear.

The concentration technique of Sovenyi & Molnar (1990) for the detection of myxosporean blood stages was used, and the resultant fractions examined fresh or after Giemsa staining. Heparinised micro-haematocrit tubes were half filled with fresh blood taken from the caudal artery of each fish. Lymphocyte separation medium (LSM) was introduced from the opposite end of the tube via a syringe, care being taken to ensure that a small air bubble of 1-2mm in length was interspersed between the blood and the LSM. The tube was plugged at the LSM side and centrifuged in a haematocrit centrifuge for 2 min until the lymphocyte layer was sharply delineated from other blood constituents. The tube was then scratched using a diamond knife and broken just below the lymphocyte layer. The lymphocyte fraction was examined fresh or after the preparation of a smear and subsequent Giemsa staining.

Examination of the kidney

Kidneys were examined by means of fresh squash preparations of anterior, mid and posterior kidney, and by the preparation of Giemsa stained impression smears. For smears, the kidney of each fish was removed whole, blotted dry on a piece of clean dry filter paper, and placed firmly onto a clean slide in three separate places. Small steaks from the anterior, mid and posterior kidney were also taken, blotted dry and dabbed several times onto a second slide.

Examination of other organs

Small pieces of gills, liver, spleen, gall bladder, urinary bladder and brain were squashed between a coverslip and a slide and examined routinely. Particular attention was paid to any gross changes in organs, such as the presence of cysts or focal necrosis.

<u>Histology</u>

Samples of anterior, mid and posterior kidney, liver, spleen, skin, gills, eyes, gall bladder, brain and urinary bladder were fixed in 10% neutral buffered formalin for at least 48 hours. Tissues were then trimmed, processed routinely in a Histokinette 2000 processor and embedded in paraplast wax. Sections were cut at 5-7μm thickness and stained routinely with haematoxylin & eosin. Where necessary, areas of interest were recut and sections stained with PAS (Periodic Acid Schiff), Giemsa and Mallory's trichrome (Drury & Wallington, 1980).

All blood smears, kidney impression smears and smears prepared using the blood concentration technique were air dried and fixed in absolute 100% methanol for 30 seconds. They were subsequently stained in 10% Giemsa (10% stock v/v in distilled water) for 30-60 min. Slides were washed under running tap water for 1 min, blotted dry with paper towels, and examined under oil or mounted in pertex.

2.2.3   Microscopical examination of samples

Fresh squash preparations were examined at magnifications of x200-x1000 using bright field, phase contrast or Nomarski optics on a Leitz SH Lux compound microscope, a Leitz Wetzlar Orthomat or an Olympus BH2 compound microscope respectively. Histological sections and Giemsa stained smears were examined using bright field optics on a Leitz SH Lux compound microscope.

Drawings were made freehand, or using a Leitz SH Lux compound microscope fitted with a drawing tube.

Light and phase-contrast photographs were taken with an Olympus BH2

microscope with dedicated 35mm camera (C.35AD). Nomarski interference contrast photographs were taken on a Leitz Wetzlar Orthomat with dedicated 35mm photomicrographic camera.

Mature spores were photographed fresh or after being embedded in 1.5% agar (Lom, 1969b). Measurements were taken from enlarged photographs of known scale, or directly from fresh preparations using a calibrated eye-piece graticule.

2.2.4 Transmission electron microscopy

Small pieces of anterior, mid and posterior kidney were immersed in Karnovsky's fixative for 4-6 hours at 4°C. Following post-fixation in 0.5% osmic acid in cacodylate buffer for one hour, tissues were dehydrated in an acetone series and embedded in Araldite epoxy resin. Resulting blocks were cut as 1μm semi-thin sections and stained with methylene blue. Ultrathin sections were cut of areas of interest, stained with uranyl acetate/lead citrate and examined in a Philips 301 transmission electron microscope at 60kV.

2.3 RESULTS

2.3.1 Extrasporogonic stages

Light microscopy

Extrasporogonic stages were the first stages to be detected each year in salmon parr. They were found in both the blood and the interstitial tissues of the kidney, by means of fresh tissue squash preparations and Giemsa-stained blood and kidney smears. In blood films, parasites were particularly concentrated around the periphery of the

smear. Histologically, extrasporogonic stages could also be found in the blood vessels and sinuses of the spleen and liver. The concentration technique of Sövenyi & Molnar (1990) was found to be effective in increasing the rate of parasite detection from the blood. Extrasporogonic stages were wholly extracellular, apart from individual parasites engulfed by host macrophages. The engulfment of extrasporogonic stages in the kidney interstitium was a common finding at the light and TEM level.

In fresh tissue preparations of the blood and kidney, live parasite primary cells were round or irregular in shape. They possesed a clear cytoplasm containing inner, secondary cells and occasional refractive granules. Secondary cells were more easily distinguished under phase-contrast. Parasites of all sizes appeared to be non-motile.

Giemsa-stained blood smears and kidney imprints were the preferred means of studying the extrasporogonic parasites and their development, as this method resulted in flat preparations in which cell numbers could be studied.

Extrasporogonic stages were found in far higher numbers in the kidney interstitium than in the blood. Stages in both the blood and kidney, and indeed those seen in the spleen and liver, were identical in morphology, size range, and secondary cell number, although some apparently degenerate parasites were seen in the kidney (chapter 5).

Parasite stages from such preparations were arranged into a presumed developmental sequence (fig.2.1 & 2.2). The earliest extrasporogonic stages in epidemiological terms in stained smears measured around 5μm in diameter, and consisted of an outer, primary cell which contained in its cytoplasm a single, darker staining, secondary cell. This was easily distinguished from later, released secondary cells. In Giemsa-stained smears, the cytoplasm of the primary cell stained a very light

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fig 2.1 Extrasporogonic stages from Giemsa stained blood and kidney impression smears (Giemsa, bar=10μm)

A: very earliest stage of infection seen epidemiologically, consisting of a primary cell containing a single secondary cell. Note the fine cytoplasmic projections of the primary cell membrane

B-E: more advanced stages with larger numbers of secondary cells

F-G: two extrasporogonic stages with less than ten secondary cells, but with triple formations already present (**arrows**)

H: larger stage with 18 secondary cells

I: released secondary cells, each with a single, tiny, tertiary cell inside (**arrows**)
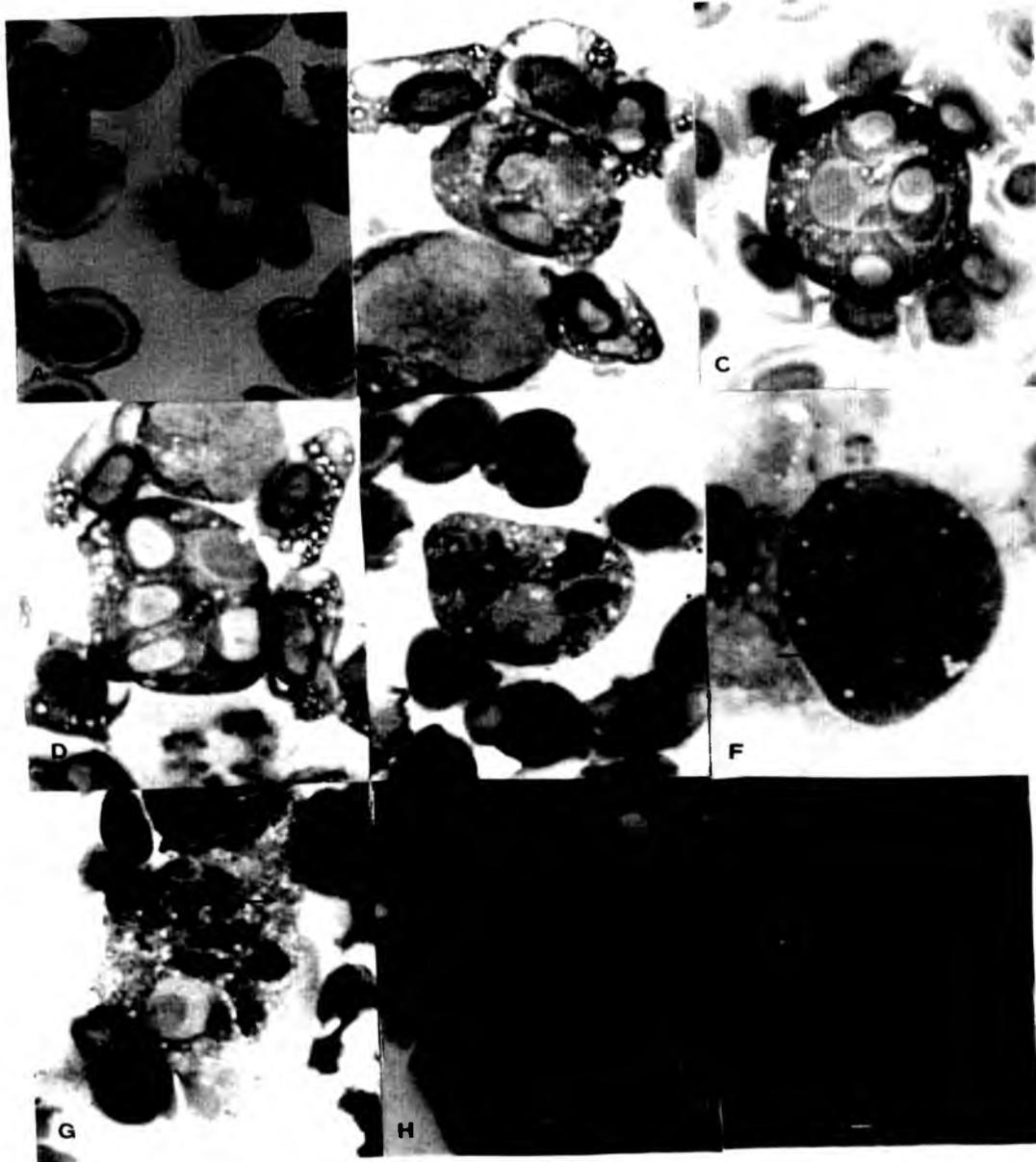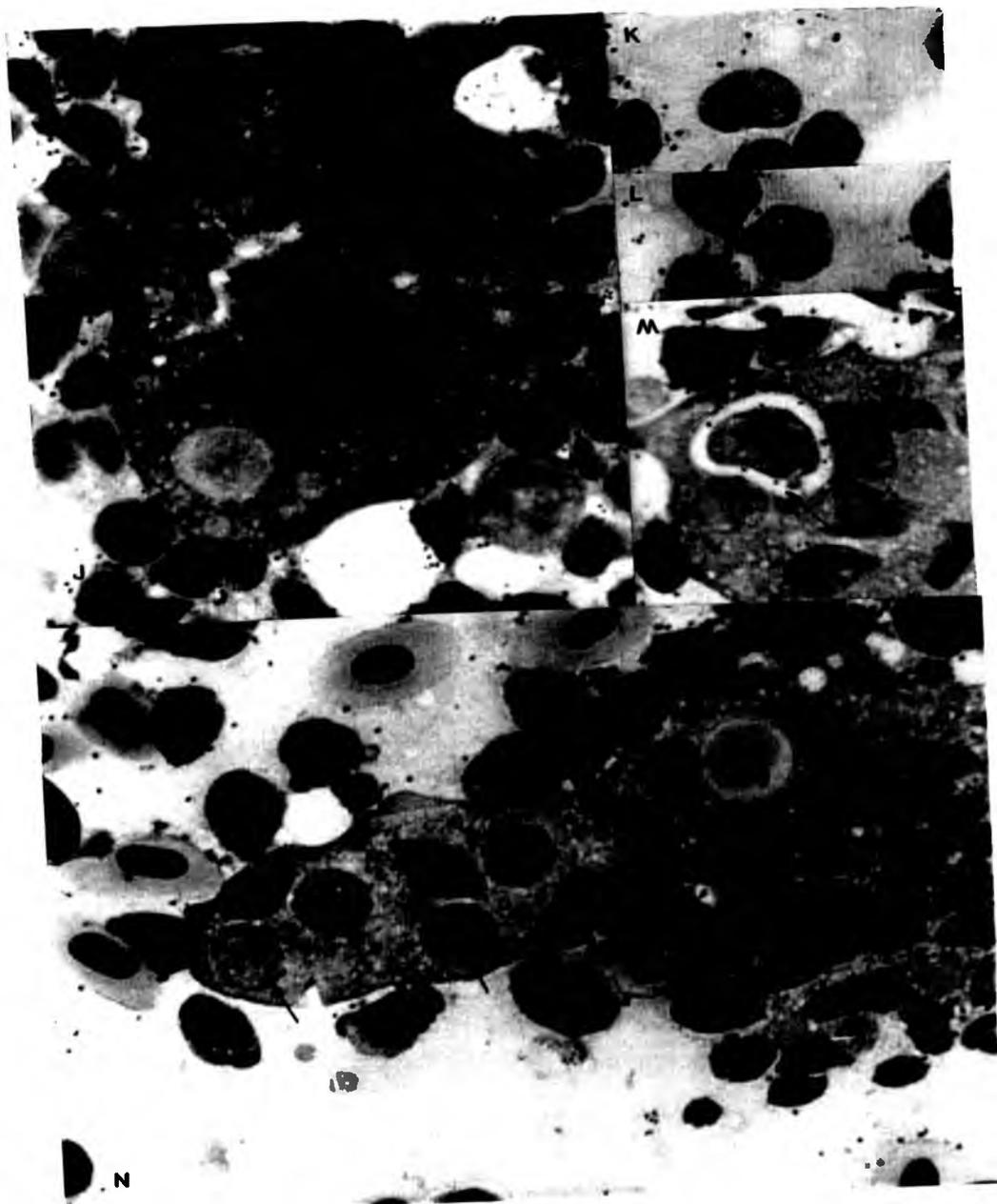
fig.2.2 Drawings of extrasporogonic stage development
A: early stage with cytoplasmic projections and folds
B: dividing stages
C-E: larger primary cells, some secondary cells contain on or two teriary cells
F: large primary cell containing numerous triple forms
G: lone triple form
H: lone secondary cell with one tertiary cell
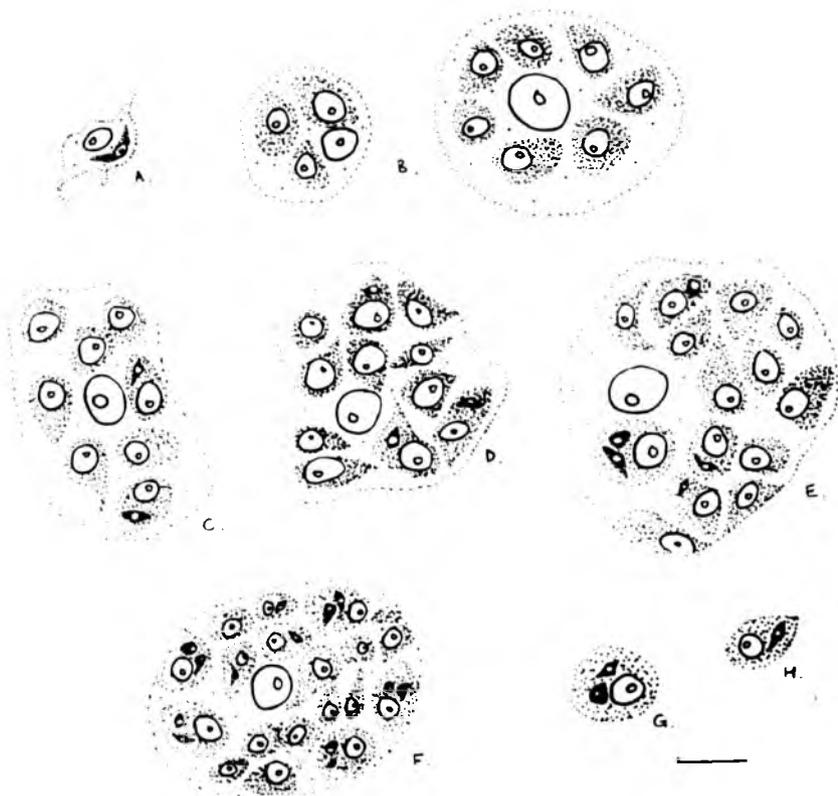
blue; its nucleus stained very light pink, with a darker staining eccentrically positioned nucleolus. Secondary cells were oval or spindle-shaped with a darker staining cytoplasm; their nuclei also stained more intensely and possessed an eccentric nucleolus.

In what was determined from epidemiological observations to be very early infections, the outer membrane of the primary cell of the earliest parasite stages seen was irregular, with cytoplasmic infoldings and projections (fig.2.1). This feature appeared to be characteristic, and was not seen in more developed stages or released secondary cells.

As the infection progressed, the number of secondary cells within primary cells increased. Later, parasites containing up to one hundred and twenty secondary cells were not uncommon, both in the circulating blood and the kidney interstitium. Such stages measured up to 70μm in diameter. Secondary cells recently released from ruptured primary cells (fig.2.1), were small ($3-5\mu$m) and often possessed one or two tiny inner cells (tertiary if within the primary cell, secondary when released). The now primary cell then evidently underwent a period of growth prior to the onset of repeated secondary cell production, as some primary cells containing only one or two secondary cells were $15-20\mu$m in diameter (fig.2.1). The secondary cells of these primary cells were much larger than those of later, more developed primary cells with more secondary cells.

The secondary cells of some extrasporogonic stages frequently contained one or two, tertiary cells in their cytoplasm (fig.2.1). The small nuclei of these cells stained an intensive pink, but their very thin layer of cytoplasm was frequently difficult to distinguish from that of the secondary cells in which they were enveloped. Secondary cells with two tertiary cells were strongly reminiscent of the "triplets" or triple

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formations of Baska & Molnar (1988) and appeared to represent the end product of the extrasporogonic stage. These structures often seemed to be isolated from the primary cell cytoplasm in a vacuole (fig.2.1) to a much greater and more noticeable extent than equivalent secondary cells with one or no tertiary cells. Single tertiary cells within secondary cells were tiny, difficult to see and stained less strongly than when two tertiary cells were present.

There appeared to be no precise, fixed developmental sequence or pattern, in terms of the number of secondary cell divisions or the number of secondary cells present within the primary cell. Equally there was no fixed point in development in terms of secondary cell number at which the first tertiary cells were present in secondary cells, or when the first triple formations were seen within the primary cells of a particular parasite. Although they were more frequent in large primary cells with more secondary cells, triple forms were observed in extrasporogonic stages with less than 10 secondary cells (fig.2.1). Single tertiary cells in secondary cells could be seen in parasites with three secondary cells or more (fig.2.1).

An extrasporogonic stage primary cell could therefore contain one to one hundred and twenty secondary cells, each with zero, one or two tertiary cells. Freed triple forms (fig.2.1) were easily distinguishable, by their small size, from primary cells containing two secondary cells.

Single secondary cells with one, two or no tertiary cells were abundant in smears, presumably having been released from ruptured parasites (fig.2.1). It could not be determined whether released secondary cells with only a single tertiary cell were able to become triple forms by another division, or whether they were destined to become new extrasporogonic primary cells. Similarly, there was no means of telling whether

fig.2.3 Possible fates of released secondary cells: **A**, further extrasporogonic proliferation; **B**, migration to the kidney tubules to initiate sporogony
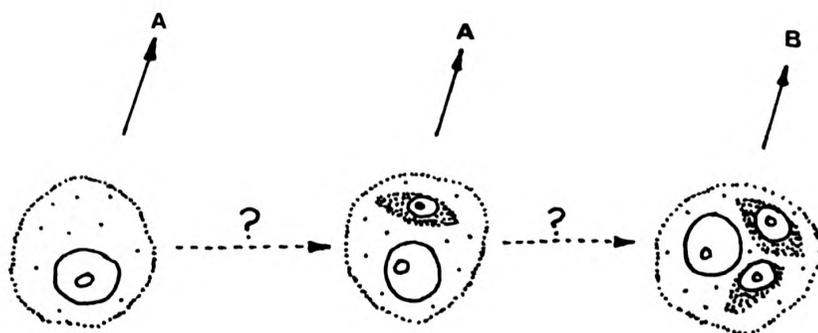
fig 2.4   Histological section of the kidney to show a large extrasporogonic stage (arrow) in very close
           association with the basal lamina of a kidney tubule Primary cell nucleus (arrowhead) is clearly
           visible. (H&E, bar=10μm)
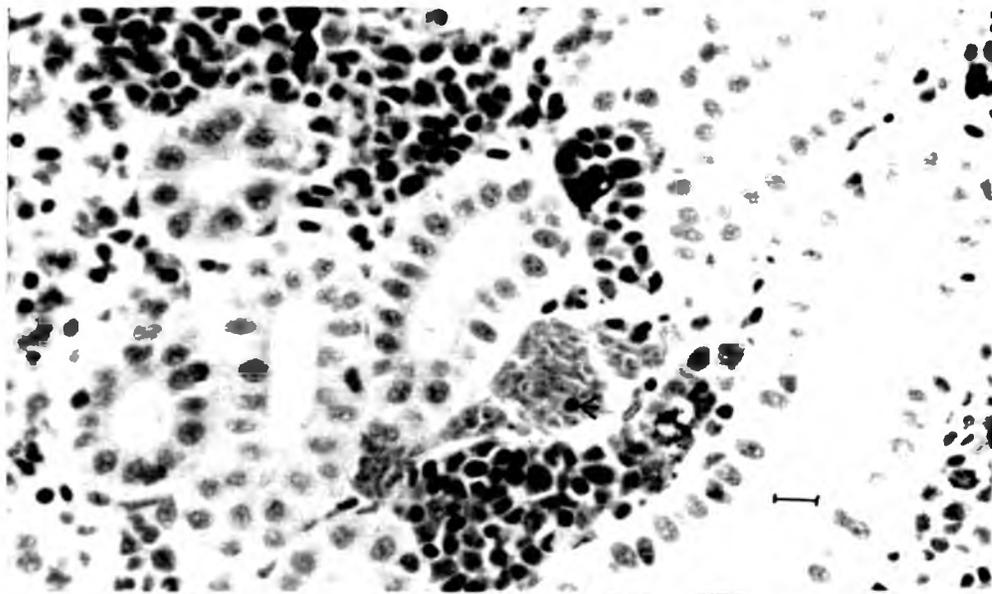
fig 2.5   Histological section of the kidney to show an extrasporogonic stage closely associated with the basal
           lamina of a kidney tubule. The primary cell nucleus is out of the plane of the section (□), but a
           triple form is clearly visible, comprising a secondary cell in a vacuole in the primary cell cytoplasm
           (arrowhead), itself containing two tiny tertiary cells (arrow) (H&E, bar=10μm)

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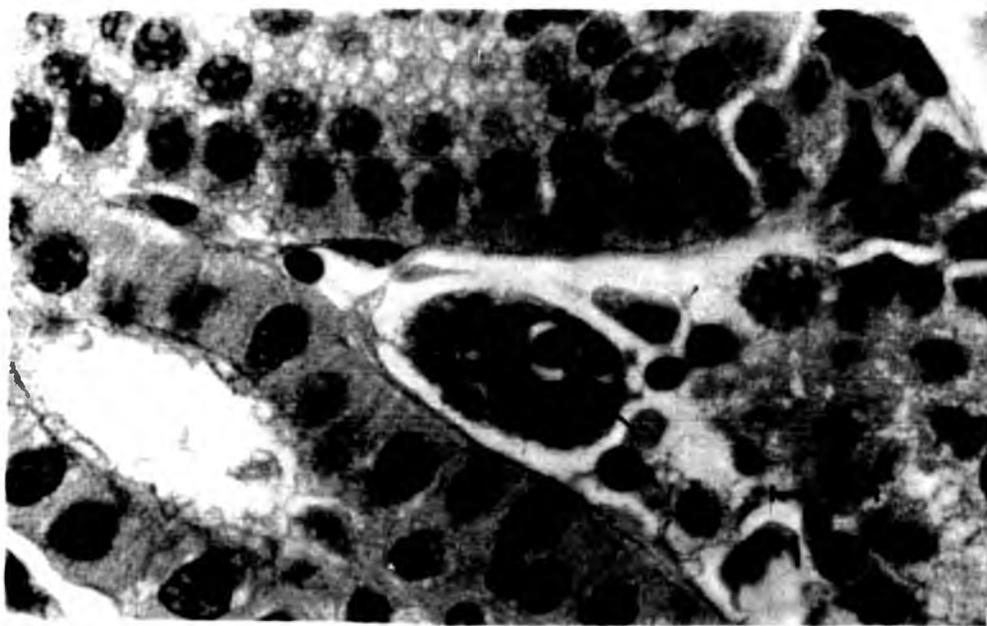
fig 2.6   Histological section of liver showing several extrasporogonic stages (arrows) in a blood sinus. (H&E, bar=10μm)
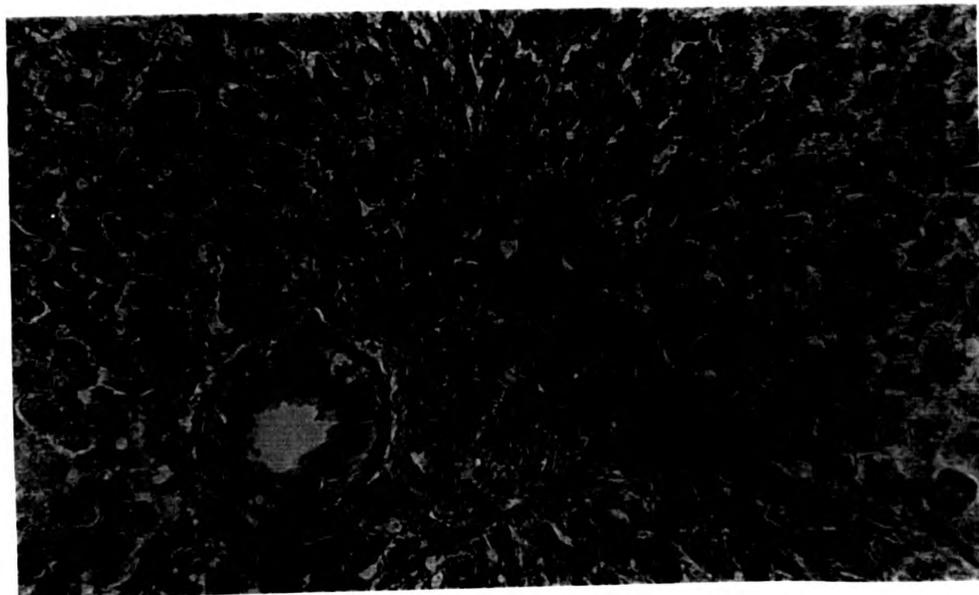
secondary cells released without any tertiary cells were able to produce one or two tertiary cells after release and thus go on to become sporogonic triple forms or whether they became new primary cells (fig.2.3).

In histological sections, the parasites were easily distinguishable in H&E and Giemsa stained preparations (fig.2.4). Primary cell nuclei were very characteristic in appearance, often surrounded by a halo or clear area. They possessed a nucleolus with, occasionally, a second clear area in its centre. Secondary cell numbers were impossible to count from sectioned material, but triple formations could be detected in some parasites (fig.2.5). From histological material, it was also evident that extrasporogonic stages in the spleen and liver (fig.2.6) were wholly restricted to blood sinuses, vessels, and in the case of the kidney, the interstitial tissue. This confirmed their wholly extracellular nature. Extrasporogonic stages were more abundant in the kidney, compared to all other tissues, even the blood, confirming fresh and impression smear observations. In addition, from histology it was clearly apparent that extrasporogonic stages, as well as having a bias towards the kidney tissue, were frequently found adhering to the outer basal lamina of the kidney tubules in large numbers (fig.2.4).

In semi-thin sections, small extrasporogonic stages were seen in large numbers in glomerular capillaries.

Ultrastructure

Seen at the ultrastructural level, the extrasporogonic primary cell was bounded by a single unit membrane. Its cytoplasm was uniformly granular and contained numerous free ribosomes along with large numbers of mitochondria which possessed

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few, irregular plate-like cristae. Well organised Golgi apparatus were present occasionally and rare cisternae of rough endoplasmic reticulum (fig.2.7 & 2.8). The cytoplasm of, especially larger, extrasporogonic primary cells often featured numbers of apparently empty membrane-bound vacuoles (fig.2.9, 2.13 & 2.14). These occasionally contained a very electron lucent material (fig.2.15). Some very small double membrane bound inclusions contained a more dense accumulation of ribosomes than the primary cell cytoplasm, appearing almost identical to the cytoplasm of secondary cells, but containing no nucleus or other organelles. No electron dense "haplosporosomes" were seen in the cytoplasm of primary cells.

The structure of the primary cell nucleus appeared to differ markedly between the small extrasporogonic stages containing very few secondary cells and the more developed forms with many secondary and tertiary cells. In early stages the nucleus, separated by a double membrane from the primary cell cytoplasm, was small and exhibited a granular, but evenly stained appearance. Invariably there was an eccentrically positioned, very electron dense nucleolus, often closely opposed to the nuclear envelope (fig.2.7). The nucleus was often encircled by rough endoplasmic reticulum. There were few organelles in the primary cell cytoplasm of early stages apart from mitochondria, dense ribosomes, and occasional Golgi (fig.2.8).

In larger extrasporogonic stages the nucleus became greatly enlarged with the nucleoplasm assuming a generally more uneven, less granular staining property. The nucleolus became enlarged in even greater proportion, and less compact. Portions of the nucleolus frequently contained small lucent areas and always a single, larger, central lucent area. This feature was also detectable in histological sections (fig.2.9). Rough endoplasmic reticulum was no longer found only encircling the nucleus, and there was

an increase in the number of organelles, such as mitochondria and Golgi in the cytoplasm. A less dense concentration of ribosomes was evident.

In early primary cells, secondary cells were very small, dense and possessed little cytoplasm (fig.2.7). Endoplasmic reticulum was often concentrated around the nucleus of the secondary cell. The nucleus invariably contained a much darker staining eccentrically positioned nucleolus.

The cytoplasm of secondary cells in larger extrasporogonic stages contained a more dense accumulation of free ribosomes, fewer mitochondria, and only rare Golgi, in comparison with that of the primary cell (fig.2.9 & 2.10). All secondary cells were delimited by two unit membranes; one bounding the primary cell cytoplasm, the other that of the secondary cell. All secondary cells were thus wholly contained within a tightly fitting "vacuole" in the primary cell cytoplasm. Occasionally two, but never more, secondary cells were found in a single vacuole, perhaps indicating that they were the products of a recent division (fig.2.10). Some secondary cell membranes were found to be incomplete (fig.2.11). In some sections two nuclei were found sharing the same cytoplasm, being surrounded by as yet incomplete cytoplasmic membranes (fig.2.11). These may represent developing tertiary cells being formed by an endogenous cleavage. Some secondary cells showed infoldings of their cytoplasm to the extent that they almost appeared to partition the cell (fig.2.9). Rarely, two secondary cells were found in very close apposition, with one cell seeming to be in the process of engulfing the other (fig.2.12).

Conversely, two secondary cells were often seen apparently in the process of pulling apart, having previously been together within a single vacuole (fig.2.13 & 2.14).

Cells recently parted often showed pseudopodia-like extensions of their cytoplasm.

Tertiary cells were elongate or spindle shaped. As a result, only a very thin layer of cytoplasm lay between the nuclear membrane and the tertiary cell membrane on either side. The cytoplasm contained very few features other than dense ribosomes and occasional mitochondria (fig.2.15 & 2.16). Fully formed tertiary cells were delimited by a double membrane from the secondary cell cytoplasm, although, as with secondary cells, some less developed tertiaries were incompletely membrane-bound. The tertiary cell nuclei were small and irregularly stained, with some dense areas of chromatin, and other clearer areas. This gave them a more uneven appearance compared to secondary cell nuclei. Tertiary cell nuclei again showed infoldings of the nuclear membrane, and always contained a more densely staining nucleolus.

Some macrophage engulfed extrasporogonic stages were found, particularly in the kidney blood sinuses (fig.2.7), along with apparently degenerate or degenerating forms where the cytoplasm appeared extremely disrupted, and the internal secondary and tertiary cell membranes ill-defined (chapter 5). Surrounding host tissue appeared intact and well fixed, suggesting that this was not artefact.

Means of entry to the kidney tubules

Small extrasporogonic stages could be found penetrating between two adjacent cells of the basal epithelium of the kidney tubule of the host (fig.2.17). A few early sporogonic stages appeared to have reached the lumen via this route in some sections, as large portions of their cytoplasm remained between adjacent epithelial cells whilst the remainder of the cell lay in the tubule lumen (fig.2.18). Large extrasporogonic stages

fig 2 7   Early extrasporogonic stage in a blood sinus The primary cell cytoplasm (Pc)contains ribosomes,
mitochondria (M) and cisternae of rough endoplasmic reticulum (RER) (arrow). The nucleus (Pn)
has granular evenly stained chromatin and a densely staining eccentric nucleolus. Two secondary
cells (Sc) are present (uranyl acetate/lead citrate, mag x16,000)
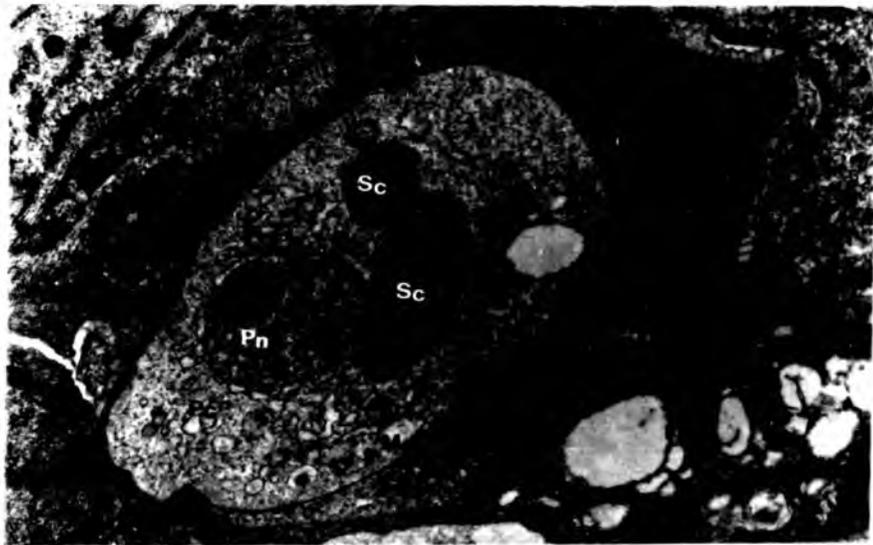
fig 2.8   Later extrasporogonic stage with several secondary cells (Sc). The primary cell cytoplasm (Pc) is
studded with ribosomes, occasional mitochondria (M) and other inclusions and is uniformly granular.
Note the absence of 'haplosporosomes'. Golgi (G) are present in stack form in both the primary cell
cytoplasm and that of one of the secondary cells (uranyl acetate/lead citrate, mag x10,000)

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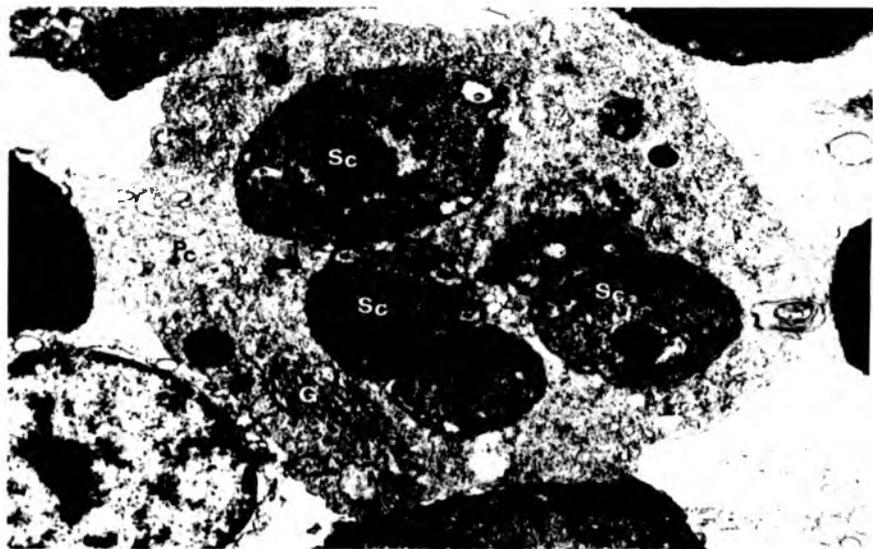
fig 2 9   Later stage containing many secondary cells (Sc), and one tertiary cell (Tc). The nucleus (Pn) of
the primary cell has a markedly different structure from that of less developed forms. It is much
larger, with very large nucleolus (arrow) which has a central electron lucent area (*). The secondary
cells have a more dense accumulation of ribosomes in their cytoplasm along with mitochondria (M).
In one cell the cytoplasm contains a deep fold (arrowhead). (uranyl acetate/lead citrate,
mag x13,000)
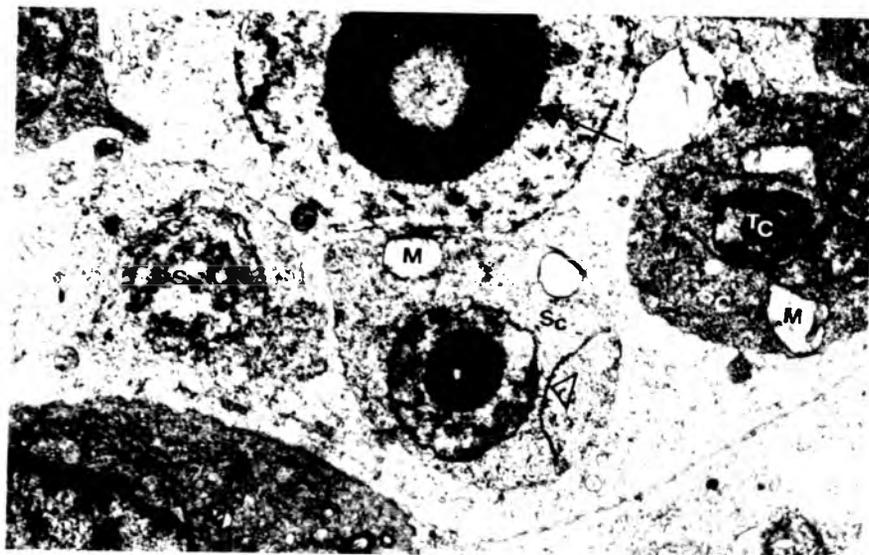
fig 2.10 Two secondary cells (Sc) lie together within a single vacuole (arrows) in the primary cell cytoplasm
(Pc). The secondary cell cytoplasm contains a denser accumulation of ribosomes than that of the
primary cell. Both primary and secondary cell cytoplasm contain numerous mitochondria (M).
Some cell membranes appear incomplete (arrowhead). (uranyl acetate/lead citrate, mag x26,000)
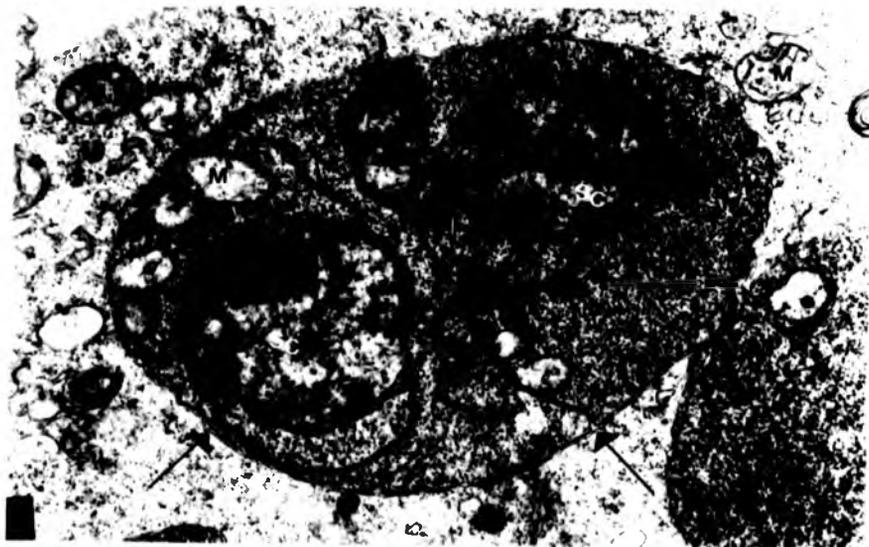
fig 2.11 Two secondary cells (Sc) within a single vacuole in the primary cell cytoplasm. The nuclei (N) of these two cells appear to be in the process of being enveloped by membranes (arrows) and may thus represent tertiary cells being formed within secondary cells. Note the staining difference between the cytoplasm of the secondary cells and that of the primary cell due to increased ribosome density. Numerous mitochondria (M) are present, some of which are enlarged and swollen. (uranyl acetate/lead citrate, mag.x24,000)

fig 2.12 Possible engulfment of one secondary cell (Sc₁) by another (Sc₂). Incomplete membranes are again present. Large numbers of mitochondria (M) are present in the primary cell cytoplasm (Pc). The cell being engulfed has a deep cytoplasmic fold (arrow). (uranyl acetate/lead citrate, mag.x18,000)

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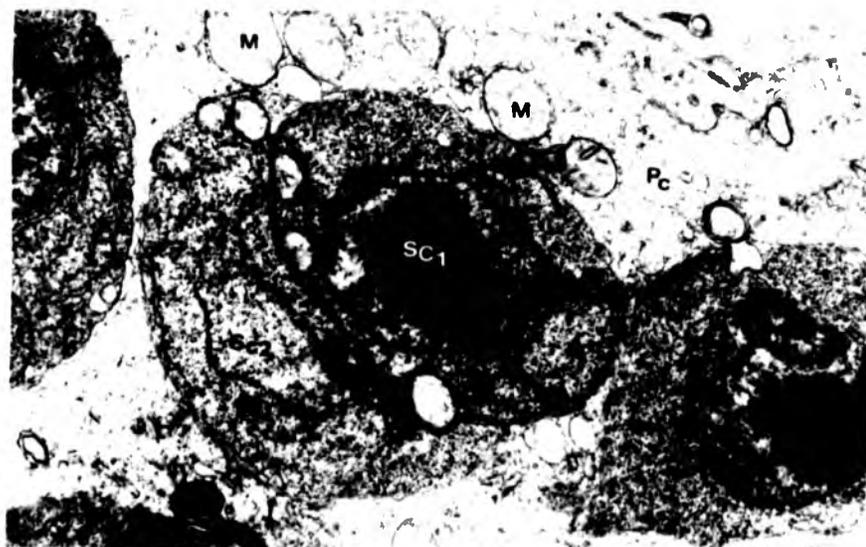
fig 2.13 Secondary cells (Sc₁) and (Sc₂) apparently pulling apart having lain within a single vacuole (arrows). The primary cell cytoplasm (Pc) appears disrupted, containing empty vacuoles (*) and large swollen mitochondria (M). The nucleus of one secondary cell is encircled by RER (arrowhead). (uranyl acetate/lead citrate, mag.x16,000)
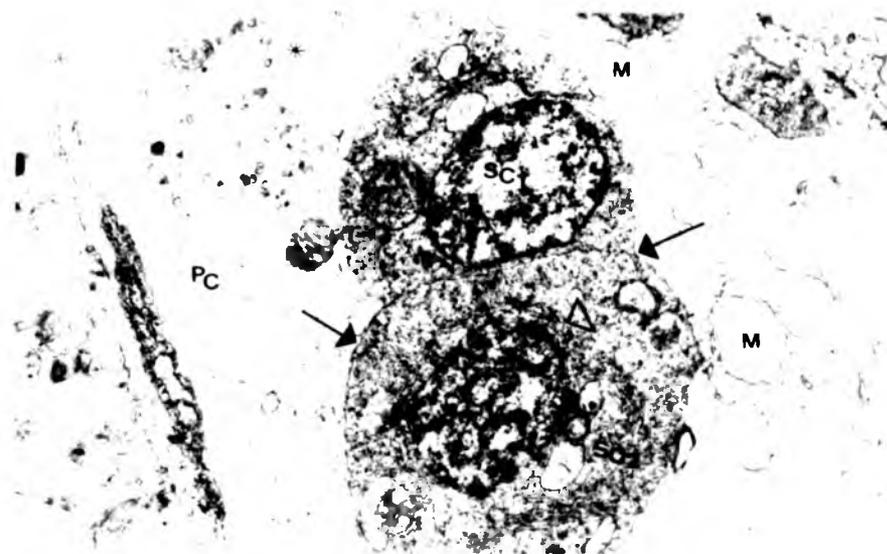
fig 2.14 Secondary cells (Sc₁, Sc₂) in close apposition in the primary cell cytoplasm. This could represent an early stage of engulfment or a late stage of pulling apart. (uranyl acetate/lead citrate, mag.x16,000)
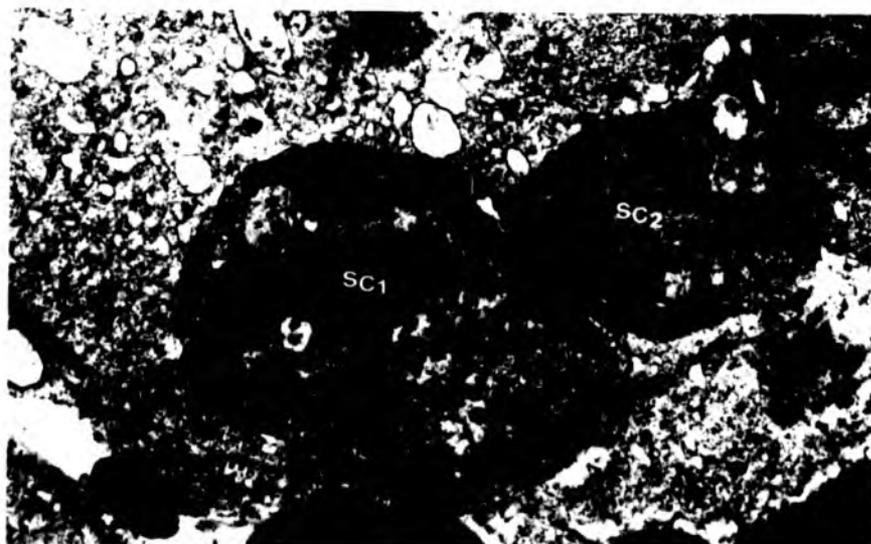
fig 2.15 Tertiary cell (Tc) within a secondary cell (Sc). Note the uneven staining of the tertiary cell nucleus (Tn). The cytoplasm of the tertiary cell (Tc) contains a single mitochondrion and is more densely studded with ribosomes than that of the secondary cell. The secondary cell contains numerous mitochondria (M). Many of the mitochondria in the primary cell cytoplasm are particularly swollen. (uranyl acetate/lead citrate, mag x18,000)
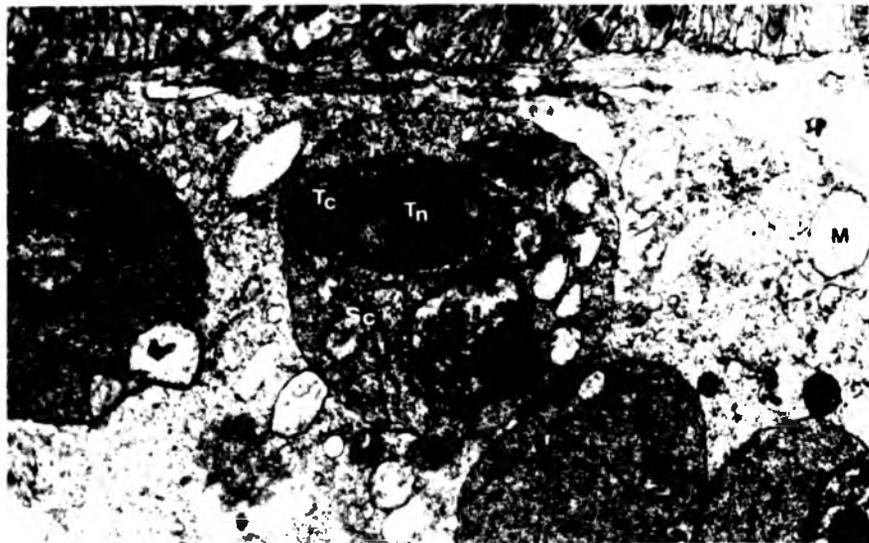
fig 2.16 Spindle shaped tertiary cell (Tc) within a secondary cell (Sc). Note again the uneven staining characteristic of the nucleus (Tn) compared to that of the secondary cell (Sn). No organelles are present in the tertiary cell cytoplasm other than dense ribosomes. Only a very thin layer of cytoplasm is present in the tertiary cell, especially around the nucleus (arrows). The secondary cell nucleus is encircled by RER (arrowhead). (Pc, primary cell cytoplasm) (uranyl acetate/lead citrate, mag.x24,000)
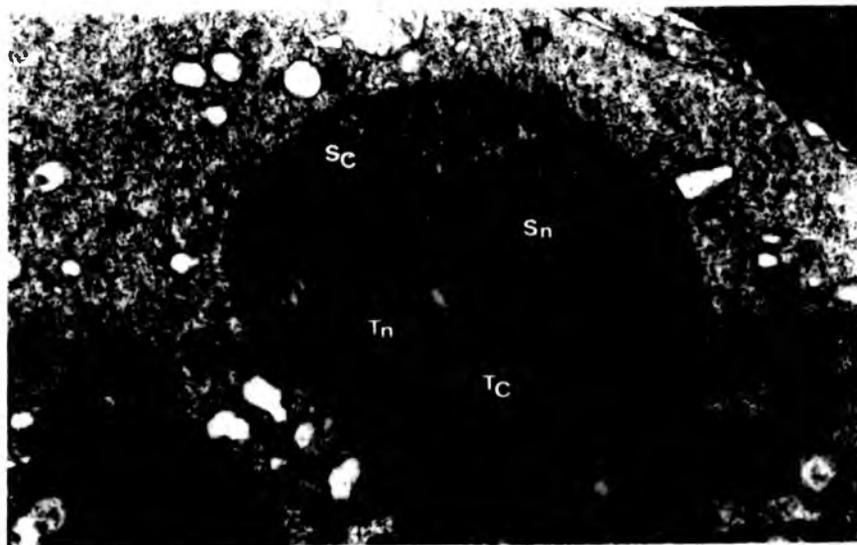
fig 2 17 Small extrasporogonic stage (*) penetrating between adjacent kidney tubule epithelial cells (Kc). The parasite is close to reaching the lumen (L). (uranyl acetate/lead citrate, mag x8,500)
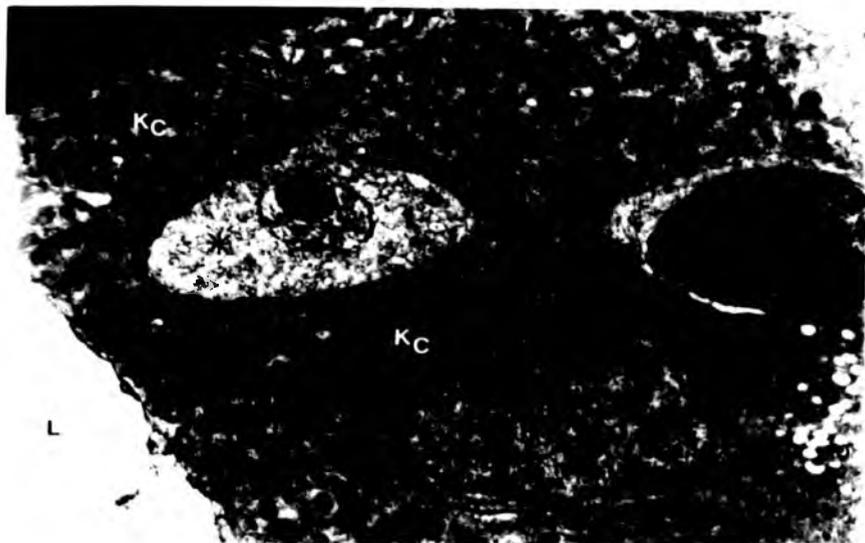
fig.2.18 Early stage in the tubule lumen (L) with a large portion of cytoplasm (C) wedged between two cells of the tubular epithelium (Kc), perhaps indicating entry to the tubule via this route. (uranyl acetate/lead citrate, mag.x7,500)

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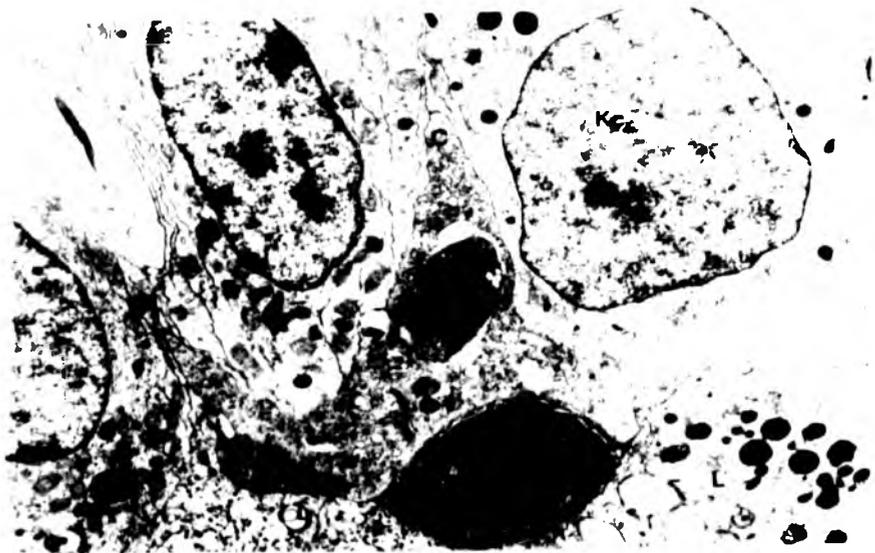
fig.2.19 Large numbers of small extrasporogonic stages (*) lodged in a glomerulus. Some stages show degenerative changes (arrows). (uranyl acetate/lead citrate, mag.x3,800)
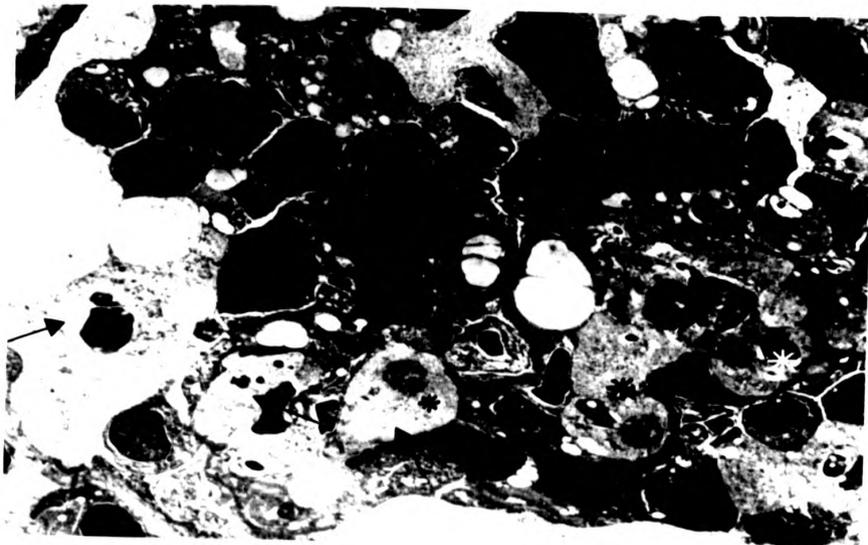
fig.2.20 Fresh squash preparation of a kidney tubule heavily infected and occluded by sporogonic stages at various stages of development (mag.x400)

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were frequently found closely associated with the outer wall of the kidney tubule. Numerous small extrasporogonic stages were found in TEM sections of glomeruli (fig.2.19).

Some small parasite stages in the kidney tubule lumen and Bowman's space showed characteristics of both extrasporogonic and sporogonic stages, and could thus be regarded as transitional forms. They appeared identical in cell complement to early released extrasporogonic secondary cells containing two tertiary cells, but differed in terms of the relative density of cytoplasmic organelles such as mitochondria (see below).

2.3.2 Sporogonic stages

Light microscopy

Sporogonic stages first appeared in the lumena of the kidney tubules three to four weeks before mature spores were seen (chapter 4). Extrasporogonic stages were still to be found in the circulating blood and kidney interstitium. Tubules infected with sporogonic stages were often completely occluded by parasites at different stages of development (fig.2.20). No signs of inter- or intracellular sporogonic developmental stages were found in the interstitial tissues or kidney tubule epithelial cells.

In fresh preparations pseudoplasmodia and mature spores were almost always restricted to the first, proximal third of the tubule, up to and including the Bowman's space. Rarely, mature sphaerospores were found in fresh squashes of the urinary bladder. It was notable that whilst many tubules were uninfected, containing no sporogonic stages, those which were infected often contained very many stages.

Individual pseudoplasmodia in the kidney tubules were up to 40μm in size. They

were often oval in shape or slightly elongate, but generally had no regular defined form. They contained large numbers of refractile bodies, often grouped together at either pole of the pseudoplasmodium, as well as apparently clear areas (Fig.2.21).

The two spores within each pseudoplasmodium developed slightly asynchronously, as did the two polar capsules of each spore. When measuring and describing spores it was found to be vital to differentiate between mature and immature spores, as they displayed markedly different morphological characteristics and dimensions (see chapter 3). The differences between mature and immature spores were especially evident when spores were observed under phase contrast or Nomarski microscopy.

All measurements were taken from fresh squash preparations, as formalin fixed samples showed marked shrinkage (chapter 3).

The six cells comprising each spore were clearly visible under phase contrast and Nomarski microscopy, as were their nuclei (fig.2.22 & 2.23). Development was always disporous (fig.2.24); the valvogenic cells of each spore as it matured were often seen to pull away posteriorly from the cytoplasm of the pseudoplasmodium.

Occasionally, pairs of aberrant spores were found in fresh preparations of kidney tissue. Such spores comprised three polar capsules, three shell valves and three sporoplasms (fig.2.24).

Giesma-stained impression smears of kidney tissue were employed to study the course of sporogonic development (fig.2.25 & 2.26). Staining properties differed somewhat from those exhibited by extrasporogonic stages, in that the cytoplasm of the internal cells within the outer envelope cell, or pseudoplasmodium, was often difficult

fig 2 21 Fresh squash preparation of a kidney tubule showing numerous dividing and differentiating pseudoplasmodia, but, as yet, no recognisable spores. Note refractile granules. (bar= 10μm)
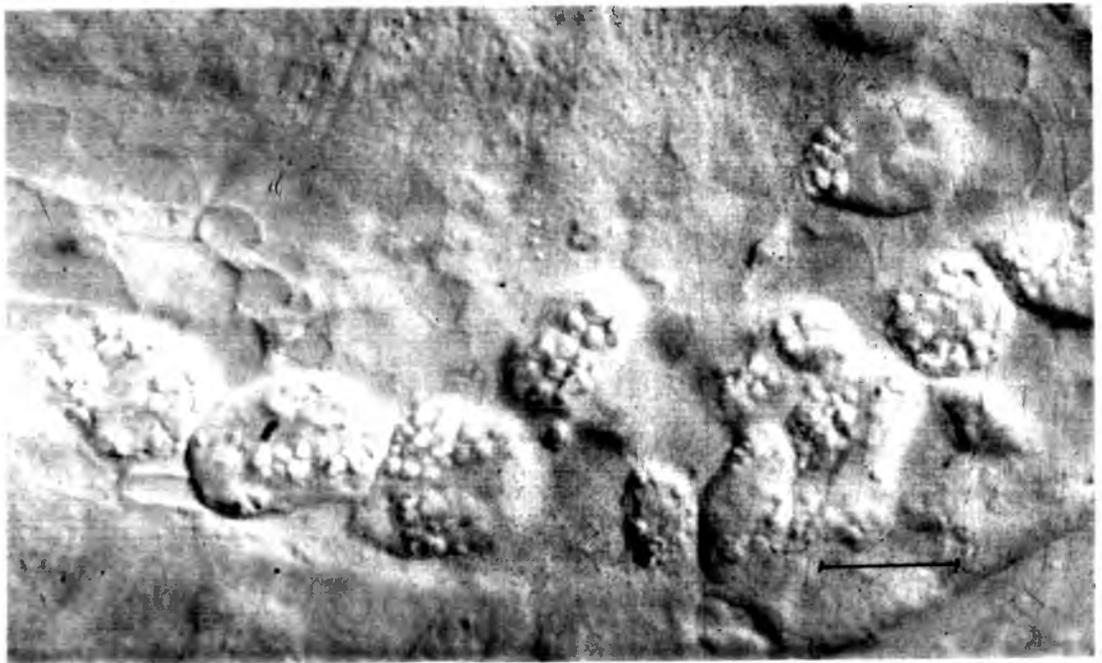
fig 2 22 Fresh squash of the kidney to show two almost mature spores within a disporous pansporoblast. Shell valves and polar capsules are clearly visible. (Nomarski interference contrast, bar=10μm).

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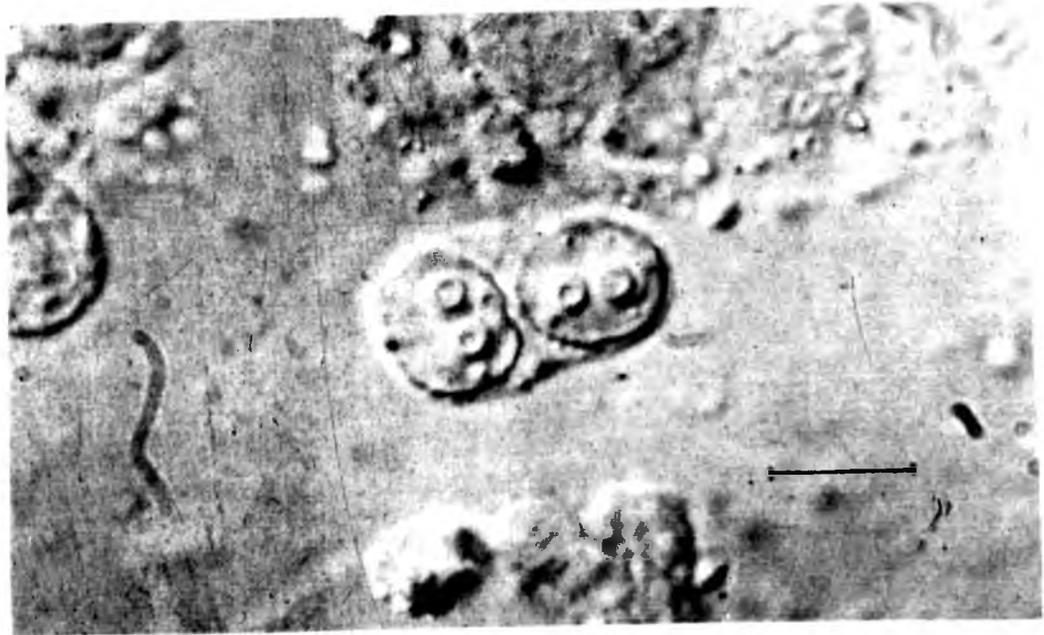
fig.2.23 Phase contrast photomicrograph of a maturing spore from a fresh squash of the kidney. Not the vacuolating cytoplasm of the valvogenic cell, the two prominent polar capsules, and the nuclei of the two sporoplasm cells (bar=10μm)
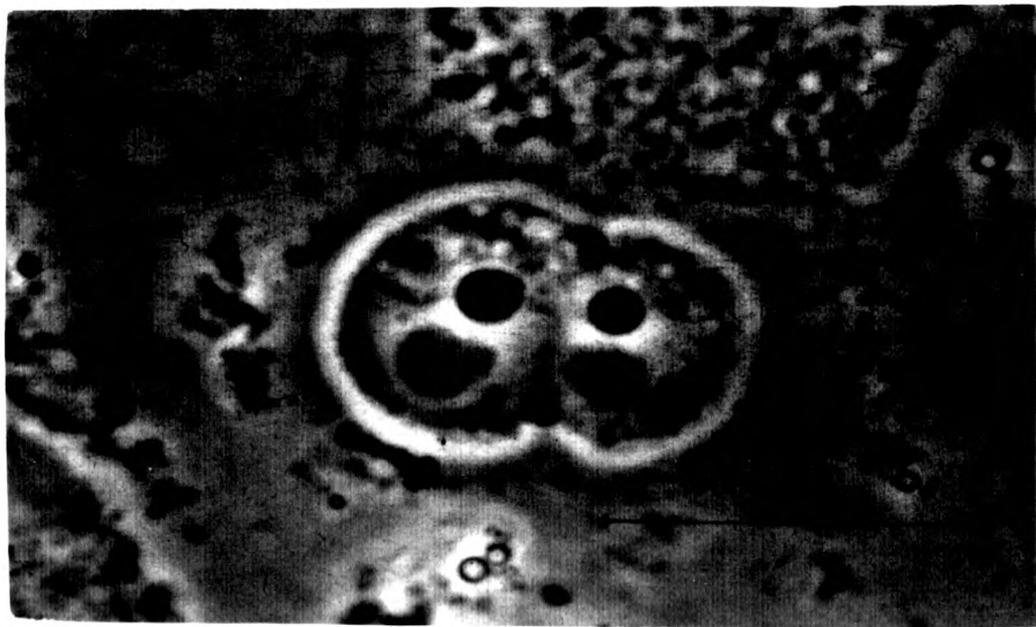
fig.2.24 Aberrant *Sphaerospora* spore with three polar capsules, three shell valves and three sporoplasm cells. (Nomarski interference contrast, bar=10μm)

to distinguish from that of the pseudoplasmodial cell itself. Fewer numbers of sporogonic stages were seen by this method than would be expected from the number of parasites seen in fresh preparations of the tissue concerned. This was probably because sporogonic stages, as will be shown by TEM studies, were attached to the inner wall of the tubular epithelium of the host, and therefore often not dislodged when an impression smear was made.

The earliest sporogonic stages seen consisted of an outer pseudoplasmodial cell containing two, inner, sporoblastic cells (fig.2.25A-B). Subsequently the pseudoplasmodium grew in size and the sporoblastic cells divided repeatedly (fig.2.25C-G). The most developed stages, in terms of cell numbers, contained twelve sporoblast cells within the envelope cell (fig.2.25I-K). All sporoblastic cells were not the same size, and some cells in the process of division were seen (fig.2.25G). Sometimes division product cells appeared to remain closely together, sometimes they separated. Invariably the sporoblast cells within a single pseudoplasmodium divided to reach the twelve cell stage prior to the onset of any visible signs of cell differentiation. Eventually two separate clusters of six cells within the pseudoplasmodium grouped together (fig.2.25). The two groups then differentiated to form a single spore, each of which comprised two capsulogenic, two valvogenic and two sporoplasm cells.

External tube formation could clearly be seen in capsulogenic cells, the capsular primordia and external tubules appearing as clear areas within the capsulogenic cell cytoplasm (fig.2.25H-J). At this point, the valvogenic cells became thin, elongate, and encircled the other four constituent cells of each spore (fig.2.25I-K). Once mature, polar capsules stained a very intense red (fig.2.25K-L). Sporoplasm cells remained barely changed within the enveloping valvogenic cells, from early cell differentiation onward.

Giemsa stained kidney impression smears (bar=10μm)

A-B: Pseudplasmodia containing two sporoblast cells and resembling extrasporogonic triple forms

C-F: Increasing numbers of sporoblast cells

G: Two adjacent pseudoplasmodia. One contains five sporoblast cells, the other only two. Both pseudoplasmodia contain sporoblast cells of different sizes.

H-J: Pseudoplasmodia containing twelve sporoblast cells. Developing capsular primordia and external tubes can be seen in the capsulogenic cells (arrows)

K: Pseudoplasmodium containing two almost mature spores. The six cells comprising each spore are clearly visible. Capsulogenic cells no longer feature external tubes and primordia, but mature polar capsules which stain very intensly.

L: Mature spore. The six cells comprising the spore are clearly visible, as are their nuclei. Polar capsules are intensly stained
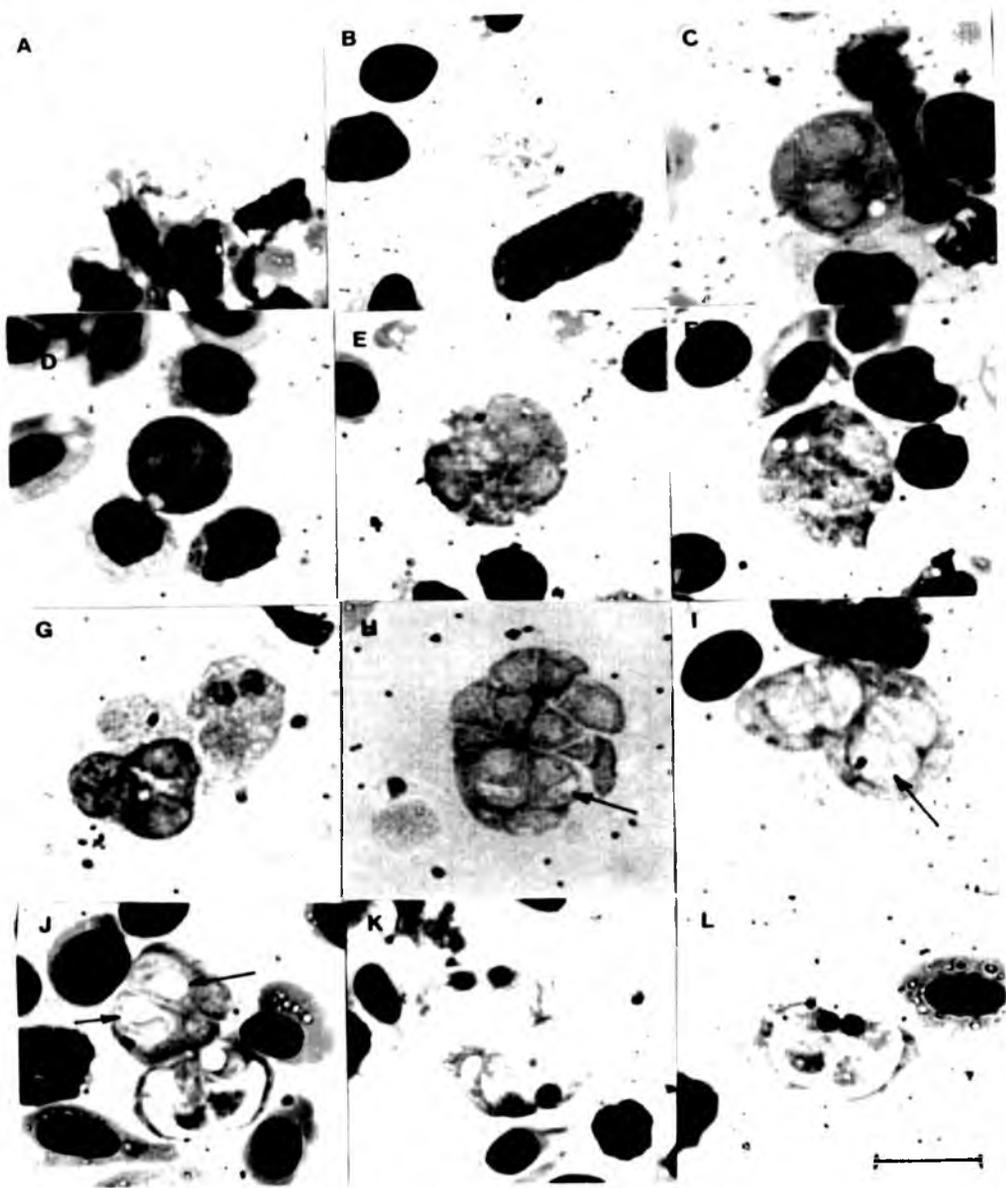
fig.2.26 Drawings of development of sporogonic stages of salmon *Sphaerospora* (bar=10μm):

A-B:	Early triple forms
C-G:	Dividing pseudoplasmodia (note differences in cell sizes)
H:	Twelve sporoblast cell stage. Note early capsular primordia in capsulogenic cells
I:	Two almost mature spores within the pseudoplasmodium

10μm
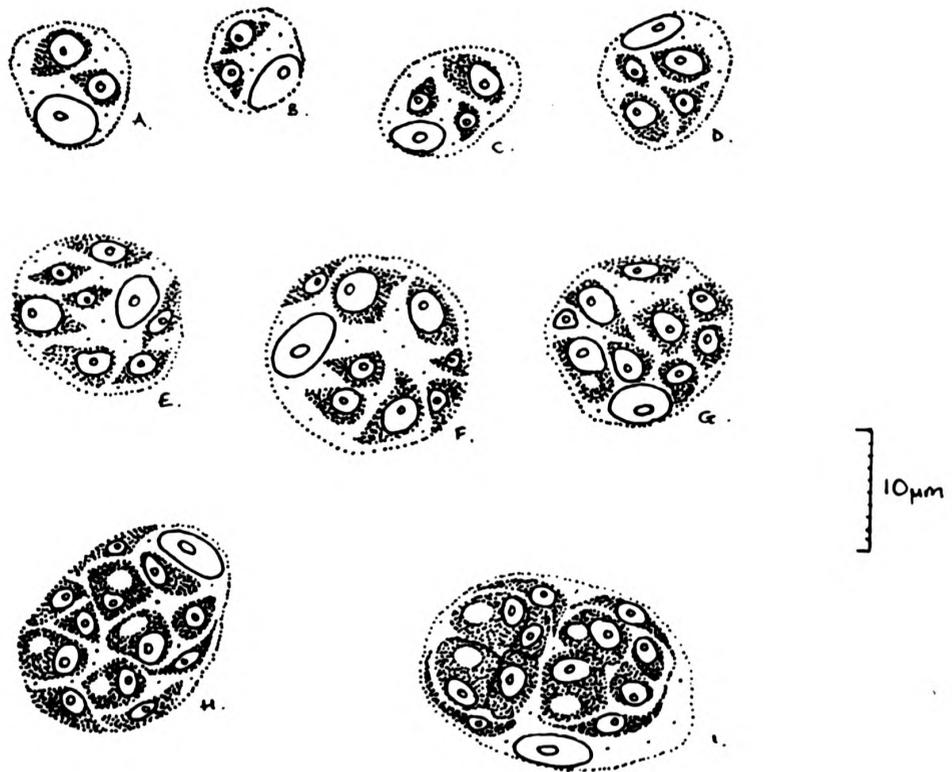
Histological observations showed that sporogonic stages were restricted to the kidney tubule and Bowman's space of the glomerulus. No sporogonic stages were seen in the interstitial tissues of the kidney, or in renal epithelial cells confirming fresh smear observations. In H&E sections, pseudoplasmodia containing developing and mature spores appeared to associate with the inner wall of the kidney tubular epithelium via numbers of "processes" or "holdfasts" and were thus suspended in the tubular lumen (fig.2.27). However, this did not agree with fresh preparation observations, whereby the entire lumen was seen to be completely blocked by pseudoplasmodia. Material fixed for TEM also showed complete blockage of the tubuli and a much more intimate host-parasite association, leading to the conclusion that these fine "thread-like" processes were caused by the parasites shrinking away from the inner microvillus wall of the tubule during formalin fixation. In addition it appeared that many spores and sporogonic stages "drop out" of sections, since in kidneys where fresh squash preparations had shown many tubules blocked with pseudoplasmodia, the equivalent histology revealed many spaces, and fewer spores. Giesma-stained sections were useful in demonstrating the presence of mature polar capsules in spores (fig.2.28), whilst Casson's stain demonstrated that parasites at different stages of development could be found within the same tubule. Casson's selectively stains carbohydrate, and so reveals the degree of maturity of individual spores; valvogenic cells appeared to lay down increasing amounts of carbohydrate as they form the spore valves (fig.2.29). PAS staining confirmed that two sporoplasms were present in each spore.

Ultrastructure

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fig 2.27 Histological section of the kidney stained with H&E. The 'holdfasts' suspending individual pseudoplasmodia in the lumen of the tubule can be clearly seen (arrows). These appear to be a result of formalin fixation. (bar=10μm)

fig.2.28 Histological section of the kidney stained with Casson's stain to demonstrate the variation in maturity of sporogonic stages in single kidney tubules. The most mature spore stages stain yellow (Y), with intermediate stages red (R), and less developed sporogonic stages purple/blue (B) (bar=10μm)
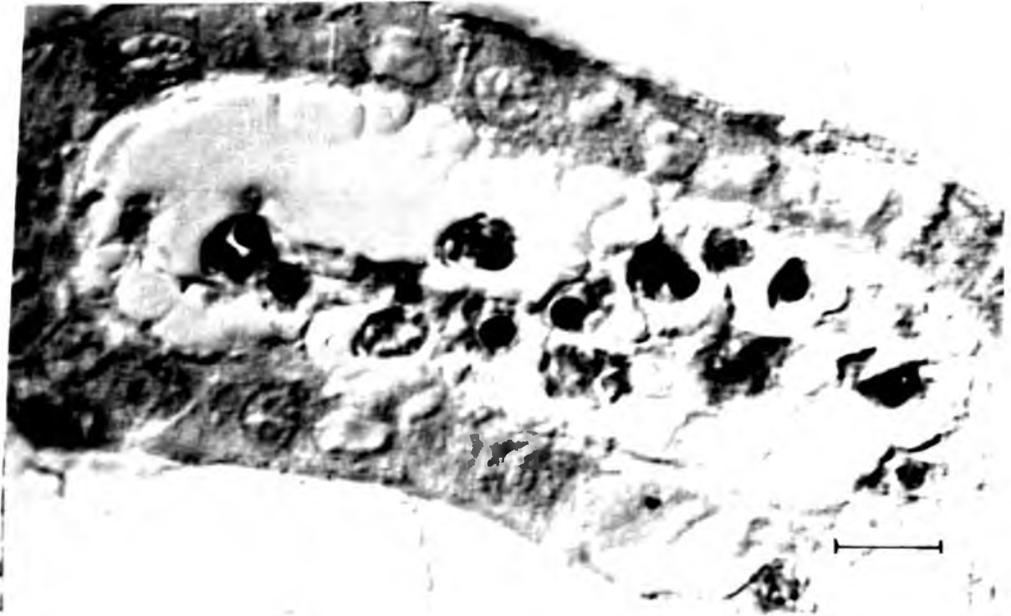
fig.2.30 Early sporogonic stage in the tubule lumen. Note Golgi (G) in the pseudoplasmodial cytoplasm (Pc) and numerous mitochondria (M). The pseudoplasmodial nucleus (Pn) lacks a nucleolus in this section. Inner sporoblastic cells (Sc) contain numerous mitochondria (*). Note the similarity in structure and appearance to extrasporogonic tertiary cells within a secondary cell, especially that between the staining appearance of the sporoblastic cell nucleus and that of the tertiary cells in extrasporogonic stages (Figs 10 & 11). (uranyl acetate/lead citrate, mag.x13,600)
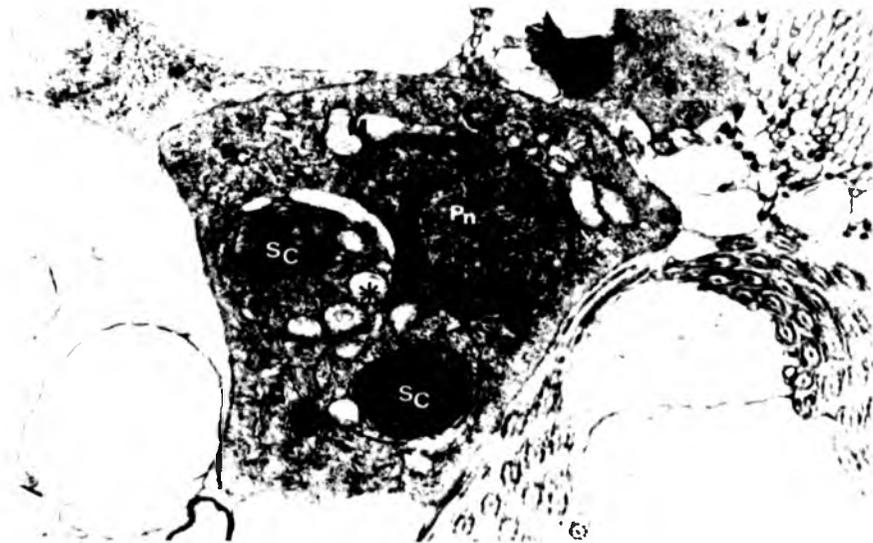
fig 2.31 Early sporogonic stage in the tubule lumen. The pseudoplasmodial cell cytoplasm (Pc) contains very large numbers of mitochondria (M) and other inclusions (*). Golgi (G) are also present. One sporoblast cell has Golgi and a nucleus with eccentric nucleolus (Sn). The other features incomplete membranes (arrows) and a pseudopodium-like cytoplasmic projection (arrowhead). (uranyl acetate lead citrate, mag x16,000)
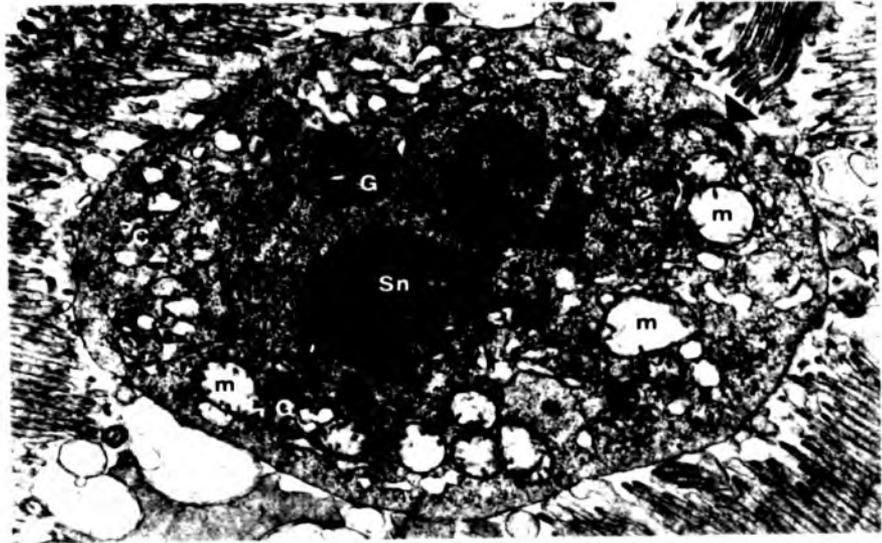
fig.2.32 Early sporogonic stage in the kidney tubule lumen virtually identical ultrastructurally to an extrasporogonic triple form. The pseudoplasmodial cell nucleus (Pn) is present and there are many areas of Golgi (G) in the pseudoplasmodial cytoplasm along with numerous mitochondria (M) and inclusions (*). Inner, sporoblastic cells (Sc) have a more unevenly staining nucleus and very few cytoplasmic organelles other than rare mitochondria. (uranyl acetate lead citrate, mag x18,000)

The earliest sporogonic stages observed in ultrathin sections were found in the lumena of the kidney tubules, and the Bowman's space. They consisted of a single enveloping pericyte or pseudoplasmodium, enclosing two inner cells, or sporoblasts (fig.2.30, 2.31 & 2.32).

The pseudoplasmodial cell cytoplasm of early stages in the kidney tubule lumen was bound by a single unit membrane and characteristically contained very large numbers of mitochondria (fig.2.31 & 2.32). These were often of two distinct types- large mitochondria with a loose electron lucent matrix and few sparse cristae, and more compact forms, with a denser matrix and better formed cristae in larger numbers (fig.2.31). Numerous stacks of Golgi were present, along with some ribosomes, endoplasmic reticulum, and occasionally other membrane bound inclusions containing amorphous material (fig.2.31). The nucleus was uniformly granular with an eccentric nucleolus. In later pseudoplasmodia, the cytoplasm retained the same basic structure, but fewer mitochondria and Golgi were present.

Sporoblast cells had a cytoplasm more densely studded with free ribosomes than that of the pseudoplasmodium, giving them a darker staining appearance. Their cytoplasm contained mitochondria and occasional Golgi, but few other organelles. The cytoplasm was often folded in upon itself and occasionally was pulled out into pseudopodia-like projections into the pseudoplasmodial cytoplasm (fig.2.31). Sporoblast cell nuclei possessed an eccentric nucleolus. The remainder of the nucleus was less uniform than that of the pseudoplasmodial nucleus, having occasional areas of clumped chromatin. Some sporoblast cells were smaller than others within the same pseudoplasmodium, confirming light microscope findings.

In heavier infections the complete lumen of an infected tubule was occluded by

sporogonic stages of differing degrees of maturity. Mature spores were found in the same tubules as newly established early stages. The most advanced plasmodia, containing almost mature spores, were positioned towards the centre of infected tubules (fig.2.33).

Many pseudoplasmodia adhered to the microvilli of the tubular epithelial cells. At points, thin cytoplasmic processes interdigitated with the microvilli, thus presumably strengthening their attachment to the inner wall of the tubule (fig.2.34). Stages further towards the centre of the tubule were not attached to the microvilli, but wedged tightly together and lacked projections (fig.2.35).

Sporoblast cells were enclosed by two unit membranes within the cytoplasm of the pseudoplasmodium, one belonging to the cell, the other to the pseudoplasmodium; they were thus separated from the pseudoplasmodial cytoplasm and enclosed in their own tightly fitting vacuole in a similar fashion to extrasporogonic secondary and tertiary cells within primary cells. However, as development proceeded, the two groups of six cells which went on to form a spore each came to lie in a single vacuole in the pseudoplasmodial cytoplasm. Some sporogonic cells were traversed by microtubular bundles (fig.2.39, 2.43 & 2.45).

As sporoblast cells divided it was impossible to identify their future fate. At the twelve cell stage however the first signs of differentiation became apparent. The first sporoblastic cells to become clearly differentiated were those destined to become capsulogenic cells. The nuclei of such cells contained an eccentric nucleolus; the cytoplasm however was characteristic. Invariably it contained large numbers of

fig 2.33 Kidney tubule lumen completely occluded by sporogonic pseudoplasmodia. Note the space left by a mature spore (*) and the extensive small vacuoles (arrows) in the cytoplasm of the host tubular epithelial cells. (uranyle acetate/lead citrate, mag.x2,400)
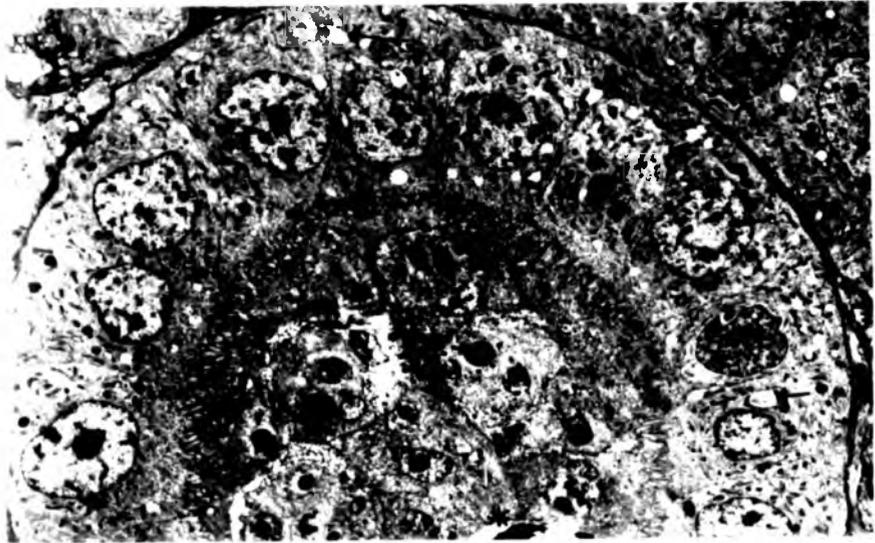
fig 2.34 Interdigitation of the pseudoplasmodial cytoplasm (Pc) and bounding membrane with the microvilli of the host tubular epithelium (Kc). At some points the parasite/host interface is almost confluent (arrows). (uranyl acetate/lead citrate, mag.x25,000)

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interdigitating with the host via pseudopodium-like projections of pseudoplasmodial cytoplasm (arrows). Those towards the centre have no point of attachment to the host but are tightly packed together in an almost tissue like arrangement. Note the mitochondria (M), some of them very elongate (arrowhead). Many pseudoplasmodia contain internal sporoblastic cells. Some of these can be discerned as future capsulogenic cells by the presence of dilated cisternae of RER (*). (uranyl acetate/lead citrate, mag.x7,000)
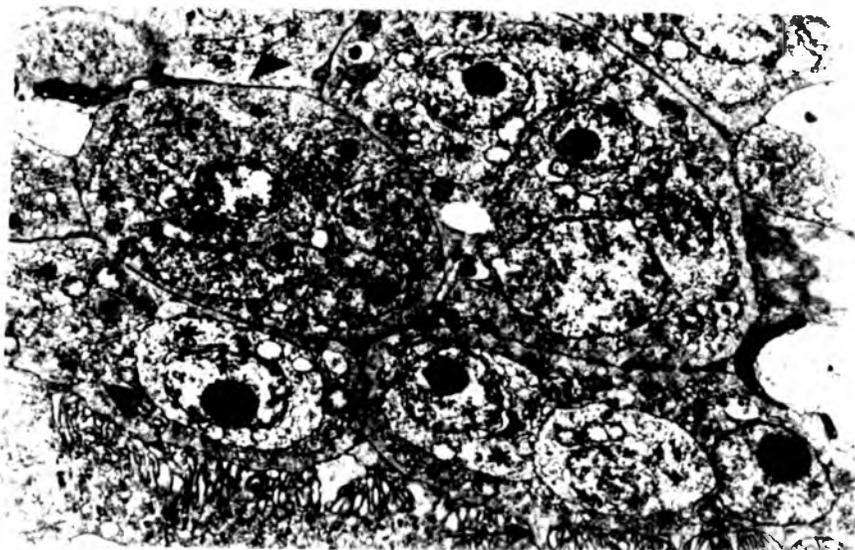
fig.2.36 Assortment of developmental sporogonic stages in the kidney tubule lumen. One pseudoplasmodium (P₁) has only a single sporoblast cell in the plane of the section. In all early stages the pseudoplasmodium is characterised by large numbers of mitochondria. At the bottom left is a more mature parasite. The capsulogenic cell (C) contains three cross-sections of the external tube of the capsular primordium in different planes (arrows). A dark central core present in the external tube represents the developing filament. Note the dilated cisternae of RER in the capsulogenic cell cytoplasm. Valvogenic cells (V) are beginning to envelope the internal cells of the spore, but have yet to join to form the spore suture. The nucleus (Vn) of one valve cell is visible. Sporoplasm cells (S) can be differented by their internal location and more densely stained cytoplasm. (uranyl acetate/lead citrate, mag.x7,500)
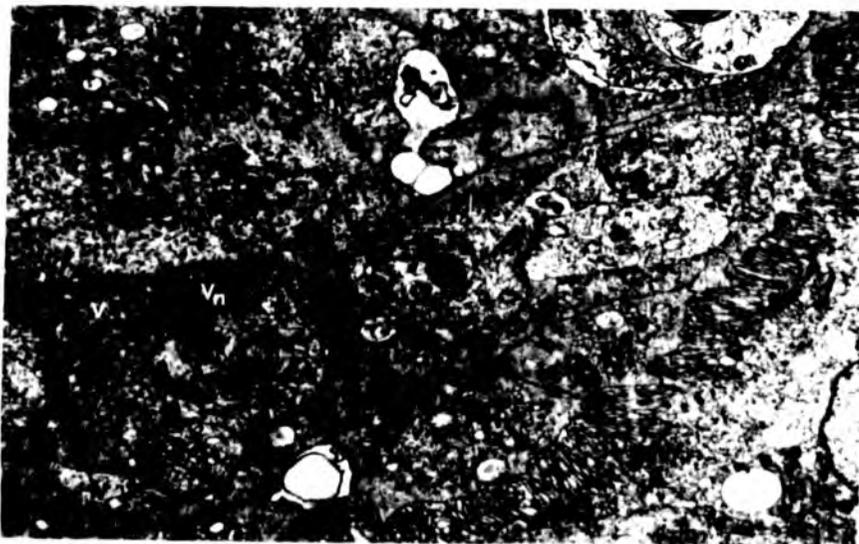
fig 2.37 Early sporogonic stage in which only a single sporoblast cell is evident. The pseudoplasmodial cytoplasm (Pc) features many well-formed mitochondria (M), other inclusions (*) and some cisternae of RER. Internal cell cytoplasm has less well formed mitochondria (arrow) and is less densely staining. Golgi (G) is present. (uranyl acetate/lead citrate, mag x18,500)
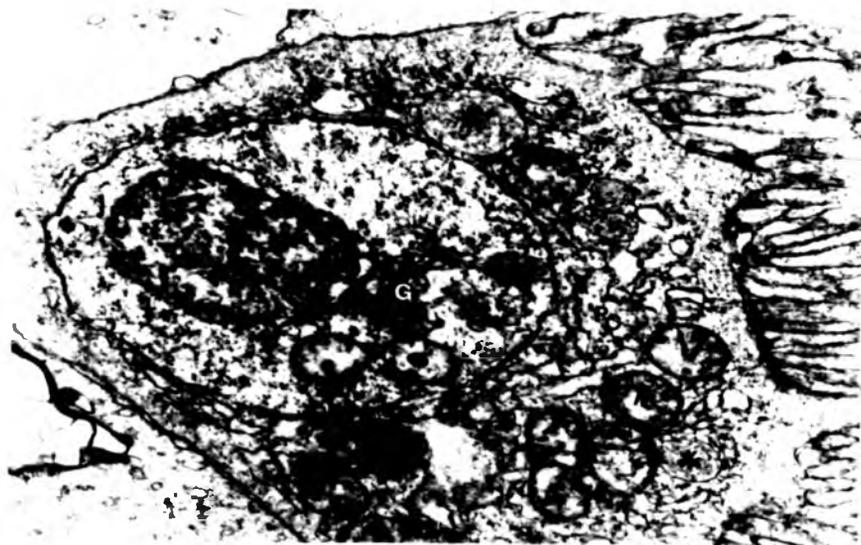
fig 2.38 Various sporogonic developmental stages in the tubule lumen. An early stage (1) has an interdigitating pseudoplasmodial cell (Pc) with Golgi, mitochondria and RER. Two internal cells appear closely apposed (arrow), and may be pulling apart after a recent division. A maturing spore (2) has capsular primordia (*) in each capsulogenic cell (C). One has an external tube forming from the capsular primordium. Sporoplasm cells (S) lie internally and are more electron dense, with the valvogenic cells (V) encircling the two capsulogenic and two sporoplasm cells, and joining at a suture (arrow). A mature spore at the top right has extremely electron dense spore valves with no signs of cellular detail (arrowhead). (uranyl acetate/lead citrate, mag x7,200)

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fig 2.39 Sporogonic stage at the division stage with internal sporoblastic cells of different sizes. One small cell (S_1) contains Golgi (G) and mitochondria (M). The nucleus (Sn) of another (S_2 is traversed by microtubules, indicating active division. An early capsulogenic cell (C) contains well-developed dilated cisternae of RER in the cytoplasm and numerous mitochondria. Two cross-sections of external tube are evident (arrowhead). Note the increased electron density at the host/parasite interdigitating interface (arrows). (uranyl acetate/ lead citrate, mag x17,000)

fig 2.40 Early sporogonic stage. The pseudoplasmodial cell (Pc) contains inclusions (*) and mitochondria (M). The early capsulogenic cell contains a cross section of the external tube with an electron dense core (arrow). Along with its adjacent sporoplasm cell (Sp), it is in the process of being enclosed by two valvogenic cells (V_1, V_2). The nucleus of one valvogenic cell (Vn) is visible. (uranyl acetate/lead citrate, mag.x12,000)
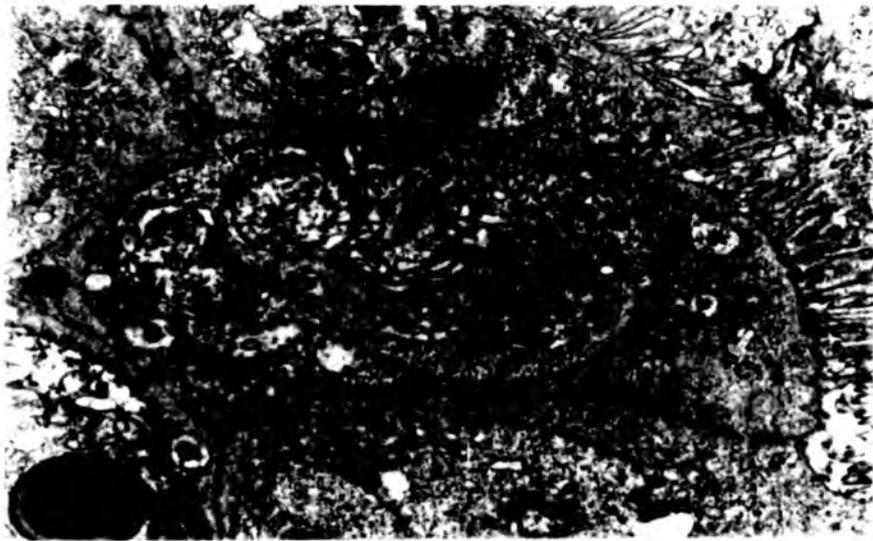
fig 2.41 External tube (**arrow**) forming from a bulbous capsular primordium (*). Note the electron lucent and dense layers forming the primordium, which pass out into the external tube, and the cross section of the external tube (**arrowhead**). Two cross sections of the external tube contain a very electron dense core, representing the developing filament, the other does not (uranyl acetate/lead citrate, mag.x18,000). Inset: regularly spaced microtubules forming a sheath around the external tube. These have been related to the active invagination of the tube as the filament becomes coiled within the capsule (see text). The external tube has a wall composition identical to that of the primordium. (uranyl acetate/lead citrate, mag.x32,000)
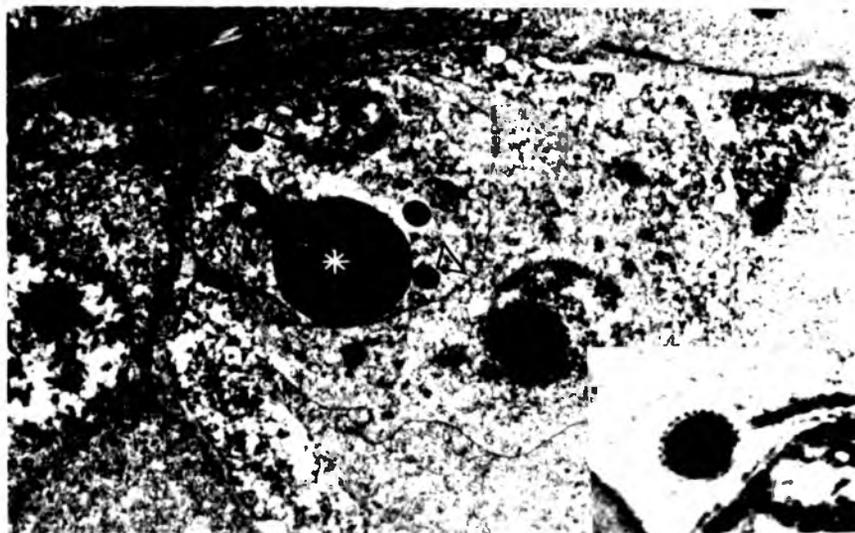
fig 2.42 Maturing spore. The capsulogenic cells (C_1) contain numerous cross sections of the external tube. Note the large inclusions present in the capsulogenic cell cytoplasm (*). Discrete very electron dense particles are beginning to accumulate in the sporoplasm cells (**arrow**). Valve cells (V_1, V_2) encircle the other cells forming the spore, and retain cytoplasmic and nuclear integrity. The pseudoplasmodium (**Pc**) contains the capsulogenic cells (C_1) of its second spore, not yet enclosed by valvogenic cells, reflecting the asynchronous development of the two spores of a pseudoplasmodium. **Pn**, pseudoplasmodial cell nucleus. (uranyl acetate/lead citrate, mag.x13,000)

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pseudoplasmodial cell (Pc) contains many mitochondria (M). Capsulogenesis is at different stages. One capsule (C_1) has helically coiled windings of the polar filament within. In another, (C_2), the external tube is seen at an unusual section. The nucleus of one sporoplasm cell (S_1) is traversed by a bundle of microtubules. Two valvogenic cells (V_1, V_2) surround each spore and are joined at a suture (arrow). Valvogenic cells are beginning to pull away from the pseudoplasmodial cytoplasm (*). (uranyl acetate/lead citrate, mag x8,500)
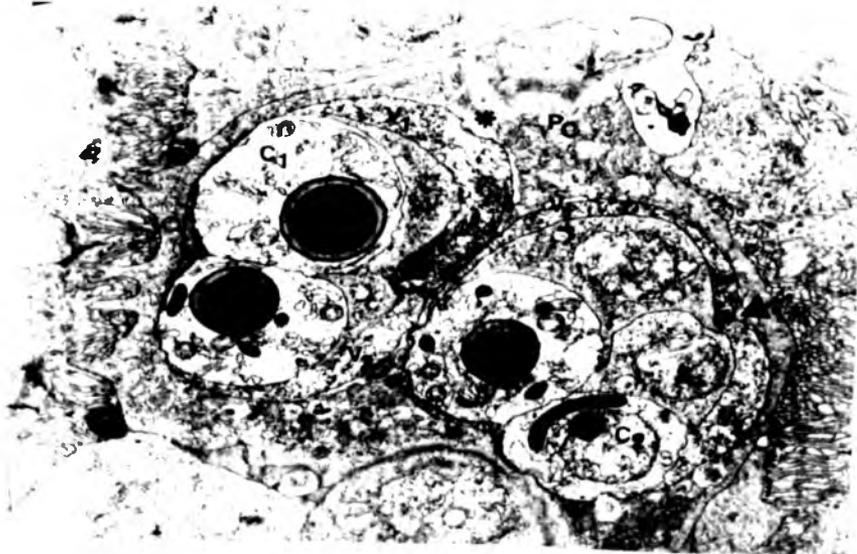
fig.2.44 Detail of fig 2.43 to show the very thin anterior layer formed by the valvogenic cells (arrow) and the thickened suture between the two valve cells where they join at either pole of the spore (arrowhead). Note especially the two thickened junctions (J) connecting the valve cell to the capsulogenic cell either side of the discharge canal being formed by the valvogenic cell. Note that although the developing capsule is far from this point in the cell, in mature capsules it always comes to lie adjacent to the discharge canal. Sporoplasm cell (S) contains electron dense particles. (uranyl acetate/lead citrate, mag.x21,000)
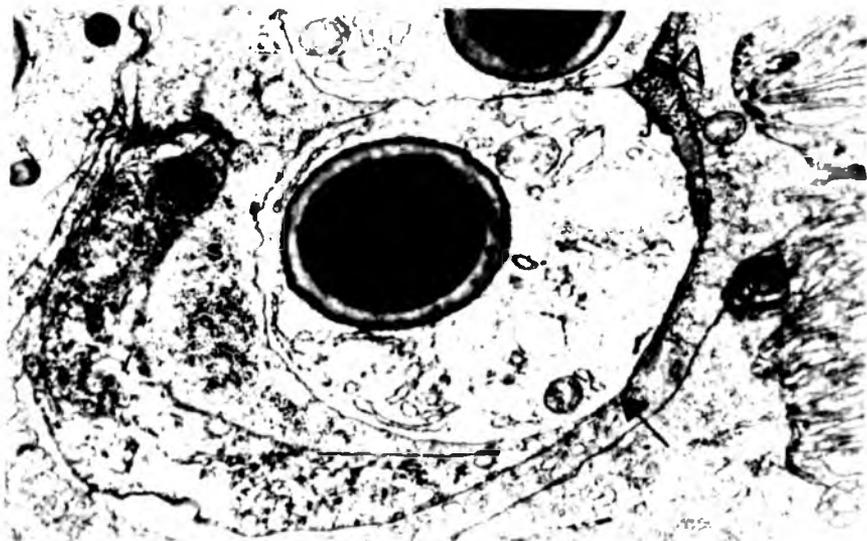
fig 2.45 Detail of fig 2.43 showing microtubular bundle (**arrowhead**) across the sporoplasm cell nucleus (**Sn**). Notice the unusual external tube structure in the lower capsulogenic cell (**C**), and the well defined membranes separating all individual cells forming the spore (**arrows**). The valvogenic suture (**Vs**) is thickened and electron dense. (uranyl acetate/lead citrate, mag x23,400)
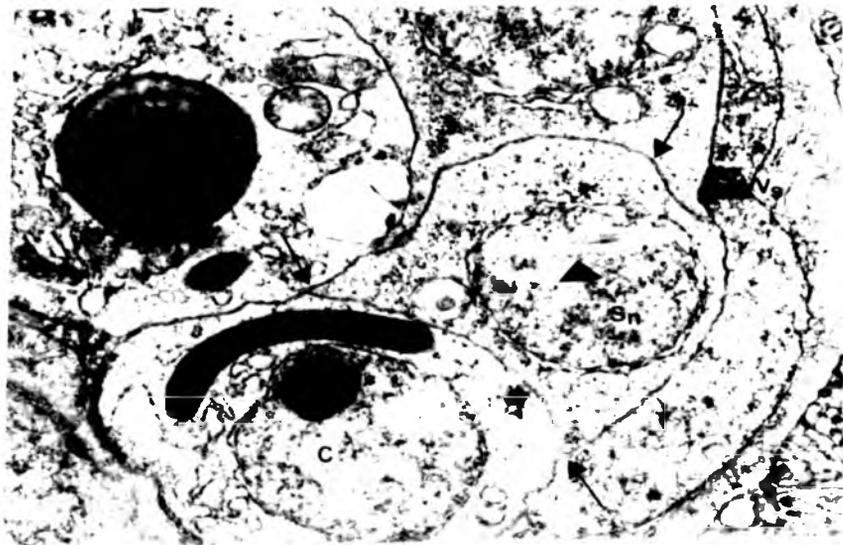
fig.2.46 Almost fully mature capsule. The electron lucent wall is confluent with the outer wall of the helically coiled invaginated polar filament (arrow). Note the valvogenic-capsulogenic cell junctions (arrowheads) either side of the prominent discharge canal (Dc) formed by the valvogenic cell. The stopper structure (Sp) of the capsule lies directly below this canal. (uranyl acetate/lead citrate, mag.x32,500)
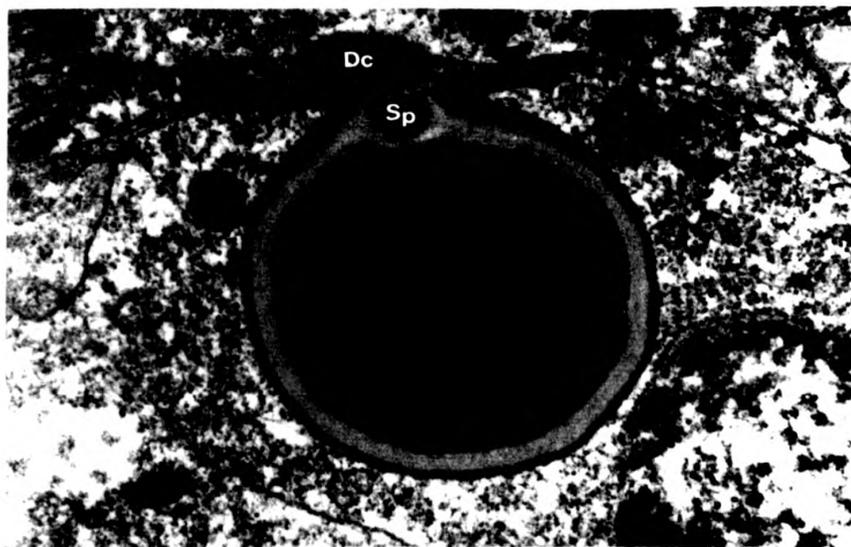
fig 2.47 Almost mature spore. The valvogenic cells (V_1, V_2) are joined at the raised and thickened suture line (Vs). The valvogenic layer is particularly thin anteriorly, but thicker posteriorly. The valvogenic cell cytoplasm appears to be becoming degraded, losing cytoplasmic structure and integrity, becoming vacuolated, and pulling away from the pseudoplasmodial cytoplasm (X). The discharge canals (D) of the polar filaments are evident along with the valve-capsulogenic cell junctions (arrows). Sporoplasm cells contain the accumulation of discrete, tiny electron dense bodies (*) characteristic of maturing sphaerospores from Atlantic salmon. The sporoplasm cells are deeply folded (arrowhead). (uranyl acetate/lead citrate, mag.x15,000)

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fig.2.48 Possible means of tertiary cell production: **A**, by endogenous cleavage whereby the secondary cell nucleus divides, then one of the daughter nuclei remains in the secondary cell cytoplasm, whilst the second becomes surrounded by a double membrane to separate it from the secondary cell cytoplasm in a vacuole; **B**, by the engulfment of one secondary cell by another
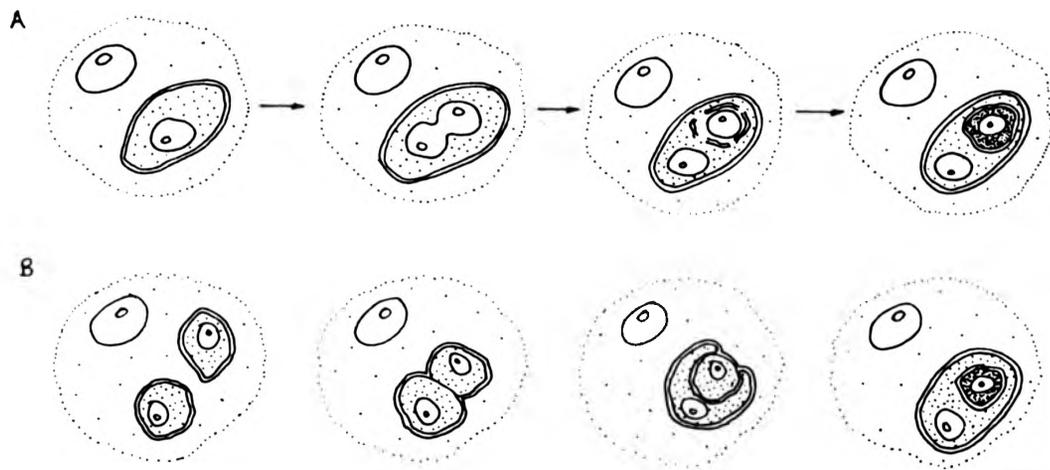
distended cisternae of rough endoplasmic reticulum, some mitochondria, but hardly ever Golgi (fig.2.35, 2.39 & 2.40). Free ribosomes were also present, along with occasional, membrane-bound inclusions of amorphous material.

Capsulogenesis was slightly asynchronous within a single spore, and began with the formation of a bulb- or pear-shaped capsular primordium, wholly membrane-bound within the cytoplasm of the cell (fig.2.41). The primordium was bounded by a very thin electron dense membrane, and consisted of an electron lucent, thicker, outer region and a more electron dense central core. From the primordium grew a narrow external tube which could be seen in a variety of oblique, transverse and longitudinal sections throughout the cytoplasm (fig.2.41-2.45). A maximum of five cross-sections of external tube were seen in the cytoplasm of any single capsulogenic cell. The external tube possessed identical lucent and dense layers to those of the primordium. At its centre however, appeared an extremely electron dense material (fig.2.40, 2.41 & 2.42). Around the outside of the tube lay a sheath of regularly spaced, longitudinally running microtubules (fig.2.41 inset).

The extremely electron dense material of the centre of the external tube later appeared within the capsular primordium as coils of the polar filament, H- or S-shaped in cross-section. The filament contained both lucent and dense regions, the lucent area being continuous at the anterior end of the capsule with the lucent outer layer of the capsule itself (fig.2.46).

The nucleus and cytoplasm of the capsulogenic cell changed as capsulogenesis neared completion. Dilated cisternae of rough endoplasmic reticulum were no longer present, and the nucleus became heterochromatic. Sometimes electron dense globular inclusions appeared.

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At the end of capsulogenesis the very uppermost point of the polar capsule tapered very slightly, directly opposite the slightly raised discharge canal that had, in the meantime, been formed by the valvogenic cell. This was always slightly less electron dense than the rest of the valvogenic cell cytoplasm, and formed a cylindrical channel from the inner valvogenic cell wall where it contacted the capsulogenic cell, to the outer cell wall of the valvogenic cell, which on spore release would presumably be in contact with the external environment (fig.2.44, 2.46 & 2.47). At this position on the capsule a plug formed where the capsule wall turned down into the capsule itself to form the filament. The mouth of the capsule thus became plugged directly opposite the discharge canal (fig.2.46).

Two interesting structures were seen during capsulogenesis at either side of the point where the discharge canal was later to be formed by the valvogenic cell. Here the valvogenic and capsulogenic cells formed a desmosome-like thickened junction reminiscent of that which joined the valvogenic cells together to form the spore suture (fig.2.43, 2.44, 2.46 & 2.47). These junctions became progressively more thickened as the spore matured. The discharge canal was formed by the valvogenic cell directly between these two structures (fig.2.46 & 2.47). Capsular primordia were often seen to be developing well away from this point, sometimes even at the other end of the capsulogenic cell (fig.2.44), but mature capsules were always found with their plug directly between the two thickened junctions and thus directly opposite the discharge canal (fig.2.46).

Valvogenic cells became easy to distinguish from other sporogonic cells at the onset of capsulogenesis, when the capsulogenic cells had become clearly differentiated.

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At this point the two valve cells were seen to spread thinly around the four other cells comprising the spore, enclosing them completely (fig.2.36, 2.40, 2.42 & 2.44). The two cells were joined together by a desmosome-like junction at either end of the spore (fig.2.45 & 2.47). This junction was thickened at both poles of the spore by an electron dense substance, and was slightly raised anteriorly.

The valvular cytoplasmic layer was invariably very thin anteriorly, but thicker posteriorly. The valve cell nuclei, if present in the section, were usually to be found in the posterior portion of each cell (fig.2.44). In early valvogenesis the cytoplasm contained numerous mitochondria, occasional Golgi, and free ribosomes. The nucleus was uniformly granular with an eccentric nucleolus (fig.2.42). However, as the spore matured, both outer and inner membranes of the valve cells became more electron dense and thickened. Vacuoles became extensive in the cytoplasm, particularly posteriorly, and the cytoplasm itself lost all organelles, becoming degenerate with clumps of electron dense material in an electron lucent or clear matrix (fig.2.47). Polar filament discharge canals formed opposite the plug of the polar capsule, between the two areas of cell junctions described above. Increased vacuolation of the valve cell cytoplasm posteriorly eventually caused the now almost mature spore to "pull away" from the cytoplasm of the pseudoplasmodial cell (fig.2.47).

In fully mature spores, the valvogenic cells had become a thin, very dense, structureless, and presumably very resistant "spore coat", with no nuclei or visible cytoplasmic details. As such they became very difficult to section, as fixatives and infiltration media appeared to fail to penetrate (fig.2.38).

The two uninucleate sporoplasm cells were distinguishable by their position on

the inside of the spore, encircled by the valvogenic cells, and by being distinct from capsulogenic cells.

Their cytoplasm contained a much higher number of free ribosomes than both valvogenic or capsulogenic cells, making them darker staining than other sporogonic cells (fig.2.36, 2.38 & 2.43). Other cytoplasmic features included mitochondria, and accumulations of discrete, tiny, extremely electron dense particles (fig.2.44 & 2.47). No sporoplasmosomes were seen. Occasionally, the cytoplasm was infolded upon itself (fig.2.47). The sporoplasm cell nucleus appeared as scattered chromatin but lacked a nucleolus (fig.2.45). On one or two sections it was traversed by a bundle of microtubules (fig.2.47).

Spore formation was often asynchronous in each pseudoplasmodium. Whilst one developing spore was encircled by valvogenic cells and possessed differentiating capsulogenic cells, its partner often had undifferentiated sporoplasmic and capsulogenic cells, and had yet to be surrounded by valvogenic cells (fig.2.42).

2.4 DISCUSSION

2.4.1 Extrasporogonic stages

Light microscope observations

The results of the present investigation have demonstrated that the myxosporean species under study has two distinct life cycle stages in Atlantic salmon. The first is an extrasporogonic phase in the blood and kidney interstitium, the second a sporogonic one in the kidney tubules.

Extrasporogonic proliferative stages of *Sphaerospora* sp. are being reported with

an increasing frequency from a range of fish species. Such reports have illustrated that *Sphaerospora* sp from a number of fish species, despite having very similar spores, have distinctly different extrasporogonic stages in the blood (Lom, *et al*, 1985b; Baska & Molnar, 1988; Kepr & Trsova, 1989). These authors have suggested that individual fish species might have their own distinct, host specific, *Sphaerospora* sp and that extrasporogonic stage morphology could become an important criterion for species differentiation, and thus a valuable taxonomic feature. Extrasporogonic stages have been defined as stages which proliferate, without the formation of spores, before, or at the same time as the sporogonic phase takes place, at a site other than that in which sporogony occurs (Lom, 1987; Lom *et al*, 1991).

The salmon extrasporogonic stages, like all other *Sphaerospora* sp extrasporogonic stages described to date are associated with the blood. The salmon *Sphaerospora* sp. extrasporogonic stages are, however, found in far greater numbers in the kidney interstitial tissue compared to the circulating blood. The K-stages of *S. renicola*, are similarly found in large numbers in the blood vessels of the swimbladder wall (Csaba *et al.* 1984). A number of examples exist of extrasporogonic stages that are found in high numbers in the kidney interstitium. Hedrick *et al*, (1988) found that extrasporogonic stages of *S. elegans* showed such a preference for the kidney interstitium of sticklebacks, but could also be found in the blood, conflicting with the observations of Lom *et al* (1991), who had found them restricted to the *rete* of the eye. The extrasporogonic stages described by Cone & Cusack, (1991) from brook trout were also restricted to the kidney interstitium and a similar situation exists in PKX infections in salmonids. A definite bias existed in kidneys infected with the salmon *Sphaerospora*, in that more of the largest, more developed primary cells with very high numbers of

secondary cells, were found in the kidney interstitium than in the blood. However, even though larger stages were comparatively more rare, the full range of primary cell size and secondary cell number was seen in the blood.

It may be that the apparent preference of *Sphaerospora* extrasporogonic stages for areas such as the kidney interstitium is a purely passive phenomenon due to the entrapment of relatively large parasites in a network of narrow blood vessels or sinuses. However the *rete* stages of Lom *et al* (1991) appear to have adaptations, such as cytoplasmic projections which assist their maintainance in that site.

In histology, a very high proportion of salmon extrasporogonic stages in the kidney interstitium were seen to be adhering to, or in close proximity to, the basal lamina of the epithelium of the kidney tubule. Lom *et al*, (1983a) encountered extrasporogonic C-stages of *S. renicola* in the kidney interstitium in the same site. Extrasporogonic stages may therefore show some kind of positive 'attraction' to the kidney and, more specifically, the kidney tubule outer wall. This might be reflective of the tubule lumen as the site of sporogony. Branson (1987) reported that extrasporogonic stages of the salmon *Sphaerospora* were more common in the anterior, haematopoetic areas of the kidney. This contrasts with the present study, where although present throughout the anterior, mid and posterior kidney, extrasporogonic stages were more common posteriorly due to this apparent attraction for the outer wall of the kidney tubules.

In the salmon, extrasporogonic stages were present, rarely, in the sinuses of the blood vessels of the spleen and liver. Hedrick *et al*. (1990) found that the blood borne extrasporogonic stages of *S. ictaluri* were systemic, being seen in the blood vessels and sinuses of the kidney interstitium, liver, brain and even between the dermis and the

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skeletal muscle. Supamattaya *et al.* (1991) reported extrasporogonic stages of *S. epinephali* in the kidney interstitium and liver arteries of cultured grouper. Odening (1989) found *S. renicola* K-stages in numerous organs as well as the swimbladder. The number of organs and tissues involved according to these reports seems to indicate that they reflect the blood borne nature of extrasporogonic stages rather than any parasite preference for the organs concerned.

The period of growth prior to the onset of repeated secondary cell division shown by the extrasporogonic stages from salmon after their release from ruptured primary cells has also been shown in both *S. renicola* and *S. epinephali* (Csaba, 1976; Lom *et al*, 1983a; Csaba *et al*, 1984; Supamattaya *et al*, 1991).

Although showing very similar staining characteristics in Giemsa-stained smears, *Sphaerospora* extrasporogonic stages from salmon in this study are distinctly different in a number of ways from those of other species of *Sphaerospora* described to date. Most notable is the large size of the most developed primary cells of the salmon parasite, and also their high number of secondary cells (table 2.5).

Of the larger extrasporogonic stages documented in the literature, those described by Baska & Molnar (1988) from white bream and asp of 20-60μm, with up to 40 secondary cells; by Hedrick *et al.* (1990) from channel catfish infected with *S. ictaluri*, of up to 25μm with 50 or less secondary cells; and those of the K-protozoan swimbladder stages of *S. renicola* in common carp of up to 30μm with a maximum of 46-60 secondary cells (Csaba *et al.*,1984; Dykova *et al.*, 1990) are the largest described to date. However, these are still significantly smaller than those from the salmon, which are up to 70μm, and none of the others described contain as many secondary cells i.e.

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table 2.5   Features of extrasporogonic stages

Host	Sphaerospora sp	Size (μm)		No. of secondary cells	No. of tertiary cells
		min.	max.		
common carp	S. renicola C-stage	5	15	1-8	0:1
common carp	S. renicola K-stage	5	30	1-60	0:1:2
gudgeon	Sphaerospora gobionis	4	16	1-8	0:1
roach	Sphaerospora sp.	4.5	15	1-8	0:1
tench	Sphaerospora sp.	4	17	1-8	0:1
rudd	Sphaerospora sp.	7	15	1-8	0:1
blue bream	Sphaerospora sp.	7	15	1-8	0:1
white bream	Sphaerospora sp.	7	60	1-40	0:1
asp	Sphaerospora sp.	7	60	1-40	0:1:2
bleak	Sphaerospora sp.	15	30	1-8	0:1:2
native chub	Sphaerospora sp.	-	-	1-10	0:1
stickleback	Sphaerospora sp.	6.3	12.5	1-9	0
channel catfish	Sphaerospora ictaluri	-	25	1-50	0:1
grouper	Sphaerospora epinephali	2	11	1-16	0:1
stickleback	S. elegans/M. gasterostei	8	22	1-18	0:1:2
brook trout	unknown	5	30	1-36	0

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up to 120.

The extrasporogonic primary cell of the salmon parasite only ever contains a single nucleus, as do those of most others in the literature. However Baska & Molnar (1988) found primary cells with 2 to 4 nuclei in bleak, and Lom *et al.* (1985b) found two nuclei in some blood stages of *Sphaerospora galinae*. Ultrastructural studies of PKX have reported multinucleated primary cells (Kent & Hedrick, 1986; Feist & Bucke, 1987). Such details may prove to be relevant in the differentiation of taxa, once further extrasporogonic stages are discovered and their morphology becomes clearly established as a valuable taxonomic feature. The localisation of the secondary cells within a single vacuole in the primary cell cytoplasm, seen in some extrasporogonic stages from bleak by Baska & Molnar (1988), was not seen in the salmon parasite.

It is notable that some extrasporogonic stages produce many more secondary cells than others. There seems to be a clear distinction between extrasporogonic stages in which there is a precise end-point in development as far as secondary cell numbers and cell composition are concerned, and those in which there is no such fixed point. For example, the end point of the *S. renicola* C-stages appears to be a primary cell containing eight secondary cells, each containing a single tertiary cell (Csaba, 1976; Lom *et al.*, 1983a). The most developed primary cells of extrasporogonic stages from gudgeon similarly contain eight secondary cells each with one tertiary cell (Lom *et al.*, 1985b), whilst in *S. epinephali* from grouper (Supamattaya *et al.*, 1991) the end product issixteen secondary cells.

The extrasporogonic stages from salmon appear to have no precise end point in secondary cell formation, and can be found with any secondary cell number up to 120. This is more in keeping with the type of secondary cell proliferation shown by the

extrasporogonic stages from brook trout with up to 36 secondary cells (Cone and Cusack, 1991), those of *S. ictaluri* with up to 50 (Hedrick *et al.*, 1990), white bream and asp with up to 40 (Baska and Molnar, 1988), or the *S. renicola* swimbladder stages with up to 60 (Dykova *et al.*, 1990).

Even in extrasporogonic myxosporean stages with a precise end-point in terms of cell numbers, it has been noted by a number of authors that the means and order in which cell divisions occur is often asynchronous and variable. Such variation is even shown by individuals of the same parasite population in a single host. Lom *et al.* (1983a) found irregular sequences in the development of the C-stage of *S. renicola* compared to the very rigid scheme outlined by Csaba (1976). Later, Dykova *et al.* (1990) studying the K-stages of the same parasite, showed that sometimes tertiary cells could be found in primary cells with very few secondary cells. On rare occasions triple formations could even be seen in such very early developing forms. They therefore noted that K-stage morphology was extremely varied, with large numbers of different combinations of size, shape, number and development of secondary cells being encountered. This agrees with findings from the salmon parasite where extrasporogonic stages with just three secondary cells could contain single tertiary cells. Two triple forms were also seen in a stage with only ten secondary cells. Although such findings were quite rare, such parasites appeared normal in all other respects. This suggests that rather than being inviable and representative of developmental error, they were intact, viable and simply demonstrative of irregular developmental sequences. This kind of variability and irregularity of development appears a normal characteristic feature of not only *Sphaerospora* life cycles, but those of myxosporeans in general (Lom *et al.*, 1985b; Lom, 1987). Considering the relatively few detailed reports of extrasporogonic stages

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and their development, it is perhaps possible that as more such stages are discovered and studied a more complete picture might emerge.

In some species with extrasporogonic stages, no tertiary cells were described (Cone & Cusack, 1991), whilst in others they were only visible under TEM (Supamattaya *et al.*, 1993). In a number of instances, one tertiary cell is found in secondary cells e.g. *S. renicola* C-stages (Csaba, 1976; Lom *et al.*, 1983a), *Sphaerospora* spp. from blue bream, white bream and asp (Baska & Molnar, 1988), and gudgeon, roach and tench (Lom *et al.*, 1985b). In other species the situation is more like that shown by salmon extrasporogonic stages, with some secondary cells having two tertiary cells e.g. bleak *Sphaerospora* sp. (Baska & Molnar, 1988) and *S. renicola* K-stages (Csaba *et al.*, 1984; Dykova *et al.*, 1990).

Intracellular extrasporogonic developmental stages were never seen in salmon infected with *Sphaerospora*. Such stages are to be found in *S. renicola* development at certain times of the year (Dykova, Lom & Grupcheva, 1983). Infected cells undergo hypertrophy before fusing together to form a large syncytium containing many parasite stages. However these spontaneously degenerate and are subsequently attacked by the host response. This has led to some workers believing that these stages are a 'blind alley' in the parasite's development (Lom & Dykova, 1985; Lom, 1987). Other authors, however, consider them to be unrelated to *S. renicola*, being viable developmental stages in the life cycle of *Hoferellus cyprini* (Molnar & Kovacs-Gayer, 1986b).

The end products of the extrasporogonic phase of the life cycle of the salmon parasite are 'units', of a secondary cell containing two tertiary cells, produced within the primary cell. These are comparable to the 'triple formations' or 'triplets' described by Baska & Molnar (1988) from the blood of a range of fish species. The salmon 'triple

formations', when released from primary cells were identical to the earliest sporogonic stages seen in the lumen of the kidney tubule, i.e. a pseudoplasmodium containing two sporoblast cells.

Ultrastructure

The extrasporogonic stages from salmon were bound by a simple, single unit membrane.   In the salmon *Sphaerospora*, the "thin fuzzy surface coat" observed ultrastructurally in studies of the C-stages of *S. renicola* (Lom *et al.*, 1983a) and in the extrasporogonic stages of *S. epinephali* (Supamattaya *et al.*, 1991) was not seen.   A similar surface coat may be present on the extrasporogonic stages of *S. ictaluri*, since Hedrick *et al.* (1990) reported a thickened external membrane with a "brush-like" surface in light microscope preparations; however, no TEM was presented on these stages, precluding an exact comparison.

The cytoplasmic membrane of both *S. renicola* and *S. ictaluri* primary cells is often drawn out into irregular projections or folds.   This also appears to be the case in PGD stages in the gills of channel catfish (Styer, Harrison and Burtle, 1992a).   This parasite may be related to *S. ictaluri* according to Hedrick *et al.* (1990).   Pellicular folds of the primary cell cytoplasm were seen in a few salmon *Sphaerospora* extrasporogonic stages, mainly those from the blood, but only in the very least developed parasites (with only one or two secondary cells) and only during the earliest stages of infection in epidemiological terms.   Such stages were never seen in well established infections or in larger parasites, despite the fact that in established infections many extrasporogonic stages consisted of a primary cell with one secondary cell.   These latter stages almost certainly are secondary cells released from ruptured primary cells, and are not newly

established parasites. Extrasporogonic stages with pellicular folds were not seen in any TEM sections. Lom *et al.* (1983a) related these folds, and the fuzzy surface coat, to the undulating membranes and movement associated with live C-stages of *S. renicola*. Hedrick *et al.* (1990) related the presence of folds in *S. ictaluri* to: (a) motion; or (b) nutrient uptake. Salmon *Sphaerospora* extrasporogonic stages observed in fresh preparations were non-motile. Perhaps the very earliest extrasporogonic stages of the salmon parasite with such cytoplasmic folds have a motile capability, and represent the infective agent which has recently established in the fish. Such a stage, entering the fish via the epithelia of the skin, gills, fins or buccal cavity (Markiw, 1989a), might need to be motile in order to reach the vascular system. The cytoplasmic projections of the primary cell of extrasporogonic stages described from stickleback (Lom *et al.*, 1991), differ from the above reports since they seem to represent an adaptation to securing the parasite to the endothelium of the *rete* of the eye of the host.

The cytoplasm of all *Sphaerospora* extrasporogonic primary cells studied ultrastructurally is characterised by a uniformly granular appearance and the presence of free ribosomes and mitochondria of a typical myxosporean form i.e. swollen with a pale matrix and few, often plate-like, cristae. Variability in structure of the mitochondria and their appearance such as that described in the present study appears to be a feature of myxosporeans (Current & Janovy, 1977; Yamamoto & Sanders, 1979; Lom *et al.*, 1982). The sparse amounts of cisternae of rough endoplasmic reticulum seen in salmon *Sphaerospora* primary cells is similar to that observed by Lom *et al.* (1991) from the *rete* stages of *S. elegans* in sticklebacks and that of the PKX primary cell described by Kent & Hedrick (1986). However, in the C-stages of *S. renicola* (Lom

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et al., 1983a), such rough endoplasmic reticulum is concentrated around the primary cell nucleus. This was seen only in very early stages of the salmon parasite, with few secondary cells. In *S. epinephali* (Supamattaya *et al.*, 1991, 1993), rough endoplasmic reticulum is present in an unusual "double ring" form not seen in salmon *Sphaerospora* or described in other reports.

In salmon extrasporogonic primary cells, Golgi are often present, and are always arranged in well organised "stacks". This contrasts with the diffuse Golgi of the K-stages of *S. renicola* (Dykova *et al.*, 1990).

The primary cells of *S. epinepheli* were found to contain electron dense, 188-145nm, sporoplasmosome-like bodies (Supamattaya *et al.*, 1991, 1993). These features were not seen in the cytoplasm of secondary or tertiary cells. Very similar structures have long been known in PKX primary cells as 'haplosporosomes', and are similarly localised in the primary cell only (Seagrave *et al.*, 1980; Kent & Hendrick, 1986; Feist & Bucke, 1987). Their nature and function remains unknown. No such bodies were seen in the primary cells of *Sphaerospora* from salmon.

No signs of phagocytic activity were encountered at the primary cell surface of the salmon extrasporogonic stages, contrasting with the findings of Dykova *et al.* (1990) from the K-stages of *S. renicola*.

The dense large lipoidal inclusions of Supamattaya *et al.* (1993) were not seen in salmon extrasporogonic stages. Although some large inclusions in the cytoplasm were present, these appeared empty, or contained very electron lucent material. Such apparently empty or electron lucent material-containing vacuoles of the primary cells of the larger salmon extrasporogonic stages are mirrored by the vacuoles and vesicles reported by Lom *et al.* (1983a) from *S. renicola* C-stages, and Lom *et al.* (1991) from

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extrasporogonic stages in sticklebacks infected with *M. gasterostei* and *S. elegans*.

The changes exhibited by primary cells as they developed from small stages containing few secondary cells to larger ones with many secondary cells, were marked, particularly in respect of nuclear structure. Small extrasporogonic stages had cytoplasmic features identical to those of secondary cells with one or two tertiary cells inside large extrasporogonic stages; especially with respect to free ribosome density, rough endoplasmic reticulum distribution, nuclear appearance and internal cell appearance. Therefore the small, least developed extrasporogonic stages described in the micrographs of the present study probably represent released secondary cells from large parasites which have ruptured.

These changes in nuclear structure in developing primary cells are comparable to those described by Dykova *et al.* (1990) from SBI stages of *S. renicola*. In this species, as newly released secondary cells began to grow as primary cells, their nuclei changed; when released, the cells possessed a compact nucleolus with clumps of heterochromatin peripherally throughout the nucleoplasm. As they developed the heterochromatin disappeared; the nucleolus became less compact, enlarged, and contained many small lucent areas. Dykova *et al.* (1990) suggested that this was evidence of intensive RNA synthesis. In the later stages of primary cell nuclear development Dykova *et al.* (1990) described the nucleolus as eventually becoming completely fragmented; this later stage was never seen in the salmon parasite. Only a single nucleus per primary cell was seen in the present study of salmon *Sphaerospora* extrasporogonic stages.

In developed primary cells of the salmon extrasporogonic stage secondary cells were wholly contained in a vacuole. This is a feature of all ultrastructural reports of extrasporogonic stages. However there are some differences between species in terms of the numbers of secondary cells found within any single vacuole in the primary cell cytoplasm. In the salmon *Sphaerospora*, the maximum number encountered was two, which is interpreted as a result of the recent division of a single secondary cell. More commonly, just one secondary cell occupied each vacuole. In *S. epinephali*, Supamattaya *et al.* (1993) never encountered more than one cell in a vacuole. However, Lom *et al.* (1991) found groups of 2-5 division products in the *rete* extrasporogonic stages from the eyes of three-spined sticklebacks, whilst Lom *et al* (1983a) described encountering whole clusters of secondary cells remaining together in the C-stages of *S. renicola*. Engulfment of one secondary cell by another, seen rarely in the extrasporogonic stages of the salmon parasite, was described by Feist & Bucke (1987) in PKX.

In salmon extrasporogonic stages some secondary cells were apparently in the process of 'pulling apart' from each other to lie in their own vacuole within the primary cell having previously shared a vacuole. Dykova *et al.* (1990) and Lom *et al.* (1983a) showed a similar phenomenon in the K- and C-stages, respectively, of *S. renicola*. The former authors, along with Lom *et al.* (1991), also describe secondary cell surface projections. These were shown by secondary cells of the salmon extrasporogonic stage, suggesting that they were evidence of recent cleavage.

Feist & Bucke (1987) indicated that, in PKX, tertiary cells may be formed by secondary cells engulfing each other. There was some limited evidence of this from the

present study. However, both Lom *et al.* (1991) and Dykova *et al.* (1990) saw, occasionally, two secondary cell nuclei in the same cytoplasm of both *S. renicola* K-stages, and the *rete* stages of sticklebacks. This suggests that tertiary cell formation is a result of an endogenous division of the secondary cell nucleus and evidence of this was also seen in salmon *Sphaerospora* stages. Sometimes more than one nucleus lay in the same secondary cell cytoplasm, but was incompletely membrane-bound, being, along with a portion of cytoplasm encircled by a cytoplasmic membrane. Additionally, tertiary cells were invariably very much smaller than secondary cells and possessed comparatively little cytoplasm. This seems inconsistent with their being engulfed secondary cells. It could be that in the salmon parasite, both methods of tertiary cell production, engulfment and endogenous division are utilised (fig.2.48). This might be another example of the irregularity of developmental pathways shown by myxosporeans. As a group, and as individual species, they seem to be able to achieve the same developmental result via a range of developmental pathways (Lom, 1987).

All reports of *Sphaerospora* extrasporogonic stages note a marked increase in the numbers of free cytoplasmic ribosomes in secondary cells compared to primary cells, giving them a more densely staining characteristic. In salmon *Sphaerospora* stages, rough endoplasmic reticulum was concentrated around the nucleus, often almost encircling it. This was described by Dykova *et al.* (1990) from the secondary cells of extrasporogonic K-stages of *S. renicola*. Another common feature is a reduction in the number of mitochondria in the secondary cell cytoplasm compared to that of the primary cell, perhaps because of the presumed role of the primary cell in nutrient uptake and maintainance in the host.

Microtubules feature as common structures in the secondary cells of

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extrasporogonic stages indicating that these cells are active in division (Lom *et al.*, 1991; Lom *et al.*, 1983a), but were not seen in salmon stages despite the fact that they were very obviously dividing. Microtubules were seen in sporogonic stages of the salmon *Sphaerospora*.

Deep infoldings of the nuclear and cytoplasmic membrane were shown by Lom *et al.* (1983a) and by Dykova *et al.* (1990), in both C- and K-protozoan stages of *S. renicola*. This was a commonly observed phenomenon in salmon *Sphaerospora* extrasporogonic stages, sometimes giving the impression that two nuclei existed in close apposition within the same cytoplasm. Careful tracing of the nuclear membranes showed that usually the nucleus was actually folded over itself, the two actually being lobes of the same nucleus. Two nuclei were present in some secondary cells, when tertiary cells were being formed, but these were easily distinguished from folded nuclei by their incomplete membranes.

There is, in the salmon parasite, and most other extrasporogonic stages described to date, a distinct lack of difference between the appearance and staining intensities of the cytoplasm of the secondary and tertiary cells, perhaps indicating why tertiary cells are often difficult to distinguish in Giemsa-stained preparations under the light microscope (Supamattaya *et al.* 1991).

Sphaerospora extrasporogonic stage tertiary cells featured very small amounts of cytoplasm around the cell nucleus. The cells lay within vacuoles in the secondary cell cytoplasm, in the same manner as secondary cells lie in that of the primary cell. The cytoplasm contained few organelles other than mitochondria. In most extrasporogonic stages, including those of the salmon *Sphaerospora*, PKX, *S. epinephali*, and *S. renicola*, the tertiary cell cytoplasm contains dense free ribosomes. However,

fig.2.49 The possible role of the valve cell-capsulogenic cell junctions during capsulogenesis.

A   capsulogenic cell (C) being enveloped by valvogenic cell (V). Thickened cell junction forms, and the capsular primordium (CP) has an early stage of external tube growth

B   The distal end of the fully extended external tube attaches at the thickened junction. Polar filament discharge canal/ aperture begins to be formed by the valvogenic cell

C   External tube invaginates into the capsule to form the polar filament. This causes the capsule to be 'pulled up' to a position directly below and opposite to the discharge canal

D   Mature polar capsule containing the polar filament, with its apex directly opposite the discharge canal of the mature spore
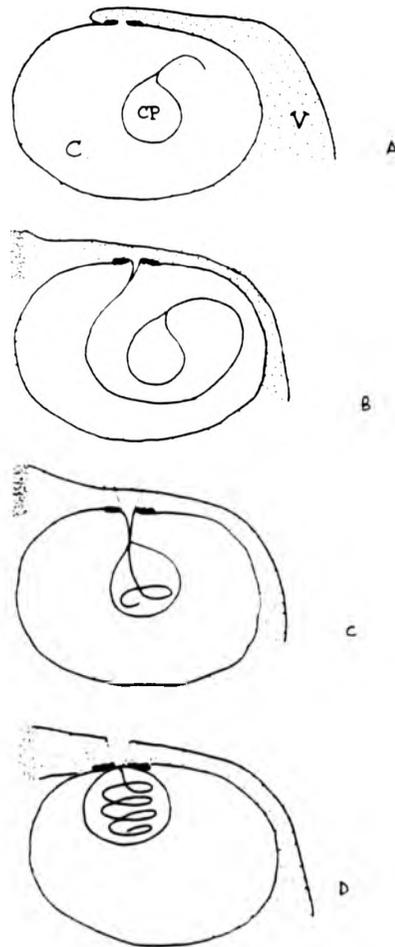
stickleback *rete* stages have tertiary cells with a more electron lucent cytoplasm than secondary cells (Lom *et al.*, 1991).

The tertiary cells of salmon *Sphaerospora* extrasporogonic stages possess incomplete nuclear and cytoplasmic membranes, as do those of *S. renicola* (Lom *et al.*, 1983a; Dykova *et al.*, 1990); however they lack the microtubules seen in the tertiary cells of *S. renicola* (Lom *et al.*, 1983a; Dykova *et al.*, 1990) and PKX (Feist & Bucke, 1987).

Molnar (1988c), in a study of the ultrastructure of K-stages, described a lack of nuclear and cytoplasmic membranes in some cells and made a number of controversial statements concerning the development of these stages according to Dykova *et al.* (1990). The latter authors found intact membranes and attributed Molnar's findings to poor fixation of material leading to poor quality electron micrographs.

The link between the blood and kidney interstitial extrasporogonic stages and later sporogonic forms seen in the kidney tubule lumen of the salmon appeared to be the 'triple formations' produced by the extrasporogonic primary cell. Triple formations in the renal tubules had extremely high numbers of mitochondria in the outer cell cytoplasm compared with those in the blood and interstitial kidney tissue. This difference may perhaps be due to the need for a respiratory burst associated with transport to, entry into, and establishment in the kidney tubule. Perhaps such a burst is also required at the onset of sporogony.

2.4.2   Entry to the kidney tubules

In the salmon *Sphaerospora*, two possible routes of entry into the kidney tubules for extrasporogonic triple forms were apparent: via the glomerulus into the Bowmans space and thence to the tubules; or by passing between the cells of the kidney tubular epithelium.

The large numbers of small extrasporogonic stages seen trapped in the capillaries of the glomerulus might cause them to rupture, and thus provide a route into the tubules. The damage and degeneration sometimes seen in the glomeruli of affected tubules also suggests that this is the normal route. Supamattaya *et al.* (1993) and Molnar & Kovacs-Gayer (1986a) showed similar glomerular disruption and suggested this means of entry. The fact that often the earliest sporogonic stages lay in the upper portion of infected tubules supports this view.

Semi-thin sections and TEM demonstrated small extrasporogonic stages between adjacent epithelial cells of the tubules. However, such stages were relatively uncommon. Similar findings were described by Kent & Hedrick (1986) and Lom *et al.* (1983a) for PKX and *S. renicola* K-stages respectively. Kent & Hedrick (1986) suggested that this was the main route of entry into the tubules for PKX.

Many extrasporogonic stages in the interstitium were surrounded by host cells or were degenerate (chapter 5). Few such stages appeared likely to survive to penetrate the epithelial wall. Secondary cells must enter the interstitium with the circulating blood. It may be that these, or those released from primary cells in the interstitium close to the tubule wall, may be able to enter the lumen through the epithelium of the tubule wall, rather than continue to circulate in the blood until they reach a glomerulus.

In infected salmon some kidney tubules in fresh preparations were massively

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infected with sporogonic stages, whilst adjacent tubules contained no parasites whatsoever. Various explanations are possible.

Once a small number of stages have become established in a tubule, they somehow might actively attract further parasites to that site. Perhaps some tubules provide more suitable physiological conditions for sporogony than others. Alternatively, the process may be entirely passive; a damaged glomerulus being a much easier route into a tubule for subsequent presporogonic stages. It may be that only a few stages establish in a particular tubule, but then undergo further proliferation at the site of sporogony. (Lom, 1992, pers. comm.). This would be a presporogonic, rather than an extrasporogonic proliferation, since extrasporogonic stages must proliferate at a site other than that at which sporogony occurs (Lom, 1987). However, no evidence for such a proliferation was seen in the present study.

2.4.3   Sporogonic stages

<u>Light microscope observations</u>

The process of sporogony in the salmon parasite was very similar to that in other *Sphaerospora* sp described to date. The appearance of the differentiating pseudoplasmodia of the salmon parasite, containing refractile granules is identical to that in other species (Clifton Hadley & Feist, 1989; Lom *et al.*, 1985b; Supamattaya *et al.*, 1991; Odening, Walter & Bockhardt, 1988; Dykova & Lom, 1982; Odening, 1987; El-Matbouli & Hoffman 1992; Feist *et al.*, 1991). Very few species of *Sphaerospora* show both disporous and monosporous development. These are *S. scardinii* (El-Matbouli & Hoffmann, 1992), *S. epinephali* (Supamattaya *et al.*, 1991, 1993) and PKX (Hedrick *et al.*, 1988). The salmon species was always disporous, and *S. carassii* is for example

always monosporous (Desser *et al.*, 1983a). This may be an important species character.

Dykova & Lom (1982) showed that a similar means of pseudoplasmodium sporoblast cell division occurred in *S. renicola* sporogonic stages to that which occurs in the salmon *Sphaerospora*. They too found that some sporoblast cells were smaller in size than others and that sometimes the division products of initial sporogonic cells stayed apart but sometimes stayed together, before reaching the two six cell spore forming unit stage.

External tube formation at the light microscope level was described by a number of authors from Giemsa-stained smears (Butschli, 1892; Auerbach, 1909; Stempell, 1919; Awerinzew, 1941) (all cited by Lom & de Puytorac, 1965). Recent papers have described the structure and development of sporogony at the TEM level. Often it is difficult to see all the cells in a particular parasite in TEM sections. This is especially so in terms of whether one sporoplasm cell or two are present; or whether, if there are two, one envelops the other. Drawings or photographs of Giemsa-stained smears of sporogonic stages are scarce in the recent literature, yet they provide additional information with respect to these features and others such as cell sizes. Their inclusion should therefore be encouraged in descriptions of sporogony.

There are relatively few *Sphaerospora* sp for which mature spores have been found in the glomerulus, as were seen in salmon *Sphaerospora* infections, have been described. These are *S. cristata* and *S. elegans* (Feist *et al.*, 1991), *S. ohlmacheri* (Desser *et al.*, 1986) and *S. truttae* (Fischer-Scherl, El-Matbouli & Hoffmann, 1986). This is perhaps a result of the glomerular route of entry for triple forms and could help to explain the pathology exhibited by a number of species (chapter 5).

Ultrastructure

Desser *et al.* (1986) showed in histological sections that very thin, elongated cytoplasmic processes attached *S. ohlmacheri* pseudoplasmodial cells to the inner wall of bullfrog tadpole kidney tubular epithelial cells. This gave the impression that sporogonic stages were almost suspended in the tubule lumen. This phenomenon was seen in histology of the salmon *Sphaerospora*, but fresh and glutaraldehyde fixed samples showed that the means of attachment was far more intimate than this, with pseudopodia-like interdigitating ramifications of the pseudoplasmodial cell cytoplasm being wedged tightly between the microvilli lining the inner wall of the tubular epithelium. It seems likely that formalin fixation causes pseudoplasmodia in the tubule lumen to contract away from the lining microvilli of the inner wall of the hosts tubular epithelial cells, giving rise to the less intimate attachment seen in histological material. All ultrastructural reports of *Sphaerospora* spp. infecting the kidney tubules described to date have described this more intimate means of attachment, and it seems likely that *S. ohlmacheri* sporogonic stages share this feature. The strength of this attachment is evidenced by the unexpectedly low numbers of sporogonic stages released onto impression smears of the kidneys for Giemsa-staining, in relation to the high level of infection in the fish.

The tight adherence of salmon *Sphaerospora* pseudoplasmodia to each other in the centre of the lumen when no available microvillar attachment is possible, in a tissue like arrangement, is similarly a common feature (Supamattaya *et al.*, 1991, 1993; Hamilton, 1980; Lom, Dykova & Lhotakova, 1982; Lom, Körting & Dykova, 1985a; Desser *et al.*, 1983). Despite this contact, no reports have described cell junctions

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between the parasite and the host (Lom *et al.*, 1985b), although Lom *et al.* (1982) and Supamattaya *et al.*, (1991) both mention an increase in electron density at the tip of the host microvilli and the plasmalemma of the pseudoplasmodial cell at their contact point. Areas of increased electron density were seen across areas of the pseudoplasmodial outer membrane where it was in contact with host microvilli (fig.2.39).

Pseudoplasmodial cell structure is comparable with that of other species studied, although the lipoidal inclusions commonly seen in, for example, *S. epinephali* and in *S. renicola* (Supamattaya *et al.*, 1993; Lom *et al.*, 1982), were rare in the salmon parasite. The membrane-bound vesicles described in the present study could perhaps be lysosomes (Branson, 1987). Typical Golgi, as stacks of cisternae are also reported in the literature. Lom *et al.* (1982), discussing *S. renicola* sporogonic stages, also noted and discussed the variability in the size and structure of the mitochondria seen in their studies, a prominent feature of the salmon parasite sporogonic stages. The abortive plasmodia and the rod-shaped organelle of unknown function described by Lom *et al.* (1985a) from *S. galinae* infections of tench were not seen in salmon *Sphaerospora*. Large numbers of mitochondria in the pseudoplasmodial cytoplasm are probably required for maintainance in the host, providing energy required for sporoblast cell division and sporogenesis, and to enable uptake of nutrients from the host. It has been hypothesised that the latter is achieved by the incorporation of useful nutrients excreted by the host and later destined to be reabsorbed further down the tubule (Molnar, 1980a).

Asynchronous development in terms of individual pseudoplasmodia, individual spores within pseudoplasmodia and capsulogenesis within each spore is a common feature of ultrastructural studies. Why this phenomenon is so ubiquitous amongst

Sphaerospora spp and indeed other myxosporeans is unknown, and no authors appear to have suggested a functional reason.

The presence of microtubules in some sporogonic cells of the salmon *Sphaerospora*, along with pseudopodia-like cytoplasmic projections are indicative of recent cell division. This is to be expected in sporogonic stages since division is taking place to increase the number of sporoblast cells from two to twelve prior to their differentiation. That sometimes these division products stayed together, or sometimes remained in their own vacuoles was reported by Lom *et al.* (1982) for *S. renicola*. In the *S. renicola* pseudoplasmodium divided cells often shared a common vacuole. It would seem reasonable to assume that the cells which are going to produce one of the two spores within the pseudoplasmodium should come to lie in the same vacuole. Therefore, two cells remaining together on division will go on to form the same spore, whilst those which part will form different ones. This hypothesis is supported by the fact that, at both light and electron microscope levels, once fully divided to the 12 cell stage, the cells become polarised within the pseudoplasmodium into two 6 cell spore forming units each of which lies in its own vacuole in the cytoplasm.

Folded cytoplasms and nuclei were described by Lom *et al.* (1982) from sporogonic *S. renicola* cells. This too was seen in the salmon parasite. However, the presence of tertiary cells in sporogonic cells as reported for *S. renicola* (Lom *et al.*, 1982), *S. galinae* and *S. tincae* (Lom *et al.*, 1985a) was not seen in salmon *Sphaerospora* sporogonic stages. Tertiary cells have not been described as features of the sporogonic cells of any other myxosporean genera.

The events of myxosporean capsulogenesis have been described in numerous studies, and the process appears to be very standard across a number of myxosporean groups. Cisternae of rough endoplasmic reticulum are established as characteristic signs of the onset of capsulogenic cell differentiation. The changes upon capsular maturation, whereupon rough endoplasmic reticulum recedes and lipoidal inclusions appear, have also been well described (Sitja-Bobadilla & Alvarez-Pellitero, 1992b), as has the microtubular ensheathment of the external tube. Some variation exists in the number of cross sections of the external tube seen in the cytoplasm and in the number and arrangement of the coils of the inverted filament in mature capsules, but such details are minor, unrelated to the mechanics of the process itself, and probably reflect the length and appearance of the mature filament within the capsule. The use of features such as the number and arrangement of the coils of the polar filament has long been recognised as a valuable taxonomic feature (Lom, 1969b).

The two desmosome-like junctions between the capsulogenic cell and its enveloping valvogenic cell at either side of the point at which the future discharge canal forms have been shown by Desportes & Theodorides, (1982), Lom & dePutorac (1965), and, interestingly, in the actinosporean genera *Aurantiactinomyxon* (present study) and *Triactinomyxon* (Lom & Dykova, 1992c). In many respects these areas are reminiscent of the early stages of suture formation between the two valvogenic cells.

These junctions may be part of a mechanism which allows coordination of the development of valvogenic and capsulogenic cells to ensure that the discharge canals of the mature spores and the plugs of the polar capsules align correctly. Perhaps, although there is no direct evidence for this, the external tube attaches here, and thus as the tube and filament become incorporated into the capsule the capsule is "pulled up" into the

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correct position. Lom *et al.* (1982) mentioned that the closed distal end of the external tube always lies in front of the future discharge channel (see fig.2.49). The increased thickening of these structures in the salmon parasite spores as they mature may indicate that they also have an important role in the attachment and maintainance of the position of the capsule in the spore at this site.

One feature highlighted by Sitja-Bobadilla & Alvarez-Pellitero (1992b) is the presence, in some myxosporeans, of amounts of Golgi in the capsulogenic cell during capsulogenesis. It is prominent in *Sphaerospora angulata (=renicola)* (Desser *et al.*,1983a), *Myxobolus cotti* (El-Matbouli *et al.*, 1990) in the genus *Sphaeromyxina* (Lom, 1969a) and has been implicated as being directly involved in capsular primordium formation. However, Golgi was not described in the salmon parasite, or *S. dicentrarchi, Heneguya exilis, Zschokella ruselli, Myxobolus* spp, *Sphaerospora carassii, Kudo lunata* and *S. galinae* (Sitja-Bobadilla & Alvarez-Pellitero, 1992b; Current & Janovy, 1978; Davies & Sienkowski, 1988; Desser & Patterson, 1978; Desser *et al.*, 1983a; Lom *et al.*, 1985a repectively). The presence of Golgi in the capsulogenic cytoplasm of some groups need not necessarily imply it has an active role in the actual process of capsulogenesis.

That valve cells do not encircle the other four cells of the spore until the onset of capsulogenesis was shown by Lom *et al.* (1982), as well as in the present study. Valvogenesis is very conserved in *Sphaerospora* spp development with the cytoplasm losing structural definition as it becomes first vacuolated, especially posteriorly, then thickened as electron dense layers are layed down as the resistant spore coat is formed. This results in a thin, but very hard, dense resistant valve, much less thick than the original valvogenic cells. This last phase in spore maturation can be traced

histologically via Casson's trichrome staining, indicating that the material laid down is probably a carbohydrate. The vacuolation process in salmon *Sphaerospora* compared exactly with that seen in Nomarsky light microscope studies. The resulting thinning and hardening of the valves explains the transitions seen from immature through to mature spores under the light microscope. The "pulling away" of the spore valves of salmon *Sphaerospora* spores from the pseudoplasmodial cytoplasm is very reminiscent of the results of Feist *et al.* (1991) from the pseudoplasmodia of *S. elegans*, but the "horns" of the posterior part of each shell valve of *S. elegans* - containing the remnants of the valvogenic nucleus - were not seen in the present study. In mature spores of the salmon parasite, nuclei appeared to degenerate in a manner similar to that shown by Lom *et al.* (1982) for *S. renicola*. The overlapping valve cell joining sutures, shown by Sitja-Bobadilla & Alverez-Pellitero (1992b) were not seen in the salmon *Sphaerospora* spores, and appear unique within the genus, if not in myxosporeans as a whole. The nature of the suture line as a cell junction was discussed in detail by Lom & dePuytorac (1965), Desser & Paterson (1978) and Desportes-Livage & Nicolas (1990). The latter authors were able to relate its nature ultrastructurally at the freeze-fracture level with metazoans.

Spores of *Sphaerospora* from Atlantic salmon have two uninucleate sporoplasm cells. Few bivalvulid myxosporeans have two sporoplasm cells (Lom *et al*, 1983b), however the presence of two such cells is a generic characteristic of *Sphaerospora* (Lom & Noble, 1984; Lom *et al*, 1985b). A single binucleate sporoplasm has been described in spores of *S. dicentrarchi* (Sitja-Bobadilla & Alvarez-Pellitero, 1992b), *S. carassii* and *S. angulata* (Desser *et al.*, 1983a).

All ultrastructural descriptions of *Sphaerospora* report a dense accumulation of

ribosomes in the sporoplasm cell cytoplasm, but none have described the accumulations of tiny discrete very electron dense particles seen in the salmon *Sphaerospora*. Their closest parallel is the so called sporoplasmosomes seen in the sporoplasm cells of many myxosporean spores including those of *Sphaerospora* spp. (Lom *et al*, 1982; Lom *et al*, 1985a; Desser *et al*, 1983a). These are much larger and more widespread throughout the sporoplasm cell cytoplasm than the accumulations of particles in the salmon parasite sporoplasm cell. Sporoplasmosomes appear very late in spore formation and sporoplasm maturation (Lom *et al*, 1985a; Sitja-Bobadilla & Alvarez-Pellitero, 1992b), and it could be that their apparent absence from the salmon parasite is simply a function of not seeing mature enough spores in sections. However, Supamattaya *et al*. (1993) did not see sporoplasmosomes. Lom *et al*. (1985a) saw 'globular-like' inclusions unlike normal sporoplasmosomes in the sporoplasm cells of *S. tinacae*, indicating that there may be variability between sporoplasmosomes in different species.

In *S. tincae*, Lom *et al*, (1985a) described an amorphous opaque substance filling the spaces between the sporoblast cells in the developing spore. In salmon sphaerospores, as in most other reports, the four inner cells of the spore remain tightly together within the valvogenic cells, leaving no spaces.

Aberrant spores

Aberrant spores, with three capsules, sporoplasms and valves were sometimes found in the present study of the salmon *Sphaerospora* spp. Similar spores in *Sphaerospora* spp. were described by Feist *et al*. (1991), Arthur & Lom (1985) and Lom & Noble (1984). Kudo, (1922) described similar deformed spores of *Leptotheca ohlmacheri* (quoted by Shulman, 1966). Shulman (1966) and Arthur & Lom (1985)

showed that such spores frequently developed in disporous pseudoplasmodia, agreeing

with our findings. It may be that an abnormal series of divisions leads to the production

of 18 sporoblast cells instead of the normal twelve, and that upon differentiation, two

nine, rather than six cell, spore forming units produce two aberrant spores (see diagram).

2.4.4   Life cycle summary

A diagram summarising the lifecycle of the salmon parasite based on light and

EM observations is presented in fig.2.50

fig 2 50 Diagrammatic summary of the development of salmon *Sphaerospora* in the fish

SPOROGONIC

EXTRASPOROGONIC

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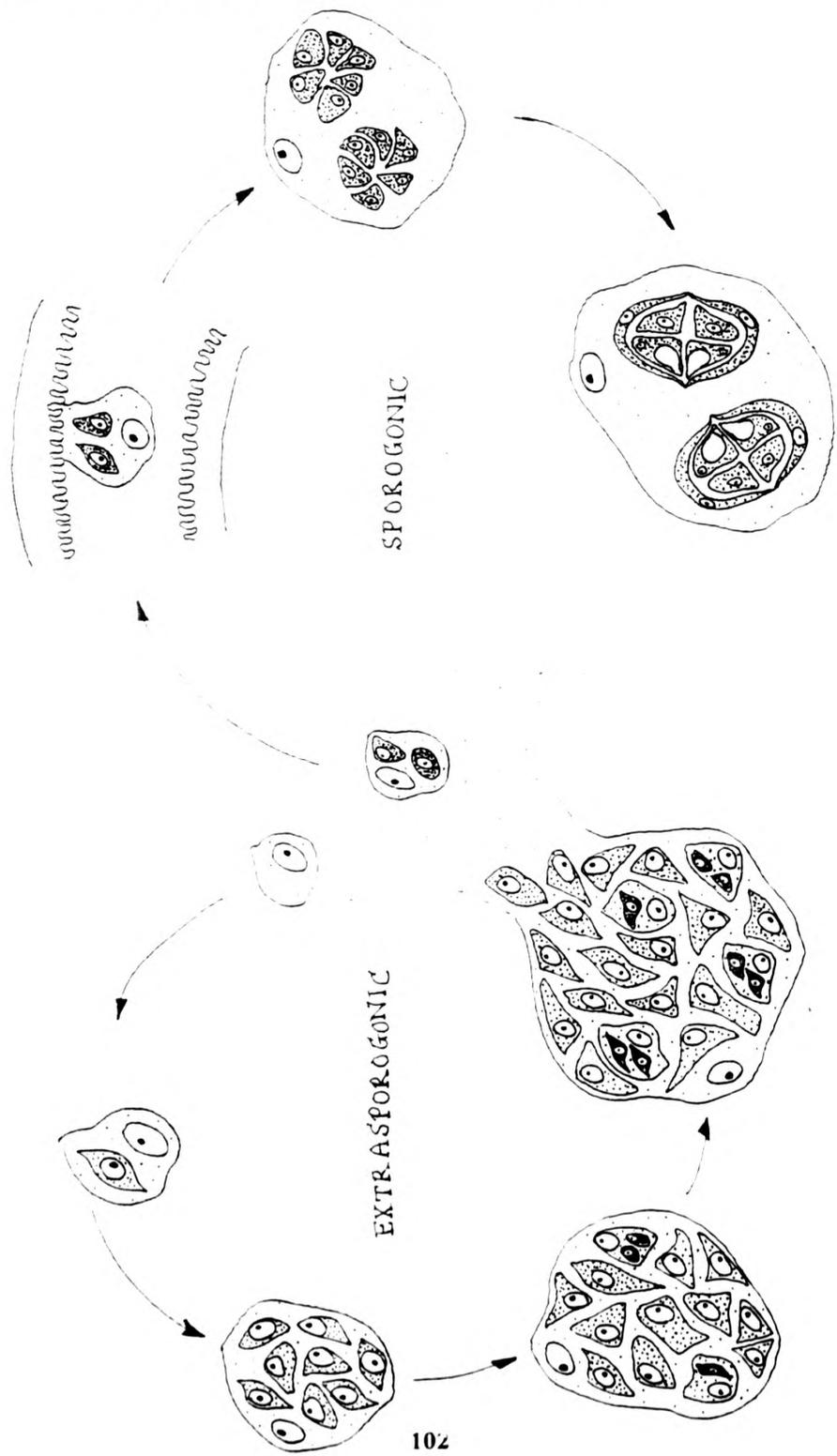
Chapter 3
Specificity & identification

3   SPECIFICITY AND IDENTIFICATION

3.1   INTRODUCTION

3.1.1   Specificity

It is important to attempt to obtain an accurate generic and specific identification

for the salmon myxosporean under study, particularly due to the possibility of

commercial and economic impacts upon the Atlantic salmon farming industry. It is also

necessary to compare the parasite with other important myxosporeans of salmonids, such

as PKX and *Myxobolus cerebralis* which are very serious pathogens. A full

characterisation of the species is important to achieve so that comparisons can be made

with other occurrences, and its safe discrimination assured in the future. Without such

an identification, comparisons with similar and related species in the literature become

more difficult; these are important in increasing our knowledge of species and their

epidemiology, pathology and host range. However the identification of myxosporean

species is problematic for a range of reasons such as a lack of information on host

specificity, tissue specificity, geographic range, epidemiology, ultrastructure,

development and, in some cases, even on basic details of spore morphology and

dimension. These problems are further compounded by an historic inability to

experimentally transmit myxosporeans.

Lom & Dykova (1992d) pointed out that a wide degree of host specificity and

zoogeographical distribution is displayed by myxosporeans. The extent to which these

three factors are expressed is not taxonomic, with individual species of a given genus

having very different levels of expression of each.

Some species, such as *Chloromyxum mucronatum*, *Thelohanellus hovorkai* or

kidney infecting members of the genus *Sphaerospora*, are regarded as being very host

specific (Lom *et al.*, 1985b; Shulman, 1966; Molnar & Kovacs-Gayer, 1986a) whereas others appear polyxenous, such as *Myxobolus muelleri* (Shulman, 1966), *Kudoa thyrsitis* (Barja & Toranzo, 1993) or *Chloromyxum fluviatile* (Lom & Dykova, 1992b).

Some species have a very wide natural geographical range e.g. *Myxidium incurvatim, Myxidium lieberkuehni,* and *Sphaerospora elegans* (Shulman, 1966; Lom & Dykova, 1992d), whilst others are limited to restricted areas or water systems, such as *C. shasta* (Johnson, Sanders & Fryer, 1979). Some species, such as *M. cerebralis* have acquired a wide geographical range as a result of man's activities (Hoffman, 1990)

Other myxosporeans are specific for certain tissues such as *Myxobolus cerebralis, Myxobolus pseudodispar* or *Myxidium incurvatum,* whilst species such as *Myxobolus muelleri* or *Myxidium giardi* are less specific. Tolerance to salinity is rare, most myxosporeans being either freshwater or marine, yet *Myxobolus exiguus* has been found in different hosts in both environments (Shulman, 1966), as has *Sphaerospora elegans.*

Shulman (1966) considered it vitally important when commenting upon myxosporean infections to establish the level at which specificity was described. For instance, at a high taxonomic level, although a few species have been described from invertebrates, cartilaginous fish, amphibians and reptiles, almost all myxosporeans are specific to teleost fish.

Shulman considered the number of myxosporeans specific to a single host species to be exaggerated by the fact that many authors had, and continued to, describe new species on the basis of host species as a single factor without critical assessment.

In the former Soviet Union he showed that less than 10% of species infected different orders of fish, whereas over 40% infected representatives of the same genus

or family. Most species with wide host specificity crossing different orders of fish were marine.

Specificity of *Sphaerospora* spp

Shulman (1966) described myxosporeans infecting the urinary bladder, uriniferous tubules and the blood as exhibiting narrower host specificity. In the case of kidney tubule species, he hypothesised that such specificity was due to the nature of the chemical composition of the contents of the tubules, ureters and bladders.

Kidney infecting species of the genus *Sphaerospora* appear to show a very high degree of host specificity, with many known from a single host (Shulman, 1966; El-Matbouli & Hoffmann, 1992). The majority of species have been reported from cyprinid hosts (Arthur & Lom, 1985) in which, even when spore forms are very similar, extrasporogonic stages show marked species specific morphological differences (Lom *et al.*, 1985b; Baska & Molnar, 1988). However, the situation has become complicated by authors citing the proposed restricted host specificity of *Sphaerospora* spp. in itself as a reason for creating a new species, and so there is little definite information on host specificity in the genus *Sphaerospora* (Bucke *et al.*, 1989).

The problem of host/geographic/tissue specificity might be eliminated by cross infection experiments. This is becoming possible as laboratory based life cycles using oligochaete hosts become established. However successful direct fish to fish transmission has been achieved with *Sphaerospora* spp. which have blood-borne extrasporogonic stages:

Molnar (1984) and Molnar & Kovacs-Gayer (1986a) transmitted K-stages via IP injection and obtained spores, although clinical SBI was not induced. C-stages

established in naive fish via IP injection failed to do anything other than cycle in the blood, leaving their role in the life cycle unproven until Molnar (1988b) showed that 13 days post injection they became detectable and after 1 month C-stages, K-stages, acute SBI, spores and sporogonic stages were all found in the fish. The author suggested that after 3 to 4 weeks of cycling, C-stages transformed into K-stages. C-stages continued to cycle simultaneously with, and even after, spore production.

Molnar & Kovacs-Gayer (1986a) found that C- or K-stages could not establish in silver carp, grass carp, tench, roach or gibel carp and thus established a narrow host specificity for *S. renicola*.

Fish to fish transmissions have been achieved with PKX. Clifton-Hadley, Bucke & Richards (1984) and D'Silva, Mulcahy & deKenkelin (1984) transmitted PKX between rainbow trout by IP injection. Kent & Hedrick (1985b) transmitted PKX with IP-injected homogenates of blood and spleen and not only obtained blood and interstitial kidney stages of PKX in experimental fish but also intraluminial sporogonic stages.

Schafer (1968) and Bower (1985) all succeeded in maintaining *C. shasta* spores, sporoblasts and trophozoites in the body cavity of salmonids by the IP injection of such stages taken from the digestive/intestinal tract of naturally infected fish. Ibarra, Gall and Hedrick (1992) achieved such passages of *C. shasta* stages using cryopreserved material, but pointed out that the stages transmitted were not true infections but that the 'infection' was caused by the transfer of pre-spore stages that continued their development to mature spores in the recipient fish.

3.1.2 Variations between host species

A number of myxosporean species infect a range of hosts of the same family. There is increasing evidence that in such cases, different host species and different strains of a single host species may show differential susceptibility to infection. This has important consequences for stocking and rearing programmes (Buchanan, Sanders, Zinn & Fryer, 1983; O'Grodnick, 1979). Data on variation in susceptibilty according to host species exists for a number of pathogenic myxosporeans, especially those of salmonids. However an experimentally established host range may not necessarily be indicative of that in natural conditions. Differences in prevalence and intensity of infection in wild fish samples from the same water body might not reflect differences in the actual susceptibility of a host species, but rather be indicative of ecological or behavioural differences between hosts which render them more or less likely to encounter infective stages. Where differences have been reported from fish held at the same hatchery there would seem to be a strong indication of differential susceptibility.

Myxobolus cerebralis appears to be specific to salmonids. Seventeen species of the genera *Oncorhynchus, Salmo* and *Salvelinus* have been shown to be positive for infection (Halliday, 1976). Hoffman, Dunbar & Bradford (1962) showed that salmonids exhibited a differential susceptibility to *M. cerebralis*. Brown trout and rainbow trout were the least and most susceptible respectively, with brook trout intermediately so. Brown trout may act as important carriers of infections (Hoffman & Putz, 1969). O'Grodnick (1979) found that brown trout did not become diseased even in highly infective waters. Lake trout and coho salmon were usually refractive, brook trout, sockeye and chinook salmon intermediately so, with rainbow trout always most susceptible. Clinical disease was restricted to rainbow trout, brook trout, chinook and sockeye.

PKX infects a range of salmonid hosts, both in the USA (Hedrick, Kent, Foott, Rosemark & Manzer, 1984; MacConnell & Peterson, 1992) and Europe (Clifton-Hadley, Bucke & Richards, 1984; Ellis, McVicar & Munro, 1985; Bucke *et al.*, 1991). It has also been found in grayling, roach and northern pike (Seagrave, Bucke, Hudson & McGregor, 1981). High mortalities have been experienced in hatchery populations of rainbow trout from the USA, when Atlantic salmon in the same tanks were uninfected (Ferguson & Needham, 1978). In Canada, Brown, Thonney, Holwell & Wilson (1991) found that arctic charr, *Salvelinus alpinus*, suffered severe PKD at a site where *Salmo salar*, *Oncorhynchus mykiss* and *S. salar ouananiche* were cultured without problems. The authors proposed that the charr lacked resistance since they were from the Fraser River, where no PKD is found, whereas continual exposure had resulted in the other species, particularly the ouananiche, developing a genetic resistance to infection.

Bucke *et al.* (1991) found that sporogonic stages were more common in some salmonids susceptible to PKX, but noted that extrasporogonic stages were present in all. They hypothesised that the extrasporogonic stage of PKX might be relatively non-host specific whilst the sporogonic stage was highly specific.

Amandi, Holt & Fryer (1985) found that parasitism by *Myxobolus insidiosus* varied between sites, hatchery populations, wild populations and between five *Oncorhynchus* spp. Susceptibility differences were actually inherent resistance differences since some so called "refractory" species could be infected by increasing their exposure time to infective waters.

Udey, Fryer & Pilcher (1975) found coho salmon better able to counteract infections of *C. shasta* than rainbow trout, especially at lower temperatures. There have been a number of conflicting interpretations on the specificity of *C. shasta* to different

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salmonids, probably due to varying susceptibility of different strains of the same host species (Johnson *et al.*, 1979).

3.1.3 Variations within host species

Conflicting results on the specificity of *C. shasta* have been attributed to variations in susceptibility of different strains of the same host fish species; for example in rainbow trout (Schafer, 1968; Johnson, Sanders & Fryer, 1979; Johnson, 1975; Ibarra *et al.*, 1991), chinook salmon (Zinn, Johnson, Sanders & Fryer, 1977) and coho salmon (Hemmingsen, Holt, Ewing & MacIntyre, 1986). By producing all possible crosses between three stocks of varying susceptibility, Hemmingsen *et al* (1986) found that the susceptibility of progeny was always intermediate between that of parental stocks, proving that it was a genetically inherited trait. The selection pressure exerted by the parasite was considered the cause of resistance of stocks in infected areas. Buchanan *et al*. (1983) proposed a similar evolution of resistance amongst populations historically exposed to *C. shasta* and considered the survival of released rainbow trout to be dependent upon strain susceptibility to ceratomyxosis.

O'Grodnick (1979) showed lake trout *Salvelinus namaychush* to be refractory to *M. cerebralis*, a species which Hoffman & Putz (1969) had found susceptible.

Similar variations were shown by Amandi *et al*. (1985) for *Myxobolus insidiosis* and by Ellis *et al*. (1985) for PKX. In the latter case, Norwegian Atlantic salmon were found to be much more susceptible to PKX than native Scottish stocks.

3.1.4 Problems of specific identifications

Spore morphology

Many problems of myxosporean identification and taxonomy relate to the morphology and dimensions of mature spores representing the major criterion for differentiating taxa (Lom & Arthur, 1989). Vegetative, pre-spore stages of the myxosporean life cycle such as trophozoites have few taxonomically valuable features (Lom and Dykova, 1992d). It appears, however, that the morphology of extrasporogonic stages in the life cycles of *Sphaerospora* or *Chloromyxum* may prove to be species specific (Lom *et al.*, 1985b; Baska & Molnar, 1988). Although this could provide additional characters to aid the discrimination of closely related species no consideration appears to have been given to the possibility that, as with spore stages, there might be a significant degree of variation in the structure of extrasporogonic stages according to host species.

The reliance upon spore morphology in myxosporean classification stems from the historical lack of knowledge of the life cycle. Only recently has the need for an alternate oligochaete host in the life cycle of some, if not all, myxosporeans been established (Markiw & Wolf, 1983; El-Matbouli & Hoffmann, 1989). The historic inability to experimentally infect fish has meant that cross infection experiments to determine the extent of host specificity, tissue specificity and geographical range of most species have been impossible to perform. The need for such experiments has long been recognised (Meglitsch, 1957; Mitchell, 1977; Kovacs-Gayer & Molnar, 1983).

Myxosporean spores have a relatively simple structure presenting few features or parameters to utilize taxonomically (Meglitsch, 1957; Mitchell, 1977; Hine, 1979; Lom & Arthur, 1989). In addition many species were initially very poorly described, and have been little studied since (Mitchell, 1977). This has led to their subsequent reidentification becoming both subjective and arbitrary (Lom & Arthur, 1989; Lom &

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Dykova 1992d). Currently accepted criteria for specific identifications were in many cases not given in the original description of many species, with host fish often the only available guideline for reidentification. Cone (1992) described two possible scenarios which resulted when attempting to identify a species by relating a contemporary description to a poor early one. One was that many poorly described species from the past would go unrecognised and as a result disappear into taxonomic extinction; the other was that new finds would be misidentified, leading to false and exaggerated impressions of the host specificity and geographical distribution of a species. Arthur (1992) considered that the use of species names of taxa initially poorly characterised, confused not only host specificity and geographical range information, but also led to conflicting data on the morphology and morphometrics of their spores. He thought it a priority to redescribe such species from material from the type host and location, and to refrain from using their names in the meantime.

The status of the taxonomy of a number of genera, such as *Myxobolus* and *Myxidium*, has been described as chaotic (Lom & Noble, 1984). In such genera few species are well defined, and a lack of basic data on spore variability, host and site specificity and even spore morphology exists. This makes safe species identifications very difficult (Hine, 1979; Lom, Dykova, Horner, Hoffman & Durham, 1992; Athanassopoulou & Sommerville, 1993a). It is therefore likely that in most genera many unrecognised synonyms, species described without specific name, and many distinct species under the same name exist (Lom & Arthur, 1989).

Myxosporeans have been, and continue to be, described from a small amount of material from one host, one host organ, or one geographic location, despite these being

no decisive justification for the creation of new species (Lom & Dykova, 1992d). The erection of new species due to minute deviations in spore size or morphology between host species, tissues or locations takes no account of spore variation, despite much evidence that many differences may be a product of natural variability (Davies, 1968; Lom, 1969b; Hine, 1979). Recently, Athanassopoulou & Sommerville (1993a) proposed that *Myxidium pfefferi* and *Myxidium rhodei* were synonymous after finding considerable such variation.

The validity of many species is therefore questionable, and has led to proposals that a greater understanding of myxosporean biology and the factors influencing spore variation will lead to much synonymisation, with perhaps many characters currently used even for generic identifications, being relegated to species level or below (Davies, 1968; Mitchell, 1977; Li & Desser, 1985).

Considerable evidence exists throughout the literature that spores of a single myxosporean species vary widely in morphology and dimension in response to a range of factors. Size of spores is often an important variable but variations in shape as well as polar capsule and valve number are also common as are changes in spore morphology as spores mature (Athanassopoulou & Sommerville, 1993a). Without knowledge of the factors which influence spore morphology, inaccurate species descriptions can result. Shulman (1966) believed that many very rarely described or encountered species may be modified forms of common, widely distributed species.

Polymorphic variation in spore morphology and size of a given myxosporean species may exist between different host fish species infected. For instance, spore size

and range of organs infected by *Myxobolus muelleri* varied between *Leuciscus cephalus, L. leuciscus* and *Rutilus rutilus* (Davies, 1968). In *Anguilla australis*, spores of *Myxidium giardi* (=*M. zealandicum*) were large and uniform, whereas in *Anguilla dieffenbuchli* they were often small and irregular (Hine, 1979). Yet large spores from *A. dieffenbuchli* and small spores from *A. australis* were indistinguishable. Differences between spore size in different salmonids infected by *Myxobolus insidiosus* were shown by Amandi *et al.* (1985), in different host species there being a difference in the range of extent of this variation. Spore variations have been attributed to the biochemical and physiological suitability of the host for myxosporean development.

Marked variation within myxosporean species may also result from tissue induced polymorphism according to the tissue location within a single host in which development occurs. Meglitsch (1957) regarded organ specificity as a reflection of physiological requirement or potentialities. Such variation has been shown in *Myxobolus muelleri* (Davies, 1968), *Myxidium giardi* (Hine, 1978; 1979; Copland, 1981), *Myxidium rhodei* (Kepr, 1987; Athanassopoulou & Sommmerville, 1993a) and *Myxobolus punctatus* (Mukherjee & Haldar, 1981). The latter authors considered this an adaptive change due to new physiological and biochemical environmental conditions associated with the tissue concerned, and considered that where new species had been described on the basis of spore differences, especially in different organs of the same host species, such a distinction was unwarranted.

In other species of myxosporean, trophozoite/cyst structure and ultrastructure can vary according to the site invaded, although spores are identical. For example *Henneguya* sp. cyst manifestation in channel catfish (McCraven *et al.*, 1975; Current & Janovy, 1978; Current, 1979). Komourdjian, Hulbert, Fenwick & Moon (1977) found

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similar cyst variation in *Myxidium giardi* infections of eels, whilst Pulsford & Matthews (1982) showed site dependent ultrastructural differences in trophozoites of *Myxobolus exiguus* infecting grey mullet.

Spores may vary markedly in individual cysts within an individual host. Thélohan (1895) found *Myxobolus ellipsoides* spores of widely different size, with and without caudal processes and with varying numbers of polar capsules in single cysts. Li & Desser (1985) found some spores in cysts of *Thelohanellus* sp with two polar capsules as in *Myxobolus*. Shulman (1966) described many findings where single cysts contained spores resembling other species or genera. Sometimes these were deformed or degenerate but often appeared intact and viable. Shulman (1966) said that tri- and tetra-valvular *Ceratomyxa* and *Leptotheca* spores were common, in cysts there being a percentage ratio of normal to abnormal spores. Increased polar capsule and valve number in some spores from a cyst are especially common in the marine environment and can lead to the appearance of spores of two different genera in the same cyst (Cheung & Nigrelli, 1990; Egusa, 1986; Kovaleva, 1992). This raises the question of how genetically distinct many genera actually are.

In freshwater, spore variation in individual fish or single cysts has been shown by Shulman (1966), Davies (1968), Komourdjian *et al.* (1977) and Hine (1978, 1979). Lom & Dykova (1992b) found a remarkable degree of variation in striation pattern in individual spores of *Chloromyxum fluviatile*.

Environmental conditions such as season (Booker & Current, 1981; Davies, 1968) or pollution (Kalavati & Narasimhamurti, 1983) may also cause variation in the structure of both trophozoites and spores.

This considerable evidence for variation in both trophic forms and spores, between and within host species, host site of infection, cysts and individual hosts, phenotypic variation must be regarded a characteristic of many myxosporean species spores. Hine (1979) warned against the description of new species based on material from one cyst, fish species or site, as this may lead to "a proliferation of species of dubious validity".

The overall situation led Lom & Arthur (1989) to produce comprehensive guidelines for the characterisation of new species and for the redescription of poorly outlined existing ones. They emphasised that what was required to help unravel the current status of taxonomy in the field was,

"...a meticulous time-taking morphological analysis of species - of their spores and vegetative stages, taking into account their variability..."

3.1.5 Objectives

The objective of this part of the study were to attempt to identify the salmon parasite under study by standard morphological means according to spore dimension and morphology, and other features such as extrasporogonic stage morphology. Cross infection experiments would attempt to establish the host range of the parasite

3.2 MATERIALS AND METHODS

3.2.1 Spore characterisation & species identification

Mature and developing spores were characterised and measured according to the guidelines of Lom & Arthur (1989). Measurements taken are illustrated in fig.3.1. Ten spores were measured from fresh smears of kidney tissues from each of ten infected

Atlantic salmon from farm A, and ten from farm B. Spores, both mature and immature, were drawn on a Leitz microscope with attached drawing tube, and photographed under bright field, phase contrast and Nomarsky interference-contrast optics on an Olympus BH2 with dedicated camera or a Leitz Orthomat. The agar monolayer technique for the photography of myxosporean spores was used (Lom, 1969b).

A small number of trout, the progeny of returning sea trout raised at the hatchery at farm C, were available for study in the summer of 1992. These fish had been bred for ranching puposes, but were also being used for an unrelated scientific study. Six fish were examined according to the standard procedures outlined in chapter 2, with 60 spores measured, ten from each of the six fish as shown in fig.3.1.

One hundred spores, ten from each of ten formalin-fixed kidneys from Atlantic salmon at farm A, were measured in order to provide a direct comparison with fresh material and with the literature.

3.2.2   Antigenic comparison with PKX

Histological material comprising Atlantic salmon kidneys infected with extrasporogonic and sporogonic stages of the parasite under study was provided for testing with monoclonal antibody and lectin probes specific for PKX. The immunohistochemical testing was performed by Mercedes Marin using the antibodies, lectins and methods described by Marin, Adams, Richards, Castagnaro & Hedrick (1993).

3.2.3   Experimental infections

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fig.3.1   Measurements taken from *Sphaerospora* spores from Atlantic salmon for taxonomic purposes:

a: spore length
b: spore thickness
c: spore width
d: polar capsule diameter
e: polar filament coil number

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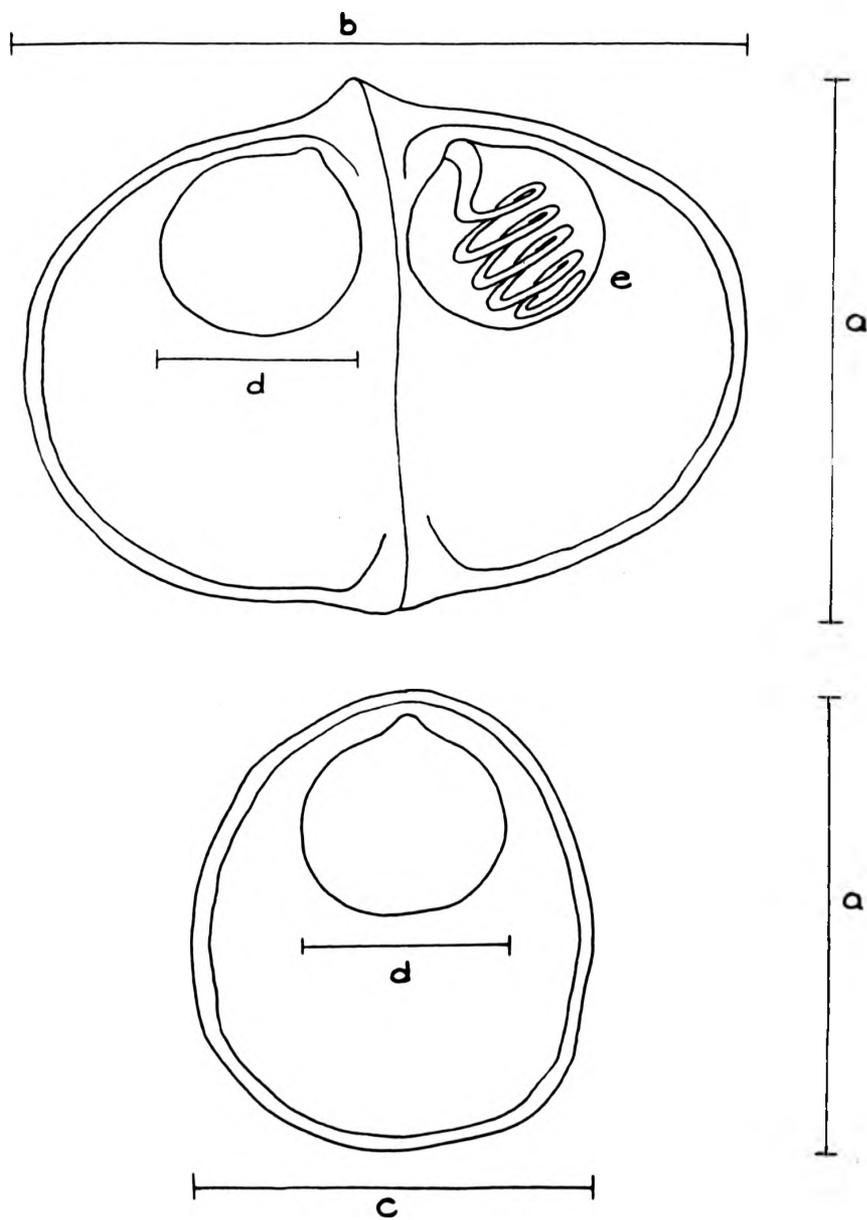
<u>Donor fish</u>

Experiments 1 & 2: infections with sporogonic stages, 1993

15 Atlantic salmon parr were obtained from farm C in April, 1993. The kidney tubules of these fish were infected with differentiating pseudoplasmodia, sporogonic stages and mature spores at a high prevalence and intensity. Extrasporogonic stages had not been seen in this cohort of fish since September 1992.

Experiment 3:   infections with extrasporogonic stages, 1992

10 Atlantic salmon parr were obtained from farm A in mid August 1992. Epidemiological observations had indicated a high level of prevalence and intensity of extrasporogonic stages of salmon *Sphaerospora* at this time in the fish cohort.

Experiments 4, 5, 6 & 7:   infections with extrasporogonic stages, 1993

40 Atlantic salmon parr were obtained from farm A in early August 1993, and ten fish used for each experiment. Epidemiological observations had indicated a high level of prevalence and intensity of extrasporogonic stages at this time in the fish cohort.

<u>Recipient fish</u>

Recipient fish for all experiments were obtained from hatcheries with bore-hole water supplies from which *Sphaerospora* had never been recorded. These fish were retained in 40l flow through tanks for seven days on charcoal filtered mains water at 12°C and fed *ad libitum* with a commercial pelleted feed. Before each experiment, eight fish were sacrificed and examined for the presence of myxosporean infection by the methods outlined in chapter 2. None were found to be infected.

Preparation and administration of blood and kidney homogenates

Kidneys, infected with sporogonic or extrasporogonic stages as appropriate, were removed under sterile conditions from a number of donor fish. A small sample of the mid-portion of each kidney was removed and later scanned to confirm the presence of the relevant stages by fresh and Giemsa-stained smears (see chapter 2). The remainder of each kidney was placed in a pre-weighed sterile universal tube containing a small volume of sterile phosphate buffered saline (PBS) at 15°C. PBS was then added to the universal to give a ratio of approximately 1g of tissue to 10ml of PBS. The kidney was then pressed forcibly through a 100μm nylon mesh filter with a sterile spatula to disrupt the tissue and create an injectable suspension.

Blood from donor fish was obtained from the severed caudal arteries of sacrificed donor fish using heparinised glass capillary tubes. A subsample of blood from each fish was examined fresh and used to prepare a Giemsa-stained blood film (see chapter 2) to scan for the presence of parasites. The remaining volume was pooled, and mixed 1:1 with PBS to form an injectable suspension.

All suspensions were prepared fresh shortly before the commencement of the infection procedure and retained in sterile universal tubes on ice during the course of the experiment.

Samples of all homogenates were scanned under phase contrast microscopy prior to injection to ensure that viable, intact parasites of the stage required were present.

Recipient fish were individually anaesthetised using benzocaine. According to the experiment concerned they were then IP injected with 0.4ml kidney homogenate or 0.4ml blood suspension using a sterile syringe fitted with a 21 gauge needle, or 0.4-0.6ml of kidney suspension was introduced into their stomachs via a sterile plastic 1ml

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pipette. All injected fish were panjet marked to ensure their future discrimination from control fish. Status of control fish differed according to experiment, and is detailed below.

Control and experimental fish for each experiment were held in the same tank, and maintained in 40l flow-through tanks supplied with charcoal filtered mains water at ambient temperature (~12°C). Fish were fed *ad libitum* with a commercial pelleted diet.

Experimental design

Experiment 1:   IP-injection of sporogonic stages to Atlantic salmon, 1993

Kidney homogenate from Atlantic salmon, containing numerous pseudoplasmodia, immature and mature spores, was IP-injected into 25 naive recipient Atlantic salmon of ~30g in April, 1993. Fifteen control fish from the same stock received an injection of 0.4ml PBS.

Experiment 2:   Oral administration of sporogonic stages to Atlantic salmon, 1993

The same homogenate used in experiment 1 was introduced by plastic pipette orally into the stomachs of 25 naive Atlantic salmon of ~30g on the same date as in experiment 1. Control fish received a similar oral administration of 0.4ml PBS.

Experiment 3:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1992

Kidney homogenates from Atlantic salmon infected with extrasporogonic stages were IP-injected into 30 naive Atlantic salmon of ~15g in mid-August 1992. Fifteen

control fish from the same stock received an injection of 0.4ml PBS.

Experiment 4:   IP-injection of extrasporogonic stages from blood to Atlantic salmon, 1993

Blood suspensions from extrasporogonic stage-infected Atlantic salmon were IP-injected into 25 naive Atlantic salmon of ~20g in early August 1993. Ten control fish from the same naive stock received an injection of 0.4ml PBS.

Experiment 5:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1993

Kidney homogenates from the same infected fish stock used in experiment 4 were IP-injected into 25 naive Atlantic salmon of mean weight 21g on the same date as in experiment 4. Control fish were those used in experiment 4.

Experiment 6:   IP-injection of extrasporogonic stages from kidneys to rainbow trout, 1993

Kidney homogenates from Atlantic salmon infected with extrasporogonic stages were IP-injected into 50 rainbow trout of mean weight 18g in early August 1993. Fifteen control rainbow trout received an injection of 0.4ml PBS.

Experiment 7:   IP-injection of extrasporogonic stages from kidneys to brown trout, 1993

Kidney homogenates from Atlantic salmon infected with extrasporogonic stages were IP-injected into 39 brown trout of mean weight 26g in early August 1993. Fifteen

control brown trout received an injection of 0.4ml PBS.

Where possible 5 fish, depending on the level of mortalities experienced, were sampled on each sampling date. Experiments 1 and 2 were sampled on days 14, 28, 42, 70 and 120 post injection. All other experiments were sampled at 28, 42, 70 and 120 days post injection.

Some experiments were terminated before the full course had run due to depletion of stock as a result of *Aeromonas* infection (see results). Control fish were sacrificed at the termination of each experiment. All fish sampled, plus all intervening mortalities in both control and experimental fish, were examined using the standard examination procedure for the detection of the parasite as outlined in chapter 2.

3.3   RESULTS

3.3.1   Spore characteristics

Degree of maturity

Immature and mature spores were found to vary markedly in dimensions and morphology. These differences were especially evident when spores were observed under phase contrast or Nomarski microscopy, since more structures, such as nuclei became visible. Immature spores were considerably broader and deeper than mature ones. The sutural ridge was much more prominent, especially towards the posterior of the spore, and the developing polar capsules were much further apart than when mature (fig.3.2 & 3.3). Immature spore valves were much thicker and were extensively vacuolated posteriorly; the spore coat of each spore as it matured was often seen to pull

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away posteriorly from the cytoplasm of the pseudoplasmodium.

Mature spores

Mature spores were smaller, more rounded and compact, with a smooth outer shell valve coat. They were subspherical, 7.4 (6.5-8.4)μm in length, and 9.9 (8.8-11.2)μm in thickness perpendicular to the plane of the suture. There was only a very slight sutural ridge anteriorly, a small intercapsular appendix and the suture joining the two valves appeared much less prominent and distinct compared to immature forms. Shell valves were thin and no longer vacuolated. Polar capsules measured 2.4 (1.9-3.3)μm in diameter, were of equal size and did not converge at the apex of the spore. There were typically five, sometimes four coils of the polar filament, which were most commonly arranged with their central axes parallel to the plane of suture (fig.3.2 & 3.3).

Concentrated urea was used to extrude the polar filaments of fresh spores. Polar filaments were ~30μm long on average, but often incompletely everted.

All six sea trout from farm C were found to be positive for *Sphaerospora* sporogonic stages. Mature spores were of similar range to those of the salmon *Sphaerospora* (table 3.1). Infections were of a similar intensity to those of Atlantic salmon held at the same site.

Formalin fixation

Formalin fixation was found to cause a degree of shrinkage of mature spores. Such spores were similar in morphology to fresh spores but were 6.8μm(6.0-8.0μm) in

fig.3.2 Photomicrographs of (a) immature, and (b) mature *Sphaerospora* spores from Atlantic salmon (bar=10μm)
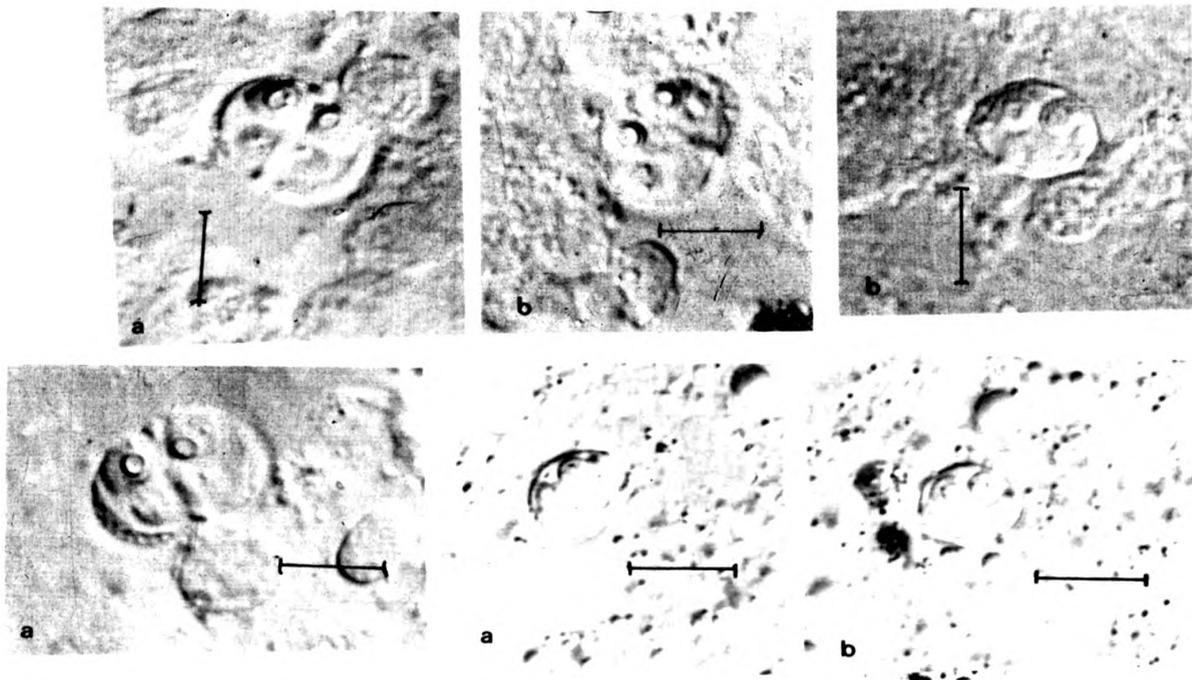
fig.3.3 Drawings of (a) immature, and (b) mature *Sphaerospora* spores from Atlantic salmon (bar=10μm)

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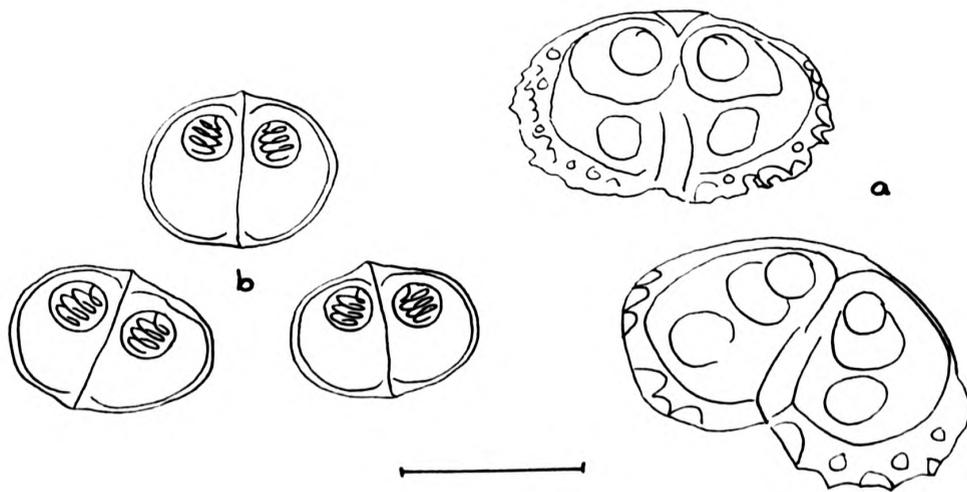
table 3.1   Comparison of spore dimensions for the *Sphaerospora* sp. of the present study from naturally infected Atlantic salmon and sea trout, and experimentally infected Atlantic salmon and brown trout.

SPECIES	LENGTH	THICKNESS	CAPSULES
Atlantic salmon (natural exposure)	7.35(6.51-8.37)	9.90(8.84-11.16)	2.35(1.86-3.26)
Atlantic salmon (IP-injection)	7.75(7.00-8.00)	9.59(9.30-10.30)	2.14(1.86-2.79)
sea trout (natural exposure)	7.41(6.79-7.91)	10.10(8.84-12.50)	2.39(2.00-2.50)
brown trout (IP-injection)	7.13(6.80-8.20)	10.75(9.00-12.25)	2.40(2.00-3.00)

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length and 8.5μm$(7.4-9.5\mu$m$)$ in thickness perpendicular to the plane of the suture. Polar capsules were 2.2μm$(1.5-3.0\mu$m$)$ in diameter. Formalin fixed spores are therefore around 8% smaller than fresh ones.

3.3.2   Antigenic comparison with PKX

Neither monoclonal antibody probe Mab12 or lectin GS-1 bound to extrasporogonic or sporogonic life cycle stages of the salmon myxosporean. Control slides infected with PKX were positive to both the Mab12 and GS-1.

3.3.3   Experimental infections

Results of infection experiments

All eight fish subsampled from each recipient stock prior to each experiments were uninfected. Remaining control fish from all experiments were negative for infection at the termination of the relevant experiment.

Experiments 1 & 2: IP-injection and oral administration of sporogonic stages from kidneys to Atlantic salmon, 1993

All donor fish for this experiment were infected at high intensity with mature spores, immature spores and developing pseudoplasmodia, though intensity values were not measured. Fish receiving kidney homogenate orally or via IP-injection were negative for myxosporean parasites at all sampling dates. The experiments were terminated at 120 days when remaining control and experimental fish were sacrificed.

Experiment 3:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1992

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The prevalence and intensity of infection of the donor fish for this experiment was not calculated, but the prevalence of extrasporogonic stages in Giemsa stained smears from the epidemiological sample obtained from the same farm at the same time was 80%.

Infections were first recorded in experimentally infected fish on day 28 when both extrasporogonic and sporogonic stages were detected in 4/5(extrasporogonic) and 2/5(sporogonic) fish. The time for detectable infection to appear was therefore 14-28 days. Sporogonic stages were subsequently detectable for the rest of the experiment, and were found in 7/8 experimental fish sacrificed at the termination of the study. Extrasporogonic stages were detectable in only the 28 and 42 day samples (table 3.2).

Experiment 4:   IP-injection of extrasporogonic stages from blood to Atlantic salmon, 1993

Although the prevalence and intensity of extrasporogonic stages in the donor fish sample was not calculated, fish used were from the same stock as those used in experiments 5, 6 and 7.

Atlantic salmon were negative at all sampling dates for myxosporean infection. This experiment was terminated on day 85 due to elevated mortalities depleting the experimental stock.

Experiment 5:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1993

The prevalence and intensity of infection in donor fish was not calculated, but the fish used were from the stock described for experiments 6 & 7 below.

Detection of both extrasporogonic and sporogonic stages occurred day 28. At this point there was a higher prevalence of extrasporogonic stages than sporogonic stages. Sporogonic stages remained detectable in all experimentally infected fish at subsequent sampling dates, but extrasporogonic stages were only present at days 28 and 42 (table 3.3).

Mortalities within the experimentally infected salmon stock began at day 24. Continued increased mortality levels led to the experiment being terminated on day 85.

Experiment 6:   IP-injection of extrasporognic stages from kidneys to rainbow trout, 1993

Eight of the 10 donor Atlantic salmon were infected with extrasporogonic stages, three at high levels of intensity (4+/5+ - see chapter 4), in Giemsa stained smears.

Eight rainbow trout sampled at 28, 42 and 70 days were all found to be negative for myxosporean infection. The experiment was terminated on day 117 when the remaining 16 fish were found to be negative for infection. Ten mortalities during the study were all negative for infection.

Experiment 7:   IP-injection of extrasporogonic stages from kidneys to brown trout, 1993

Ten of 14 donor fish were infected with extrasporogonic stages, four at high levels of intensity (4+/5+), in Giemsa stained smears.

Both extrasporogonic and sporogonic stages were first detected in experimentally infected brown trout on day 28 post-infection. Both life cycle stages were present in the samples taken at 42 and 63 days (table 3.4).

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table 3.2   **Experiment 3**:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1992

DAYS POST-INFECTION	SAMPLE	EXTRASPOROGONIC STAGES	SPOROGONIC STAGES
14	5	0	0
28	5	4	2
42	5	3	4
70	5	0	3
120	8	0	7
TOTAL EXAMINED	28	7	14
MORTALITIES	2	0	2

table 3.3   **Experiment 5**:   IP-injection of extrasporogonic stages from kidneys to Atlantic salmon, 1993

DAYS POST-INFECTION	SAMPLE	EXTRASPOROGONIC STAGES	SPOROGONIC STAGES
28	5	3	1
42	5	2	4
70	3	0	3
85 *	3	0	3
TOTAL EXAMINED	16	10	11
MORTALITIES	9		

table 3.4   **Experiment 7**:   IP-injection of extrasporogonic stages from kidneys to brown trout, 1993

DAYS POST INFECTION	SAMPLE	EXTRASPOROGONIC STAGES	SPOROGONIC STAGES
28	5	3	1
42	5	3	4
63 *	3	2	3
TOTAL EXAMINED	13	8	8
MORTALITIES	26		

High levels of mortality amongst experimentally infected brown trout began on day 31 and continued unabated thereafter, leading to a premature termination of the experiment on day 63. *Aeromonas salmonicida*, the causative agent of furunculosis, was isolated from bateriological samples from mortalities in experimentally infected rainbow trout, brown trout and Atlantic salmon stocks in 1993. It was therefore held to be responsible for mortalities in these stocks, although rainbow trout appeared to experience fewer mortalities. Control fish also suffered lower levels of mortality. Farm A has endemic furunculosis, and it is therefore almost certain that this disease was transmitted to the experimental stock with the injected kidney homogenate.

Comparative parasite morphology

Extrasporogonic stages from experimentally infected Atlantic salmon and brown trout appeared to be identical morphologically to those from naturally infected Atlantic salmon. All shared the same staining characteristics and appearance, with no parasite deviating morphologically from the range of developmental forms found in naturally infected Atlantic salmon at the farm from which the donor fish were obtained. However, the full range of secondary cell number seen in such natural infections was not seen in experimentally infected fish (fig.3.4). A maximum of 63 and 40 secondary cells were seen in the most developed primary cells observed in experimentally infected Atlantic salmon and brown trout respectively. The intensity of infection in experimentally infected fish was markedly lower than that seen in the equivalent donor fish, with typically only around ten parasites per kidney impression smear.

Mature spores produced in Atlantic salmon were of similar size and morphology to those from naturally infected stocks. Table 3.1 compares spore dimensions of

fig 3.4 Stages of salmon *Sphaerospora* from experimentally infected brown trout. A, extrasporogonic stages containing five and fourteen secondary cells. B, extrasporogonic stage containing fourteen secondary cells; C, pseudoplasmodia in the lumen of a kidney tubule; D, two maturing sphaerospores. A & B, Giemsa stained kidney impression smear; C & D, fresh squash preparations of infected kidney (bar=10μm)
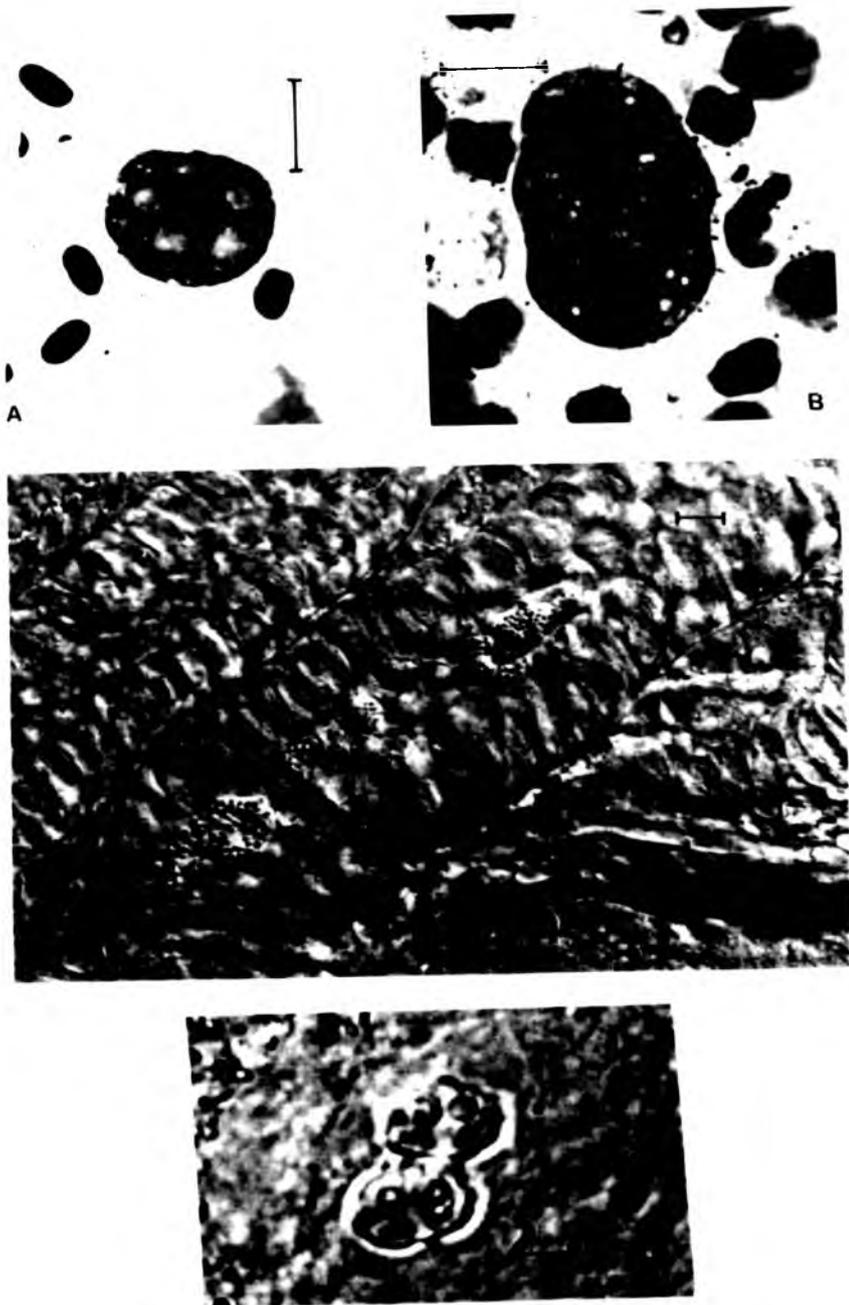
experimentally infected Atlantic salmon from the 1992 and 1993 experiments 3 and 5, and those from experimentally infected brown trout (experiment 7) with the measurements from naturally infected Atlantic salmon from farm A.

3.4 DISCUSSION

3.4.1 Generic identity

The morphology and dimensions of the mature spores of the salmon parasite indicate that it could belong to one of two myxosporean genera: *Sphaerospora* (Thélohan, 1892) of the family Sphaerosporidae, or *Leptotheca* (Thélohan, 1895) of the family Ceratomyxidae.

In their taxonomic scheme for the Myxosporea, Lom & Noble, (1984) made it clear that distinctions between the two were often arbitary; indeed a number of myxosporean species initially described as *Leptotheca* have been subsequently assigned to *Sphaerospora* (see Arthur & Lom, 1985). The two genera are distinguished by the morphology of their mature spores. Spores of *Sphaerospora* are more rounded, with similar valvular and sutural diameters, whilst those of *Leptotheca* are broader, with valvular diameter exceeding that of the suture.

The generic description of *Sphaerospora* (Lom *et al.*, 1985b) stated that the valvular diameter of the mature spore should not 'significantly' exceed its sutural diameter. From table 3.1, the mean values of these measurements for spores from salmon in the present study are:-

> **Valvular diameter** 9.90μm
>
> **Sutural diameter** 7.35μm

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The mean valvular diameter of spores from salmon thus exceeds the mean sutural diameter by approximately 25%.

According to Lom & Noble (1984), in *Leptotheca* spp., the length of the individually prolonged shell valve must 'significantly' exceed half of the axial (sutural) diameter of the spore.   In effect this comparison is the same; instead of comparing valvular diameter with sutural diameter, half the sutural diameter is compared with half the valvular diameter (See fig.3.5).   Using the values for the salmon parasite:

Length of individual shell valve = 4.95μm

Half of sutural diameter = 3.68μm

For the salmon parasite spores, shell valve length exceeds half the axial length, again by approximately 25%.

In terms of spore measurements and morphology the distinction between the two genera is therefore dependent upon a definition of significant.   Lom & Noble, (1984) described the transition in spore form from *Sphaerospora* through *Leptotheca* to *Ceratomyxa* as gradual.   Tripathi (1948), though recognising that *Ceratomyxa*, *Leptotheca* and *Sphaerospora* formed a continuous and overlapping series with no natural segregation, considered their merging to be impractical and therefore imposed an arbitrary division based upon length and breadth to 'avoid ambiguities'.   For historical reasons the two genera are therefore placed in separate families within the subfamily Variisporina; *Sphaerospora* in the Sphaerosporidae, and *Leptotheca* in the Ceratomyxidae (Lom & Noble, 1984).

There are a remarkable number of other similarities in the definitions of these

fig.3.5 Measurement parameters for distinguishing of *Sphaerospora* spp. from those of the genus *Leptotheca*. For *Sphaerospora*, the valvular diameter of the spore (**a**) should not exceed its sutural diameter (**b**), whilst for *Leptotheca*, the length of the individually prolonged shell valve (**c**) must significantly exceed half of the sutural diameter (**d**)
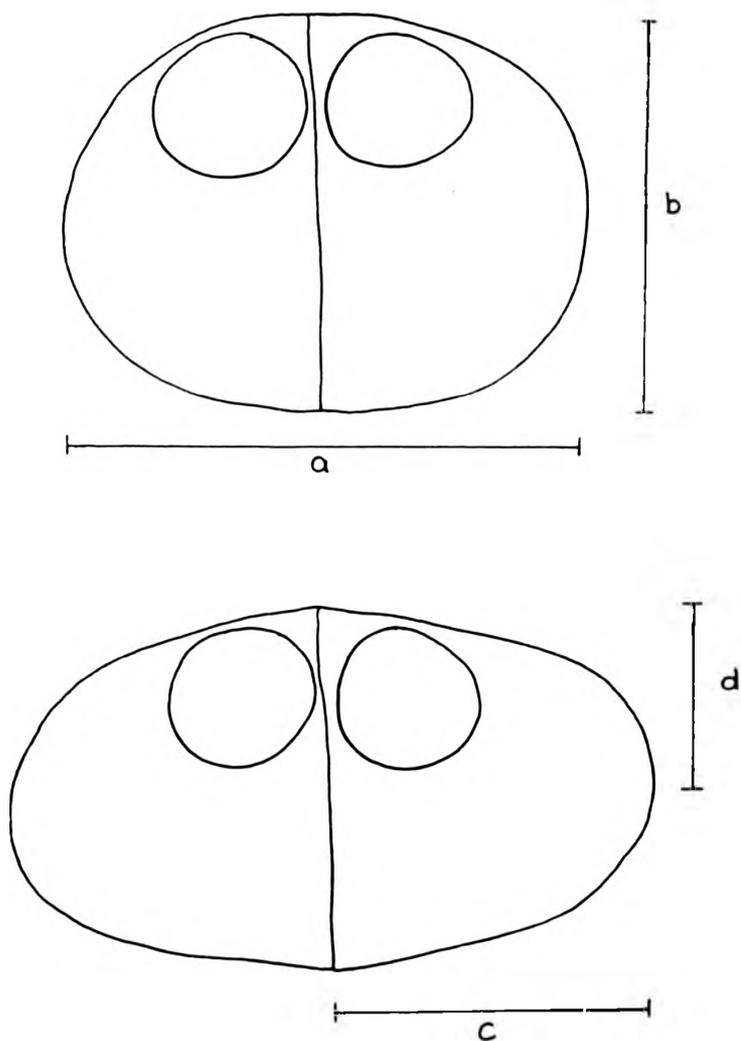
Sphaerospora and *Leptotheca*. These include two uninucleate, or a single binucleate sporoplasm, disporous trophozoites (pseudoplasmodia) and a coelozoic location in the urinary system (though both genera have a few histozoic species). Major differences are that *Leptotheca* spp. have been described mainly from marine hosts, whilst the majority of those of *Sphaerospora* are freshwater; and that no *Lepthotheca* spp have been shown to have extrasporogonic developmental stages (though this could be reflective of a lack of study).

Supamattaya (1991), also considering the *Leptotheca/Sphaerospora* problem, proposed that the ratio of the sutural length (SL) to the valvular length (VL) of the spores would aid in separating the two genera and proposed the following guidelines:-

SL : VL < 1.60 = *Sphaerospora*

SL : VL 1.25 - 2.58 = *Leptotheca*

A clear degree of overlap obviously exists between spores with a ratio of 1.25-1.60, which means that the method fails to differentiate species, and thus must be considered of very limited taxonomic value. The SL : VL ratio for the salmon spores is 1.35. Consequently the parasite lies within the range of both *Leptotheca* and *Sphaerospora* sp.

Table 3.5 shows the spore dimensions of species of *Leptotheca* and *Sphaerospora* with similar spore morphology and dimensions as those of the salmon parasite, concentrating on those from salmonid host species. Also shown is the relationship between the valvular and sutural diameters.

table 3.5   Sporogonic stages of *Sphaerospora* and *Leptotheca* species from salmonid hosts, or with dimensions similar to those of the parasite under study from Atlantic salmon (**continued overleaf**)

		Sphaerospora sp. (present study)	*S. truttae* (Fischer-Scherl et al., 1986)	*S. truttae* (Lom & Dykova, 1989)	*S. truttae* (Walter et al., 1991)	*L. renicola* (Thelohan, 1895)
host		*Salmo salar*	*Salmo trutta*	*Salmo trutta*	*Salmo trutta* *Thymallus thymallus*	*Scomber scombrus*
organ		kidney tubules	kidney tubules renal epithelium	kidney tubules	kidney tubules renal epithelium kidney interstitium	kidney tubules
length	min	6.51	6.58	-	5.00	-
	max	8.37	8.68	-	8.00	-
	mean	**7.35**	**6.84**	**7.20**	**6.30**	**8.00**
thickness	min	8.84	8.22	-	7.00	-
	max	11.16	10.11	-	10.00	-
	mean	**9.90**	**8.81**	**10.10**	**7.80**	**10.00**
valve/suture (+/- % difference)		+25%	+29%	+40%	+24%	+25%
valve:suture ratio		1.25	1.29	1.40	1.24	1.25
capsules	min	1.86	-	-	1.80	-
	max	3.26	-	-	2.50	-
	mean	**2.35**	-	-	**2.00**	-
filament coils		5	4	-	5	-
pseudoplasmodium		disporous	disporous	-	disporous	disporous

table 3.5   continued

		L. subsphaerica (Zaika, 1963)	S. colomani (Baska, 1990)	S. krogiusi (Konovalov & Shulman, 1965)	PKX (Hedrick et al., 1988)	S. oncorhynchi (Kent et al., 1993)
host		Coregonus autumnalis Thymallus thymallus	Acipenser ruthenus	Oncorhynchus spp. Salvelinus alpinus S. leucomenes Salmo mikiss	Oncorhynchus mykiss	Oncorhynchus nerka
organ		kidney tubules ureters	kidney tubules glomerulus	urinary bladder	kidney tubules	kidney tubules
length	min	7.00	8.40	5.80	-	8.20
	max	8.40	8.70	7.50	-	10.20
	mean	-	8.60	-	6.10	9.10
thickness	min	9.80	9.70	7.50	-	10.20
	max	10.50	10.50	11.50	-	11.20
	mean	-	10.20	-	7.10	10.40
valve/suture (+/- % difference)		+32%	+19%	+42%	16%	13%
valve:suture ratio		1.32	1.19	1.42	1.16	1.13
capsules	min	-	3.70	2.70	-	2.9
	max	-	4.00	3.60	-	3.3
	mean	2.8	3.90	-	2.00	3.0
filament coils		-	5-6	-	-	4-5
pseudoplasmodium		disporous	disporous	disporous	mono/disporous	monosporous

All parasites in table 3.5 have a valvular diameter in excess of sutural diameter. This is to be expected for the *Leptotheca* species, as this is supposedly a diagnostic criterion for the genus. However, all *Sphaerospora* species in table 3.5 also show a valvular diameter in excess of sutural diameter, the former being between 16 - 42% larger than the latter. Indeed, *S. krogiuisi*, *S.truttae* and the salmon species exceed the percentage difference exhibited by the two 'genuine' *Leptotheca* species, *L. renicola* and *L. subsphaerica*. At what point therefore does this difference become 'significant' and thus assign a species to *Leptotheca* rather than *Sphaerospora*, as defined by Lom *et al.* (1985b)? All but the PKX spores, themselves probably immature and therefore of undetermined genus, lie within the uncertain band. Indeed from the appearance of immature salmon *Sphaerospora* spores it is clear that this ratio changes as spores mature.

When developmental similarities and the evidence for considerable phenotypic variation in spore form within single myxosporean species are taken into account, it seems questionable that sufficient differences exist between the *Leptotheca* and *Sphaerospora* to justify their retention in different families. It seems reasonable therefore to propose that the two genera represent a continuum, certainly in relation to spore form and dimension, and that consideration should be given to their synonymisation, with *Leptotheca* Thélohan, 1895 becoming a junior synonym of *Sphaerospora* Thélohan, 1892. However, further study into the relationships between the genera would be required to confirm their similarities, especially more developmental studies on *Leptotheca* life cycles to see if they too have extrasporogonic phases, and also on their host specificty.

It is proposed that the salmon parasite be classified as a *Sphaerospora* sp for two reasons:-

(1)   all *Leptotheca* spp. have been described from marine fish (Lom & Noble, 1984)

(2)   no reports of *Leptotheca* spp. have described extrasporogonic proliferative stages, wheras the presence of such stages are a feature of *Sphaerospora* spp. included in the generic definition (Lom *et al*, 1985b).

Spore variation during development

Arthur & Lom (1985) presented evidence that mature and immature spores differed significantly for *Sphaerospora araii*. Immature spores were much wider than mature ones, with a more prominent sutural ridge, and still vacuolating cytoplasm. Such findings are very similar to the observations in salmon *Sphaerospora*. Similar differences were also seen in spores of *S. molnari* (Lom *et al*., 1983b), *S. epinephali* (Supamattaya, 1991), *S. truttae* (Fischer-Scherl *et al*., 1986) and *L. subsphaerica* (Shulman, 1966). This is a very important developmental variation to recognise, especially since spore morphology is the major taxonomic feature for species identification. If not appreciated it could lead to problems in species of *Sphaerospora* in which development of all sporogonic stages was synchronous. In such a species, a single sample taken when spores were immature would result in a very false impression of spore morphology and dimensions, compared with that given by mature spores. The relevance of the need to discriminate mature from immature spores was emphasised by Athanassopoulou & Sommerville (1993a) from a study of *Myxidium* spp. infections of

roach. Without such discrimination the same species might be identified as a different one. However in the salmon *Sphaerospora*, a range of spore forms of different levels of maturity were found at all times that spore stages were present.

It is clear from the present study that the description of myxosporean species from formalin fixed material is to be avoided. Such material undergoes a marked shrinkage in response to fixation, and thus all species descriptions should be based on measurements taken from fresh preparations. This shrinkage does however appear to be consistent with respect to each measurement parameter (length, width, thickness), with ratios of length to thickness remaining very similar to those exhibited by fresh material. In the present study, formalin fixation caused an 8% reduction in dimensions, whereas Walter,Odening & Bockhardt (1991) found that the spores of *S. truttae* shrank by 25-35% when fixed in Bouin's fixative. The use of fixed spores in cases where material is otherwise unavailable or precious has been condoned (Arthur & Lom, 1985; Lom & Arthur, 1989). However, their use could lead to subsequent confusion concerning the spore dimensions, and thus lead to misidentifications or the erection of new species by later workers describing fresh material of the same species.

3.4.2   Host specificity

Extrasporogonic stages provide a means of experimentally infecting naive fish without knowledge of the correct alternate host. Successful two host life cycles have been achieved for only a few myxosporean species to date (chapter 6), meaning that few opportunities for attempting cross infection experiments are available, and no reports exist yet of such experiments.

In the present study, farm managers were reluctant to allow fish of other species,

and from other sources, to be exposed on site so ruling out attempts to determine host specificity by exposure. Much information on myxosporean specificity has been obtained by this method in the past either by exposure of fish in separate tanks (Hoffmann & Putz, 1969; Sanders & Fryer, 1970; O'Grodnick, 1979), or by holding stocks of a variety of fish species in pond polyculture (Molnar & Kovacs-Gayer, 1986c). However there remains a problem in such exposure experiments, in that several myxosporean infections may be cycling in the wild, and so an experimental fish could be exposed to several different infective agents. Although infections generated by the transfer of extrasporogonic stages ensure that experimental fish are only exposed to a single myxosporean species, ideally exposure to the infective agent would also have been employed in the present study had it been practicable.

Experimental infection

The results of the transmission experiments indicated that spores were not directly transmissable from donor to naive salmon orally, or via IP-injection. However, extrasporogonic stages were successfully transmitted to naive salmon by the IP-injection of extrasporogonic stages present in the kidney of donor salmon. Both the extrasporogonic K-swimbladder and C-blood stages of *S. renicola* (Molnar, 1984, 1988b; Molnar & Kovacs-Gayer, 1986a), and extrasporogonic PKX stages in the kidney interstitium (D'Silva *et al*, 1984; Clifton-Hadley *et al*, 1984; Clifton-Hadley & Feist, 1989) have been transmitted in a similar fashion between host fish. In the present study, transmission via the IP-injection of blood was not successful. Perhaps the relatively low density of salmon *Sphaerospora* extrasporogonic stages in the blood, compared to the kidney, of the naturally infected fish used as donors (see results & chapter 2) explains

this finding. Kent & Hedrick (1985b) were able to infect rainbow trout with PKX stages from the blood and spleen of infected fish, even though PKX extrasporogonic stages, like those of salmon *Sphaerospora* have a predeliction for the kidney interstitium.

The resultant intensity of infection generated by IP-injection, even of kidney homogenates, was lower than that in the donor fish, even in salmon to salmon transmission. Molnar & Kovacs-Gayer (1986a) transmitted K-stages of *S. renicola* to carp but clinical levels of SBI did not result. Probably only a percentage of injected extrasporogonic stages become established in naive hosts, so accounting for the failure of low level, blood borne extrasporogonic stages to infect fish compared to high intensity kidney homogenates.

Some experimentally injected fish failed to go on to develop detectable levels of infection, and showed an identical appearance post-mortem to unexposed controls. This could indicate differential susceptibility within the population, or that some fish did not receive a sufficient number of parasites to produce an infection.

Molnar (1984; 1988b) and Molnar & Kovacs-Gayer (1986a) found that stages of *S. renicola* became detectable in experimentally infected carp within one to two weeks. This compares with four weeks for *Sphaerospora* to become detectable in experimentally infected salmon. A similar, four to five week period before the detection of extrasporogonic stages in IP-injected fish has been shown for PKX (D'Silva *et al*, 1984; Clifton-Hadley & Feist, 1989). This time may be related to the temperature of the water used to maintain the fish, this being lower for salmonids than for carp. It is notable that this four week period is similar to that identified in chapter 4 as the pre-patent period before naturally infected fish showed detectable levels of parasites. Many

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experimentally infected fish, however, were found to have sporogonic stages in the kidney tubules coincident with the first appearance of extrasporogonic stages. This is much sooner for sporogonic stages than would be expected from data obtained for natural infections (chapter 4), where sporogonic stages did not generally appear until 17-31 days after the first appearance of extrasporogonic stages. Kent & Hedrick (1985b) found sporogonic stages 8-12 weeks post experimental infection with PKX from the blood and spleen of donor fish. Clifton-Hadley & Feist (1989) in experimental infections with the same organism from the kidney, found PKX extrasporogonic stages within five weeks, but sporogonic tubular forms were not found until nine to ten weeks. Molnar (1984) showed that sporogonic stages in naive carp infected with swimbladder K-stages of *S. renicola* appeared just one or two days after extrasporogonic K-stages were first detected. However, when C-blood stages of the same organism were transmitted to naive fish, sporogonic stages did not appear until two weeks after the first detection of C-stages. The extrasporogonic stages injected into naive fish in the present study were obtained from donor fish sampled in August, the peak prevalence and intensity period for such stages (see chapter 4), just prior to the point at which sporogonic stages became detectable in naturally exposed fish. Injected homogenates therefore contained well developed, later extrasporogonic stages which contained triple formations, as well as individual secondary cells and triple forms. It could be therefore that some *Sphaerospora* stages were already infective for the kidney tubules on injection, and thus sporogonic stages appeared simultaneously with extrasporogonic stages. This could also explain the difference in the results of Molnar (1984) and Molnar (1988). Molnar (1984b) injected K-stages of the *S. renicola* extrasporogonic cycle; these stages produce large numbers of triple forms (Dykova *et al.*, 1990) and

hence sporogonic stages appeared virtually coincidentally with extrasporogonic forms. Molnar (1988b), however injected C-stages, which cycle continuously, producing only secondary cells. It might be that it is not until these transform into K-stages in the swimbladder that triple forms begin to be produced in such infections, and so sporogonic stages appeared much later than the first appearance of extrasporogonic stages. In experimental PKX infections it could be that since very few stages reach the kidney tubule anyway, their detection is delayed compared to the detection of extrasporogonic stages.

The salmon *Sphaerospora* extrasporogonic stages were successfully transmitted to brown trout, in which sporogonic stages and spores also appeared in the kidney tubules at the first sampling date. The intensity of the resultant infections was rather lower than that demonstrated by salmon to salmon transmissions. This might indicate that brown trout are less susceptible to the parasite than are Atlantic salmon. However, the naturally exposed sea trout at farm C were infected at a similar prevalence and intensity as Atlantic salmon on that site. The most likely explanation is a difference in the number of parasites present in the homogenate injected into the brown trout compared to that used in the salmon experiments.

Attempts to infect rainbow trout by IP-injection of extrasporogonic stages were unsuccessful, despite the numbers of parasites contained in the injected homogenate. This indicated that rainbow trout may not be a suitable host for the salmon *Sphaerospora*. Branson (1987) found rainbow trout uninfected at a farm site where salmon were infected with *Sphaerospora* in the UK, supporting the experimental evidence of the present study. Fischer-Scherl *et al* (1986) found no infections of *S. truttae* in rainbow trout and brook trout in the same German water body in which the

parasite was present at a high prevalence in brown trout. Odening *et al* (1988) found *Sphaerospora* sp in rainbow trout but did not establish its specific identity, although its finding was often coincident with that of PKX. Ellis *et al.* (1985) found strain differences in PKX susceptibility in rainbow trout from Scotland compared to Norway. It is not therefore entirely certain whether rainbow trout are refractory to infection by the *Sphaerospora* sp. under study, or whether host strain differences play a role.

3.4.3 Specific identity

Sporogonic stages

It is suprising that, considering the extent of aquacultural, recreational and scientific interest in the salmon in Scotland over many years the parasite has been reported so rarely. Occasional spores of *Leptotheca* sp were reported from the kidneys of juvenile Atlantic salmon by Wootten & Smith (1980) from a freshwater smolt unit in eastern Scotland (Almondbank, Tay system). However, of the 625 fish examined, the parasite was only noted in 4 of the fish over the two year sampling period. Kennedy (1974) also listed spores of a *Leptotheca* sp from Atlantic salmon kidneys in Scotland, as part of a description of the parasite fauna of British freshwater fish. Considering the *Leptotheca/Sphaerospora* identification taxonomic problem discussed earlier, it seems likely that these two reports are of the same species under study here. However neither authors give any morphological or measurement data for the spores, simply noting their presence.

Given the apparent high degree of host specificity of *Sphaerospora* sp. proposed by Baska & Molnar (1988), Lom *et al.* (1985b), Arthur & Lom (1985), and

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experimentally demonstrated by Molnar & Kovacs-Gayer (1986a) for *S. renicola* and outlined by Shulman (1966), it is most relevant to discuss the salmon *Sphaerospora* in relation to other reports of *Sphaerospora* spores from salmonid fish (table 3.5).

Only two non-salmonid infecting myxosporean species are similar in spore dimensions to the salmon *Sphaerospora*. Of these *S. colomani* infects sterlet, *Acipenser ruthenus*, has a limited geographic distribution, and has some unusual developmental stages in the Bowman's capsule (Baska, 1990; Baska, 1992). *Leptotheca renicola* is a parasite of a purely marine fish, the mackerel, *Scomber scombrus*.

There are two reports of the genus *Leptotheca* from salmonid fish. One of these, *Leptotheca krogiusi*, was assigned to the genus *Sphaerospora* by Lom & Noble (1984). As described by Shulman (1966) it shows a degree of similarity to the salmon *Sphaerospora* and is found in a very wide range of *Onchorhynchus*, *Salvelinus*, and *Salmo* host species. Only sporogonic stages were described. They were of similar dimensions and morphology to those of the salmon species, showed disporous development, and similar, almost spherical polar capsules. However, they were restricted to the urinary bladder within the fish, the number of sporoplasm cells is unclear, and spores had valve cell outgrowths and protruberances not seen in the spores of the salmon *Sphaerospora*.

Leptotheca subsphaerica spores have been reported from the kidney tubules, urinary bladders and ureters of grayling and coregonids where they developed in disporous pseudoplasmodia (Shulman, 1966). Spores showed similar changes in appearance as they matured to those of the salmon *Sphaerospora*, and were of a similar size. However, the distinct anterior point of the spore suture and their pyramidal appearance in the plane perpendicular to the suture are clear differences.

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Bennet & Wolke (1986) gave a brief report of sporogonic myxosporean stages in the kidney tubules of a single adult Canadian Atlantic salmon held in fresh water. This was based on histological material only, but appeared to be a *Sphaerospora* species from their poorly preserved TEM photographs. In the USA, Meyers and McPherson (1985) found what appeared to be a sporozoan parasite in histological sections of the glomerulus of returning Chinook salmon, *Oncorhynchus tshawytscha*, but no details of its morphology were given.

Fischer-Scherl *et al.* (1986) described *S. truttae* as a new species in brown trout in Germany. A number of features of this species are identical with those of the salmon *Sphaerospora* species under study. These include disporous development, sporogonic stages and spores present in the tubules and Bowman's capsules and immature spores wider than mature ones. In addition this species was never encountered in rainbow trout or brook trout in the same water body. Spore measurements, although on average slightly smaller than those of the salmon *Sphaerospora*, generally lie within the same range (see table 3.5). However, for *S. truttae*, Fischer-Scherl *et al*, (1986) showed sporogonic stages intracellularly in the epithelial cells of the kidney tubules. Despite an intensive search, the authors found no extrasporogonic stages in *S. truttae* infections, although one fish was infected with PKX (Fischer-Scherl, 1992, pers. comm.; Fischer-Scherl *et al*, 1986). The spores of *S. truttae* from brown trout described by Lom & Dykova (1989) lie more or less within the same range of measurements as those of the salmon *Sphaerospora*. Walter *et al.* (1991) included grayling as a new host species for *S. truttae*. Development in both grayling and brown trout was disporous. Spores were, in terms of mean measurements, 16-27% smaller than those of the salmon *Sphaerospora* under study, and from other reports of *S. truttae* (Fischer-Scherl *et al.*, 1986; Lom &

Dykova, 1989), but were similar in range. Four coils of the polar filament were present. Fish were coinfected with *Chloromyxum truttae*, but never with PKX, despite its presence in the water body. Spores were again found intracellularly in renal tubule cells, and, in addition, in well defined vacuoles in the interstitium.

Although there are minor differences in mean spore size, the spores of *S. truttae* from the above reports and those of the salmon *Sphaerospora* under study are broadly similar. Spore measurements from brown trout experimentally infected with salmon *Sphaerospora* in the present study differed, by being slightly less long but slightly wider on average, from those in the salmon itself, illustrating that a degree of spore variation within a single myxosporean species between different hosts may be common. Such minor differences within individual hosts, sites and organs could be attributed to phenotypic variation due to factors associated with the physiology or biochemical suitability of the host species concerned (Athanassopoulou & Sommerville, 1993a).

However, the finding of spores intracellularly in renal epithelial cells, and in the kidney interstitium by Fischer-Scherl *et al* (1986) and Walter *et al.* (1991) is in direct contrast to the findings from naturally infected or experimentally infected farmed salmon, from brown trout experimentally infected with the salmon *Sphaerospora*, or from sea trout held at farm C, where salmon suffer high prevalence and intensity of *Sphaerospora*. This seems an important developmental difference between reports of *S. truttae* and the present study of salmon *Sphaerospora*.

Reports of *S. truttae* have yet to include any ultrastructural descriptions of sporogonic development, making that comparison impossible. No reports of *S. truttae* have described the extrasporogonic stages detailed here from the salmon and experimentally infected brown trout; extrasporogonic stages of *Sphaerospora* spp. can

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be very different morphologically in closely related species of fish, with almost indistinguishable spores of *Sphaerospora* in their kidneys (Lom *et al.*, 1985b; Baska & Molnar, 1988).

Extrasporogonic stages

Cone & Cusack (1991) described myxosporean extrasporogonic stages from impression smears of the kidneys of brook trout in Canada. These were 5-30μm in diameter and contained only up to 36 secondary cells, many less than the most developed *Sphaerospora* extrasporogonic stages from salmon. No parasites were found in blood smears and no tertiary cells were seen in secondary cells. *Sphaerospora* spores were not present in any fish and there were no wild salmonids in the water supply to the infected site. Spores of *Chloromyxum truttae* were found in the gall bladder of one infected fish, though the relationship, if any, between these and the extrasporogonic stages was not apparent. Lom *et al.* (1985b) mentioned possible extrasporogonic stages in brown trout and grayling from Czechoslovakia, but had insufficient material to provide a description.

Relationship between salmon *Sphaerospora* and PKX

PKX is a parasite of great economic importance. Not only can it infect the Atlantic salmon and a range of other salmonids, but also its extrasporogonic and sporogonic sites of development, the kidney interstium and the kidney tubule lumen respectively, are the same as those of the salmon *Sphaerospora*. It is therefore important to establish its relationship, if any, to the parasite causing salmon sphaerosporosis, and in addition ensure that the two can be safely differentiated. PKX

for some reason fails to undergo full sporogonic development in the kidney tubules of its host. As a result few, if any mature spores are produced. By contrast, in the salmon *Sphaerospora*, the extrasporogonic stage is followed by a sporogonic phase in which very large numbers of mature sphaerospores are produced. Although smaller extrasporogonic stages of the two parasites look very much alike in Giemsa-stained blood and kidney smears, in PKX infected fish, primary cells contain one to just seven secondary cells, compared to the 100+ of the salmon *Sphaerospora*. At the transmission electron microscope level PKX is further differentiated by the presence of characteristic 'haplosporosomes' in its primary cell, a feature not present in the salmon parasite studied here. Both the lectin and the monoclonal antibody probes of Adams, Richards & Marin, (1992) and Marin, Adams, Richards, Castagnaro & Hedrick, (1993) failed to bind to either extrasporogonic or sporogonic stages of the salmon parasite, yet are specific for both stages of PKX. This indicates antigen differences between salmon *Sphaerospora* extrasporogonic and sporogonic stages, and those of PKX. A similar result was outlined by Rafferty & Mulcahy, (1988), although their report contains a number of confusing errors (They established the specificity of a PKX antiserum by comparing it against "tissues from brown trout heavily infected with *Sphaerospora renicola*" - either they tested their serum against *S. renicola* from carp, or *Sphaerospora truttae* from brown trout). The severe pathology associated with PKX infections is not shown by salmon *Sphaerospora* (chapter 5), and, in addition, no clinical PKD has ever been reported from any of the sites in the present study.

If spores of the salmon *Sphaerospora* were to be found coincident with PKX infections they might appear to be one and the same organism. A precise generic identity of the PKX organism has never been made, due to the fact that the rare spores

which are seen in fish following PKX infection are invariably immature, possibly due to incomplete valvogenesis (Kent & Hedrick, 1986). However the most mature stages look very like *Sphaerospora* spores, and frequently authors have reported *Sphaerospora-*like organisms in other host fish in infected waters (Hedrick *et al*., 1988). That they cannot produce spores has lead to the suggestion that salmonids may be unnatural hosts (Seagrave *et al*., 1980), and that a natural host exists in the environment, since if mature spores are not produced in salmonids, presumably the parasite would be unable to continue to cycle each year. Bucke *et al*. (1991) identified intralumenal myxosporean stages and immature spores of, presumably, a *Sphaerospora* species in the kidney tubule lumen of Atlantic salmon, brook trout and grayling associated with PKD infections in the UK. Again, no measurements were provided, probably due to the lack of fully mature spores. Interestingly, despite high levels of PKD in rainbow trout held at a farm site on the same waters, no such sporogonic stages were seen in this species. Similar immature myxosporean forms were found in UK brown trout by Clifton Hadley & Feist (1989).

On occasion, mature spores have been described from salmonids coincident with PKX, but the relationship between the two not definitely confirmed (Odening, Walter and Bockhardt, 1988) No clinical PKD has ever been recorded from the sites sampled in the present study. Very recently Kent, Whitaker & Margolis (1993) described a new species, *Sphaerospora oncorhynchi*, from adult sockeye salmon in Canada and related this finding to the fact that young rainbow trout at a farm served by the same water body became infected with PKX. This relationship still awaits further study however. The spores of this species were 8.2-10.2μm in length and 10.2-11.2μm in thickness perpendicular to the suture. These are significantly larger than those of the

salmon *Sphaerospora*, and develop in monosporous pseudoplasmodia.

From table 3.5 it can be seen that immature spores of PKX from the kidney tubules of rainbow trout in the USA are much smaller (Hedrick *et al.*, 1988) than those from the salmon *Sphaerospora* in the present study. Also, pseudoplasmodia of PKX contain up to three nuclei, a feature never seen in the salmon parasite. PKX spores are almost always found in monosporous pseudoplasmodia (Kent & Hedrick, 1986), although sometimes the latter are disporous (Hedrick *et al.*, 1988).

Summary on specific identity

Until more information is forthcoming on the development of *S. truttae* a definite, specific identification of the salmon *Sphaerospora* should not be made. Evidence for the salmon *Sphaerospora* being *S. truttae* is restricted to the similarity of their spores and the salmonid nature of their hosts. The lack of any reports of extrasporogonic stages of *S. truttae* or of the ultrastructure of this parasite prevents a detailed comparison. The presence of sporogonic stages of *S. truttae* intracellularly in the renal epithelial cells and also in the kidney interstitium is at variance with our observations on the salmon *Sphaerospora*. It might be considered that, without further information on *S. truttae* extrasporogonic stages and ultrastructure from the type host and locality, these differences are too major to warrant assigning the salmon *Sphaerospora* to *S. truttae*.

It is important to emphasise that there may be a range of *Sphaerospora* spp. or *Sphaerospora*-like myxosporeans which infect the urinary system of salmonid fish. In addition to the salmon *Sphaerospora* of the present study, there are PKX, *L. krogiusi*,

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L. subsphaerica, S. truttae and the extrasporogonic stages of Cone & Cusack (1991) from brook trout, there are the *Sphaerospora* spores of Odening *et al.* (1988) and Hedrick *et al.* (1988) from rainbow trout in PKX infected waters, those of *S. oncorhynchi* (Kent *et al.*, 1993) from *O. nerka* in PKX infective waters. The inter-relationships, if any, between these species remain largely unknown. It may be that some salmonid species are hosts to a number of different, distinct *Sphaerospora* species, and that the distribution of these *Sphaerospora* species may overlap. Hedrick (pers. comm. to Feist) is of the opinion, for example, that there may be a range of species of *Sphaerospora* infecting brown trout. A greater understanding of the life cycle of salmonid *Sphaerospora* spp. is needed to allow cross infection experiments by exposure to the correct infective agent. More knowledge of their extrasporogonic stages, few of which have to date been reported, is also necessary before their identities can be definitely determined.

Chapter 4
Epidemiology

4 EPIDEMIOLOGY

4.1 INTRODUCTION

The level of a myxosporean infection in a particular host and given locality is a result of the interaction of a great many factors, both physiological and ecological (Lom & Dykova, 1992d; Bucher, Hofer & El-Matbouli 1992). These include environmental factors such as temperature, season, geographic location, pollution and salinity; and host factors such as age, availability, density, susceptibility, time exposed, immune response, previous infection status, maturity and sex.

Many hypotheses to explain the reasons for the variations in patterns of epidemiology in myxosporeans were constructed without knowledge of the need for an alternate host in the life cycle. This introduces a whole new range of host related and seasonal factors for consideration in the study of myxosporean epidemiology and may help to explain some aspects of the host-parasite relationship which were previously difficult to understand.

Brummer-Korvenkontio, Valtonen & Pugachev (1991) noted that there were few studies of the seasonal dynamics of myxosporean infections. Such data was often limited to a record of the percent prevalence of the parasite concerned in a fish population or area at a single time, and gives no impression of seasonal aspects of the life cycle. However the host-parasite relationship is not static, but dynamic, and for a number of reasons may change over time. More detailed studies of seasonal variations and epidemiology have been described for economically important, pathogenic species infecting fish in intensive culture (Wyatt, 1978; Ching & Munday, 1984a; Amandi *et al.*, 1985; Foot & Hedrick, 1987). Cultured fish represent ideal populations for study since they provide a cohort which can be followed from hatching, and regularly sampled over a prolonged period.

4.1.1.　　　Prevalence and intensity

The prevalence of myxosporean infection, that is the percentage of sampled hosts found to be infected, often reaches 100% in fish populations, even in the wild. Conversely it has sometimes been found to be very low (Lom & Dykova, 1992d). In temperate areas prevalence is often seasonally variable, especially in the freshwater environment, with temperature considered a major factor in myxosporean infection. Seasonal epizootics due to myxosporeans are therefore common (Mitchell, 1977; Athanassopoulou, 1990).

The intensity of myxosporean infection in fish populations and individuals is rarely recorded, probably due to difficulties in assessing infection levels (Kovacs-Gayer & Molnar, 1983). Intensity values are often, though not always, at their highest when the parasite is at its most prevalent in the host population (Alvarez-Pellitero & Gonzalez-Lanza, 1985; Gonzalez-Lanza & Alvarez-Pellitero, 1985). Molnar (1979b) noted that both prevalence and intensity increased with increased fish exposure to waters infective for *Myxobolus pavlovskii*, and Morado & Sparks (1986) noted a similar phenomenon for *Kudoa* sp infections in Pacific whiting. Intensity levels can vary markedly between different susceptible host fish species inhabiting a water body (Molnar 1979a), and is usually considered an effect of host specificity (See Chapter 3). Variation is also seen between infection intensity in individual fish of the same species of host in a population. This overdispersed distribution is common in parasite populations.

In farmed populations carrying *Sphaerospora* spp infections, individual fish have been reported to be either very heavily infected or not at all (Molnar 1979a; 1980a). This appears to be due to the fact that extrasporogonic proliferative stages ensure that any fish in which the parasite establishes becomes highly infected. In the wild, however, sphaerospores are scarce even in infected fish (Lom *et al*, 1985b; Baska & Molnar, 1988) indicating that perhaps the

extent to which extrasporogonic proliferation can occur is limited. Of course, other factors such as the stress status of farmed fish or their stocking density may dispose such populations towards higher levels of infection.

4.1.2.   Seasonal patterns

Brummer-Korvenkontio *et al* (1991) showed very few clear seasonal patterns of prevalence or intensity in a study of three myxosporean parasites of roach from Finnish lakes.

Where seasonal patterns have been recorded, there is considerable variation between species. The prime influence on prevalence of infection appears to be that of environmental factors on the release and presence of the infective (presumably actinosporean) stage of the life cycle (Foott & Hedrick, 1987; Alvarez-Pellitero & Sitja-Bobadilla, 1993). However, although many authors described changes of and peaks in prevalence and intensity at certain times of the year, these have usually referred to the presence of trophozoites or, more frequently, spores and so may not necessarily be reflective of when fish actually became infected. Due to the historic lack of knowledge of the nature of the infective agent in almost all myxosporean infections, there is an absence in most species of any understanding of the period over which infection can occur or of changes in the abundance of the infective stage in the environment. Obviously therefore we can have no idea how these factors are reflected in terms of the detection, prevalence and spore production of the myxosporean species concerned. In addition, in a number of myxosporean species, such as PKX (Ferguson & Ball, 1979; Ellis, McVicar & Munro, 1985; Foott & Hedrick, 1987; Clifton-Hadley & Feist, 1989; Kent, 1992) and *M. cerebralis* (El-Matbouli, Fischer-Scherl & Hoffmann, 1992b), it has been shown that there may be a pre-patent period of four weeks or more from initial infection to when that infection becomes detectable. This must be borne in mind when interpreting

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prevalence and intensity data and trying to relate it to the host-parasite relationship.

Three broad categories of seasonal pattern of myxosporean infection were briefly outlined by Kovacs-Gayer & Molnar (1983):-

All year round infectivity

In this type of life cycle, spores are present at any time of the year, spore production being assumed to be relatively rapid. Kashkovskii (1966) demonstrated that the waters of a Russian reservoir were constantly infective for *Myxidium rhodei*, several parasite generations occuring each year (cited by Shulman, 1966). Molnar (1979b) showed a similar cycle for *Myxobolus pavlovskii*. However both these myxosporean species still showed prevalence and intensity changes during the course of the year. Shulman (1966) related these in *M. rhodei* to seasonal aspects of host feeding behaviour in terms of the chances of ingesting 'aged' spores from the sediments, whilst Molnar (1979b) related an increasing intensity of infection simply to time exposed to infective waters.

Single peak one year life cycles

A large number of myxosporeans show an annual life cycle, where characteristically, host fish become synchronously infected by the parasite during a restricted period each year. The cycle in the fish itself is frequently short, with a single peak in mature spore production, and subsequent release into the environment. In some species however, especially those which inhabit histozoic sites with no possible means of spore release until the death of the host, spores, once formed, may persist in the fish for a long period of time (Lom & Dykova, 1992d).

Many myxosporeans species showing annual life cycles are, in temperate climes,

initially detected in naive fish in the summer months when temperatures are higher. They subsequently increase in prevalence over the summer period, then decrease during the autumn and winter (El-Tantawy, 1989). Molnar (1982) showed that the fish phase of the life cycle of *Thelohanellus nikolskii* in common carp spanned just 8-10 weeks, yet only a single generation of the parasite occurred each year. Similar summer based life cycles are shown by a range of species such as *Sphaerospora colomani* in the sterlet *Acipenser ruthenis* (Baska, 1990), *Thelohanellus hovorkai* from common carp *Cyprinus carpio* (Molnar & Kovacs-Gayer, 1986c), *Myxobolus cyprini* in carp (Molnar & Kovacs-Gayer, 1985), *Myxobolus ellipsoides* in *Tinca tinca* (Gonzalez-Lanza & Alvarez-Pellitero, 1985) and *Chloromyxum inexpectatum* from sterlet (Baska, 1990).

In particular myxosporeans of wild and cultured salmonids seem to show this type of pattern of seasonal occurrence, with release of the infective stage apparently timed to coincide with the appearance of 0+ fish in the wild: for example, *Myxobolus cerebralis* infects young salmonids, as its trophozoites infect the cartilage around the head prior to ossification. The highest levels of actinosporean infective stages (Markiw & Wolf, 1983) are present in the Spring when potentially susceptible juvenile host fry are present. Mature spores develop by the end of Summer, but new infections do not occur until the following Spring when newly hatched susceptible fry are available.

The infective stage of *Ceratomyxa shasta* is present from late March to mid-November in Canada (Johnson *et al.*, 1979), with outbreaks of ceratomyxosis in salmonids related to a rise in water temperature over 10°C. Ching & Munday (1984a) found peaks of prevalence, and accelerated rates of disease and time to death, as temperature increased above the 10°C threshold.

Myxobolus insidiosus, a parasite of *O. tshawytsca*, is infective in waters from February,

with disease not appearing until the first week of May (Wyatt, 1978). Amandi *et al.*, (1985) showed that older fish carrying the spores of this species died in the Autumn but, despite this release, new infections did not occur in susceptible fish until the next February.

Outbreaks of PKD, caused by the myxosporean extrasporogonic stage, PKX, occur from late Spring until early Autumn associated with water temperatures over 15°C (Ferguson & Ball, 1979; Clifton-Hadley *et al.*, 1984). By exposure experiments, Hedrick *et al.*, (1985) showed that in the USA, this period corresponded to the restricted period each year for which the infective stage was present. Sentinel fish exposed to infective waters became infected from May through to November, peaking in June. In the UK, a similar epidemiological picture was established, although fish at low temperatures later in the season did not become clinically diseased (Ferguson, 1981; Clifton-Hadley, Richards & Bucke, 1986). This might not relate simply to the presence of PKX, since the disease is dependent upon the response of the host as well as that of the parasite. As the host response in fish is very temperature dependent, host factors have a very important role in the epidemiology of the disease condition. Foott & Hedrick (1987) attributed the limited window for infection to a temperature effect that they proposed might affect the emergence or presence of the infective agent, an endogenous parasite rhythm, or a change in host susceptibility.

Foott & Hedrick, (1987) showed that the appearance of sporogonic stages of PKX always followed and was later than that of extrasporogonic interstitial stages. They remained present in the fish long after the extrasporogonic stages were no longer detectable. Spore stages persisted throughout the Winter, began to drop by the following August (year two), but were still present into September. Cone & Cusack (1991) found extrasporogonic stages of a myxosporean (presumably *Sphaerospora* or *Chloromyxum* sp) in brook trout only in June in Canada.

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A number of myxosporean species demonstrate annual life cycles where infections are detected in the winter months when temperatures are lower. These include *Hoferellus carassii*, a problematic species infecting goldfish (Molnar, Fischer-Scherl, Baska & Hoffmann, 1989; Fischer-Scherl, El-Matbouli & Hoffmann, 1992; Yokoyama, Ogawa & Wakabayashi, 1990), *Hoferellus (=Mitraspora) cyprini* in goldfish (Ahmed, 1973), *Myxobolus artus* in carp skeletal muscle (Ogawa, Delgahapitiya, Furata & Wakabayashi, 1992), *Ceratamyxa labaricus & Ceratomyxa diploidae* in cultured Mediterranean sea bass in Spain (Alvarez-Pellitero & Sitja-Bobadilla, 1993) and *Myxobolus dujardini* in northern squawfish in the USA (Mitchell, 1977). It is difficult to find explanations for greater levels of prevalence and intensity in the colder months of the year. Lom (1970; pers. comm. to Chubb), by raising the temperature at which pike and perch infected with *Henneguya psorospermica* were held, achieved a host induced drop in infection level. He attributed this phenomemon to the effect of temperature on the host's ability to mount an effective immune response.

Some species have life cycles with two peaks of spore production each year, though again, rarely have hypotheses to explain such findings been proposed. Shulman (1966) showed such cycles for *Myxobilatus legeri* and *Myxidium lieberkuehni*, whilst Kovacs-Gayer & Molnar (1983) found a similar developmental cycle in *Myxobolus baselamellaris* in carp. In Spain, a number of myxosporeans in a range of hosts show a bimodal pattern of seasonality (Gonzalez-Lanza & Alvarez-Pellitero, 1984; Alvarez-Pellitero & Gonzalez-Lanza, 1985). *Sphaerospora renicola* has a two peak life cycle, though there is confusion between the studies of different authors (Dykova & Lom, 1982; Pavlaskova & Strelkov, 1987; Grupcheva, Dykova & Lom 1985; Sedlaczek, Friede, Kluss & Vinzelberg, 1990; Odening *et al.*, 1988).

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4.1.3 Factors affecting seasonality

Many coelozoic myxosporean species with annual life cycles produce few spores in small plasmodia, Shulman (1966) therefore hypothesised that such species would have evolved to limit the time period in which these relatively low numbers of spores were produced, coincident with periods when many potential naive hosts were present. In the case of some species, this period would be when fish became crowded due to spawning behaviour and was also just prior to when new young potential hosts hatched. Mitchell (1977) recorded high prevalences of *Henneguya* species in farmed channel catfish in the spring and suggested a timing to coincide with the hatching of juvenile fish. Sitja-Bobadilla & Alvarez-Pellitero (1993) showed that *Sphaerospora testicularis* developed as *Dicentrarchus labrax* matured. They considered that drops in fish condition associated with spawning might compromise the immune response and explain high levels of infection.

Shulman (1966) also related infection peaks to fish behaviour, noting that *Myxobolus sandrae* in pike perch produced mature spores in the winter coinciding with fish hibernation, as did *Henneguya creplini*.

The hypotheses developed to explain seasonality must now be qualified by the progress in knowledge of myxosporean life cycles outwith the fish which indicate that many, if not all species have an alternating life cycle, with spores ingested by oligochaete alternate hosts requiring approximately three months to become infective actinosporean stages. Such hypotheses may indeed have masked the influence of the alternate host cycle. Thus it would be expected that spore maturation and release would be timed to allow for a suitable length of time for ingestion and development within the alternate oligochaete host to occur. Both seasonal factors and those associated with the behaviour and biology of the alternate host are

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therefore likely to influence the emergence of the infective actinosporean stage, its rate of development in the oligochaete host and the period for which it is released and thus present in the water supply. Of course, this does not rule out a role for fish behaviour in relation to the epidemiology of infections.

Site of myxosporean development has been shown to influence seasonality. Bond (1938) for example, showed that *Myxosoma (Myxobolus) subtecalis* in the kidney and brain of *Fundulus heteroclitus* showed no seasonal variation, whereas infection on the fins was seasonal. Prevalence of *Myxobolus muelleri* in the muscle and kidney of roach has been shown to remain constant, but to vary seasonally in the gills and subcutaneously (Mitchell, 1977). Such site dependent variation may relate to spore release, with histozoic species infecting the brain or muscle tissue being unable to achieve spore release until the fish dies or is eaten, even though there might be a seasonal pattern in the presence of their infective stages.

Some seasonal variations of infection have been thought to result from myxosporeans having an intrinsic natural timing, unrelated to external factors. In such cases the parasite appears able to show variations in the timing of the progress of infection, and developmental stage present, even when the fish host is held under constant experimental conditions. Such 'endogenous rhythms' have been proposed for PKX (Foott & Hedrick, 1987) and *Sphaerospora renicola* (Odening *et al.*, 1988). Seasonal patterns in all species of fish infected by a particular myxosporean parasite in a given environment are often the same. PKX shows an identical epidemiological picture in rainbow trout, brown trout and salmon for example (Ellis *et al.*, 1985) as does *M. cerebralis* in a range of salmonids. However, Lom (1970; pers.

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comm. to Chubb) noted that spores of *Henneguya psorospermica* matured at different times in pike (autumn) compared to perch (early spring).

A major epidemiological influence appears to be the size and age of fish. Numerous studies have demonstrated marked variations in prevalence and intensity of infection in different age classes of the same fish species in the same environment. Some reports show that previously infected hosts can be reinfected (for example, *M. funduli* in *Fundulus kansae* (Knight, Janovy & Current, 1977)), or that prevalence and intensity are higher in older fish (Cone & Anderson, 1977; Molnar & Kovacs-Gayer, 1985; Morado & Sparks, 1986; Sitja-Bobadilla & Alvarez-Pellitero, 1993). The latter authors proposed that this was due to an increasing probability of fish encountering infective stages over time. Obviously, if fish do not develop acquired resistance to infection by a particular myxosporean as they age, then they are likely to accumulate infection due to increased time exposed to infective waters. A number of myxosporean species, however, appear to infect very young hosts in their first year (Ahmed, 1973; Mitchell, 1977; Egusa & Nakajima, 1981; Molnar, 1982; Kovacs-Gayer & Molnar, 1983; Lom *et al.*, 1985; Molnar & Kovacs-Gayer, 1985; Molnar & Kovacs-Gayer, 1986a; Baska & Molnar, 1988). This seems especially true in species with one year life cycles and in particular, salmonid infecting species such as PKX, *C. shasta* and *M. cerebralis*.

Some authors have indicated a general drop in prevalence with increasing age of fish sampled (Brummer-Korvenkontio *et al.*, 1991; Gonzalez-Lanza & Alvarez-Pellitero, 1984; Molnar, 1979b; Molnar & Kovacs-Gayer, 1986c). Reasons for such decreases have included age related physiological factors, age related changes in fish behaviour making them less likely to encounter infective stages, or a decline due to the death of infected hosts within the

population. However there is considerable evidence that in some species, fish infected by myxosporean parasites in their first year subsequently become refractory to reinfection, and thus immunity increases with time within a year class. Such immunity has been shown for PKX (Ferguson & Ball, 1979; Ferguson, 1981; Foott & Hedrick, 1987; Kent, 1992), *M. cerebralis* (Hoffmann, El-Matbouli & Hoffmann-Fezer, 1992) and *S. renicola* (Odening, Walter & Bockhardt, 1988)

Few influences have been recorded for myxosporean infections in terms of host sex, although *Sphaerospora testicularis*, being specific to the testicular gonadal tissue of *Dicentrachus labrax*, only infects males.

Bucher *et al.*, (1992) showed that the local prevalence of *Zschokella nova* in bullhead *Cottus gobio* rose in a river, associated with high levels of pollutant discharge. Pollution-related eutrophication might lead to a multiplication of potential oligochaete alternate hosts. However, Brummer-Korvenkontio *et al* (1991) found no increase in the prevalence of a number of myxosporeans in a polluted lake in Finland compared to an oligotrophic one, despite the polluted lake harbouring a high tubificid population.

Shulman (1966) described salinity as a "...substantial influence..." on myxosporeans in general. Seasonal effects on wholly marine myxosporeans, though they exist, have been less studied. It would seem that often, the more constant temperatures afforded by the marine environment lead to all year round infections (Suppamattaya, 1991; Sitja-Bobadilla & Alvarez-Pellitero, 1993; Alvarez-Pellitero & Sitja-Bobadilla, 1993). A particularly interesting aspect of the influence of salinity on myxosporeans relates to anadromous members of the salmonidae, which may become infected in their juvenile freshwater stages, as growers in the

sea, or again in freshwater when returning to breed. Several papers indicate that these transitions may have an effect on myxosporean infections. Shulman (1966) noted that spores of *M. cerebralis* survived for one year, but not more, in Pacific salmon species following their migration to the sea. Another parasite, *Henneguya zschokkei*, only infected Pacific salmon in the freshwater environment. Returning adults which harboured infection upon leaving fresh water were no longer infected on return, and remained so unless in fresh water for a long period of time. Ching & Munday (1984b) showed that fish infected with *Ceratomyxa shasta* died earlier and faster in seawater than freshwater controls as a result of impaired osmoregulation leading to death by osmotic shock due to seawater stress. They did not report any changes in the parasite itself following transfer to sea water.

Bailey, Margolis & Workman (1989) found that the prevalence of spores of *Myxidium salvelini* in the kidney tubules of sockeye salmon, *O. nerka*, dropped from approximately 85% to 0% in ten weeks in the sea, although plasmodia without spores remained. Higgins, Margolis & Kent (1993), in an experimental study found a similar drop, but when fish were transferred back to freshwater, spore prevalence rose to approximately 80% due to a resumption of sporogony by pre-spore plasmodial stages. Such arrested development was shown to occur after up to 25 weeks in the sea. Wild fish would be in the marine environment for around 2 years, therefore it was not known whether this phenomenon could occur naturally. The interdigitating adherence of the *Myxidium salvelini* plasmodia to the tubular epithelium of the kidney enabled pre spore stages to remain in the tubular lumen and thus was responsible for their maintenance in the kidney until transition back to freshwater produced suitable physiological conditions for sporogony to recommence.

Kent (1992) noted that PKX persisted upon the transfer of ranched rainbow trout (steelhead) to sea. Although he provided no figures relating to the time the parasite persisted,

he related its presence to osmoregulatory problems, suggesting that in the wild the parasite might be responsible for poor fish survival rates after migration.

The spores and trophozoites of other myxosporeans remain in fish after their transfer to sea and even upon return to freshwater. These include *Myxobolus articus* and *Myxobolus neurobius* (Nagasawa, Urawa & Awakura, 1987; Margolis, 1982- cited by Higgins *et al.*, 1993). However these inhabit locations which do not experience major physiological changes upon the migration of the host from freshwater to the marine environment. This is in marked contrast to the changes experienced by coelozoic species resident in the kidney.

4.1.4. Objectives

The objectives of this part of the study were to investigate the epidemiology of the extrasporogonic and sporogonic phases of the life cycle of *Sphaerospora* infections of farmed Atlantic salmon in Scotland. This was undertaken to determine the time for which each stage was present, when the respective stages first appeared and how the prevalence and intensity of infection varied over this time. This would involve the routine sampling of the prevalence and intensity of infection of *Sphaerospora* at two farms for comparative purposes, and the investigation and sampling of other sites where their management strategies gave further insights into the epidemiology of the parasite.

4.2   MATERIALS AND METHODS

4.2.1 Sampling programme

Two of the Atlantic salmon smolt producing farms described in section 2.2, farm A and farm B, were selected for the epidemiological study which covered the period

from January 1991 through to July 1993. Samples were taken every two to four weeks from the single year class of fish present on each site, from their hatching through to their transfer to sea. Thus two complete year classes, that of 1991/1992 and that of 1992/1993, were studied at both farms. The same tank of fish was followed, when possible, throughout this period at each farm. When fish were transferred into different tanks for grading purposes, the smaller grade were followed due to the higher potential commercial value of larger fish to the farm. Samples consisted of 20-25 fish on each sampling date, but later in the growing season, the increased value of individual fish led to less fish, for example 15-20, being sampled. Fish were randomly netted from the same tank cohort of fish on each occasion and transferred as described in section 2.2.

4.2.2 Examination procedure

Fish from each sample were examined as soon as possible, usually immediately upon return from the site. If necessary, fish were maintained in 40l tanks on charcoal-filtered, aerated mains water at ambient temperature until they could be studied, but this period never exceeded two days.

All fish were subjected to the standard procedure for parasitological examination outlined in chapter 2.

4.2.3 Prevalence and intensity calculation

Prevalence was calculated as the percentage of the total number of fish examined found to be infected with salmon *Sphaerospora* stages. Positive verification of infection was demonstrated by the recognition of one or more individual *Sphaerospora* parasites of any developmental stage in material prepared by any of the standard procedures used.

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Prevalence was determined separately for extrasporogonic and sporogonic stages. In 1991/1992, prevalence of the spore stage was determined in terms of the presence of mature spores only, whereas in 1992/1993 sporogonic stages at any stage of development were used.

Intensity of infection with extrasporogonic stages for individual fish was calculated in a semi-quantitative manner from Giemsa stained impression smears of the entire kidney prepared in a standard manner as in chapter 2. This was found to be representative of the numbers of parasites seen in fresh preparations of the equivalent tissue. Kidneys were scored as follows according to the mean number of extrasporogonic stages per field from 40 random viewing fields at x250 magnification. Category 1+ refers to slides where 40 fields yielded no parasites, but more detailed scanning of the slide showed the parasite to be present.

Intensity index	Extrasporogonic stages
1+	detectable infection
2+	1 or less parasites per field
3+	2 or less parasites per field
4+	3 or less parasites per field
5+	more than 3 parasites per field

In contrast to extrasporogonic stages, Giemsa stained kidney impression smears were found to be unrepresentative, with far fewer parasites being seen in smears

compared to fresh preparations of the same material. This was probably due to the efficiency of the interdigitating relationship between the cytoplasm of the sporogonic pseudoplasmodial cell and the microvilli of the tubular epithelium inner wall (chapter 2), leading to fewer sporogonic stages being released using the impression smear technique. Histological preparations were found to be equally unrepresentative of the levels seen in fresh squash preparations, as many stages appeared to be lost from sections during processing and cutting. In addition, it was clear from fresh observations that the intensity of infection was not simply related to the number of tubules infected, but also to the number of individual pseudoplasmodia and spores in each infected tubule (see chapter 2). Where tubules carried infections, the number of sporogonic stages in them was almost always high. Kidneys were therefore scored in terms of the proportion of total tubules containing sporogonic stages on a scale of 1 to 10 (10 being all tubules infected). This figure was then multiplied by a subjective intensity factor of between 1 and 5, which reflected the number of sporogonic stages in infected tubules. The resulting figure, ranging from 1 to 50 was scaled down by a factor of ten then rounded off to the nearest whole number to give an intensity index of 1+ to 5+ comparable with that for extrasporogonic stages.

4.2.4 Experiment to determine date of first infection and pre-patent period

This experiment was designed to establish when fish first became infected with salmon *Sphaerospora* at farm A, and to compare this date with that at which the earliest extrasporogonic stages of infection became detectable in routine samples.

Sampling for this experiment began in late April 1992, and was carried out coincidentally with the routine epidemiological sampling programme. In addition to the

routine sample of 20-25 fish, a further group of ten fish were obtained. On each date, each group of ten fish was retained separately on charcoal filtered aerated mains water at ambient temperature in 40l tanks in the laboratory and fed *ad libitum* with a commercial pelleted diet. When extrasporogonic stages were first detected in normal epidemiological samples, retained fish from all previous sampling occasions were examined to determine whether they were infected.

4.2.5   Experiment to determine the time for which spores were retained

This experiment was designed to determine how long mature sphaerospores remained in infected fish. Sampling at farm C established that fish had been infected by extrasporogonic stages of the salmon *Sphaerospora* in their first summer, and had contained mature sphaerospores by October 1991 at a prevalence of 100%. Thirty of these fish were sampled in May 1992 prior to their transfer to sea as S_2 smolts, and retained on charcoal filtered mains water at ambient temperature in 40l tanks at the Institute of Aquaculture and fed *ad libitum*. Two fish were sampled from this population each month and examined for the presence of extrasporogonic and sporogonic stages of salmon *Sphaerospora*.

4.2.6   The effect on sporogonic stages of salmon *Sphaerospora* of the transfer of fish to sea water

The final freshwater sample of fish from farm A in November 1991 were found to be infected with sporogonic stages of salmon *Sphaerospora* at a prevalence of 90%. Following their tranfer to marine rearing facilities in the same month, as $S_{1/2}$ smolts, ten fish from this cohort were sampled at four, eight and sixteen weeks and examined

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according to standard procedures to determine the effects of sea water on the morphology, prevalence, and intensity of spores and sporogonic stages in the kidney tubules.

4.2.7 Study of *Sphaerospora* infections of Atlantic salmon in their second year

Three groups of fish were utilised to ascertain the nature of the effect of infective stages of salmon *Sphaerospora* on second year fish of differing previous infection history. This would establish whether previously exposed fish could be reinfected.

Group 1, a reinfection group, were the 1991/1992 cohort of potential S_2 Atlantic salmon, age 1+, from farm C; these fish had been found to be infected with *Sphaerospora* at a 100% prevalence and high intensity during their first summer on site (August, 1991). Two samples were taken from this group during the summer of 1992, their second summer on site (mid-July, 15 fish; mid August, 12 fish). These fish were subjected to standard examination for extrasporogonic and sporogonic stages in order to determine whether a second exposure to the infective agent of salmon *Sphaerospora* led to the establishment of infections.

Group 2, a control group, consisted of 0+ Atlantic salmon parr of the 1992/1993 cohort at the farm C, in their first year on site. These were chosen to establish that salmon *Sphaerospora* infective stages were present on the farm, and caused infections during the summer of 1992. Ten of these fish were sampled on the same two dates in July and August 1992 as the fish from group 1.

A third group of fish, group 3, were chosen to ensure that any difference seen in the epidemiology of infection between groups 1 and 2 was not simply due to an age

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effect, such as increased immunocompetance in 1+ compared to 0+ fish. This cohort, group 3, were potential S_2 Atlantic salmon smolts, age 1+, which had been transferred to farm D in January 1993 from a site in the South-West of Scotland with no history of *Sphaerospora* infection. Six fish were available for study in the July of 1993; their second summer, but their first at a site infective for salmon *Sphaerospora*. These fish were examined according to standard procedure to determine whether fish not previously exposed to salmon *Sphaerospora* could become infected in their second year.

4.2.8   Hatchery temperature data

Hatchery temperature data for the two farms under study was collected from hatchery records over the period of the study. Mean weekly water temperatures were calculated and plotted to enable a comparison of the epidemiology of infection with salmon *Sphaerospora* to be made.

4.3   RESULTS

4.3.1   Parasite distribution

A comprehensive sampling programme was not undertaken to determine the entire distribution of the salmon *Sphaerospora* under study. However, a map illustrating sites routinely sampled, or visited occasionally and found to be positive indicates that across Scotland the parasite may be a common feature of the parasitofauna of farmed Atlantic salmon, being found in a range of discrete river systems (fig 4.1).

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4.3.2 Prevalence and intensity

The variations in percent prevalence of infection of both extrasporogonic and sporogonic stages of *Sphaerospora* over time, for single year classes of salmon at farms A and B, are plotted in figs. 4.2 and 4.3. On all occasions, infections were first detected from Giemsa-stained kidney impression smears, rather than by observations of fresh squashes, stained blood smears, concentration techniques or histology. Data for mean intensity of infection at each sampling date, and range of intensities within the population at each sampling date is shown in figs. 4.4 and 4.5. In general, mean intensity levels appeared to follow the patterns shown by prevalence, however there are marked differences, especially in range, between the two farms studied.

Figs. 4.6 and 4.7 provide the hatchery temperature records for the two farms over the period of the epidemiological study.

Prevalence and intensity of extrasporogonic stages from farms A & B

At both farms, extrasporogonic stages were prevalent for a limited, restricted period of time in each cohort.

Although fish at both sites were transferred onto river water in February/March each year, they did not appear to become infected for three to four months. Extrasporogonic stages were first detected in routine samples in late June to mid July each year at both farms. Actual sample dates for first detected infections are given in table 4.1. Conceivably, infections could have been detectable the day after the previous sample was taken and so this date is also provided. The latter dates indicate that the earliest stages of detectable infection at both farms could have been present from mid June to early July.

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Fig.4.1 Sample sites positive for salmon *Sphaerospora*
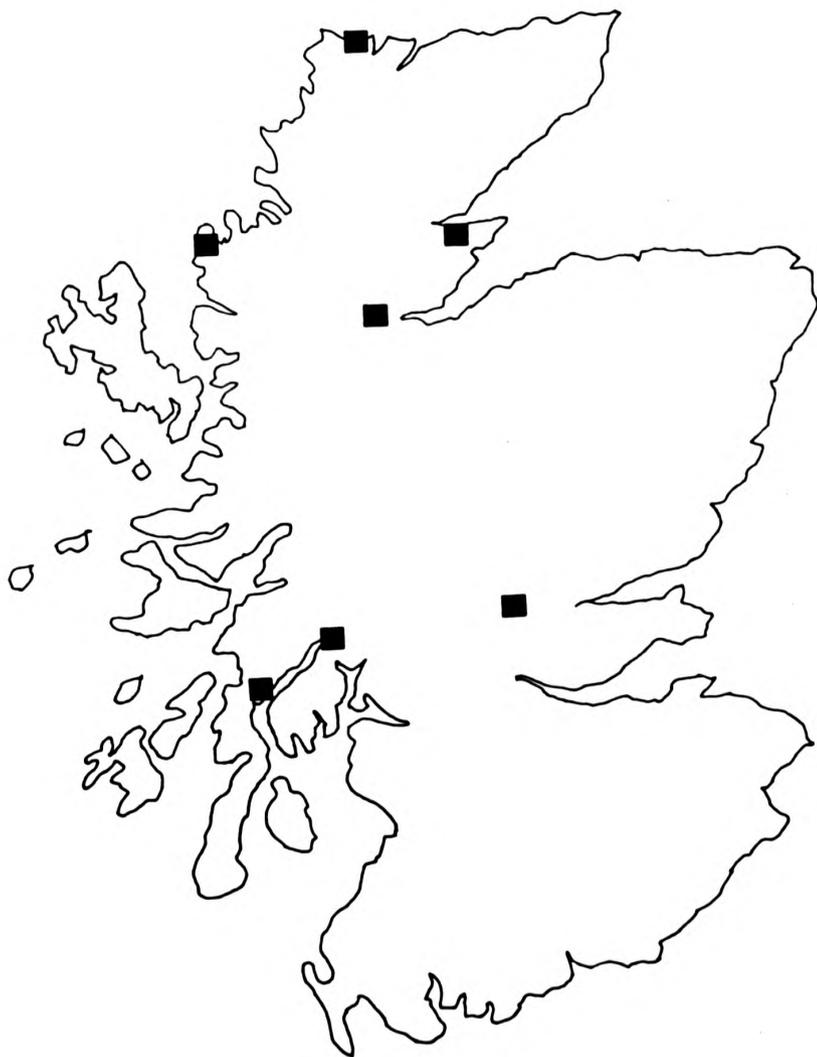
fig.4.2 Prevalence of salmon *Sphaerospora* at farm A, 1991-1993.

Extrasporogonic stages □
Sporogonic stages ■

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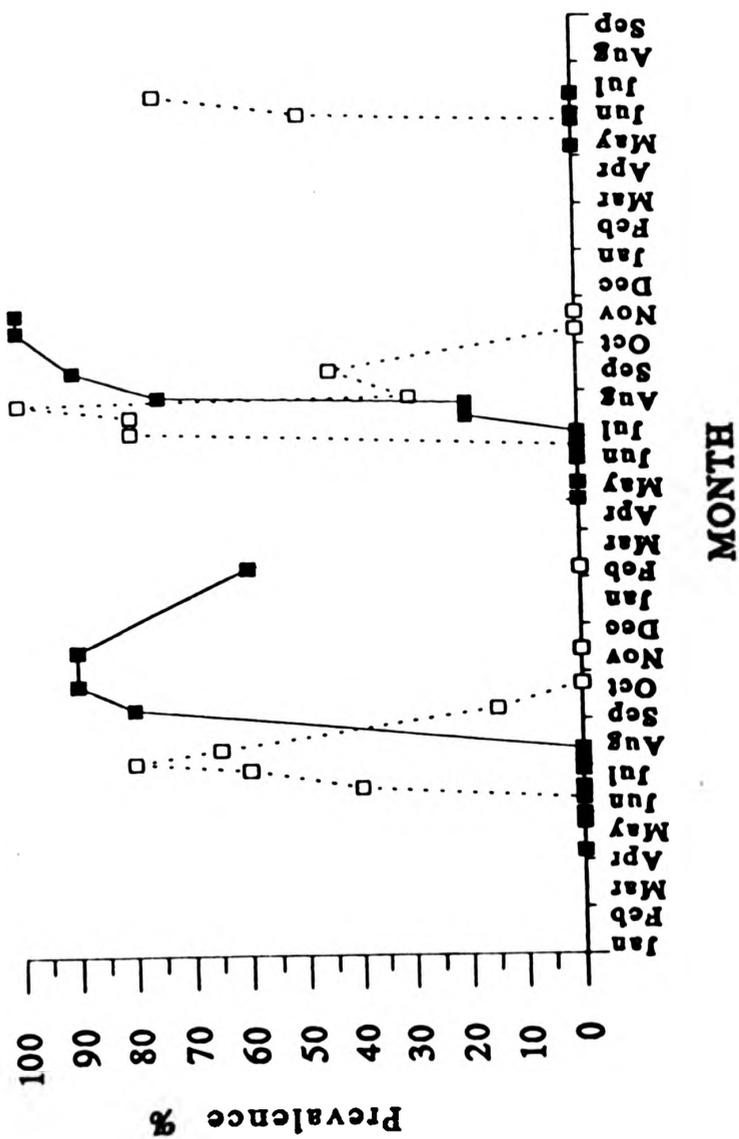
fig.4.3 Prevalence of salmon *Sphaerospora* at farm B, 1991-1993

Extrasprogonic stages ☐
Sporogonic stages ■
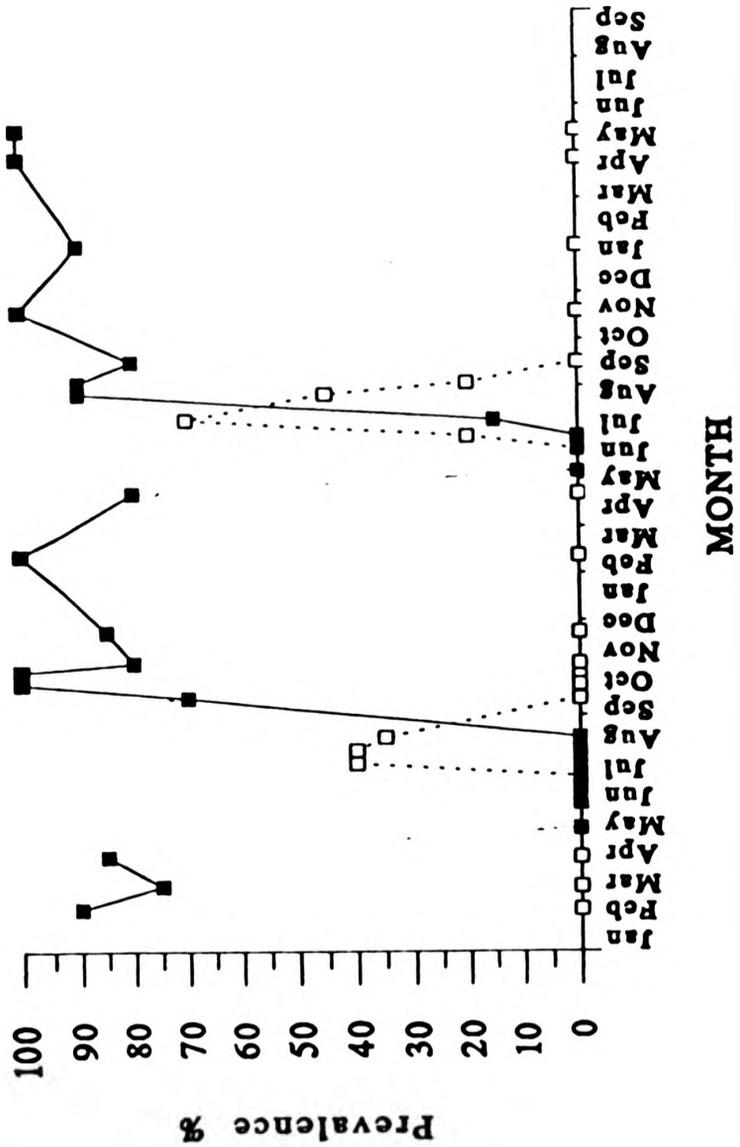
fig.4.4 Mean intensity and intensity range for salmon *Sphaerospora* at farm A, 1991-1993

Extrasporogonic stages ▫
Sporogonic stages ▪
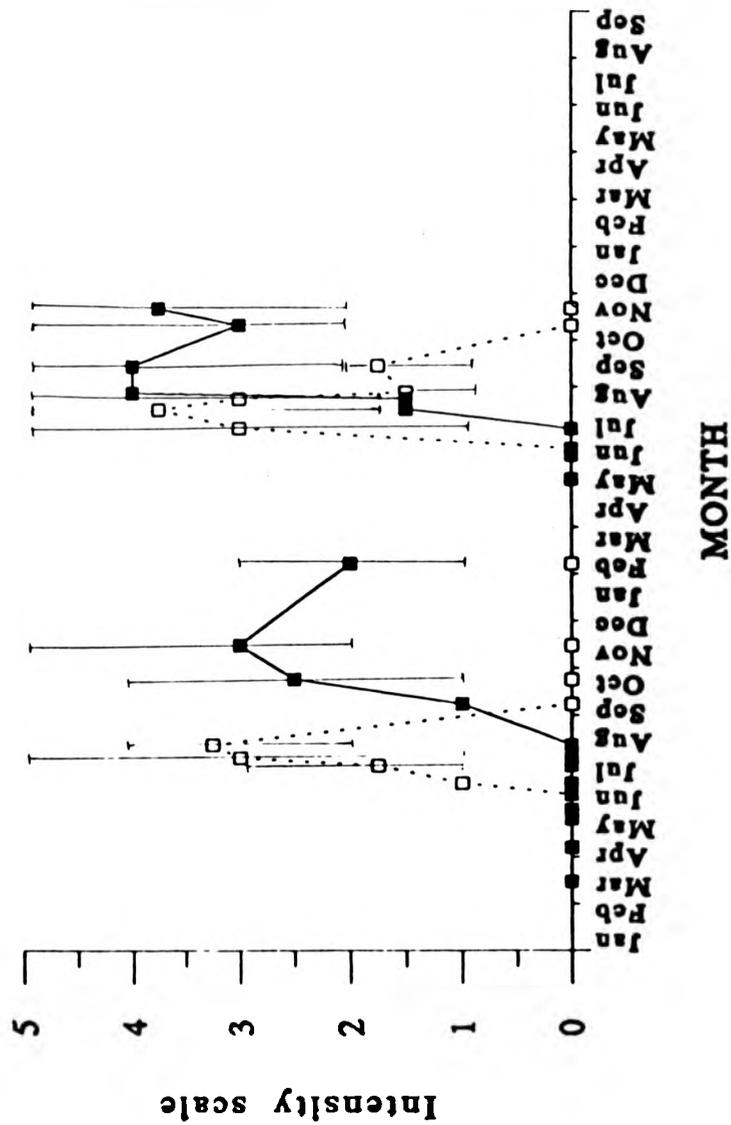
fig.4.5 Mean intensity and intensity range for salmon *Sphaerospora* at farm B, 1991-1993

Extrasporogonic stages ☐
Sporogonic stages ■
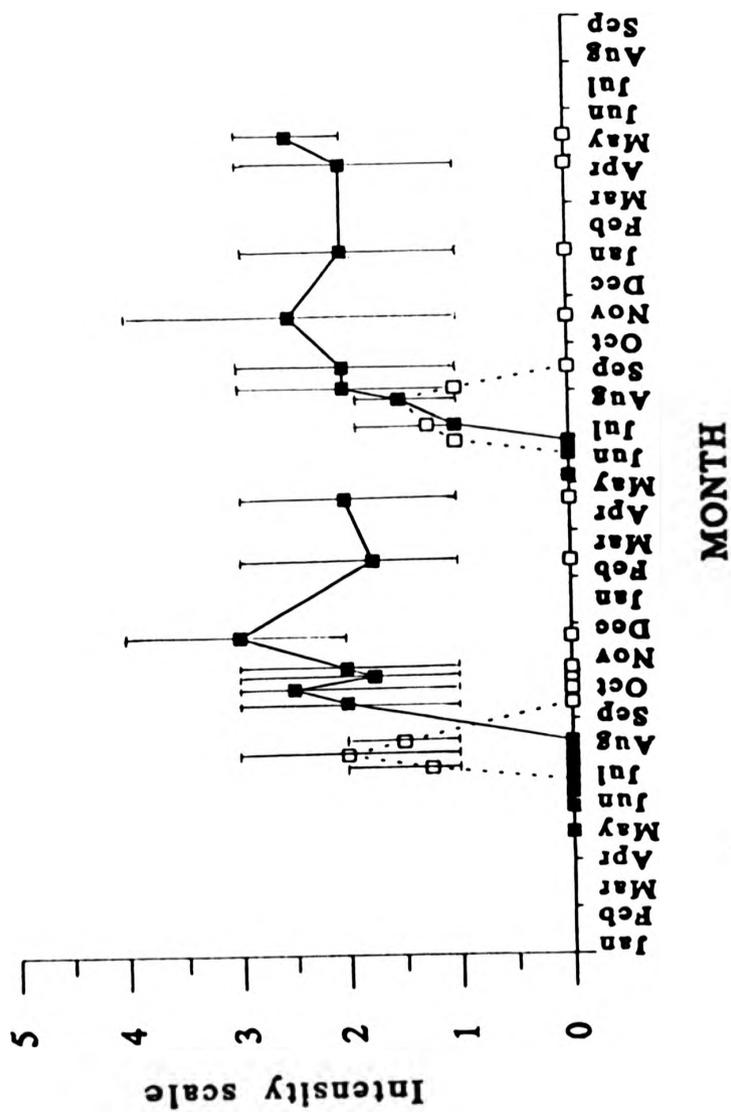
fig.4.6 Mean hatchery temperature at farm A, 1991-1993

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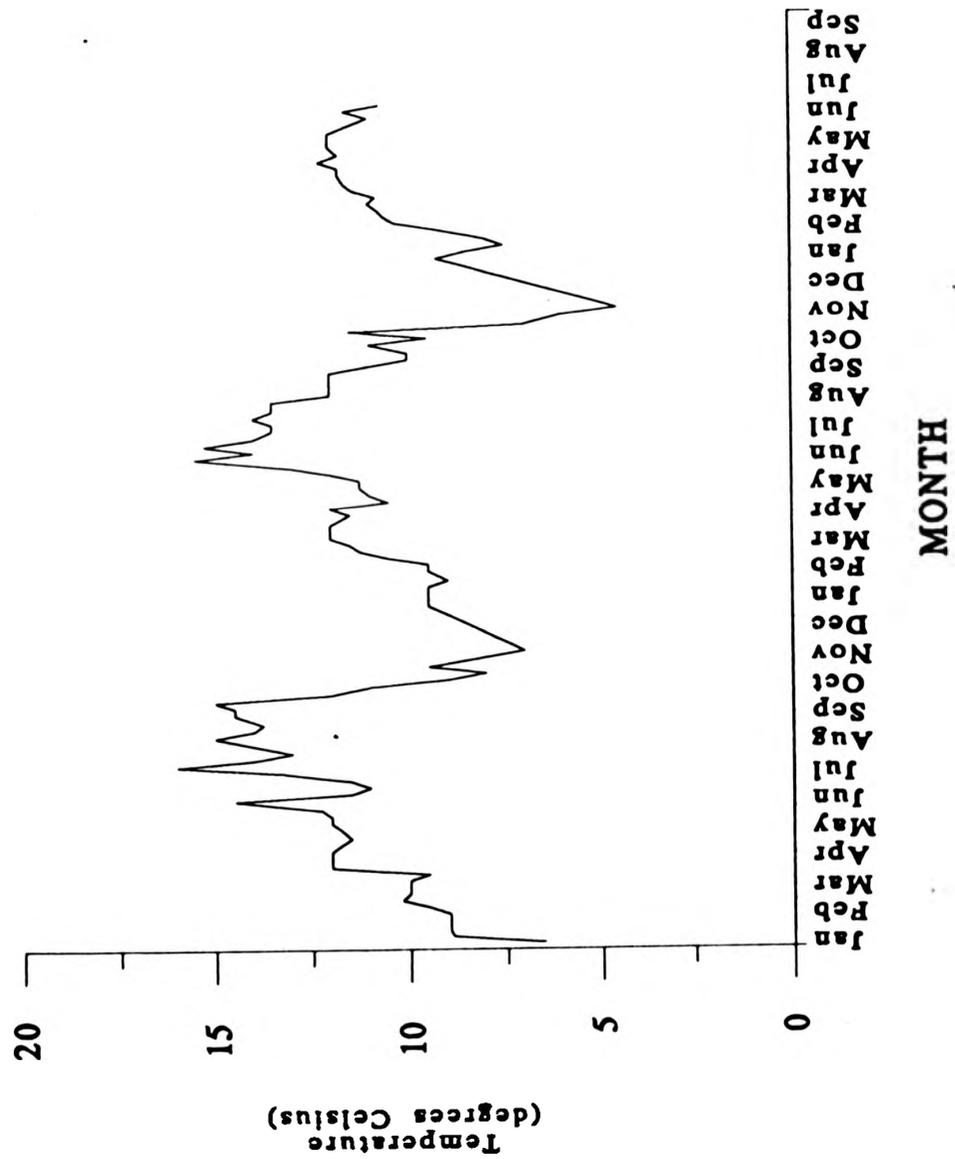
fig.4.7 Mean hatchery temperature at farm B, 1991-1993

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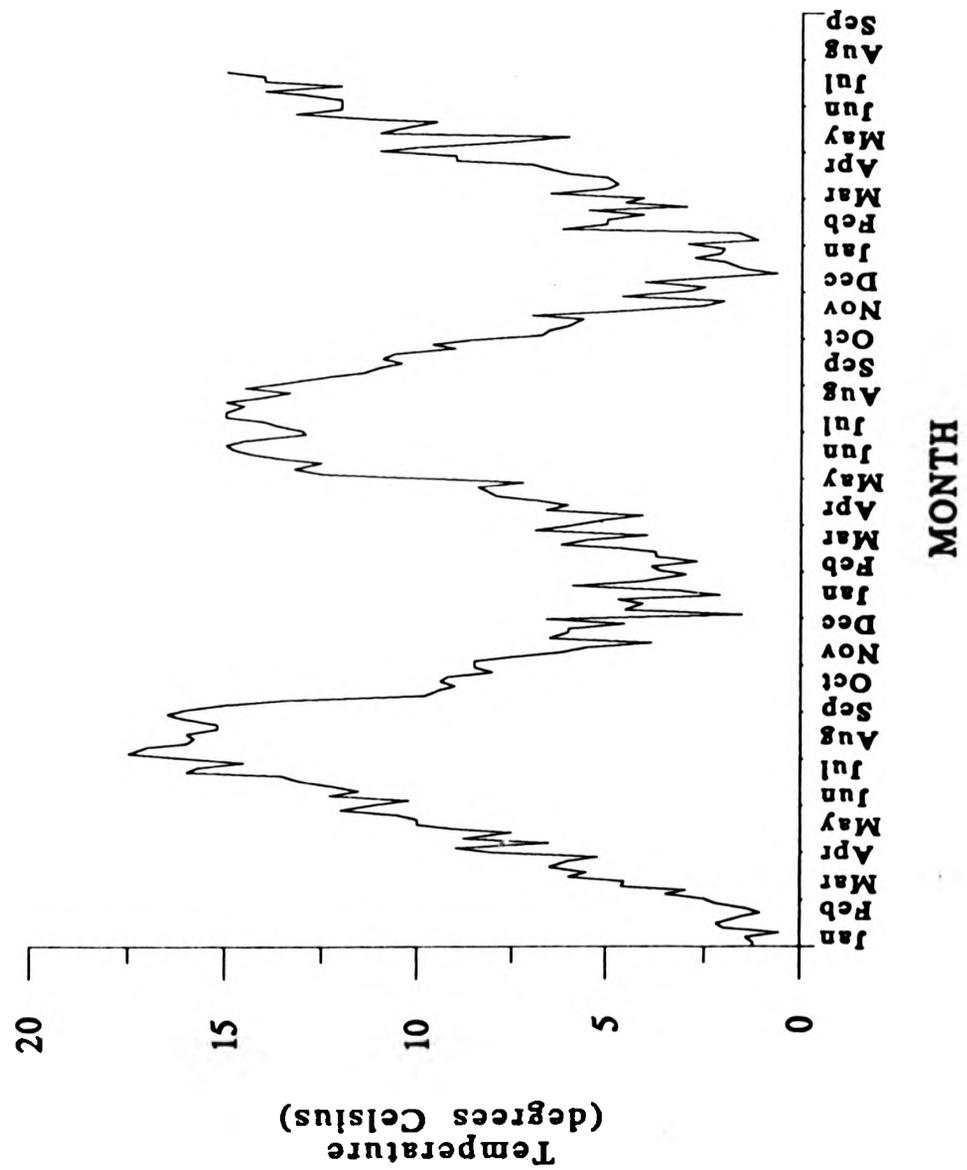
The first routine sample to be found to be infected from the 92/93 cohort at farm A was that obtained on 09.07.92. However, when all retained samples of fish from experiment 4.2.4, designed to determine the date of first infection and the pre-patent period, were examined on 10.07.92, the sample of 24.06.93 was found to be infected at a prevalence rate of 80% (8/10 retained fish). Stained slides from the routine, non retained sample from 24.06.92 were subjected to re-examination, but still found to be negative for infection. The retained sample prior to 24.06.93, that of 10.06.93, was not infected at later examination (10.07.92).

Thus it seems that the infection must have been present for some period of time in the salmon prior to the point at which it was detected in routine samples. In the case of the fish at farm A in 1992, this appeared to be between 15 and 28 days. If such a figure is applicable to other years and sites, the experiment appears to indicate that the earliest possible time at which fish have first become infected each year is up to a month before the earliest date at which extrasporogonic stages can be detected. This is around mid-May at the very earliest.

Prevalence of extrasporogonic stages invariably rose very rapidly, peaking each year in July and August. At farm A, peak prevalence reached 80% and 100% in late July 1991 and early August 1992 respectively. At farm B, prevalences were lower, especially in 1991, where prevalence peaked at only 40% in late July. In 1992 peak prevalence was 65% in mid July.

Extrasporogonic stages were last detected in mid September at farm A in both 91/92 and 92/93, but at farm B last detection was in mid to late August. Actual dates of samples are given in table 4.2, along with the date of the first negative sample. Extrasporogonic stages were thus detectable in routine samples for around 8 to 12 weeks

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at farm A in the 91/92 year class and for the same period at both farms in the 92/93 year class, but only 3/4 weeks in the 91/92 year class at farm B. If we include the pre-patent period in which extrasporogonic stages could have been present, the range increases to up to 20 weeks (table 4.3).

Peak mean intensities of extrasporogonic stages of infection and the greatest range of intensity in single samples at both farms and in both years occurred in July and August, reflecting the peak prevalence within the population. In early infections, when the prevalence of extrasporogonic stages in the cohort was low, the mean intensity was also low, as was the range of intensity levels in the sample. Late in the period over which extrasporogonic stages were present, when prevalence was again low, so was mean intensity and its range.

Mean intensity at farm A peaked at around 3+ or 4+ in both cohorts studied, whereas at farm B, this figure was between 1+ and 2+ each year. The differences between the two farm sites were even more notable in terms of intensity range within each sample; at farm A, some fish carried the highest intensity level (5+) in both years, whereas at farm B the most heavily infected fish attained an intensity of just 2+ or 3+. At both farms, even when the prevalence of infection in the population was as high as 100%, and mean intensity was high, some fish still harboured very low level infections (1+) on almost all sampling dates.

Prevalence and intensity of sporogonic stages from farms A & B

In the 1991/1992 year classes at both farms, sporogonic stages were only recorded in terms of the presence of mature spores, whereas in samples from the

1992/1993 cohort of fish, presporogonic pseudoplasmodia as well as mature spores were included. Exact sample data is given in table 4.4.

Sporogonic stages rapidly rose in prevalence from first detection, to reach a peak prevalence of around 100% in September in each of the two year classes at both farms. This was similar to the earlier rapid rise for extrasporogonic stages. Prevalence of sporogonic stages in routine samples then remained at or around this level until the fish were transferred to sea, in October/November as $S_{1/2}$ smolts at farm A, or April as S_1 smolts at farm B. The drop in prevalence at farm A in February 1992, followed fish transfer to sea in November 1991.

Mean intensity values for sporogonic stages at both farms reached maximum levels in September or October each year, after which there appeared little or no further change in intensity until fish were transferred to sea cages. However, subjectively, the proportion of mature to immature spores in infected tubules increased over this period. At farm A mean intensity levels and their range at a given sampling point were invariably higher than those at farm B. No further samples could be obtained from October/November onwards each year at farm A, as at this point all fish were transferred to sea as $S_{1/2}$'s. (However it seems reasonable from the data obtained from farm B to assume that sporogonic stages at farm A would remain at a similar mean intensity if fish were held on freshwater for longer). Again, as with extrasporogonic stages it was clear that even when prevalence and mean intensity were high within the cohort, some individuals carried relatively low level infections of 1+ or 2+.

table 4.1   First detection of extrasporogonic stages at farms A & B

FARM	COHORT	DETECTED	PREVIOUS SAMPLE
A	91/92	27.06.91	15.06.91
A	92/93	09.07.92	17.06.92
B	91/92	18.07.91	04.07.91
B	92/93	30.06.92	16.06.92

table 4.2   Last detection of extrasporogonic stages at farms A & B

FARM	COHORT	LAST SAMPLE	FIRST NEGATIVE
A	91/92	19.09.91	15.10.91
A	92/93	13.09.92	27.09.92
B	91/92	15.08.91	25.09.91
B	92/93	26.08.92	20.09.92

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table 4.3   Period for which extrasporogonic stages were present in Atlantic salmon
cohorts at farms A & B

FARM	COHORT	WEEKS PRESENT	POSSIBLE WEEKS PRESENT
A	91/92	11-12	up to 18
A	92/93	9-10	up to 15
B	91/92	3-4	up to 12
B	92/93	8-9	up to 15

table 4.4   First appearance of sporogonic stages (1992/1993), and mature spores (1991/1992; 1992/1993) in Atlantic salmon at farms A & B

FARM	COHORT	SPOROGONIC STAGES	DAYS POST INFECTION	MATURE SPORES	DAYS POST INFECTION
A	91/92	-	-	19.09.91	84
A	92/93	09.08.92	31	26.08.92	48
B	91/92	-	-	25.09.91	69
B	92/93	17.07.92	17	16.08.92	47

The experiment to determine the time for which spores were retained studied the 'potential S_2' fish from the 1991/1992 hatch at farm C. These fish, held on infection free waters at the Institute from May 1992 onwards, were found to retain spores up until May 1993, when they were last examined. The prevalence within these fish remained at 100% throughout this time. Intensity of infection appeared, subjectively, to be constant throughout.

The sample taken four weeks after their transfer to sea water in November 1991, found that the fish studied to determine the effects of host transfer to sea on salmon *Sphaerospora* at farm A retained sporogonic stages at a prevalence of 100% (10/10 fish) and no apparent changes were seen in spore and pre-spore stage structure. However, after two months at sea, in early February 1992, the prevalence had declined to 60% (6/10 fish), and the mean intensity of infection and its range had also dropped markedly from 3+(2+-5+) to 2+(1+-3+). Fresh smear and histological observations indicated that only mature spores remained in infected tubules, differentiating pseudoplasmodia being absent. These spores appeared particularly 'compact' and were no longer retained within pseudoplasmodia attached to the inner wall of the tubule, but rather were free in the tubular lumen (fig.4.8). In April 1992, after four months in sea water, sporogonic stages and mature spores were absent (0/7 fish).

The study of *Sphaerospora* infections of salmon in their second year found that the fish of group 1, the potential S_2 salmon of the 1991/1992 cohort at farm C, negative for infection by extrasporogonic stages during both the July, (0/15 fish) and August, (0/12 fish) of 1992. These fish had been found to be 100% infected by extrasporogonic stages in the summer of 1991. Group 2, the previously unexposed, 0+ 1992/1993

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fig 4 8 Effects of sea water on spores and sporogonic stages Early sporogonic stages are no longer present. Maturing and mature spores appear to no longer be surrounded by pseudoplasmodial cells. They therefore have no attachment to the lining of the tubular epithelium of the kidney, but rather are free in the lumen. (H&E, bar=10μm)
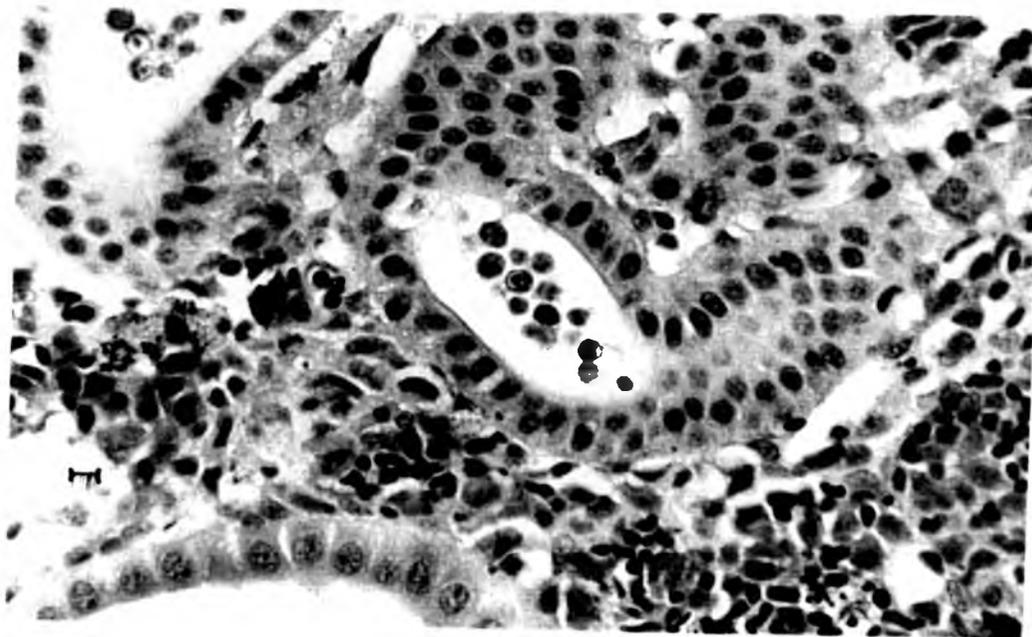
cohort of salmon in their first year at farm C on the same water supply, were infected at 100% prevalence by extrasporogonic stages on both sampling dates. The fish from group 1 contained large numbers of mature spores on both dates. However, these were considered to be a result of their infection as 0+ fish in the summer of 1991.

Group 3, the potential S_2 fish at farm D, became infected with extrasporogonic stages in the summer of 1993 with a prevalence level of 83% (5 of 6 fish). One fish was infected at very high intensity (5+), the other four at a mean of 2.25 (range 1-4).

4.4   DISCUSSION

The epidemiology of the salmon *Sphaerospora* at the two farms studied was very similar each year and was reminiscent of that shown by a number of other salmonid infecting myxosporeans such as *Myxobolus cerebralis* and *Ceratomyxa shasta*. However its closest similarity is with PKX (Ferguson & Ball, 1979; Clifton-Hadley *et al.*, 1984; Hedrick *et al.*, 1985; Clifton-Hadley *et al.*, 1986). The present study indicates that the salmon *Sphaerospora* has an annual life cycle which is timed to ensure the infection of naive 0+ hosts in early summer. The first signs of infection, extrasporogonic stages, became detectable in the fish cohort at similar times in both years, usually between the end of June and mid-July. This timing appeared to be very consistent not only from year to year but also between farms. The prevalence of extrasporogonic stages rapidly rose in all cohorts indicating that all fish within the population became infected at a similar time. This initial synchronous wave of infection of 0+ fish has similarly been shown in *C. shasta* (Ching & Munday, 1984a), *M. cerebralis* (Shulman, 1966) and PKX (Foott & Hedrick, 1987), and suggests that a synchronous release of, presumably actinosporean, infective stages takes place in the wild source of infection.

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4.4.1   Prevalence and intensity data

Although farms A and B differed in the mean intensity level at which they became infected, at both the salmon *Sphaerospora* reached a prevalence of 100% each year. However there were differences between the peak prevalence figures for the two stages of the life cycle, extrasporogonic and sporogonic, even within the same fish cohort. In only one instance, (1992/1993, farm A), did the extrasporogonic stage reach a prevalence of 100%, whilst at farm B, especially in the 91/92 cohort extrasporogonic stage prevalence was much lower. This could, in part be due to the difficulties of detecting very low level infections; at low levels of mean infection intensity, several slides of a single kidney may have to be scanned to detect the presence of extrasporogonic stages and the earliest stages of infection are difficult to detect due to their small size and low number of secondary cells. The intensity of infection at farm A was usually higher than that at farm B, making infections easier to detect at this farm. At both farms, early extrasporogonic stage infections are characterised by very low prevalence and intensities of parasitism both in mean and range. Hedrick *et al*. (1990) found an initial peak of 40% prevalence for extrasporogonic stages of *Sphaerospora ictaluri* in the vascular system of a sample from a population of channel catfish, but ten days later found 80% prevalence of sporogonic stages in the kidney tubules of the same cohort of fish. The 40% discrepancy in the two samples probably serves to illustrate that extrasporogonic stages are more difficult to detect, especially in low-level infections, than are spores. Foott & Hedrick (1987) found extrasporogonic stages to be at a higher prevalence in a population of fish than subsequent sporogonic stages. This was in a study of PKX infections and can perhaps be explained by the fact that few parasites in PKX infections reach the tubule lumen and go on to produce mature spores.

Several other workers have found the prevalence of sporogonic stages of *Sphaerospora* spp to be higher than that of precursor extrasporogonic stages (table 4.5). However, an age factor may be involved in these findings, since they are based on prevalence of infection in hosts of different age groups. Lom *et al.* (1985b), commented that extrasporogonic stages were much more commonly found in the youngest, smallest fish within a population of a given species. Baska & Molnar, (1988) also noted that such stages were more easily found in fingerlings. However spores are often found in a wider range of age groups (Baska, pers. comm.). The reports of Lom *et al.* (1985b), Baska & Molnar (1988), and of Kepr & Trsova (1989) were based on samples of wild and feral fish. Evidently, in such populations, irrespective of age, spores of *Sphaerospora* spp are scarce (Lom *et al.*, 1985b). It would follow therefore that extrasporogonic stages are likely to be equally, if not more rare since their presence may be restricted in time, and also to only the youngest fish. Baska & Molnar (1988) emphasised how low intensity levels within a host individual could be, providing an example in which extrasporogonic stages were detected in only one of ten blood smears taken from a single fish.

Mature sphaerospores may be present in the kidneys of fish that have been isolated from any possible source of infection for a long period of time (present study; Fischer-Scherl, pers. comm., 1992). Fish may therefore become infected by *Sphaerospora* spp at a very young age but retain mature spores resulting from that initial infection for a number of years. If fish are refractory to reinfection, as seems to be the case in a number of myxosporean species, such as the salmon *Sphaerospora*, *M. cerebralis* and PKX, new infections with extrasporogonic stages will not occur, and so in older fish extrasporogonic stages are unlikely to be found even though sporogonic

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table 4.5   Comparison of the prevalence of extrasporogonic and sporogonic stages in single host populations

AUTHORS	HOST SPECIES	PREVALENCE %	
		Extrasporogonic stages	Sporogonic stages
Lom *et al.* (1985)	Tench	5	21
Lom *et al.*, (1985)	Gudgeon	12	29
Lom *et al.*, (1991)	Stickleback	25	83

stages and spores may be present. This may suggest that immunity is developed to extrasporogonic stages rather than sporogonic ones.

Because of the very narrow window for which extrasporogonic stages are present in the fish cohort, and its rapid rise in prevalence, it is perhaps possible that the peak prevalence point may have occurred between sampling dates.

In most *Sphaerospora* spp in which extrasporogonic stages have been demonstrated, such as *S. ictaluri* (Hedrick *et al.*, 1990), *Sphaerospora* sp in native chub (Hedrick *et al.*, 1988), PKX infections (Foott & Hedrick, 1987) and in the case of the K-stages of *S.renicola* (Sedlaczek *et al.*, 1990), extrasporogonic stages always precede the appearance of sporogonic stages, are only present for a short period of time and do not persist following sporogony. This pattern is also shown by the salmon *Sphaerospora*. The C-stages of *S. renicola* appear to continue to cycle in the blood for long periods after sporogony has been completed, and may show further peaks of prevalence at a later date (Grupcheva *et al*, 1985; Molnar, 1988b). This would appear to be a result of the diphasic development of this parasite.

Mature spores are much more easily and accurately detected by scanning fresh smears of kidney tissues from the posterior tubular portion of the kidney, even at low intensities, and 100% prevalence levels were recorded in many samples from the two farms. Prevalence and intensity remained at very similar levels at both farms from late September when mature spores were first found, until the fish were put to sea. The apparently later appearance of sporogonic stages in both farms in 91/92 was due to the fact that in this year only the presence of mature spores rather than both sporogonic

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stages and spores was recorded. The rapid rise in prevalence of sporogonic stages within the fish cohorts might indicate that extrasporogonic stages have only a fixed number of proliferative cycles before they become sporogonic, or that a stimulus of some kind operates to cause extrasporogonic stages to become sporogonic synchronously. From the hatchery temperature data (figs. 4.6 & 4.7), this stimulus could perhaps be the drop in temperature below 12°C at this time. Alternatively, since there is a synchronous wave of initial infection with extrasporogonic stages, these might develop right through to sporogony without stopping, their synchronous development not being mediated by outside factors.

Lom & Dykova (1992d) summarised the options available for mature myxosporean spores to reach the external environment from the host. In some histozoic species mature spores may be shed directly into the water by the rupture of the trophozoite wall if near the body surface. Histozoic species occupying deeper tissue layers and organs may become encapsulated and destroyed by host responses either within the trophozoite or upon release. Alternatively they may remain trapped inside the host until its death and subsequent decay, or its ingestion by a predator, leads to spore release.

Coelozic species, especially those which infect the kidney such as many *Sphaerospora* spp have a simple means of release via the tubule lumen to the ureters and urinary bladder and then to the environment with the fishes urine. Lom & Dykova (1992) noted that myxosporeans of this type showed two kinds of release. Some show a limited window in time during which all spores are lost (e.g. *S. renicola*), whilst others have an indefinite release period (e.g. *Myxidium lieberkuehni*). In the present study, mature spores were, very rarely, found in fresh squashes of the urinary bladder. A true

picture of spore release as it occurs in the wild source of infection may not be given in a farm-based study. A very gradual release of mature spores over an extended time period, or a synchronous future large scale release is therefore not out of the question, and may be related to alternate host or other environmental factors.

The consistent prevalence and intensity of sporogonic infection within cohorts up to their transfer to sea could indicate that new spores are continually being produced to replace ones shed. This seems unlikely as there are no extrasporogonic stages in the fish from September onwards, and so no triple forms are being produced to establish in the kidney tubules to produce spores. Another possibility is that a further proliferative process involving existing sporogonic stages in the kidney tubules takes place to maintain the level of spores as they are released. Certainly there are, even in March and April, at least six months after the last appearance of their extrasporogonic precursor stages, numbers of very early sporogonic stages in the tubules which may represent such newly produced forms. However these could also represent stages derived directly from extrasporogonic stages whose development is somehow 'delayed', perhaps to ensure production of spores over a long time period. Such a mechanism could potentially aid the dissemination of the spores into the environment in both time and, due to fish movement, space. Alternatively it could be that spores are simply not released within the period of time that $S_{1/2}$ or S_1 cultured fish spend in fresh water and thus spores produced from early September onwards remain in the kidney tubules until transfer. In wild fish, release might take place gradually or even synchronously at some subsequent point. The retained S_2 fish from farm C, held in non infective waters at the Institute from May 1992 still contained mature spores into the summer of 1993. Spores must

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have been initially formed in these fish in the autumn of 1991 and thus were still to be found 20 months later. Fischer-Scherl (pers. comm., 1992) found that brown trout isolated from any possible source of reinfection maintained spores of *Sphaerospora truttae* for at least three years, whilst Grupcheva *et al.* (1985) found that spore stages remained in carp infected with *Sphaerospora renicola* for three years. There was, albeit subjectively, in retained fish from farm C, a drop in mean intensity after the second winter, but prevalence within the population remained at 100% throughout. Foott & Hedrick (1987) found a major drop in the prevalence of intraluminal sporogonic stages of PKX 10/11 months after their first presence, but this apparent drop might be influenced by the fact that true, fully mature, completely valved spores are rarely encountered in PKX infections (Kent & Hedrick, 1986).

Kennedy (1976) noted that the release of parasite stages was timed to occur when the probability of contact with a suitable next host was maximal, and that such a period was often restricted. These restrictions could be related to such factors as climactic conditions, next host availability or present host behaviour. Factors such as the duration and timing of reproduction and the release of infective stages, are related to the opportunities for the successful infection of the next host. When hosts are available all year round, infective stages are often released over a long period of time, whilst if they are available for one time of the year or only for a short period, then infective stages are often short lived and released for a restricted period of time. This kind of pattern presents an attractive hypothesis to explain the type of developmental cycle shown by many myxosporeans. Actinosporean stages are short lived and often released for a short period of time, particulary around the time when new, naive, susceptible fish hosts are

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available, whilst myxosporean spores, being long lived, can be released over a prolonged period and await ingestion by a suitable oligochaete host for a long length of time.

4.4.2 Source of infection

Infections of salmon *Sphaerospora* did not take place until May at the very earliest as indicated by the study of pre-patent period and initial infection. It is clear that the infection of a year class of fish on site was always synchronous.

This pattern of infection must reflect a synchronous release of mature actinosporean spore infective stages from oligochaete alternate hosts. From the hatchery temperature data at the farms concerned one possible stimulus for synchronous release could be a rise in temperature. The first signs of infection each year appear closely related to the highest hatchery temperatures of the year at both farms, particularly farm B. Extrasporogonic stages seem therefore to be present over the period temperatures exceed 12°C (fig.4.9). Three possibilities are apparent; that a temperature cue early in the year initiates actinosporean development; that one later in the year stimulates actinosporean release (fig.4.9); or perhaps even that a mass mortality of infected worms causes a sudden release over a short period of time. It is possible in the light of Kennedy's hypothesis (Kennedy, 1976) that once ingested by the oligochaete host the mature myxosporean stage spore remains dormant. Thus the worm population might accumulate myxospores over a time period before some kind of trigger, perhaps temperature, initiates the synchronous development of dormant parasites in all host worms in the water body; thus a subsequent synchronous release of actinosporeans over a restricted period of time coincident with the appearance of new naive host fry could result. No reports of such accumulations have yet been described, but the dynamics of

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fig.4.9   Hatchery temperatures, farms A and B compared Farm A, dotted line; Farm B, solid line. Although highest temperatures are similar at both farms, at farm A, the temperature exceeds 10°C earlier and for much longer than at farm B, indicating a higher average temperature at farm A. Temperatures rise abruptly at both farms in early Spring, 1, and early Summer, 2. These could possibly correspond to potential stimuli for the development and/or the release of actinosporean stages in alternate hosts.
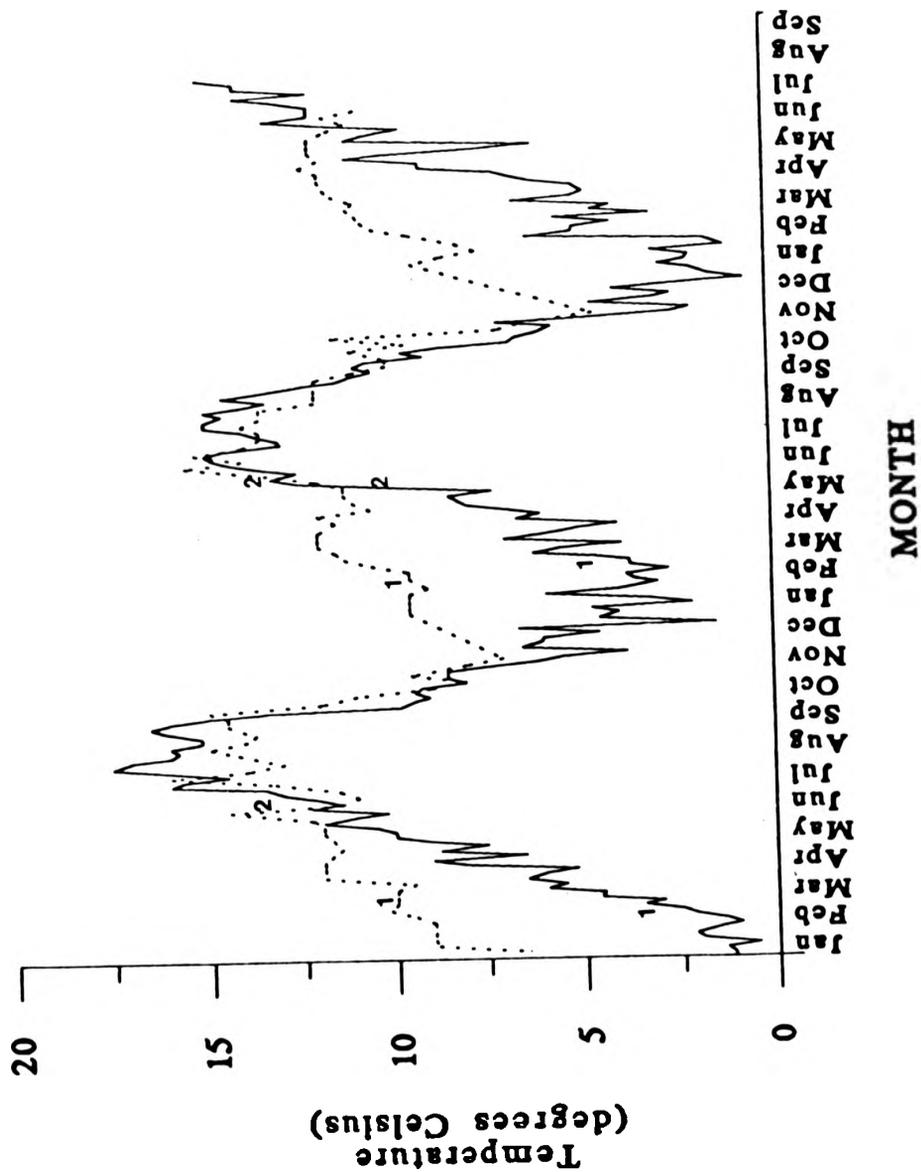
the myxozoan two host life cycle in the wild have yet to be studied. Alternatively, a synchronous release of sphaerospores from wild fish could result in their synchronous ingestion. Assuming there was little variation in the time for actinosporean development, an ensuing synchronous actinosporean release would occur.

Actinosporean spore development appears from the literature to take ~90-120 days (3/4 months) at 10-15°C. However, almost all experimental infections and studies of actinosporean development have been carried out at constant temperatures under laboratory conditions, and thus may not give a true representation of the time taken for development under ambient conditions in the wild. However, the timing of *M. cerebralis* actinosporean stage development in *Tubifex tubifex* under laboratory conditions was comparable with the gap in the life cycle, between mature *M. cerebralis* spore formation in infected fish and the infection of new host fish the following year in the wild (Markiw & Wolf, 1983). If this period of time is correct and holds true for the salmon *Sphaerospora* life cycle, development should begin in oligochaetes in Jan/Feb/Mar in order to infect the 0+ year class of salmon in May/June/July. A farm based study does not necessarily give a complete epidemiological picture of the life cycle strategy of the salmon *Sphaerospora* in wild populations. In the wild, few salmon smoltify and go to sea in their first year. Rather, they are likely to spend from two to four or five years before doing so, especially in some of the unproductive Highland rivers of the present study. Since the life cycle of the salmon *Sphaerospora* is annual in farmed fish, there must be some deposition of spores each year in the wild to ensure a successful synchronous infection of fish the following year. This release may therefore come from older Atlantic salmon parr. Brown trout may perhaps also act as reservoirs of infection, since they spend their entire lives in the rivers concerned, and

from the present study (chapter 3) they are also a potential host for the salmon *Sphaerospora*. The problems of identifying the relationship between the salmon *Sphaerospora* and *S. truttae* have already been outlined in chapter 3, but a preliminary investigation into the possibility that lochs holding large brown trout populations, and associated with rivers supplying farms, could be sources of infection revealed that the brown trout in L. Restal, connected to the the Kinglass Water which supplies farm A, certainly harbour *Sphaerospora* sp spores in their kidneys, albeit at low levels of intensity. Further studies could attempt to investigate this link. The sediments of such water bodies are undoubtedly richer in potential oligochaete hosts than the rivers supplying the farms. They therefore may have a role to play in the source of farm based infections; planktonic, floating actinospores perhaps being able to travel the long distances from loch to farm via the fast flowing rivers in the two to three days for which they are viable (chapter 6).

4.4.3 Differences between farms

In the present study, it must be appreciated that no build up of increasing levels of infection in successive fish cohorts occurs from year to year, as the parasite does not cycle on site. All infections in Atlantic salmon at farms are a result of infective stages released as a result of infections in the wild.

Mean intensity and its range within the salmon population at any time was much higher at farm A than farm B. Markiw (1986) demonstrated a positive correlation between actinosporean dose and infection level in terms of spore production for *Myxobolus cerebralis*. A similar relationship was shown by Molnar (1979b) for *Myxobolus pavlovskii*. The differences in mean intensity may therefore reflect a

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comparative difference in the numbers of infective stages of the parasite entering the farm and reaching the fish. This could result from a relative difference in the behaviour, abundance, population structure or ecology of the parasite or of either the fish or oligochaete host in the wild. Alternatively farm management factors such as tank size, shape and flow rate or fish stocking density may be involved. Farm B has the ability to mix river water with water from a small, fish-less stream. This may also mitigate against high infections. The intake point for the farm water supply could affect the number of infective stages entering the farm if the infection is focal in the river (Bucher *et al.*, 1992). Stocked fish strain may also affect susceptibility to infection. The two farms stock salmon from different egg producers. There is much evidence of stock strain variations in susceptibility to myxosporean infection, especially amongst salmonids (see chapter 3). The general health status of the fish in terms of stocking density, management efficiency, stress and other infections at each farm might also affect the degree to which they become parasitised or are able to mount an effective immune response. Perhaps local differences in environmental conditions at each farm could be responsible for differences in the size and species composition of the oligochaete population it can sustain.

The extrasporogonic stage of the salmon *Sphaerospora* is a proliferative phase, as was clearly shown by the experiment where fish were retained, whereby the infection level in fish held away from any source of cumulative reinfection rose from 0% detectable prevalence to 80% with intensity range 1+-3+. It might be assumed then, that any fish infected by salmon *Sphaerospora* would attain a high intensity infection. In some other species of *Sphaerospora* (Molnar, 1979a; Molnar 1980d) fish are either

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uninfected or infected at very high levels. This does not apply to *Sphaerospora* infections at farms A and B. Fish at farm A, especially when prevalences were high, demonstrated a wide range of intensity levels. Even when the mean intensity was high, some infected individuals in the population carried low level infections. The proliferative nature of the extrasporogonic stages must therefore in some way be mediated by other factors. Perhaps extrasporogonic primary cells can undergo only a finite number of cycles prior to all released cells becoming sporogonic; this could explain the range in infection intensities within and between farms, the final intensity of infection being dependent upon the initial dose of actinosporeans. Alternatively perhaps such stages cycle for a limited period of time in the fish or between certain temperature limits, until a temperature or other cue initiates sporogony. Perhaps a host immune response or other factor or defence mechanism causes a cessation of extrasporogonic cycling. Whatever the cause, the length of time for which extrasporogonic stages are present in the population each year is limited. This means that the more parasites initially infecting a fish, the higher the levels of intensity that can be generated in that limited time. At farm B, it is noticeable that extrasporogonic stages appear to be prevalent in the fish population for a shorter period of time than at farm A. This could be reflective of a longer release period of actinosporeans from oligochaetes at farm A. Actinosporean individuals would therefore be infecting the fish over a longer period of time at farm A than farm B. Since each of these, once established in the host may have a limited number of extrasporogonic cycles, higher intensities of infection would occur at farm A than farm B. A lower average water temperature might cause such a shorter overall period of release of infective stages, for example, and thereby lead to a shorter window for which extrasporogonic stages are

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present. At farm A, water temperatures at or above 12°C occurred for around 6 months in both the summers of 1991 and 1992, whilst at farm B they were attained for only 3-3½ months (fig.4.9).

4.4.4 Timing of development

The experiment where fish were retained at sampling dates prior to the first positive routine sample, demonstrated that a pre-patent period existed between the point at which fish became infected and the point at which infections became detectable in the population. This period is 40 days for *M. cerebralis* (El-Matbouli, Hoffmann & Fischer-Scherl, 1992). However for species with extrasporogonic stages stages this seems a little shorter; Kent (1992) gave a figure of around 3 to 4 weeks (21-28 days), Clifton-Hadley & Feist (1989) around 5 weeks (35 days), both for PKX, compared to the figure of around 15-28 days in the present study. Ferguson & Ball (1979) and Ellis *et al* (1985), both found that fish required an exposure to PKX-infective waters in May to ensure development of PKD. However, the latter authors could not identify the parasite in these May infected fish until July. Foott & Hedrick (1987) found that although retention experiments revealed that water were infective for PKX from April onwards, the first signs of infection in hatchery held fish from the same site were not apparent until June. In the present study, it could be that the parasite has an earlier stage in the fish during this time that was not detected despite the rigorous examination of all fish organs both fresh and histologically. For example, no released actinosporean sporoplasm stages were found in the epithelium of the skin or gills even during the period the infective stages were present. However it could also be that during this time the parasite

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was present at such a low intensity that its detection was not possible using the techniques employed.

Extrasporogonic stages of the salmon *Sphaerospora* are present from mid May to late September at maximum. This is around 16-18 weeks. Kent (1992) gave a figure of 20 weeks for PKX in the USA. Foot & Hedrick (1987) in the US found the first sporogonic stages of PKX around 30 days after the appearance of extrasporogonic stages. In the UK, Clifton-Hadley & Feist (1989) found first sporogonic stages of this parasite at 9 weeks (63 days) with first "spores" at 14-18 weeks (98 days). The equivalent data for the salmon *Sphaerospora* is 17-31 days for first sporogonic stages, and 48-80 days for first mature spores. Again, this switch to initiate sporogonic development could be stimulated by the temperature drop outlined above, but also there may simply be a fixed time period in extrasporogonic development from initial infection before triple formations, the stages which when released initiate sporogony, are formed.

4.4.5 Sea water effects

The continued study of infection levels after fish transfer to marine rearing facilities indicated that spores were completely lost from the kidney tubules in sea water, and that developing pseudoplasmodia became degenerate and died within four months. No fish were subsequently brought back onto freshwater to see whether sporogony could recommence as it does in *Myxidium salvelini* (Higgins *et al.*, 1993). Although this cannot be ruled out, it seems unlikely in the case of the salmon *Sphaerospora*. Higgins *et al.*, found that the pre-sporogonic plasmodial stages remained dormant in the kidney tubules, maintaining their presence by means of their intimate association with the inner

wall of the microvillus epithelium of the tubule. The pseudoplasmodial stages of salmon *Sphaerospora* have a similar means of intimate attachment to the inner tubule wall, but it was these stages which were the first to be lost in sea water, both in the differentiating presporogonic state and as the enveloping cell around mature and maturing spores. Only lone, fully mature spores seemed to persist in the tubules. It seems likely that the switch in sea water to the production of concentrated urine leads to conditions within the tubular lumen unsuitable for the survival of any stages other than mature resistant spores. These would be eventually flushed out with the urine since they have no available means of attachment to the inner tubule wall to facilitate their retention.

Since the marine phase in the life cycle of the Atlantic salmon is one year or more, it seems extremely unlikely that in the wild any *Sphaerospora* infections could remain on return to freshwater. Indeed, there appears to be no record of *Sphaerospora* spp in returning salmon. Since the salmon *Sphaerospora* appears to be a wholly freshwater parasite, the loss of mature spores in sea water must be considered as a loss to the recruitment of the population. It therefore seems certain that in wild fish there must be some release of spores into the environment before migration to sea. Perhaps this could be achieved by the sharp increase in urine production shown by *S. salar* in freshwater coinciding with smoltification prior to migration into salt water.

4.4.7 Study of *Sphaerospora* infections of Atlantic salmon in their second year

This study showed that fish previously exposed to infection by the salmon *Sphaerospora* in their first summer (group 1) were refractory to future reinfection. However, they still contained mature and developing sphaerospores from the previous

years' infections. Samples taken from S_2 fish at farm C in previous years have shown similar findings despite S_1 salmon, like the group 2 fish of the present study being heavily infected at the same time (T. Wall, Farm veterinarian, pers. comm.). This is very similar to the situation seen in fish which recover from PKD infections (Ferguson & Ball 1979; Foot & Hedrick, 1987; Kent, 1992). However in the case of PKX immunity in the second year may depend on fish having actually suffered clinical levels of the disease rather than just exposure to infection (Clifton Hadley *et al*, 1986). Similar resistance to reinfection has been shown for whirling disease, *Myxobolus cerebralis*. According to the authors, fish exposed to whirling disease are refractory to further infection due to the infective actinosporean stage of the life cycle being able to discriminate between naive and previously infected juvenile fish, presumably due to the presence of antibody in the mucus (Hoffmann, El-Matbouli & Hoffmann-Fezer, 1992). In the case of *Myxobolus cerebralis*, a further age related change, the progressive ossification of the head cartilage, makes older fish less susceptible to infection. Acquired immunity against *S. renicola* has been proposed to explain why carp older than 14 to 15 months appear no longer susceptible to infection (Odening, Walter & Bockhardt, 1988).

All fish previously infected with salmon *Sphaerospora* appeared refractory in their second year, even though some had harboured low level infections. That this resistance to salmon *Sphaerospora* was acquired rather than simply due to factors associated with increasing fish age and size was shown by the group 3 fish from farm D, which suffered high intensity levels of infection with salmon extrasporogonic stages at age 1+ in the summer of 1993. These fish had spent their first summer at a farm at

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which no salmon *Sphaerospora* had ever been recorded.

Chapter 5
Pathology

5   PATHOLOGY

5.1   INTRODUCTION

The pathogenicity of important myxosporeans of cultured freshwater fish has been reviewed by Shulman (1966), Lom & Dykova (1992d), El-Matbouli *et al.* (1992b), Dykova (1992) and Körting (1992). In the marine environment Alvarez-Pellitero & Sitja-Bobadilla (1992; 1993) summarised pathogenic species in both commercial wild fisheries and in mariculture. Of the many myxosporean species described to date, relatively few have been found to cause serious or fatal infections (Lom & Dykova, 1992d). This has been attributed to the long evolutionary coexistence of parasite and host leading to myxosporeans becoming well adapted to their hosts (Lom & Dykova, 1992a).

Dykova (1992), however, considered that there may be more pathogenic species than had previously been supposed. As fish farming increases worldwide and species are cultured for the first time, especially in the marine environment, some previously poorly recognised species, or those to whom little pathology had been associated in wild fish, may prove to be significant pathogens under culture conditions (Alvarez-Pellitero & Sitja-Bobadilla, 1992; 1993). Körting (1992) pointed out that, whereas a very restricted number of myxosporeans such as *S. renicola, M. cerebralis* and PKX are indisputably highly pathogenic and of great economic importance, epizootics caused by many other species to whom pathology has been attributed are restricted to local and occasional events. In certain circumstances, such as pond culture, myxosporeans may cycle, their prevalence and intensity within fish populations rising over time (Wyatt, 1978) to an epizootic level. In commercial fisheries exploiting wild marine stocks, myxosporean species of the genera *Kudoa, Hexacapsula,* and *Unicapsula,* some of which

table 5.1   Some economically and commercially important myxosporean parasites

SPECIES (hosts)	ENVIRONMENT	DISEASE CONDITION
Ceratomyxa shasta (salmonids)	freshwater	Ceratomyxiasis
Myxobolus cerebralis (salmonids)	freshwater	Whirling disease
PKX (salmonids)	freshwater	Proliferative kidney disease
Kudoa spp. (many fish species)	marine	Post-mortem 'milky flesh' (myoliquefaction)
Sphaerospora renicola (common carp)	freshwater	Swimbladder inflammation (SBI) of carp fry
Sphaerospora tincae (tench)	freshwater	
PGD (channel catfish)	freshwater	Proliferative gill disease
Thelohanellus nikolskii (common carp)	freshwater	
Myxobolus spp. (range of cyprinids)	freshwater	boil disease

are responsible for post-mortem musculoliquefaction have been found to be economically important (Alvarez-Pellitero & Sitja-Bobadilla, 1992). However there are very few reports of epizootics of myxosporean infection in freshwater fish in the wild (Mitchell, 1977). Although this could be due to the lack of detection of such outbreaks, it seems more likely that the normal host-parasite relationship exhibited by wild populations is reflected by an overdispersed parasite distribution; few fish are therefore likely to be heavily infected, with perhaps only the very weakest fish dying.

5.1.1 The pathology associated with myxosporean infection

The deleterious effects of myxosporean parasites can be broadly divided into three categories. In some cases the parasite itself may cause serious losses; this is the case with the salmonid myxosporeans, PKX, *M. cerebralis* and *C. shasta*. Alternatively myxosporeans may lower the aesthetic appeal of harvested fish. This is manifest by *M. cyprini*, which causes 'boil disease' of carp; *Thelohanellus nikolskii*, which forms cysts on the skin surface of common carp; or classically by the "milky-flesh" condition caused in the muscles of wild and farmed marine fish by *Kudoa*, *Unicapsula* and *Hexacapsula* spp. Thirdly, by weakening or stressing its host, a myxosporean infection may render a fish more susceptible to secondary infection by other pathogens, or narrow its tolerance limits to environmental parameters. Some *Sphaerospora* spp., such as *S. epinephali* (Supamattaya *et al*,. 1991), could be placed in this category.

Thus myxosporeans may be problem parasites of fish culture both in commercial intensive systems, and in those whose aim is to provide a cheap plentiful protein source for human populations.

<u>Host responses to infection</u>

It has been frequently demonstrated that polysporic vegetative plasmodia in genera such as *Henneguya* or *Myxobolus* elicit little or no host response as they develop. Only when mature spores have formed, and growth ceased is a granulomatous inflammation initiated (Lom & Dykova, 1992d; Athanassopoulou & Sommerville, 1993b). However some vegetative pre-spore stages, for example the extrasporogonic stages of PKX in salmonids, the K-stages of *S. renicola* in common carp or the agent of proliferative gill disease, PGD, in channel catfish provoke a vigorous host reaction (Lom & Dykova, 1992a). Mitchell (1977) claimed that an increase in pathogenicity was frequently found when a myxosporean parasite encountered a new host fish species. As an example he cited the high degree of pathology associated with *M. cerebralis* infections in rainbow trout compared with the presumed natural (European) host, the brown trout.

Individual myxosporean species have been shown to cause varying pathological manifestations according to their developmental site in the host. *M. rhodei* cysts in the renal corpuscles for example, are only recognised by the host once mature spores have developed, whereas cysts in the interstitium are rapidly destroyed prior to spore formation (Lom & Dykova, 1990d; Athanassopoulou & Sommerville, 1993b). Since SBI is caused by a specific parasite to carp, *S. renicola*, Dykova *et al.* (1990) proposed that the swim bladder was a new biotypic niche in the host to which the parasite was not yet fully adapted, thus explaining its pathogenicity.

In the past, some reports have indicated that myxosporean stages may show antigenic mimicry, and hypothesised that by acquiring host molecules they evaded an

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immune response (Pauley, 1974 for *M. cerebralis*; Siau, 1980 for *M. exiguus*; McArthur & Sengupta, 1982 for *Myxobolus* sp in eels). However Griffen & Davies (1978) detected circulating antibodies against *M. cerebralis*, as did Hoffmann *et al.* (1992). Griffen & Davies (1978) therefore attributed earlier authors inability to detect antibodies to the use of insensitive assays. They proposed that to best serve the host-parasite relationship, antigenic disparity was to be reduced, but not eliminated. Ogawa *et al.* (1992a) detected circulating antibody to developmental stages of *Myxobolus artus* in naturally infected carp. Carp injected with developmental pre-sporogonic stages provoked antibody production and were thus assumed to be recognised as antigenic. The injection of spores resulted in no antibody production, and thus spores were not considered antigenic. However Ogawa, Furata, Delgahapitiya & Wakabayashi (1992b) found that carp naturally infected with this parasite did not produce an immune response until spores had been formed, pre-spore stages producing little signs of such response. There is thus confusion concerning the mechanisms of the host response to myxosporeans. These molecular aspects of myxosporean antigenicity and host response may become clearer with the development of monoclonal antibody and immunohistochemical techniques. These have been used in studies of *C. shasta* and PKX (Bartholomew, Rohovec & Fryer, 1989; Bartholomew, Yamamoto, Rohovec & Fryer, 1990; Adams *et al.*, 1992; Marin *et al.*, 1993). Such probes may become useful diagnostic tools in the detection of antibody responses, as well as for species identifications, specificity studies, and life cycle/ epidemiological investigations.

Histopathology

Dykova (1992) summarised the present state of knowledge on fish histopathology

associated with myxosporean infections, showing that, generally, almost any fish organ could be infected and that resulting lesions could range from the barely noticeable to those provoking lethal changes.

Both Dykova (1992) and Lom & Dykova (1992d) described myxosporeans as a group as being able to induce all categories of regressive changes: (dystrophy, atrophy, necrosis); and progressive changes: (hypertrophy, hyperplasia, metaplasia) in their hosts. Proliferative granulomatous inflammation is considered a typical and characteristic host response, with phagocytosis by macrophage or in melanomacrophage centres an important host control. Lom & Dykova (1992d) listed eleven typical lesion types and provided examples of species responsible for inducing them (table 5.2). Those most pertinent to kidney infecting species of the genus *Sphaerospora* are cell/ organ hypertrophy, as demonstrated by the PKX extrasporogonic stage and by *Sphaerospora tincae* in tench; and the dilation of renal tubules and ducts by sporogonic stages.

5.1.2   Pathology of *Sphaerospora* spp.

In wild situations, where high levels of intensity of infection are rare, *Sphaerospora* spp. do not appear to affect fish severely and little or no pathology has been recorded (Supamattaya *et al.*, 1990). However, in culture systems infections may reach a level which elicits pathogenic change. Markedly different degrees of pathology have been found to occur depending on the species concerned, life cycle stage involved and the site of infection.

Pathology due to extrasporogonic stages

In culture systems, extrasporogonic stages of some *Sphaerospora* spp. appear to

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have a particularly marked pathogenic potential. Hedrick *et al.*, (1990) related *Sphaerospora ictaluri* in channel catfish, *Ictalurus punctatus*, to proliferative gill disease, PGD. Stages similar to the extrasporogonic stages of this parasite, which circulate systemically in the blood, could be found in the stroma between adjacent lamellae in the gills. Here they elicited a chronic diffuse granulomatous inflammation, with parasites often surrounded by a collar of host mononuclear cells. Affected gills became congested and mixed populations of inflammatory cells such as lymphocytes, neutrophils and macrophages infiltrated. Infected fish suffered gill swelling and necrosis, became listless and often died (Burtle *et al.*, 1991; MacMillen *et al.*, 1989). However, in the latter study, no sporogony occurred in the gills over the two months of the study, plasmodia becoming necrotic after approximately 60 days. Since sporogony of *S. ictaluri* occurs in the kidney tubules (Hedrick *et al* 1990), it can be hypothesised that stages in the gills may represent an aberrant developmental pathway.

Fish infected with the extrasporogonic K-stages of *S. renicola* show locomotory disorders such as side swimming and irregular cycling movements from the pond bottom. The presence of extrasporogonic K-stages generates "an exudative proliferative inflammation", with the swimbladder wall becoming cloudy and haemorrhagic with distended blood vessels. Later it becomes thickened and coloured, with connective tissues showing adhesions and hyperplasia. Intra- and extra-cellular localisation results in oedema and massive lymphocyte infiltration. Damage to the swimbladder has been held to cause a marked drop in hydrostatic function which can result in direct losses or lead to bacterial infections. Lesions remain after the parasite leaves the swimbladder (Dykova & Lom, 1988b; Csaba *et al.*, 1984; El-Matbouli *et al.*, 1992b). High level infections of C-stages of *S. renicola* in the blood of carp have been claimed to cause

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table 5.2   The pathology associated with myxosporean parasites (after Lom & Dykova, 1992d)

1.   Pressure atrophy due to the presence of large polysporic pseudoplasmodia
         e.g. *Myxobolus, Henneguya* cysts

2.   Induced alterations in neighbouring and infected tissues caused by polysporic plasmodia
         e.g. *Thelohanellus, Chloromyxum, Myxidium*

3.   Cell/organ hypertrophy
         e.g. *S. tincae*, PKX
         c.f. intracellular hypertrophy with contact hypertrophy.

4.   Dilation of renal/hepatic ducts/tubules
         e.g. *Sphaerospora, Zschokkella*

5.   Erosion of cartilage due to lysis/phagocytosis by plasmodia
         e.g. *M. cerebralis, M. aeglefini*

6.   Spinal deformities due to nerve cord destruction
         e.g. *M. sandrae*

7.   Replacement of host tissue by parasite stages
         e.g. myocytes with *Kudoa* spp.

8.   Destruction of host tissue by parasite stages
         e.g. intestine by *C. shasta*

9.   Necrosis of liver parenchyma by trophozoites
         e.g. *Chloromyxum cristatun* in carp

10.   Rendering of organ as non-functional
         e.g. *S. molnari* carp gills

11.   Liquefaction of musculature in marine fish
         e.g. *Unicapsula* spp., *Hexacapsula* spp., *Kudoa* spp.

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changes in blood parameters (Kudryashova & Naumova, 1978, cited by Dykova and Lom, 1988b). No other authors have reported pathogenic changes however.

The extrasporogonic stage of the life cycle of PKX in rainbow trout is a major pathogen, and the cause of economic losses in commercial culture. Gross signs of infection are dark coloration, raised mortalities, distended abdomen, anaemia, exophthalmia and a drop in stress tolerance (Clifton-Hadley *et al.*, 1984; Lom & Dykova, 1992d). At post-mortem, there is often ascites and the kidney is enlarged, grey and can show haemorrhagic areas. In heavy infections, the spleen is also enlarged and other internal organs may show pallor as a result of anaemia.

Histologically, an interstitial hyperplasia and tubular atrophy are accompanied by leucocyte infiltration. Intense defensive host reactions cause a chronic granulomatous nephritis and compromise all kidney functions. Granulomatous changes can occur in a range of other organs, such as the liver and spleen also (Ferguson & Needham, 1978; Clifton Hadley *et al.*, 1984; Lom & Dykova, 1992d). Numerous changes occur in the blood of PKX-infected fish although severe renal swelling appears necessary for anaemia (Clifton Hadley, Richards & Bucke, 1986; Foott & Hedrick, 1990). Diseased fish show anaemia despite haematopoietic proliferation, and poor food conversion and feeding rates. Anaemia is often the prime cause of death due to a drop in the oxygen carrying capacity of the blood.

Pathology due to sporogonic stages

Histozoic sporogonic *Sphaerospora* stages appear to have a greater pathogenic potential than do coelozoic spore stages. Examples of such pathogenic histozoic species include *S. molnari*, which infects the stratified epithelium of the gill filaments, branchial

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cavity and gill arches of common carp causing hyperplasia and fusion of the gill secondary lamellae. Vegetative stages and spores of the parasite cause dystrophy, necrosis and eventually disintegration of fused lamellae leading to spore release. Infection results in respiratory dysfunction and destruction of the integrity of the gills leading to secondary invasion by fungi, bacteria and ectoparasites (Molnar, 1979a; Molnar, 1980b; Lom et al., 1983b; Dykova & Lom, 1988b). Another histozoic species, *S. tincae*, has been implicated in mass mortalities of tench fry. Heavily infected fish display a swollen anterior abdomen caused by enlargement of the head kidney, which comes to occupy the entire anterior of the body cavity, having displaced other organs posteriorly. Histology shows the remaining head kidney lying at the margin, with the rest of the tissue occupied by a parasite mass held within a thin fibrous capsule (Hermanns & Körting, 1985). *S. testicularis* destroys the testicular tissues of male *Dicentrarchus labrax*, causing a very marked drop in fecundity, and is considered an agent of parasitic castration (Sitja-Bobadilla & Alvarez-Pellitero, 1990; Alvarez-Pellitero & Sitja-Bobadilla, 1992).

Coelozoic *Sphaerospora* spp. sporogonic stages appear to be less pathogenic. The most common site of development for such stages is the lumen of the kidney tubules. Sporogonic *Sphaerospora* stages in this site have in many cases been reported to cause little pathogenic change (Hedrick et al., 1990; El-Matbouli & Hoffmann, 1992). Frequently spores simply occlude infected tubules as in *S. elegans* infections of sticklebacks (Feist et al., 1991). However, in sterlet infected with *S. colomani*, the large number of pseudoplasmodia in the kidney tubules cause them to expand and so result in a flattening of the tubular epithelium (Baska, 1990).

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More major histopathological changes have been described for a number of species. *S. pectinacea* in the kidneys of perch may cause mortalities in heavy infections (Pronin & Pronina, 1985, cited by Lom & Dykova, 1992d). Dystrophic changes such as hyaline droplet formation, cytoplasmic vacuolation and pycnotic nuclei in tubular epithelial cells is a common finding for a range of species such as *S. ohlmacheri* (Desser *et al.*, 1986), *S. renicola* (Dykova & Lom, 1982; Dykova & Lom, 1988b) and *S. epinephali* (Supamattaya, 1991). The level of epithelial cell vacuolation in *S. epinephali* was extreme (Supamattaya, 1991). Desser *et al.* (1983) regarded such vacuolation at the TEM level in *S. renicola* as evidence of impaired cellular function. In the case of *S. renicola* the brush border of tubular epithelial cells disappears, and spore stages necrose leaving the lumen plugged by necrotic and calcified debris. A drop in haematopoietic and excretory function is believed to result. Molnar (1980a), however found no gross changes in *S. renicola* infected tubules apart from slight nephrosis. Glomerular damage in the form of very dilated capillaries and an enlarged Bowman's space was reported by Supamattaya (1991). In late infections the glomerular membrane was destroyed and the Bowman's space contained spores and spore debris, along with infiltrating MMC's and periglomerular fibrosis. The parasite was held responsible for rendering fish more susceptible to bacteria, viruses and other parasites. Glomerular infections of *Sphaerospora* therefore appear to have a higher degree of pathogenicity. Sitja-Bobadilla *et al.* (1992) found similar changes caused by an unidentified *Sphaerospora* sp in the kidneys of marine cultured *Sparus aurata*, with glomeruli being completely destroyed in some cases. Pathological changes were associated with haemorrhagic lesions and MMC/leucocyte infiltration. El-Matbouli *et al.* (1992b) found that *S. cristata* in *Lota lota* caused severe glomerular damage and enlargement, although no clinical signs or

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losses occured.

Some authors have described intracellular developmental stages of *Sphaerospora* spp. El-Matbouli & Hoffmann (1992) described intracellular stages of *S. scardinii* from the epithelial cells of the ureter of rudd which elicited a marked cellular proliferation, whilst in *S. renicola* infections of carp, intracellular stages in the kidney tubule epithelial cells (so called "Hoferellus" stages- see chapter 2) displace the nucleus and cytoplasm of infected cells and form a parasitic syncytium. This causes a swelling of the tubular epithelium, which becomes hyperplastic and up to three times its normal size, resulting in blockage of the lumen. Dystrophic changes result in epithelial cell disintegration and cause the parasite to be released into the tubular lumen or the interstitium. Stages in the interstitium then cause an intense granulomatous inflammation and fibroblast proliferation (Dykova & Lom, 1982; Dykova & Lom, 1988b).

Pathology of *Sphaerospora* spp. from salmonids

The sporogonic stages of *S. truttae* have been implicated in pathogenic changes in the kidneys of brown trout. Atrophy of the tubular epithelium has been observed with kidney epithelial cells appearing dislocated from the basement membrane (Lom & Dykova, 1989; Walter *et al.*, 1991). El-Matbouli *et al.* (1992b) described extensive vacuolation of epithelial cells which contained dark non-homogeneous inclusions. In heavy infections, they found the Bowman's space to be filled with masses of developmental stages and spores. Glomerular capillaries had disintegrated. Bennett & Wolke (1986) reported that *Sphaerospora* sporogonic stages in Canadian Atlantic salmon kidney tubules elicited little or no inflammatory response, and there was an

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absence of damage to the tubular epithelium and its lining microvilli at both the histological and TEM levels.

5.1.3 Objectives

The aim of this part of the study was to observe the histopathological changes and clinical signs displayed by fish harbouring *Sphaerospora* infections at farms with varying infection intensities.

5.2 MATERIALS AND METHODS

Observations of live and moribund fish and the general health and condition of stocks were made from a range of tanks at infected farms on the sampling dates for the epidemiological study (chapter 4). Farm managers were consulted concerning the past history of infections with the salmon parasite and its effects.

The tissues and organs of infected fish were examined for gross changes at post-mortem. Observations were made concurrently with those on the development of the parasite (chapter 2).

All fixed histological and TEM material obtained during the present study (see materials and methods, chapter 2) was examined to investigate histopathological changes associated with each life cycle stage of the salmon *Sphaerospora*, and how they varied throughout the course of the infection.

5.3 RESULTS

5.3.1 Clinical observations

At all infected farms, problems associated with the presence of the salmon

Sphaerospora were mainly restricted to the summer months, especially July and August. During this period, farmers reported a sudden, unexpected rise in the level of mortalities, which continued at a slightly elevated level for a number of weeks. Peak mortalities were associated with high ambient temperatures, periods of water shortage and water temperatures of 15°C and above (see chapter 4). Losses were also exacerbated by netting and grading. At farm A, this period coincided with outbreaks of furunculosis (*Aeromonas salmonicida*).

The clinical signs noted in infected fish, in addition to elevated mortality levels, were innappetance, low stress tolerance (e.g. overnight mortalities following netting or grading), a darkening in colour, and lethargic swimming movements, often at the top of the water column. Such signs were usually restricted to fish subsequently found to harbour high intensity infections (grades 4+/5+). Fish carrying medium and low level infections in the same tank often showed few clinical signs.

At post-mortem, fish harbouring low level infections showed no gross changes. Those with intermediate levels of infection often had a slight hyperplastic proliferation of the kidney which resulted in a rounding of its edges, but few other signs. Heavily infected fish were relatively rare in random samples, but more common amongst moribund and dead fish, with six of ten moribund fish sampled from a tank at farm A in late July, 1993, found to harbour infection intensity levels of 4+ and 5+. Such fish had enlarged, grey, spongy kidneys, ascites, swollen rounded spleens and pallid anaemic gills.

Outwith the summer months, infected populations no longer exhibited the clinical signs outlined above. Fish appeared to regain appetite and health, with mortality levels dropping to a normal level.

5.3.2   Histopathology

Extrasporogonic stages circulating in the blood did not appear to damage or have a pathological effect on the blood vessels. Effects on blood cells, or on blood parameters such as haematocrit readings were not measured, being outwith the scope of the present study. Histologically, low and medium level infections of the kidney interstitium (grades 1+-3+) resulted in few changes other than a rise in the numbers of eosinophilic granular cells (EGCs) in the surrounding interstitium. Higher level infections (grades 4+ and 5+) were characterised by a very large increase in EGCs in the kidney interstitium and a slight increase in melanisation (fig.5.1). A degree of kidney swelling was present, apparently proportional to the number of parasites present, and was caused by a slight haematopoetic cell proliferation and increased levels of ascitic fluid. There was no evidence of a significant decrease in the number of kidney tubules such as that seen in PKX infections. There was, however, a marked increase in leucocyte infiltration (fig.5.2), many of which were in close association with and/or in contact with the outer membrane of the primary cell of extrasporogonic stages. In TEM sections single parasites often could be found with four or five adhered leucocytes (fig.5.3). Some leucocyte-adhered stages showed degenerative changes such as the disruption of their cytoplasm, lack of cytoplasmic organelles, vacuolated cytoplasm and lack of membrane integrity in secondary and tertiary cells (fig.5.4, 5.5). Numbers of small extrasporogonic stages in the interstitium were in the process of being, or were already engulfed by macrophages. These too showed signs of degeneration.

The establishment, via the glomerulus, of triple formations in the kidney tubules appeared to be responsible for a number of pathogenic changes. These stages represent

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fig 5.1   Histological section of a kidney infected with extrasporogonic stages of salmon *Sphaerospora* (arrows). Note increased melanisation and presence of EGCs (H&E, bar=10μm)
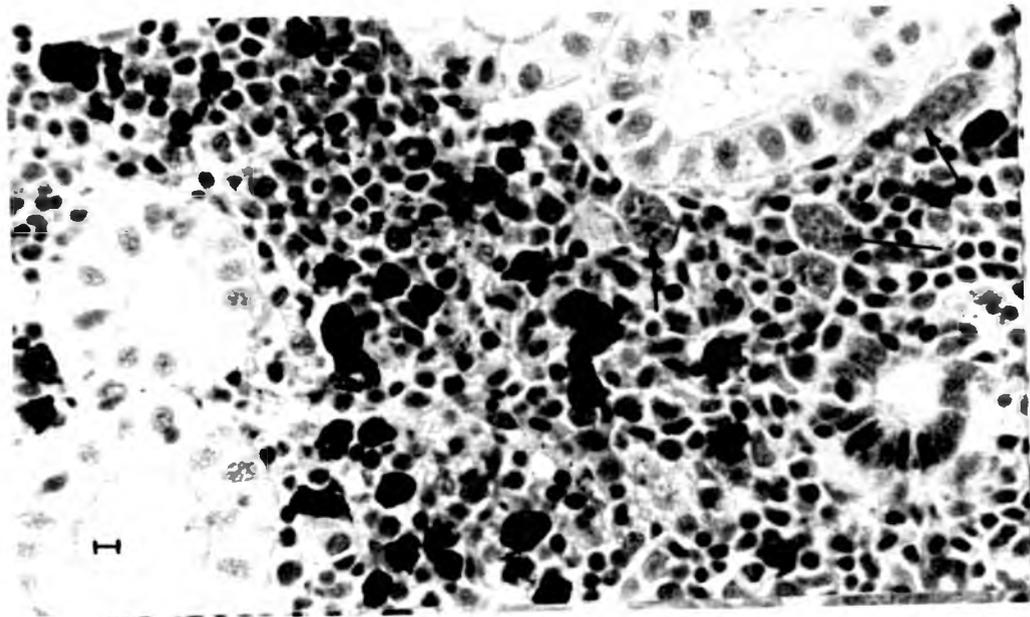

fig 5.2   Histological section. Increased leucocytes are present, some in close association with parasites. (H&E, bar=10μm)
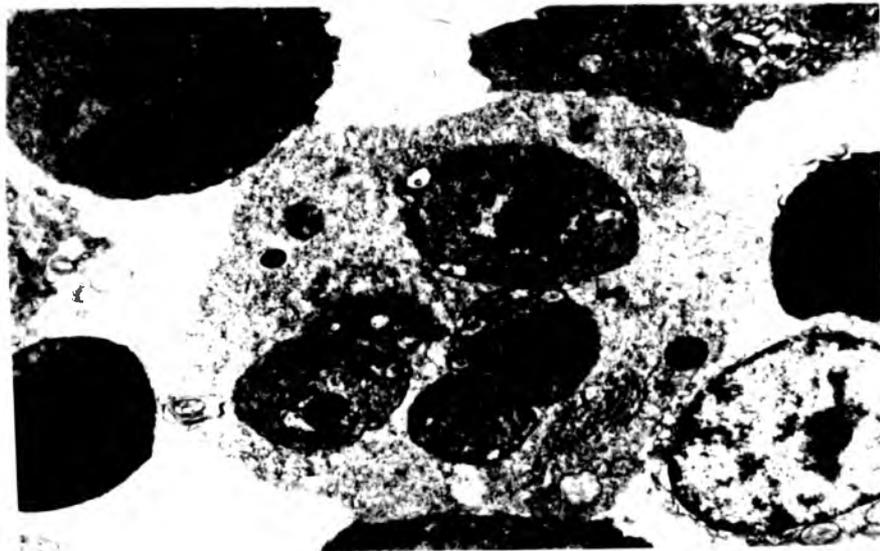
fig 5.4   Disrupted extrasporogonic stage in the kidney interstitium.   Although secondary cells are visible (S), the cytoplasm appears degenerate and contains no organelles (uranyl acetate/lead citrate, mag x2,800)

fig 5.5 Secondary cell (S) with no outer membrane (arrow) separating it from the degenerate cytoplasm of the primary cell (C). It contains a tertiary cell (T) with well defined membranes and nucleus. (uranyl acetate/lead citrate, mag. x18,000)
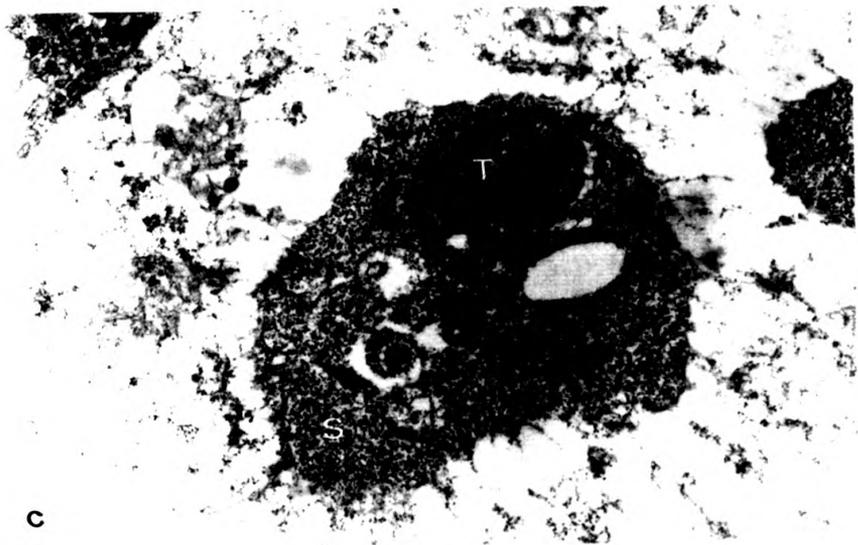
fig.5.6 Glomerulus with early sporogonic stages in the Bowman's space (arrows) (H&E, bar=10μm)

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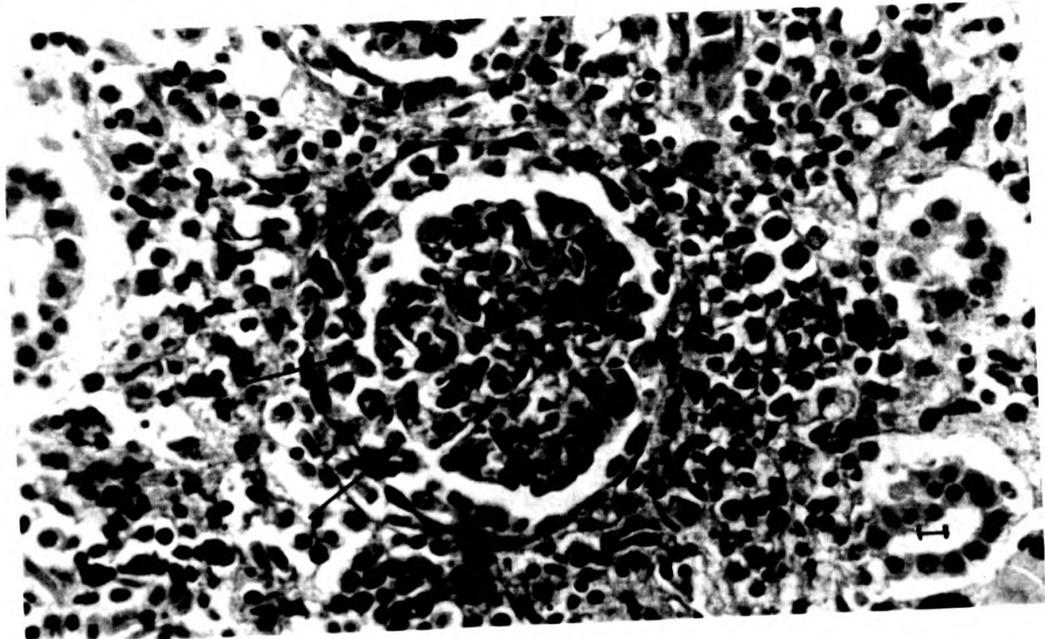
fig 5.7   Glomerulus containing large numbers of early establishing sporogonic stages and maturing spores
(**arrows**). The Bowman's space is enlarged and capillaries appear disrupted (H&E, bar=10μm)
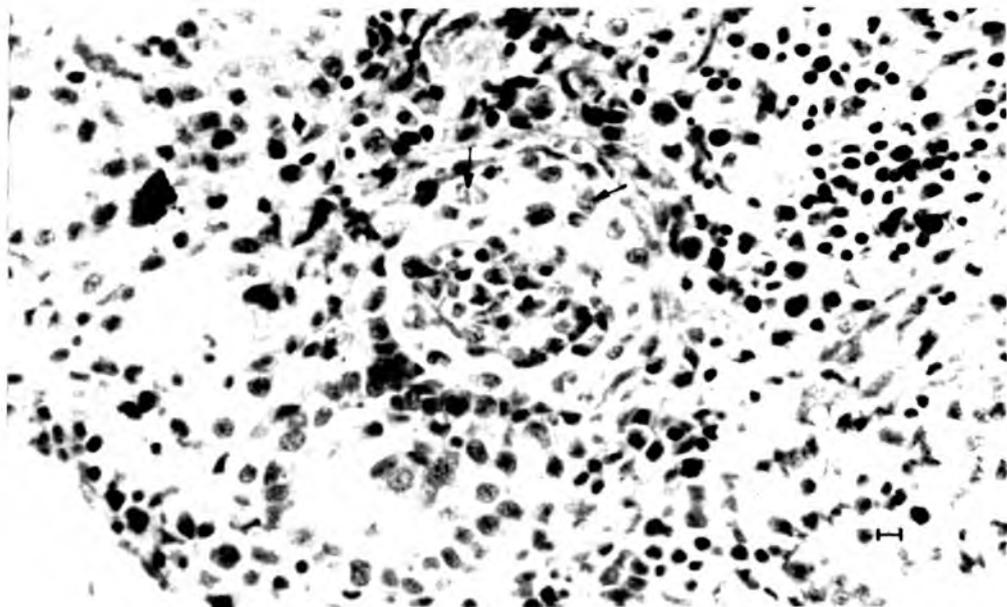
fig 5.8   Heavily damaged glomerulus with thickened capsule and degenerate capillaries.   numerous
sporogonic stages are present (**arrow**). (H&E, bar=10μm)

fig.5.9   Heavily damaged glomerulus with destroyed capillaries (**x**), and thickened Bowman's capsule
(**arrow**). (H&E, bar=10μm)

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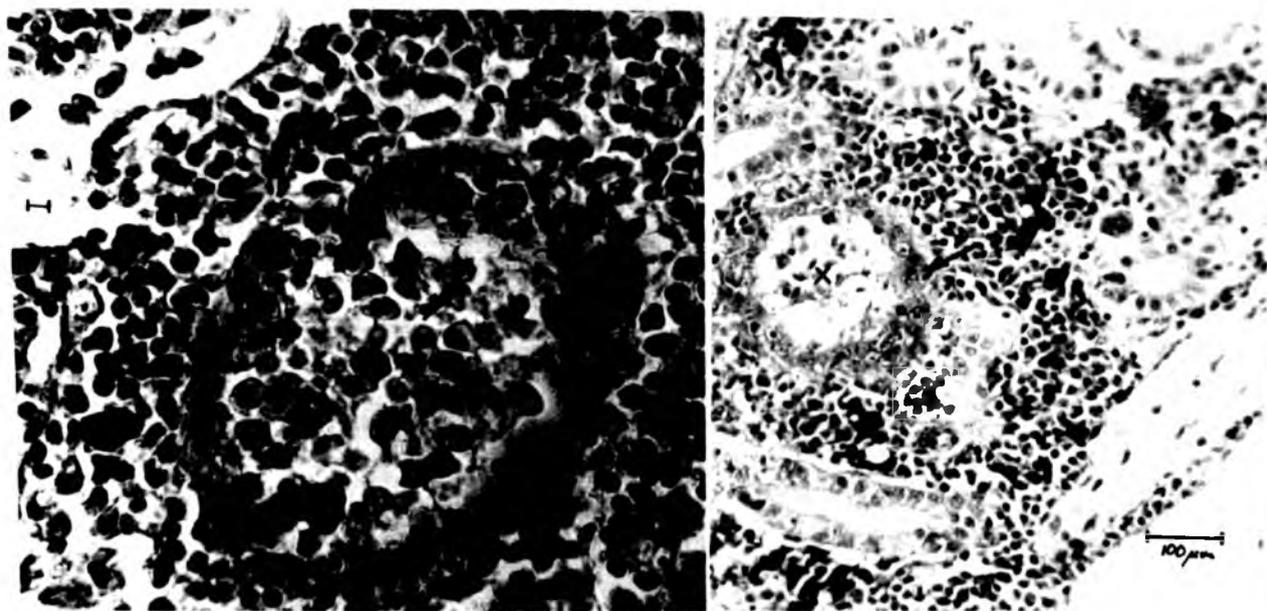
fig.5.10 Small extrasporogonic stage (Es) trapped by host leucocytes (L_1, L_2) whilst penetrating between adjacent cells of the kidney tubular epithelium of the host (K) (uranyl acetate lead citrate, mag x7,500)

fig.5.11 Sporogonic stages occluding the lumen of a kidney tubule. Tubular epithelial cells are dislocated from their basement membrane (arrow) (H&E, bar=$10\mu m$)
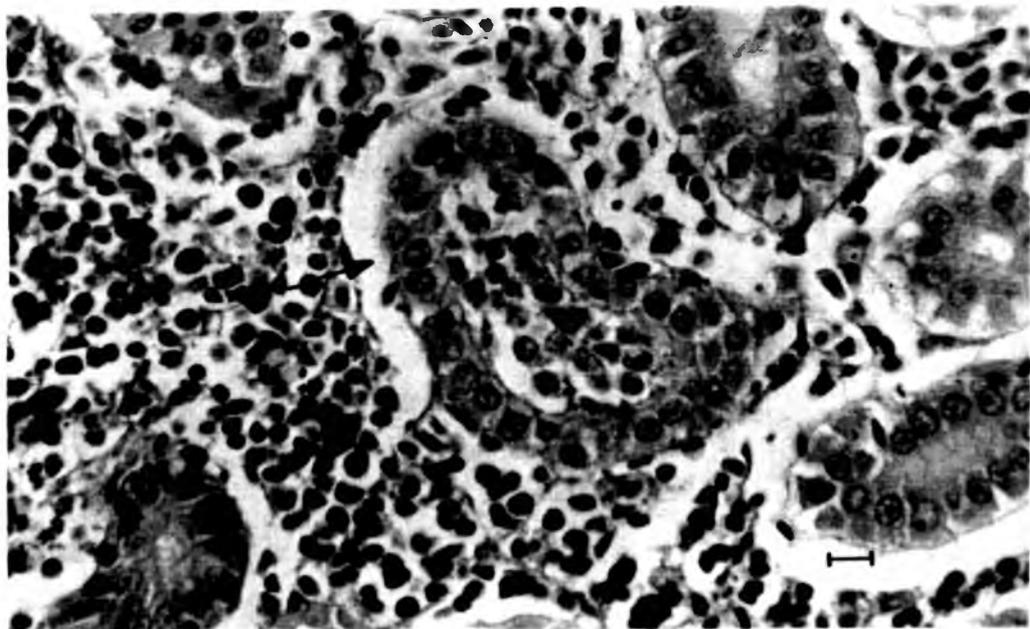
epithelial cells was mainly evident at the TEM level (fig.2.33).

There thus appeared to be three distinct phases in the histopathological changes seen in Atlantic salmon from farms with *Sphaerospora* infections. Two of these, the first and last, could be closely related to the two distinct stages, extrasporogonic and sporogonic, of the life cycle in the fish, and to the epidemiological observations of the development of the parasite. During the short period of time, (in epidemiological terms), where both extrasporogonic and sporogonic stages were present, a further phase, which appeared to be caused by the means by which extrasporogonic stages entered and established in the tubule lumen to initiate sporogony was evident.

5.4   DISCUSSION

There is increasing evidence in the literature that the extrasporogonic stages of myxosporean parasites may be not only pathogenic, but also responsible for causing diseases of economic importance (Körting, 1992). Thus in culture systems, swimbladder inflammation of carp fry, SBI; proliferative kidney disease of salmonids, PKD; and proliferative gill disease of channel catfish, PGD, are all caused by extrasporogonic stages which elicit a vigorous host response (Lom & Dykova, 1992a). However, the extrasporogonic stages of some species induce little pathology in cultured fish- the blood stages of *S. epinephali* (Supamattaya, 1991) and the C-stages of *S. renicola* for example (Dykova & Lom, 1988b). In the wild little or no pathology has been reported other than for PKX epizootics, perhaps because of the very low levels of extrasporogonic stage infection encountered (see introduction).

Lom & Dykova (1992d) questioned why some extrasporogonic stages induced such a response, when so many myxosporean pre-sporogonic trophozoites appeared to induce little or no host reaction. In the case of PKX, he believed pathogenicity to be explained by the hypothesis that the rainbow trout is an abnormal host to which the parasite is not fully adapted. This is supported by the lack of mature spore formation in rainbow trout and other salmonids. However, *S. renicola*, the causative agent of SBI, appears to be a well adapted, long established parasite of the common carp, as does *S. ictaluri*, the presumed agent of PGD, in the channel catfish. Both these organisms fully sporulate in the kidney tubules of their repective hosts. Lom & Dykova (1992d) proposed that perhaps the swimbladder of carp represented a new niche in the host that the parasite had only recently invaded, in evolutionary terms, and was thus not fully adapted to. The same hypothesis could be put forward for the *S. ictaluri* PGD stages in catfish gills.

The *Sphaerospora* sp. in the present study, however seems to be a well adapted, natural component of the parasitofauna of the Atlantic salmon. It is widespread across numerous water bodies in Scotland, long inhabited by salmon, unlike PKX, it forms fully mature spores and unless present at high infection intensities, the salmon *Sphaerospora* induces very little pathology.

Extremely low intensity infection levels with extrasporogonic stages are a characteristic feature of wild fish populations infected with *Sphaerospora* spp, despite the fact that such stages are proliferative (Lom *et al*, 1985b; Baska & Molnar, 1988; Kepr & Trsova, 1989). In the case of the salmon *Sphaerospora* it is therefore likely that in the wild, the levels of infection in individual fish would be very unlikely to reach a level capable of inducing pathogenic change. Thus it is probable that the normal host-

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parasite balance displayed by parasitic infections under natural conditions is shown in the wild, with problems only occuring when the level of intensity of parasitism is modified by the stocking densities and stresses associated with culture conditions. It would surely be non-viable, in evolutionary terms, for salmon *Sphaerospora* as a natural parasite of Atlantic salmon to have evolved to kill or seriously damage its host whilst cycling in its extrasporogonic phase, since it would then fail to sporulate and thus fail to reproduce.

Therefore it seems reasonable to suggest that as an alternative to Lom and Dykova's hypothesis of a new niche in the host (lom and Dykova, 1992d), the high level of pathogenicity shown by the K-stages of *S. renicola* in the carp swimbladder may simply be caused by an elevation of the intensity of parasitism generated by farming conditions. Under wild, natural conditions it may be that insufficient levels of parasites would reach the swimbladder to cause SBI. In carp culture systems, many hosts are present at artificially high densities in a restricted water volume and in mud bottomed ponds in which the parasite can presumably cycle annually with increasing intensity. This may cause high levels of parasitism and thereby induce significant pathology and disease, even though the organism is a natural parasite of that host. It would seem that only aspects such as the artificially high stocking densities and increased stresses apply in the case of the salmon *Sphaerospora* since all infections with this parasite come from a wild source with no cycles of increasing infection occuring in tanks.

The obsevations on clinical signs such as darkening of colour and lethargy was attributed to *Sphaerospora* infections. However these signs of general ill health in fish may also be caused by concurrent furunculosis infections. Furunculosis is endemic at

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the high intensity farm studied.

Many of the histopathological observations on kidneys infected with salmon *Sphaerospora* appear characteristic of fish responses to infectious microorganisms. These responses, such as increased blood flow and the active migration of white blood cells to affected organs, though generally protective, may on occasion initiate severe disease. Their purpose is considered to be to provide the cells and tissue fluids most suitable for the maintenance of homeostasis and resolution of infection (Roberts, 1989).

The active migration of white cells into the interstitium of the kidney is characteristic of host responses to the salmon *Sphaerospora* and has been reported as a result of extrasporogonic stage myxosporean infections in the case of PGD in the gills of channel catfish (Hedrick *et al*, 1990; MacMillan *et al*, 1989), K-stages of *S. renicola* in the swimbladder (Dykova & Lom, 1988b) and PKX in the kidney interstitium (Clifton-Hadley *et al*, 1984).

The active phagocytic action of macrophages in seeking out and enveloping salmon *Sphaerospora* stages seen at both the light and TEM level are in agreement with the observations of Lom & Dykova (1992d), who regarded phagocytosis as a very important and common host control mechanism against myxosporean infections in general.

The finding of raised numbers of eosinophilic granular cells (EGCs) was also consistent in all infections with salmon *Sphaerospora* and was particularly marked in heavily infected fish. The role of eosinophils in mammals lies in the modulation of allergenic and inflammatory reactions, and in the destruction of larger parasites.

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However these are not homologous to fish EGCs and the role of EGC's in fish is not fully understood (Roberts, 1989). Some evidence exists that a closer homologue might be to mammalian mast cells (Ellis, 1985 -cited by Roberts, 1989).

Host leucocytes were found attached to many larger, non-engulfed salmon *Sphaerospora* extrasporogonic stages in all infections. Some such parasites showed markedly degenerative changes in their cytoplasm and membranes, and so it seems likely that these host cells have an active role in parasite killing. Evidence for the presence of a specific as well as a non-specific response to infection with salmon *Sphaerospora* occur is provided by the subsequent protection against reinfection shown by fish previously exposed to the salmon *Sphaerospora* (chapter 3).

Glomerular infections of *Sphaerospora* spp. have been reported for *S. ohlmacheri*, *S. elegans*, *S. epinephali*, *S. renicola*, *S. truttae*, and *S. colomani* (Desser *et al*, 1986; Feist *et al*, 1991; Supammattaya, 1991; Bond, 1938; Fischer-Scherl *et al*, 1986; Baska, 1990). Supamattaya (1991) demonstrated parasite stages in the glomerular capillaries and Bowman's space and presumed this was the route via which extrasporogonic stages reached the tubular lumen to undergo sporogony. This appears to be the case for the salmon *Sphaerospora* extrasporogonic stages (chapter 2), and if, as is commonly held, all *Sphaerospora* spp have extrasporogonic stages, it may be that many species of the genus reach the tubules via this route. This could help to explain why a degree of glomerular pathology appears to be a common feature of *Sphaerospora* infections. Glomerular damage, similar to that shown by *Sphaerospora* in salmon has been reported to be associated with *S. epinephali* in *Epinephalus malabaricus*,

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Sphaerospora sp in *Sparus aurata*, *S. truttae* in *Salmon trutta* and *S. cristata* in *Lota lota* (Supamattaya, 1991; Sitja-Bobadilla *et al*, 1992b; El-Matbouli & Hoffmann, 1992) respectively. The disruption of infected glomeruli might be expected to have some effect upon the excretory function of the kidney, however El-Matbouli & Hoffmann (1992) found that even where glomeruli were severely enlarged and damaged no clinical signs or losses were experienced. The effects of the damage to glomeruli caused by transitional extrasporogonic/sporogonic forms of the salmon *Sphaerospora* in their host are therefore difficult to assess. Athanassopoulou & Sommerville (1993b) quantified glomerular lesions caused by *Myxidium* spp. in roach using image analysis and it may be that further studies could attempt to relate clinical signs and mortality levels to determined degrees of pathology.

The possibility that *Sphaerospora* stages may also reach the tubules by penetrating between adjacent tubular epithelial cells (chapter 2) indicated another route of entry. This may be one of the mechanisms which result in pathological changes in the tubular epithelium. In some instances the penetrating parasite stage became surrounded by leucocytes whilst still between the tubular epithelial cells. This route of entry to the tubule lumen has been shown to be predominant in PKX infections (Kent & Hedrick, 1986), but no such host response was described.

The comparative lack of pathology associated with sporogonic stages in the tubules has been described as a 'typical and consistent' feature of coelozoic *Sphaerospora* spp. (Hedrick *et al*, 1990). The slight dilation of heavily infected tubules and the flattening of their epithelial cells seen in salmon *Sphaerospora* infections has been similarly shown for *S. renicola* and *S. colomani* sporogonic stages (Dykova &

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Lom, 1988b; Baska, 1990). The complete blockage and occlusion of some infected tubules as was seen in the present study is also a common finding for *Sphaerospora* spp. Molnar (1980a) reported that myxosporean parasites in the lumen might be responsible for the uptake of excreted materials which would later be reabsorbed, and thus deprive the fish of some nutrients. He also considered that such stages in *S. renicola* infections of carp obstructed the tubules, thereby promoting the presence of hyaline cylinders in epithelial cells, and were thus responsible for nephrosis. Hyaline droplets were also found in the cytoplasm of bullfrog tadpole epithelial cells forming tubules infected with *S. ohlmacheri* by Desser *et al* (1986), as well as in the epithelial cells of the salmon kidney tubules in the present study. Hyaline droplets represent protein reabsorbed from the glomerular filtrate, and in higher animals are suggestive of glomerular lesions (Roberts, 1989). They are a common finding in both wild and cultured fish but their relationship to lesions is unclear (Roberts, 1989). However hyaline droplet formation appears to be a common feature of myxosporean infections.

Vacuolation of epithelial cells forming the walls of infected tubules was observed by Desser *et al* (1983a) in *S. renicola*, where it was considered to be evidence of impaired function. Such vacuolation was common in the epithelial cells of salmon in the present study and has been shown in brown trout infected by *S. truttae* and in *S. epinephali* infections of grouper (El-Matbouli *et al*, 1992b; Supamattaya *et al*, 1991). In the latter case, the parasite was responsible for extremely extensive vacuolation of a far greater extent than in other species, including the salmon *Sphaerospora*.

Other degenerative and dystrophic changes in the tubular epithelium of salmon infected by *Sphaerospora* sp, such as pyknotic nuclei and the dislocation of cells from the basement epithelium have been shown in *S. truttae* by Lom & Dykova (1989) and

Walter *et al*. (1991), and in *S. ohlmacheri* infections (Dykova *et al*, 1986).

However, it is evident that pathology due to the sporogonic stages of salmon *Sphaerospora* is of less importance in terms of the health of the salmon than that for which the extrasporogonic stages and the transitional glomerular stages are responsible. At no farms were sporogonic stages considered to be responsible for losses or morbidity. Their role as stressing agents, lowering host resistance to adverse environmental conditions, secondary infections or handling stresses cannot entirely be ruled out but farm managers report no unexpectedly high losses with which the parasite could be associated.

Peak morbidity and mortality levels associated with *Sphaerospora* in farm populations of Atlantic salmon occurred in July and August. These levels were directly related to the epidemiological peaks of prevalence and intensity of the extrasporogonic stages of the parasite at this time (see chapter 4). The extrasporogonic stage, and the transitional phase in the life cycle when these stages disrupt glomeruli in order to enter their sporogonic phase in the kidney tubule lumen therefore appears responsible for the pathogenicity associated with salmon *Sphaerospora*. Later, wholly sporogonic stages in the kidney tubules appeared to show a limited degree of pathology.

At farms A and C, where mean infection intensities were high, farm managers held salmon *Sphaerospora* to be directly responsible for mortalities, particularly in heavily infected fish. The finding in this study, that high levels of infection in individual fish resulted in gross signs of anaemia and kidney swelling, and histopathological changes supports this, as does the increased prevalence of heavily infected fish amongst dead and moribund individuals. It also suggests that high level

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infections might occur more commonly within the population than would be assumed from epidemiological data (see chapter 4), because many of the most heavily infected fish may die. The presence of ascites in heavily infected fish is held to be indicative of osmoregulatory imbalance which could be a result of impaired kidney function, and so it is proposed that anaemia and osmoregulatory disturbance might be the cause of death in such fish.

At all farms, however, it may be that the parasite's main pathogenic role lies in reducing the ability of individuals to respond to other stresses. The summer period when the more pathogenic stages of the salmon *Sphaerospora* exhibit epidemiological peaks in prevalence and intensity, and when farm managers consider the parasite most problematic, coincides with numerous other management problems which enhance stresses within the stock. These include the highest water temperatures of the year, low levels of dissolved oxygen and water shortages. At farm A for instance, water is often partially or wholly recirculated for periods of time during the summer months. Thus a combination of factors, the *Sphaerospora* extrasporogonic stages being one, may act at this time to decrease the overall health status of the fish. Further stresses associated with grading, netting and tank transfers might exacerbate the problem, and lead to elevated mortalies. Such decline in fish condition also favours infection by secondary invaders. It has been reported that tanks of fish with consistently heavy *Sphaerospora* infections are most vulnerable to furunculosis outbreaks at certain farms (A. Adrian, Biologist). Although this does not necessarily suggest cause and effect (since some factor associated with such tanks must predispose them towards higher levels of infection with the salmon *Sphaerospora* in the first place), general observations indicate that *Sphaerospora* infection results in a greater susceptibility. Further work to establish

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for certain such a role would involve correlating mortality of individual fish in a single tank with their infection intensity. Lower levels of PKX infections also result in mortalities as a result of stresses such as handling and grading (Lom and Dykova 1992d, Kent, 1992). PKD has therefore been described as a chronic disease made acute by further stresses (Clifton Hadley *et al.*, 1984). However, evidence exists that some sub-clinical, low level PKX infections do not predispose fish to secondary infections unless severe renal lesions are present, but rather elicit a stimulation of non-specific responses. These might even increase resistance to bacterial pathogens. Such fish are considered not to display a stress response (Foott & Hedrick, 1990).

Although no data was collected, other, hidden losses such as drops in net weight gain may compound the problems associated with the salmon *Sphaerspora*. Such losses are considered a major economic impact of PKD infection, but are difficult to assess (Clifton-Hadley *et al*, 1984). It may be that fish harbouring low and medium level *Sphaerospora* infections, which do not result in mortality, either direct or secondarily/stress induced, may be affected by such problems.

The comparison between the pathology exhibited by PKX and the salmon *Sphaerospora* extrasporogonic stages is of particular interest. The gross clinical signs, post-mortem appearance of the tissues, and the drop in stress tolerance of PKX infections (Clifton-Hadley *et al*, 1984) are very similar to those exhibited by heavily *Sphaerospora*-infected salmon. The attachment of leucocytes to the salmon *Sphaerospora* extrasporogonic stages is also exhibited during PKX infections, but the whorling of inflammatory epithelioid cells around PKX cells (Ferguson & Needham, 1978; Roberts, 1989) appears not to occur to salmon *Sphaerospora* extrasporogonic stages reflecting the difference in the magnitude of the response. A similar whorling

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reaction was seen with the PGD agent (extrasporogonic *S. ictaluri* stage) in channel catfish gills (Hedrick *et al*, 1990). In addition, the marked haematopoitic proliferation and tubular atrophy seen in kidneys affected by PKX was not seen in the present study. A degree of similarity in clinical signs, pathology and host response could be expected, since both parasites are myxosporean extrasporogonic stages infecting the same tissue site in a salmonid host. However marked clinical signs were only shown by the most intensely infected fish harbouring the salmon *Sphaerospora*. Fish with medium to low level infections showed very little pathology compared to infections of PKX of a similar level. Although the pathology associated with PKX is probably reflective of the abnormal parasite-host relationship proposed for this parasite, it is clear that for the same degree of kidney pathology to be induced, a very much higher intensity of parasitism is necessary in the case of salmon *Sphaerospora* infections than PKX. As a result overall mortality levels due to the salmon parasite are much lower than those of equivalent PKX infections.

Chapter 6
Alternate host studies

6   ALTERNATE HOST STUDIES

6.1   INTRODUCTION

The life stages of myxosporean parasites outwith the fish host have been a subject of speculation and controversy for many years. Until recently, it was generally believed that the life cycle was direct involving only a single, usually fish, host. However, there existed a great deal of uncertainty amongst workers in the field as to the means by which fish to fish transmission occurred.

Experiments confirming a direct life cycle were reported in very early studies as a result of the oral introduction of spores to fish (Thélohan, 1895; Doflein, 1898, 1899, 1909; Auerbach, 1910, 1912; Kudo, 1922 (cited by Shulman, 1966) Erdmann, 1912; Shiba, 1934 (cited by Walliker, 1968)). Such early work has rarely been repeated, with critical reviewing indicating that adequate controls and experimental procedures may not have been employed (Mitchell, 1977). Lom & Dykova (1992a) referred to a number of more recent "...trustworthy reports on direct transmission...", but if such a means of transmission is correct it is startling how very few they number. The most long standing and often quoted results are those of Uspenskaya (1978; pers. comm. to Walliker, 1966; pers. comm. to a range of scientists from the 1950's onwards) who claimed to have transmitted *Myxobolus cerebralis* to fish following the maintainance of mature spores in running spring water for three to four months. Following this 'ageing' period, spores introduced into the stomachs of trout resulted in *M. cerebralis* infections. However Uspenskaya (1992) has now questioned the security of the methodology and controls she employed. Prihoda (1983) achieved direct transmissions, whilst Johnson (1978) reported such for *Ceratomyxa* sp. in unpublished data communicated to Lom & Dykova (1992c).

Odening *et al.* (1989) successfully infected common carp with *Sphaerospora renicola* by the feeding of mature spores.

The majority of later workers have had little success when attempting direct infections of fish with mature spores (Schafer, 1968; Walliker, 1968; Wyatt, 1978; Molnar, 1979a, 1979b; Seenappa & Manohar, 1987), despite in some cases feeding tens of thousands of fully mature spores to naive fish by a variety of routes (Molnar, 1979a).

Hoffman & Putz (1969) and Taylor & Lott (1978), although unable to repeat exactly the experiments of Uspenskaya, transmitted *M. cerebralis* following a four month ageing period in pond mud. However, the former authors expressed a lack of understanding as to what factors '...alive or dead, in water or mud...' caused the change in spore infectivity, and did not rule out the need for other biotic factors for successful transmission. Although they stopped short of proposing that an alternate host might be required, such an idea had been put forward in the past (Schafer, 1968; Walliker, 1968).

In some cases a non-oral means of infection appeared to exist, so precluding the idea of infection by ingestion of aged spores. Hoffman & Putz (1971) infected pre-feeding rainbow trout sac fry by exposure to infective waters harbouring *M. cerebralis*, whilst Molnar (1979a) noted that carp initially became infected with gill sphaerosporosis at an early age whilst planktonic feeders, prior to their switch to benthic feeding.

6.1.1   Alternate hosts

<u>History and corroboration</u>

Markiw & Wolf (1983) demonstrated experimentally that a tubificid oligochaete alternate host was required to complete the life cycle of *M. cerebralis*. Wolf & Markiw

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(1984) identified that the stage infective to fish produced in, and released by, the worm was a spore of the genus *Triactinomyxon*, class Actinosporea (Myxozoa), and named it *Triactinomyxon gyrosalmo*. The worm host was later identified as *Tubifex tubifex* (Wolf, Markiw & Hiltunen, 1986) and the dynamics of the experimental production of *Triactinomyxon* spores in *T. tubifex*, from *M. cerebralis* spore ingestion to actinosporean release, outlined by Markiw (1986). Actinosporeans were first released after 104-113 days at 12.5°C, with peak release correlating almost exactly with the 3-4 month period previously attributed to myxosporean spore 'ageing'. Markiw (1989b) showed that actinosporean stages of the *M. cerebralis* life cycle cross-reacted in a fluorescent antibody test with the myxosporean stage spores. Such cross-reactions are very rare for specific antisera to myxosporean parasites.

However, Hamilton & Canning (1987) were unable to demonstrate changes in the prevalence of *Triactinomyxon* sp on the addition of *M. cerebralis* spores to *T. tubifex*, and the specific *Triactinomyxon* sp was absent from three of four farms recently diagnosed with whirling disease, yet present in an environment where worms could not have been exposed to *M. cerebralis*.

Lom (1987) reported misgivings, as did much of the scientific community at the time, not simply because such a life cycle was unprecendented, but also because the number of myxosporean species described vastly outnumbered those of actinosporeans. Hamilton & Canning (1987) quoted just 30 actinosporean species descriptions.

However, El-Matbouli & Hoffmann (1989) succeeded in reproducing the entire *M. cerebralis* life cycle by seeding tubifex worms with myxosporean spores, and further, demonstrated a similar life cycle for *Myxobolus cotti*. Triactinomyxons from worms infected by *M. cotti* spores were morphologically distinct from those generated by *M.*

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cerebralis. Ten myxosporean species of a range of freshwater fish have since been transmitted via oligochaete alternate hosts. Oligochaete families involved include the Tubificidae, Naidae and Lumbriculidae. In all instances morphologically distinct actinospores have been produced from the respective oligochaetes. Actinosporeans shown to be alternate life cycle stages of one or more myxosporean species include those of the genera *Triactinomyxon, Hexactinomyxon, Raabeia* and *Aurantiactinomyxon* (see table 6.1).

Four further species appear to be nearing confirmation of two host life cycles: In conference presentations, Hedrick , Wishkovsky, Groff & McDowell (1989) described the transmission of *Henneguya* sp and *Myxobolus* sp. to salmonids; Bartholomew, Fryer & Rohovec (1992) identified a possible alternate host, *Nais bretscheri* (Naidae), for the pathogenic myxosporean *Ceratomyxa shasta*, which released an actinosporean of the genus *Aurantiactinomyxon*; and Hedrick, Monge & Kinkelin (1992) found an oligochaete worm, *Stylaria lacustris*, in a sediment fraction infective for PKX, and established that a less than 50μm fraction passing all filters, was also infective. This 50μm fraction probably contains the actinosporean stage. El-Matbouli, Hoffmann & Fischer-Scherl (1992c) found two actinosporean species of a diameter of less than 30μm at PKD infected farms in Germany, which would obviously pass such a filter, but were unable to establish if either were the infective stage of PKX.

Actinosporean biology

The development of actinosporeans at the light microscope level was outlined by Janizewska (1955, 1957), and by El-Matbouli, Hoffmann & Fischer-Scherl (1992c). A classification scheme for actinosporeans is given in below (Janiszeweska, 1957; Levine

et al, 1980).

Class   **ACTINOSPOREA** Noble, 1980

sub class   **Actinomyxia Stolc, 1899**

      Order Actinomyxida Lom, 1980

           Family   **1. Tetractinomyxidae**

                            Tetractinomyxon spp

                  2. Sphaeractinomyxidae

                            Sphaeractinomyxon spp

                            Neoactinomyxon spp

                3. Triactinomyxidae

                    sub-family Triactinomyxinae

                            Triactiomyxon spp

                            Guyenotia spp

                            Raabeia spp

                            Echinactinomyxon spp

                            Aurantiactinomyxon spp

                  sub-family Siedleckiellinae

                            Synactinomyxon spp

                            Siedleckiella spp

                            Antonactinomyxon spp

                4. Polyactinomyxidae

                            Hexactinmyxidae spp

Mature actinosporean spores are composed of two envelopes; an endospore of one or two cells, and an epispore of six cells. Three epispore cells form the three apical polar capsules of the mature spore, and three the projections and anchors of the spore; the shape, size and form of these are generic characters. The endospore contains the sporoplasm; a syncitium containing many nuclei, the number of which is an additional species characteristic (fig.6.1).

In most actinosporean species, development occurs in the epithelial cells of the gut wall of the oligochaete. However, some species develop in the coelomic cavity. Although this distinction is generally a species charcteristic, some species develop in both sites (Janiszewska, 1955). Coelomic species reach the water following the death and subsequent putrefication of the worm, (Granata, 1924; quoted by Janiszewska, 1955), or amputation of part of the host.

The epispore floats of released actinosporeans swell as the envelope cells take in water. The spores are then able to float freely. *Triactinomyxon ignotum*, *T. magnum*, *T. dubium* and an unidentified actinosporean sp. have, for example, been reported from plankton samples (Kofoid, 1908; Doflien, 1916 - cited by Janiszewska, 1955, 1957; Dresscher & Gispen van der Weg, 1958 - cited by Marques, 1984).

El-Matbouli & Hoffmann (1991b) successfully achieved diheteroxenous transmission with fresh myxosporean spores and those that had undergone a wide variety of treatments; these included five month ageing in sterile mud, freezing at -20°C for two months, and passage through the gut of pike, *Esox lucius* and mallard, *Anas platyrhynchus*. According to El-Matbouli *et al*. (1992c) *M. cerebralis* myxospores on

fig.6.1   Generalised actinosporean spore; 1, polar capsules; 2, endospore cavity; 3, sporoplasm; 4, style; 5, epispore floats
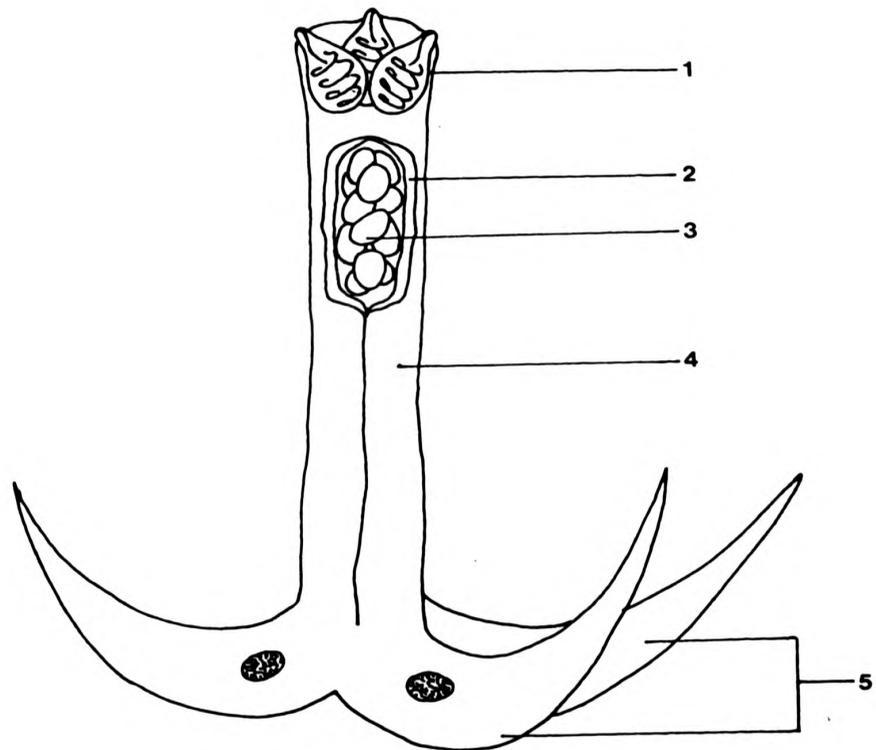
ingestion everted their polar filaments to attach to the oligochaete gut epithelial wall. The spores then hatched along their suture line and the sporoplasm penetrated the gut epithelial cell. There, a vegetative multiplication of the sporoplasm took place, with daughter cells penetrating adjacent epithelial cells until every epithelial cell in 4 to 6 segments of the worm was infected.

In a single epithelial cell, two somatic parasite cells encircled a single propagative cell. Janiszewska (1955) found two haploid propagative cells, one small, alpha, male cell and one large, beta, female cell. Each cell type then divided until there were eight alpha and eight beta cells. Alpha and beta gametes fused to form eight sporoblasts or zygotes, each of which divided and differentiated to produce a single spore. The result was a sporocyst containing eight spores packed together with their epispore processes "telescoped" together awaiting release. (fig.6.2).

Few ultrastructural studies of actinosporean development are available. Marques (1984) included some information in a doctoral thesis on the biology of the group, and later (Marques, 1987) gave an account of the fine structure of *Neoactinomyxon eiseniellae*, whilst Marques & Ormieres (1982) described *Aurantiactinomyxon eiseniellae*. Lom & Dykova (1992c) gave a comprehensive description of sporogenesis in *Triactinomyxon legeri*, a parasite of *Tubifex tubifex*, in which they compared actinosporean and myxosporean development.

Infected worms often show discolouration, opaque areas and/or a degree of swelling, making it possible to rapidly and accurately identify infected worms within a population (Wolf *et al.*, 1986; El-Matbouli *et al.*, 1992c; Lom & Dykova, 1992p; El-Matbouli & Hoffmann, 1993). However, some reports have found no such diagnostic

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table 6.1 · Reports of myxosporeans with two host life cycles

MYXOSPOREAN SPECIES	FISH SPECIES	ACTINOSPOREAN SPECIES	OLIGOCHAETE SPECIES	AUTHORS
Myxobolus cerebralis (whirling disease)	rainbow trout *Oncorhynchus mykiss* (other salmonids)	*Triactinomyxon* sp	*Tubifex tubifex* (Tubificidae)	Markiw & Wolf, (1983); El-Matbouli & Hoffmann, (1989, 1991)
Myxobolus cotti	bullhead *Cottus gobio*	*Triactinomyxon* sp	*Tubifex tubifex* (Tubificidae)	El-Matbouli & Hoffmann, (1989)
PGD (poss *S. ictaluri*)	channel catfish *Ictalurus punctatus*	*Aurantiactinomyxon* sp	*Dero digitata* (Naidae)	Styer, Harrison & Burtle, (1991)
Myxobolus pavlovskii	silver carp *Hypophthalmichthys molitrix*	*Hexactinomyxon* sp	*Tubifex tubifex* (Tubificidae)	Ruidisch, El-Matbouli & Hoffmann, (1991)
Myxobolus sp (*Zschokkella* sp *Thelohanellus* sp)	goldfish *Carassius auratus*	*Raabeia* sp (*Echinactinomyxon* sp *Aurantiactinomyxon* sp *Neoactinomyxon* sp)	*Branchiura sowerbyi* (Tubificidae)	Yokoyama, Ogawa & Wakabayashi, (1991)
Hoferellus cyprini	common carp *Cyprinus carpio*	poss *Aurantiactinomyxon* sp or *Guyenotia* sp	*Nais* sp (Naidae)	Grossheider & Korting (1992)
Myxobolus arcticus	sockeye salmon *Oncorhynchus nerka*	*Triactinomyxon* sp	*Stylodrilus heringianus* (Lumbriculidae)	Kent, Whitaker & Margolis, (1991, 1992, 1993)
Hoferellus carassii (kidney enlargement disease)	goldfish *Carassius auratus*	*Aurantiactinomyxon* sp	*Tubifex tubifex* 90% *Tubifex ignotus* 5% *Limnodrilus hoffmeisteri* 5% (Tubificidae)	El-Matbouli, Fischer-Scherl & Hoffmann, (1992)
Myxidium giardi	eel *Anguilla anguilla*	*Aurantiactinomyxon* sp	*Tubifex* sp (Tubificidae)	Benajiba & Maques, (1992)
Myxobolus carassii	golden orfe *Leuciscus idus* (other cyprinids)	*Triactinomyxon* sp	*Tubifex tubifex* (Tubificidae)	El-Matbouli & Hoffmann, (1993)

signs (Yokoyama, Ogawa & Wakabayashi, 1991).

The time taken for development from myxosporean ingestion to actinospore release varies according to species and temperature (table 6.2). Mature actinospores can be detected for a number of days prior to their first release by squashing or squeezing infected worms (El-Matbouli & Hoffmann, 1989). Markiw (1986) reported that release continued for up to one year, El-Matbouli *et al.* (1992a) for up to three months. Yokoyama, Ogawa & Wakabayashi (1992; 1993a) demonstrated circadial rhythm patterns of spore release from individual worms, with peaks between 10pm and 4am at 20°C. Release times could be manipulated by adjusting photoperiod. It was suggested that host factors and behaviour might be at least partly reponsible for this phenomenon.

Yokoyama *et al.* (1992) showed that actinospore longevity upon release was species and temperature dependent. On average spores remained intact in terms of sporoplasm presence for two to four weeks, less at higher temperatures. However, Markiw (1992b) demonstrated that although vital fluorescine diacetate dye indicated 50% viability in a four day old population of *Triactinomyxon* sp. at 12°C, they were unable to infect fish. The same number of three day old spores produced a light *M. cerebralis* infection; one and two day old spores resulted in high level infections. It therefore seems that, though seemingly intact for a considerable length of time, actinosporeans have a very limited period of time in which to encounter a suitable fish host upon their release if they are to succesfully establish an infection.

Markiw (1991) showed that rainbow trout fry exposed at only two days old became infected with *M. cerebralis* on exposure to spores of *Triactinomyxon* sp. One day old fry and eggs however, were not. Markiw (1992a) showed a dose response to actinospores in terms of the level of the resulting infection. Above doses of 100

fig.6.2   Outline of actinosporean development:(bar=10μm)

1   Binucleate stage
2   Two cells as a result of division of binucleate cell
3   One cell forms alpha-gametocyte and an encircling cell, the other the beta-gametocyte and an encircling cell
4   Encircled gametocyte
5   Alpha- and beta-gametocytes divide until there are eight of each
6   Alpha- and beta-gametocytes fuse to produce eight sporoblasts
7   Each pansporoblast divides and differentiates to produce eight spores

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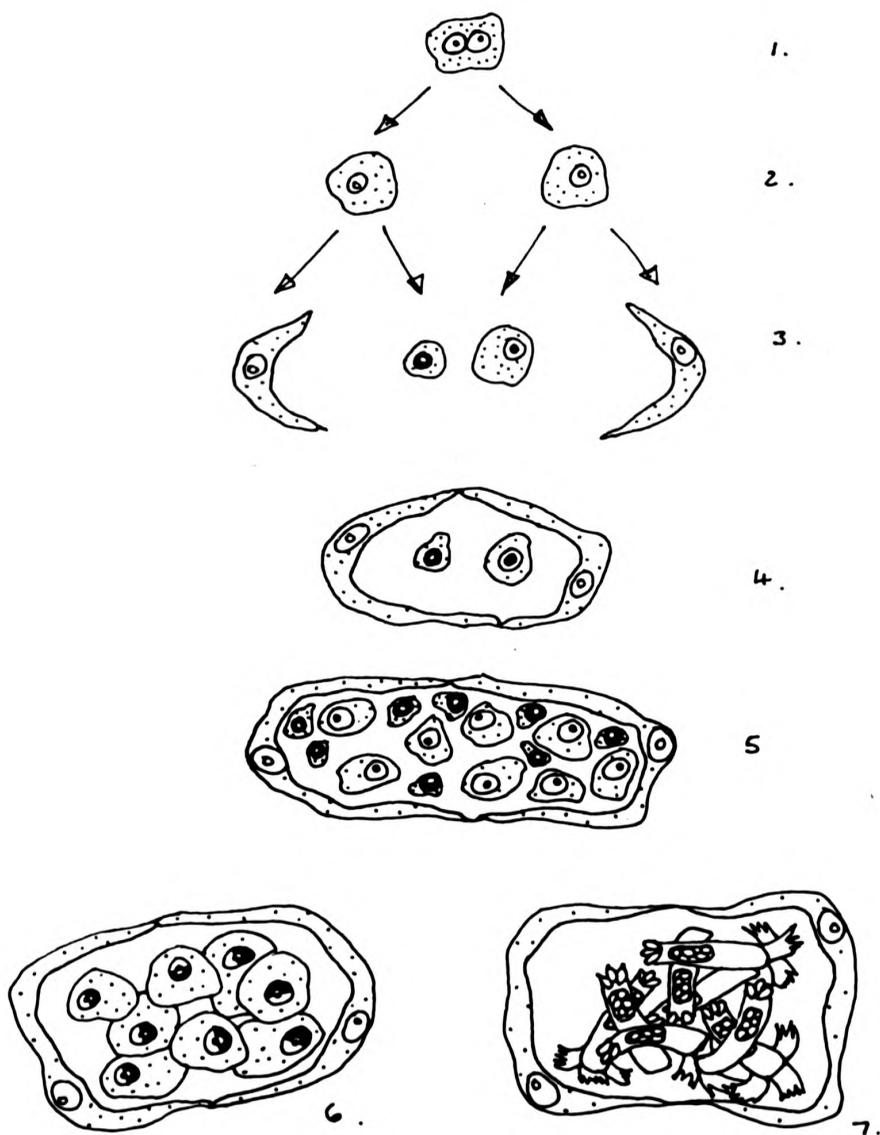
table 6.2 Time taken for actinosporean development and release

TRANSITION	TIME (days)	TEMPERATURE (°C)	AUTHORS
M. cerebralis to *Triactinomyxon* sp	104	12.5	Markiw, (1986)
M. cerebralis to *Triactinomyxon* sp	94	16-17	El-Matbouli & Hoffmann, (1989)
M. cotti to *Triactinomyxon* sp	125	16-17	El-Matbouli & Hoffmann, (1989)
H. carassi to *Aurantiactinomyxon* sp	90	-	El-Matbouli. Fischer-Scherl & Hoffmann. (1992)
M. giardi to *Aurantiactinomyxon* sp	77	-	Benajiba & Marques, (1993)
M. carassi to *Triactinomyxon* sp	91	13-14	El-Matbouli & Hoffmann, (1993)
M. pavlovskii to *Hexactinomyxon* sp	93	15-17	El-Matbouli, Fischer-Scherl & Hoffmann. (1992)

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actinospores, the number of mature myxosporean spores produced was linear according to actinosporean number, until a plateau at 100,000 actinospores per fish.

Markiw (1989b) demonstrated sporoplasms of the triactinomyxon stage in the epithelia of the fins, skin, gills, buccal cavity, oesophagus and digestive tract. This is interesting considering the widespread assumption prior to Markiw's study that infections occured orally. After a five minute exposure to rainbow trout, 60% of actinosporeans recovered from the infection tanks were "empty" of their sporoplasms and had everted their polar filaments. The sporoplasms present in fish infected with very early stages of *M. cerebralis* resembled the unidentified protozoan described by Daniels *et al.* (1976) from the epithelium of rainbow trout from waters infective for whirling disease. El-Matbouli *et al.* (1992c) showed similar routes of entry for sporoplasms of *M. cerebralis*, with a ratio of 50:50 intact to empty actinospores after a 30 minute exposure. Sporoplasms penetrated the epithelium within five minutes, and began dividing within two hours. They then could no longer be detected until trophozoites first appeared in the head cartilage at day 40. Mature myxospores of *M. cerebralis* were found by day 90.

In other reports, the first myxospores were found on day 48 (*H. cyprini*, Großheider & Körting, 1992) and at 130 days (*H. carassii*, El-Matbouli, Fischer-Scherl & Hoffmann, 1992a). Although many authors give the date of first presence of myxosporean spores, this is often the day all fish were sacrificed, rather than the first signs of myxosporean presence from regular sub-sampling of infected stock.

A few studies have looked at the stimuli required to cause the eversion of the

actinospores, the number of mature myxosporean spores produced was linear according to actinosporean number, until a plateau at 100,000 actinospores per fish.

Markiw (1989b) demonstrated sporoplasms of the triactinomyxon stage in the epithelia of the fins, skin, gills, buccal cavity, oesophagus and digestive tract. This is interesting considering the widespread assumption prior to Markiw's study that infections occured orally. After a five minute exposure to rainbow trout, 60% of actinosporeans recovered from the infection tanks were "empty" of their sporoplasms and had everted their polar filaments. The sporoplasms present in fish infected with very early stages of *M. cerebralis* resembled the unidentified protozoan described by Daniels *et al.* (1976) from the epithelium of rainbow trout from waters infective for whirling disease. El-Matbouli *et al.* (1992c) showed similar routes of entry for sporoplasms of *M. cerebralis*, with a ratio of 50:50 intact to empty actinospores after a 30 minute exposure. Sporoplasms penetrated the epithelium within five minutes, and began dividing within two hours. They then could no longer be detected until trophozoites first appeared in the head cartilage at day 40. Mature myxospores of *M. cerebralis* were found by day 90.

In other reports, the first myxospores were found on day 48 (*H. cyprini*, Grossheider & Körting, 1992) and at 130 days (*H. carassii*, El-Matbouli, Fischer-Scherl & Hoffmann, 1992a). Although many authors give the date of first presence of myxosporean spores, this is often the day all fish were sacrificed, rather than the first signs of myxosporean presence from regular sub-sampling of infected stock.

A few studies have looked at the stimuli required to cause the eversion of the

of the triactinomyxon stage of *M. cerebralis* of 2% at infected farms compared to experimentally induced figures of 20%. Hamilton and Canning (1987) found prevalences of five species of actinosporeans in all worms studied from UK samples of between 0.25% and 4.75%. However, only *Echinactinomyxon radiatum* had a prevalence above 1%, whilst *Triactiomyxon dubium* and *Aurantiactinomyxon* sp were at a level of only 0.25%.

This indicates a distinction between experimental systems where levels of up to 20% prevalence can be obtained, infected farm sites where levels may be 1% to 5%, and the wild environment, where levels are less than 0.5%. However, wild reports usually consist of surveys of the actinosporean population at a single point in time, it being possible that sampling at certain times of the year would increase the likelihood of actinosporean detection. Such seasonal variations in population prevalence have been reported by Yokoyama *et al.* (1992; 1992; 1993a), where worms from infected farms in Japan carried levels of *Raabeia* sp. infection of 0.1% to 4.1%, with lowest levels in the winter and the highest in the spring and summer.

Mixed infections of single worms are rare. This might be expected from the low chances of carrying even a single infection. Styer, Harrison & Burtle (1992b) reported mixed infections from *Dero digitata* in ponds stocked with channel catfish. Where two infections were present the two spore forms were always released at different times.

A mixed population of worms often harbour a number of genera and species of actinosporean. Styer *et al.* (1992c) reported three genera (six species) in a single pond; Hamilton & Canning (1987) four genera (five species) at one site and Yokoyama *et al.* (1991) three genera (three species) in one population of worms.

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6.1.2 Objectives

The objectives of this part of the current study were to investigate habitats in and around the farms at which Atlantic salmon became infected with salmon *Sphaerospora* for infected oligochaete worms and released actinosporean stages. Any infections and stages found were to be characterised, described and their biology studied. It was hoped that infection experiments using naive Atlantic salmon would enable the correct actinosporean stage and oligochaete host of the salmon *Sphaerospora* to be identified, and the life cycle to thus be completely established.

6.2 MATERIALS AND METHODS

6.2.1 Experimental infection of oligochaetes with *Sphaerospora* sp. spores from Atlantic salmon

<u>Source of uninfected worms</u>

Oligochaete worms were obtained from Polmaise Burn, near Cowie, Stirling by the methods outlined below for farm sampling. The burn suffers a high level of pollution as a result of nearby farming and engineering works. As a result it has been completely devoid of fish for a number of years (Forth River Purification Board, pers. comm., 1992), but supports large populations of oligochaetes, a subsample of 10 of which indicated that they were all *Tubifex tubifex*. Worms from Polmaise Burn were maintained in four tubs of 15cm diameter with a substrate of autoclaved mud of depth 2cm. 250 worms were assigned to each pot, and were maintained in dechlorinated tap-water. A complete water change was undertaken every other day, with the run off water being poured through a 20μm filter to retain any actinospores released; the material

trapped on the filter was resuspended in 4ml of dechlorinated tap water and examined under phase contrast at x250 magnification for the presence of actinosporean spores. After ten days of scanning to establish that no actinosporean infection could be detected, each of the tanks were treated as follows:

Tank 1 No treatment

Tank 2 Kidney homogenate from uninfected fish

Tank 3 Kidney homogenate from infected fish with many mature sphaerospores

Tank 4 As tank 3

All tanks were left for four days to enable the added homogenates to settle before water changes were resumed, run off waters again being continually monitored for actinosporean spore release under phase contrast microscopy at x250 magnification. The worms were maintained under the above conditions for six months, outdoors, at ambient temperatures and normal photoperiod. At three months, 50 worms from each tank were isolated individually, oneper well, in cell wells on 48-well plates, each containing 1ml of dechlorinated tap water according to the method of Yokoyama *et al.* (1991). After each well had been monitored for actinosporean release for three days, the worms were examined by squash preparations under phase contrast. At six months all remaining worms were similarly isolated for three days before being sacrificed.

6.2.2 Survey of farms and surrounding areas for actinosporeans and oligochaetes
Survey of inlets and tanks

Actinosporeans and oligochaetes were searched for in the water supplies entering

infected farms. Farm inlet water supplies to empty tanks were filtered using 30μm filters for 4 hour periods to attempt to trap incoming actinosporean spores. The material trapped on the filters was removed and resuspended in a minimal volume of water whenever filters became blocked. The water supplying tanks was also filtered through a plankton net of 250μm mesh size for four hour periods to trap oligochaetes which may have by-passed farm filters.

Water in tanks holding fish was siphoned through 3/4 inch gauge plastic tubing into 30μm filters for four hour periods to detect incoming actinosporeans and oligochaetes. Water and sediments from the bottom of tanks holding fish was siphoned through plastic tubing, filtered through plankton net bags and treated to determine whether oligochaete fauna built up in tanks. Material and worms trapped on filters were resuspended in river water and aerated prior to transport to the lab. On return all suspensions from filters were subsampled onto clean slides and scanned thoroughly under phase contrast at x250 magnification for the presence of actinosporean spores.

Survey of habitats around infected farms

Worms were sampled from a range of habitats at and around the farms harbouring *Sphaerospora* infections. Sampling was concentrated in the period from March to June when epidemiological studies indicated that the infective stage might be being released into the environment (chapter 4).

Samples of worms were obtained by several methods. Kick samples were taken using a standard Freshwater Biological Association kick net of mesh size 50μm. Samples were not subject to any time limit. In addition to kicking, substrates and stones

were disturbed and rubbed over by hand to dislodge attached invertebrates. All invertebrates were transferred to plastic bags containing stream water and aerated, prior to the sorting of oligochaetes from other invertebrates in the laboratory. Softer sediments, and those from deeper areas of streams, rivers and other habitats, were sampled by means of corers and Peterson grabs. The material obtained from such methods was transferred to laced bags of single layered household muslin. Each bag was suspended in a bucket of fresh stream water and left for a period of at least one hour. By this method, oligochaetes were found to make their way in large numbers into the water in the buckets via holes in the muslin. The contents of the bag were then examined to remove any remaining oligochates before being discarded. Many oligochaetes were frequently to be found on the inner lining wall of the muslin bag, not yet having reached the water or too large to pass through the bag mesh. Worms obtained were transfered to fresh stream water in plastic bags and aerated prior to their sorting later in the laboratory. This method appeared superficially to be the most effective in terms of the number of worms found, than kick sampling. This method was used to sample areas where the sediment substrate was very soft and fine. The apparent difference can therefore be attributed to the fact that oligochaete worms of some genera, e.g. *Tubifex* spp, have a clear preference for these substrates, and were thus found in very large numbers under such conditions.

Rivers and streams providing the water supply to infected farms were sampled in mid-April, mid-May and Mid-June 1992 and in mid-May and mid-June 1993 (fig). Samples were taken at the inlet point for water supplies to the farms, 500m upstream and 1000m upstream. Care was taken to include as many representative microhabitats

as possible; for example soft and hard substrates, deep and shallow depths, and to use a variety of sampling methods. Tributary streams off the main water course were also sampled.

The enriched sediments of the settlement pond at farm C were sampled for worms on three occasions in 1993: 14.05.93; 15.06.93; and 23.06.93. Samples were mainly taken using grabs and corers due to its fine particulate nature.

6.2.3 Examination of worms for infection

All worms were studied using the cell-well technique of Yokoyama *et al.* (1991). Worms were randomly assigned to individual cell wells on 48 well plates each containing 2ml of dechlorinated tap water in groups of five. Plates were exposed to natural temperature and light conditions outdoors, and water in each well subsampled on a daily basis prior to its being changed. Each subsample was scanned thoroughly by phase contrast microscopy at x250 magnification for the presence of actinosporeans. Worms obtained from each given sampling occasion were monitored for parasite release for a one month period. Prior to being discarded, a sample of approximately 10% of worms was squashed and examined microscopically for actinosporean stages.

When released actinosporeans were found in a well, the five worms were isolated singly in wells and left for 24 hours. Following this period, water from each was subsampled to establish which worm(s) harboured infection.

6.2.4 Identification of worm species

A sample of fifty worms were identified to obtain details of the relative species composition of the fauna of the habitats studied. Worms were initially fixed in 70% alcohol. Later they were transferred to 30% alcohol and then to distilled water before being placed individually onto slides in a few drops of Amman's lactophenol, covered with a coverslip and left for several hours to clear (see Brinkhurst, 1963). Prior to examination, slight pressure was exerted on the coverslip to flatten the mount. Mounts were examined at appropriate magnification and worms identified using the key of Brinkhurst (1963).

6.2.5 Biological studies of oligochaetes harbouring actinosporean infection

Despite the numbers of worms studied (over 2,200), few were found to harbour actinosporean infection. Infected worms were therefore very much at a premium, some species of actinosporean being found only once in the sampling programme. The identification of host species, histology, transmission electron microscopy and gut smears involve destructive techniques which necessitate the death of the worm. This was often undesirable and so it was impossible to complete the entire range of biological studies for a given species.

Species morphology and identification

Any actinosporeans found were drawn and photographed under bright field, phase contrast and Nomarski interference contrast microscopy using the methodology and equipment described in chapter 2. The measurements taken are illustrated in fig.6.3. Measurements were taken from at least 15 randomly selected spores where possible,

using a micrometer calibrated eye-piece graticule or from enlarged photomicrographs of known magnification. Spores obviously dead or senescent, and those not yet fully mature were not used for measurements. Spores were identified using the keys and diagrams of Janiszewska, (1955, 1957), Marques, (1984) and by comparison with other published reports.

Prevalence data was calculated for each sample for each species of actinosporean found, as a percentage presence in the entire worm population, regardless of worm species. A prevalence for each species in each host was difficult to calculate: the identification of all worms studied was not feasible because of the numbers involved, whilst establishing the specific identity of each infected individual worm was not always possible, due to the worm's immaturity, nor desirable, due to the destructive nature of the identification technique.

Individual infected worms were squashed between a coverslip and a slide to reveal the location of infections within the worms and the nature of the pansporoblast. Infected worms were identified following clearing as described above.

Pattern of actinosporean release

Patterns of spore release were studied by two means. Two isolated worms harbouring *Aurantiactinomyxon* sp infection were monitored to study total numbers of actinosporean spores released per worm per day. The experiment ran for a six-day period under ambient temperature and light conditions, with each worm transferred daily to a fresh well containing 1ml of water. The volume of the previous days' water was

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fig.6.3   Measurements taken from actinosporean spores

Measurements: (a)   length and width of endospore
                 (b)   length and width of epispore floats
                 (c)   length and width of polar capsules
   (d)   length and width of style

Synactinomyxon sp 1

Synactinomyxon longicauda


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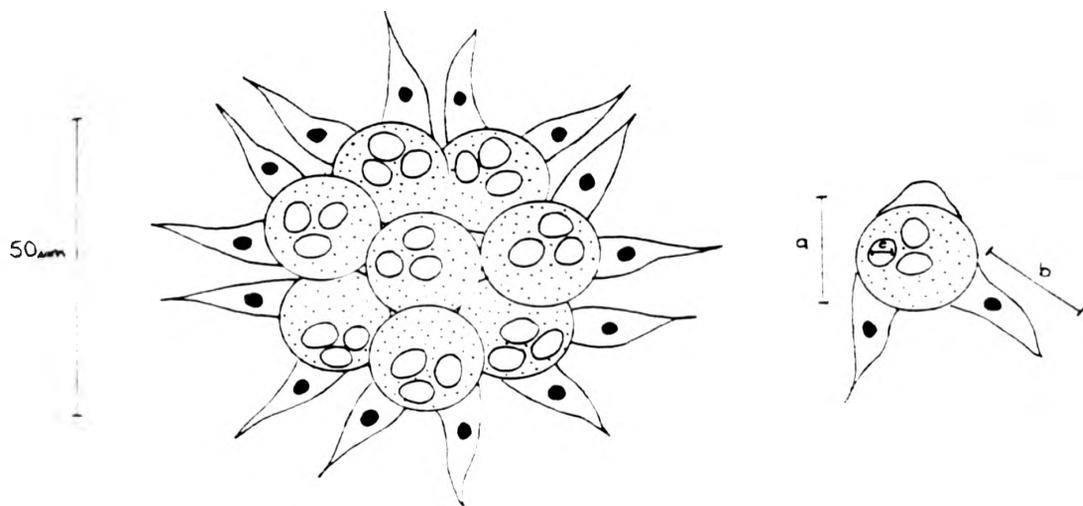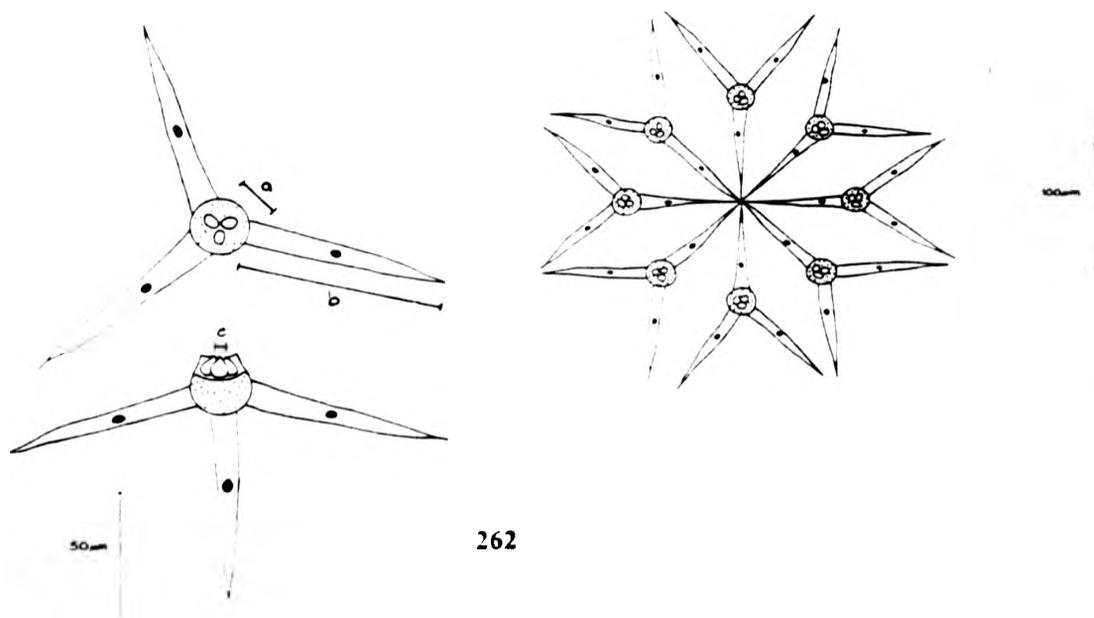
Raabeia sp
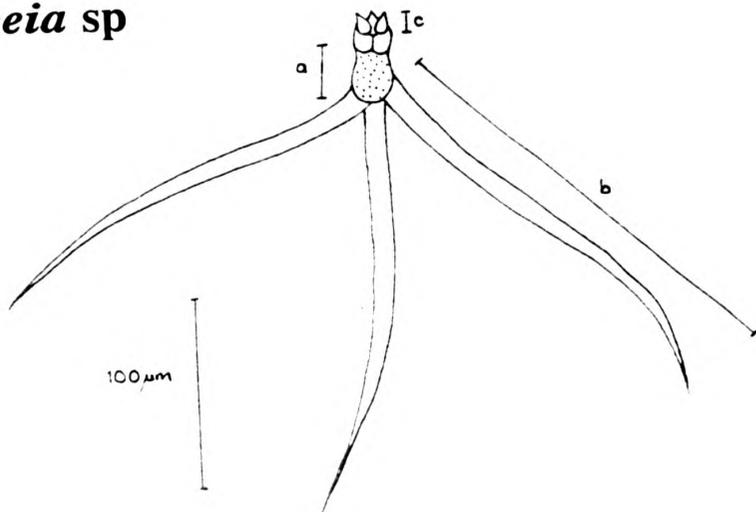
Triactinomyxon mrazeki
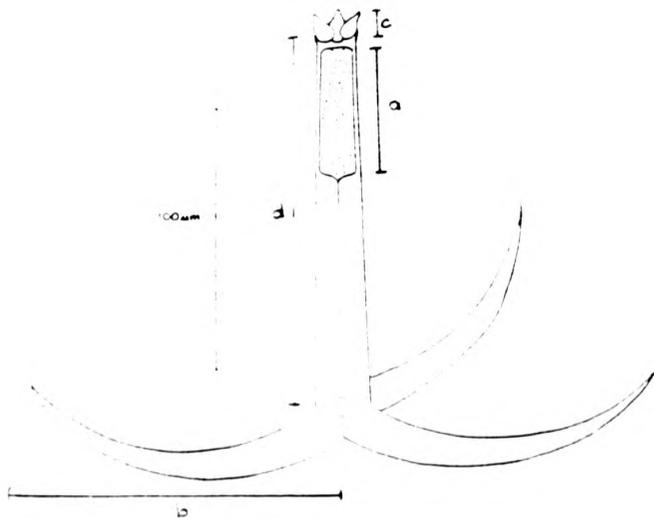
Aurantiactinomyxon sp

50μm
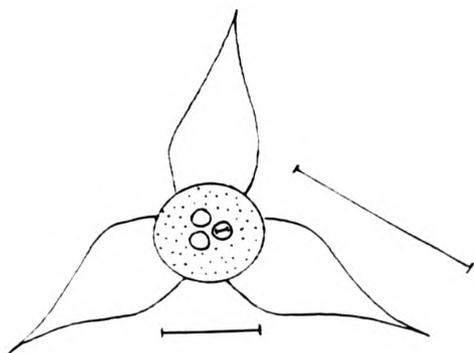
then measured and the number of actinosporeans in a subsample of known volume determined using a counting chamber. Five subsample readings were averaged to establish total daily release for each worm.

Patterns of circadian release were studied in a similar manner to that outlined above, with a single *Aurantiactinomyxon* infected worm being transferred into a clean cell well containing 1ml of water every two hours for a 48 hour period. The number of spores in five subsamples from each two hours' water was counted, averaged, and used to calculate the total actinosporean spore release into each 1ml two-hourly sample.

Stimuli for actinosporean polar filament release and spore hatching

The extrusion of the polar filaments of *Synactinomyxon* spp, *Aurantiactinomyxon* sp, and *Raaebia* sp was studied in response to the mucus of bream, brown trout and Atlantic salmon. A similar study using *Triactinomyxon* sp was not attempted due to the poor condition of the spores and the fact that they were only found on a single occassion. Water containing actinosporean spores was mixed with an equal volume of mucus obtained via skin scrapes from the fish species concerned, then placed under a coverslip and spores examined under phase contrast microscopy. Results were compared against control preparations of water containing spores from the same sample to which no mucus had been added.

Ultrastructure of *Aurantiactinomyxon* sp.

Infected worms were cut into small pieces which were immersed in Karnovsky's

fixative for 4-6 hours at 4°C. Following post-fixation in 0.5% osmic acid in cacodylate buffer for one hour, tissues were dehydrated in an acetone series and embedded in Araldite epoxy resin. Resulting blocks were cut at $1\mu m$ as semi-thin sections for histological purposes and stained with methylene blue. Where such preparations revealed the presence of actinosporean parasites, ultrathin sections were cut of relevant areas of interest, stained with uranyl acetate/lead citrate and examined in a Philips 301 transmission electron microscope at 60kV.

Water from a cell well into which many spores of the genus *Aurantiactinomyxon* had been released was syringed through an acetone resistant polyamide filter (Sartorius Ltd), pore size $0.45\mu m$, held in a plastic filter housing. The filter holding the spores was then fixed in 1% glutaraldehyde in sodium cacodylate buffer for one hour, then transferred for four hours to 3% buffered glutaraldehyde. Following washing for four hours in buffer, the filter was secondarily fixed in 1% buffered osmium for two hours, dehydrated in an acetone series and critical point dried. Pieces of filter were attached to aluminium stubs using araldite, and sputter coated with gold in an Edwards 150B sputter coater. The stubs were examined in a Philips 500 SEM for the presence of actinosporean spores.

6.2.6   Experimental infection of Atlantic salmon with *Aurantiactinomyxon* sp.

Aurantiactinomyxon sp. spores were harvested from two infected worms each day for one week and added to a static tank containing ten Atlantic salmon parr of ~15g from a farm, utilising bore hole water, that had previously been found to be uninfected with *Sphaerospora*. A second tank containing ten fish was prepared, to which no

actinosporeans were added. Fish were retained at ambient temperature.

6.3   RESULTS

6.3.1 Experimental infection of oligochaetes with *Sphaerospora* sp. spores from Atlantic salmon

No actinosporeans were found in the material filtered and subsequently examined from control tanks not exposed to Atlantic salmon *Sphaerospora* sp. spores, or those from experimental tanks to which many *Sphaerospora* spores had been seeded. Worms isolated, at three months and at six months, in individual cell wells showed no actinosporean release. Fresh squash preparations of worms at three and six months showed no signs of infection.

6.3.2 Survey of farms and surrounding areas for actinosporeans and oligochaetes

<u>Survey of inlets and tanks</u>

All 30μm filtrates from farm inlet waters, tanks and tank sediments were negative for the presence of actinosporean spores, but occasional chironomid larvae, stonefly and mayfly larvae and, rarely, occasional oligochaetes were found. These proved negative for actinosporean infections.

The filtering of siphoned sediments of occupied tanks recovered no invertebrates, the main constituents of the material retained on the filters being uneaten food and fish faeces.

<u>Survey of habitats around infected farms</u>

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Kick samples and sediment samples from farm river supplies yielded approximately 1,500 oligochaete worms in 1992 and 800 in 1993. In 1993, less effort was expended on river sampling, the study concentrating on the settlement tank environment at farm C. Worms were identified to family level, the majority being of the families Tubificidae and Naiidae. No significant difference was found in the composition of the oligochaete fauna with respect to distance from the farm inlet supplies. However tubificids were characteristic of slower flowing areas of soft substrate, whilst naiids were predominant in areas of stony/rocky substrate and faster flow. Of worms identified to species level, those of the tubificidae were mainly *T. tubifex*, whilst those of the naiidae were almost exclusively *Nais alpinus*. The relative proportions of the various oligochaete groups in the total worm population from all farms combined was approximately as follows:-

Order Lumbriculida

    Family Lumbriculidae   **5%**

Order Tubificida

    Family Tubificidae   **65%**

    Family Naiidae   **20%**

    Family Enchytraeidae   **10%**

Bias was probably introduced in the numbers of worms obtained however, since different sampling methods were used in stony habitats compared to soft sediment ones. The latter habitat yielded many more worms than the former.

Three collection trips (1993) to the settlement pond at farm C yielded numbers of oligochaete worms:-

14.05.93	720 worms
15.06.93	384 worms
23.06.93	1152 worms

The subsample of these 2,256 worms fixed for species identifications were identified to family, and in some cases generic and species level. The approximate composition of the population was:-

Tubificidae	~70%
Lumbriculidae	~25%
Naiidae	~ 0%
Enchytraeidae	~ 5%

Because a high proportion of worms were not fully mature, a precise specific identification was impossible in many cases (see Brinkhurst, 1963). Species identifications in the Oligochaeta is regarded as particularly specialised and difficult. Such identifications are rarely made on a routine basis, even by River Board personnel (Clyde River Board, pers. comm.). In many reports of two-host myxosporean life cycles worms have been sent to specialists prior to confirmation of specific identity. The help and expertise of Mr. Gresty is acknowledged in the present study.

The identification of 40 mature tubificid worms indicated that the population consisted of two species, *Tubifex tubifex* and *Limnodrilus hoffmeisteri*, in a proportion of approximately 5:1. A subsample of fifteen lumbriculids were identified as *Lumbriculus variegatus*.

6.3.3 Biological studies of oligochaetes harbouring actinosporean infection

Five species of actinosporean, of four genera, were detected from oligochaetes sampled in 1993. All five species were identified from worms from the settlement pond at farm C. One actinosporean, *Synactinomyxon* sp 1, was found in a single tubificid worm in a wild sample of May, 1993 from the river supplying the same farm. All infections were detected from worms held in cell wells. The identity of the worms is presented in table 6.3 together with their prevalence. Prevalence figures are given as: (i) prevalence of infected worms of any species sampled over the whole study; (ii) prevalence of infected worms of any species in single samples; (iii) approx. prevalence of infected worms in population of that worm species.

Species morphology and identification

All measurement data is expressed as a mean with high and low range, rather than a mean expressed with standard deviation as the former method is that used almost exclusively in the literature. Table 6.4 gives a summary of the measurement data. Although figures for extruded polar filament length are given, it is recognized that in many cases these do not fully evert, and their use in the taxonomy of myxosporean

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table 6.3   Actinosporean spp. prevalence in worm population as a whole, regardless of worm species.

ACTINOSPOREAN	NUMBER OF INFECTED WORMS FOUND (whole study)	% PREVALENCE (whole study, samples combined)	% PREVALENCE (samples where present)	% PREVALENCE 14.05.93 (720 worms)	% PREVALENCE 15.06.93 (384 worms)	% PREVALENCE 23.06.93 (1152 worms)
Synactinomyxon sp. 1	4	0.18	0.26	0.00	0.50	0.20
Synactinomyxon sp. 2	6	0.27	0.39	0.00	0.50	0.30
Raabeia sp.	1	0.04	0.09	0.00	0.00	0.09
Aurantiactinomyxon sp	11	0.49	0.72	0.00	1.60	0.40
Triactinomyxon sp.	1	0.04	0.14	0.14	0.00	0.00

species has been discouraged (Lom & Noble, 1984).

(a)   *Synactinomyxon* sp *1*

This species was found in four worms from the settlement pond at farm C with an overall prevalence of ~0.2%, although in one sample this rose to 0.5%. It was also encountered in one of ~200 wild worms (prevalence = ~0.5%) sampled in May 1993 from 500m upstream of farm C. The host oligochaete for two of the settlement tank infections were both determined from cleared preparations as *Tubifex tubifex*, but the worm host of the wild infection was not determined due to the detection of infection being by means of a fresh squash.

All eight spores of the pansporoblast of species of the genus *Synactinomyxon* are bound together by the short proceesses into a star-like figure. The sporoplasm contains many nuclei.

Some spores of the *Synactinomyxon* sp under study remained enclosed within the sporoblast membrane even when released. In such a condition, epispore processes were not evident. The whole pansporoblast was more easily recognised under bright field microscopy, than with phase contrast (fig.6.4). Fresh squashes of infected worms revealed large numbers of characteristic eight-spore groupings in a star-like arrangement (fig.6.5). When released from the pansporoblast, the epispore processes swelled. These processes were very difficult to distinguish other than by phase contrast (fig.6.6 & 6.7). Under phase, each of the two large epispore processes could be seen to contain a nucleus positioned approximately half-way down its length. Most commonly, seven spores were seen to form a circle in the centre of which the eighth

table 6.4   Summary of measurement data for actinosporean spp. from the settlement pond at farm C

SPECIES	EPISPORE LENGTH (μm)	STYLE LENGTH (μm)	ENDOSPORE DIMENSIONS (μm)	POLAR CAPSULE DIMENSIONS (μm)
Synactinomyxon sp. *1*	long:**18**(15-21) short:**5.2**(3-7)	-	**16.5**(14-19)	5x4
Synactinomyxon sp. *2*	**64.4**(55-80)	-	**21.3**(20-23) x **18.3**(16-20)	6x4
Raabeia sp.	**219.3**(170-290)	-	**12.8**(10-20) x **24.6**(19-30)	**6.4**(5-8)
Aurantiactinomyxon sp.	**25.6**(19-31)	-	**13.7**(12-15)	**2.7**(2-3)
Triactinomyxon sp.	**129**(107-153)	**130**(110-150)	**52**(45-60) x **12.4**(12-14)	7x4

spore was positioned, making it impossible to see how the net of spores was constructed (fig.6.7). However in some instances the central spore was absent and it could be seen that the third, short conical epispore process of each spore was directed centrally and attached to adjacent spores (fig.6.8). Very few single lone spores were seen (fig.6.9); these often appeared in poor condition.

The endospore of each spore measured 16.5μm (14-19μm) in diameter, was round, and contained three spherical polar capsules of 4-5μm diameter. The short epispore process linking the eight spores was 5.2μm (3-7μm) in length, whilst the mean of the long processes was 16.5μm (14-19μm) in length. The star-shaped figures formed by the eight spores were 74μm (70-80μm) in diameter. Polar filaments when extruded formed a spiral with a mean length of 70μm. The sporoplasm contained many nuclei and germ cells. (N=25)

(b)   *Synactinomyxon* sp *2*

This species occured on a total of six occasions at prevalences, where present, of between 0.3% and 0.5%. Two infected worms were *Tubifex tubifex*, whilst one was *Lumbriculus variegatus*, indicating that this species might show a low degree of host specificity. The other infected worms were not identified.

Individual spores of *Synactinomyxon* sp *2* matched exactly the generic characteristics of spores of the genus *Echinactinomyxon*. Spores of this genus have a style-less epispore whose three processes are straight, rigid and pointed, and are of equal length. However, spores were never found singly, but rather in groups of eight spores, a characteristic of the sub-family Siedleckiellinae. Spores were not grouped in the net

or web type characteristic of genera such as *Siedleckiella* or *Antonactinomyxon* (Janiszewska, 1955). Rather, all eight such spores were joined together by the tip of one of their epispore processes (fig.6.10, 6.11). Thus the species was classified as a member of the genus *Synactinomyxon* (see Marques, 1984).

The endospore of individual spores was 18.3μm ($16\text{-}20\mu$m) at its widest point, but was goblet-shaped with a rounded bottom tapering to $\sim10\mu$m wide anterior surface when viewed laterally (fig.6.12). The endospore was round if viewed apically (fig.6.12). The polar capsules were slightly subspherical measuring a mean of 6μm by 4μm. There were six coils of the polar filament in each capsule (fig.6.13). Extruded filaments were of mean 50μm in length. Epispore processes were straight and of equal length, but frequently not fully everted. Fully swollen processes were 64.4μm ($55\text{-}80\mu$m) in length and 8μm ($6.5\text{-}9\mu$m) in width before tapering to a fine point. (N=20)

(c)   *Aurantiactinomyxon* sp

This species was found in eleven worms with an overall prevalence of 0.49%, although in one sample of worms, this rose to 1.6%. The host oligochaete was identified as a member of the Tubificidae, but cleared samples were insufficiently mature to enable a precise generic or specific identification to be made.

Members of this genus have a styleless epispore with three broad leaf-like processes of equal width which curve downwards. Their bases embrace the endospore almost entirely (fig.6.14, 6.15).

Spores of the *Aurantiactinomyxon* sp under study were always seen singly when released from worms. Again epispore processes were almost invisible under bright field microscopy, but clearly evident under phase contrast. Interestingly, a proportion of

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fig.6.4 *Synactinomyxon* sp. *1*. Eight spores still retained within a pansporoblast. (a) phase contrast; (b) bright field. Polar capsules are more easily visible in (b). (bar=10µm)


a

b

fig.6.5 Fresh squash of *Tubifex tubifex* infected with *Synactinomyxon* sp. *1*. Note the large numbers of pansporoblasts containing eight spores (arrows) (phase contrast, bar=10µm)
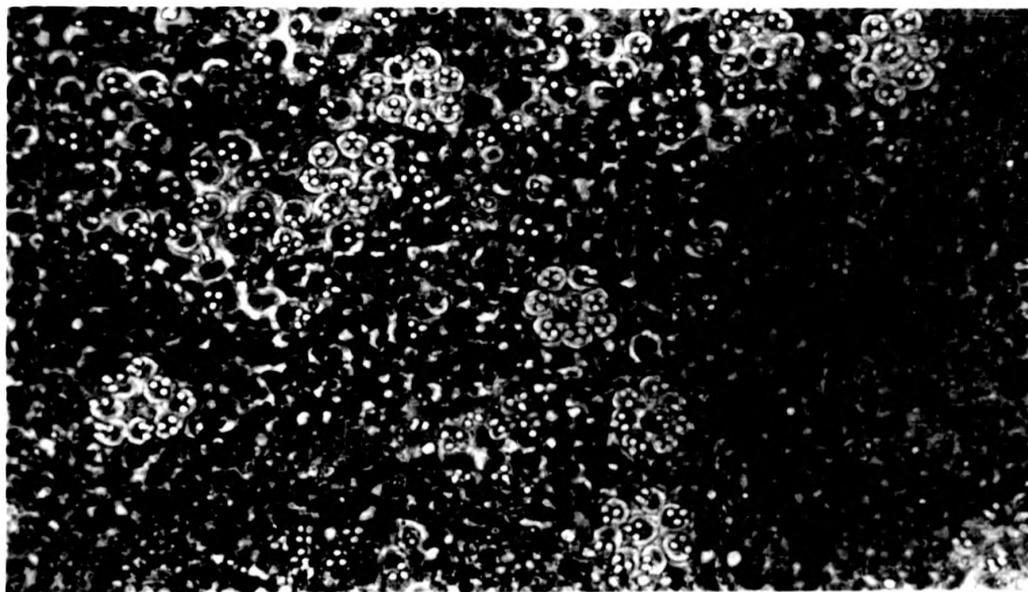
fig.6.6   Spore figure of *Synactinomyxon* sp. *1* released from a pansporoblast under bright field. Epispore processes can be seen with difficulty (**arrows**). bar=10μm)
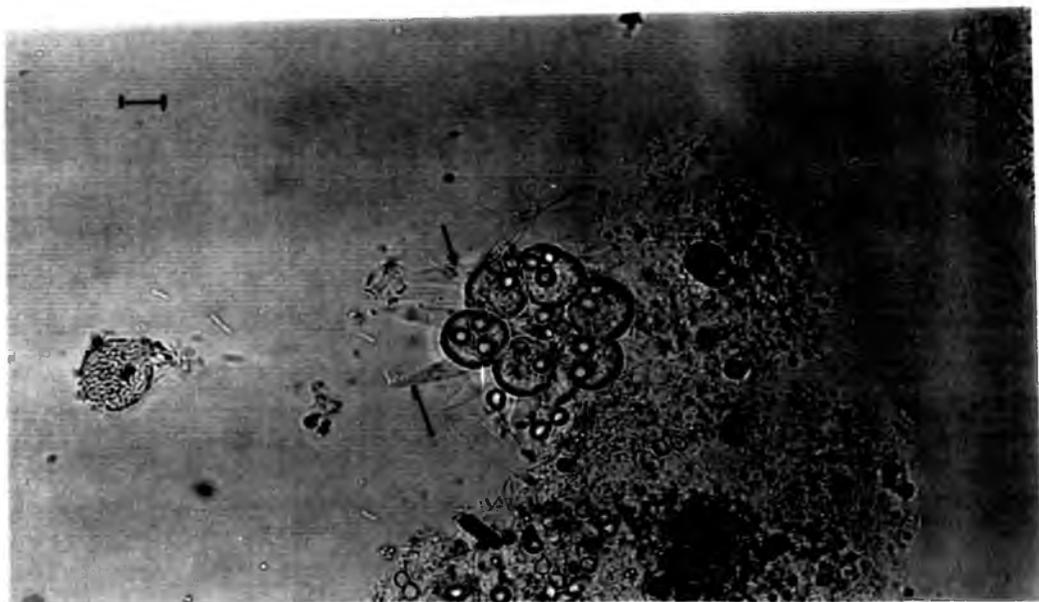

fig.6.7   Eight spore, released web or figure of *Synactinomyxon* sp. *1* under phase contrast. Epispore processes easily distinguished. Note the presence of epispore cell nuclei (**arrows**). Polar filaments have been everted due to the presence of fish mucus (see text). Endospores have a 'glowing' appearance. (bar=10μm)

fig.6.8 Spore figure of *Synactinomyxon* sp. *I* released from a pansporoblast. The eighth, centrally positioned spore is absent enabling the short conical epispore process of each spore to be seen. These are directed centrally and adhere to each other. (bar=10μm)
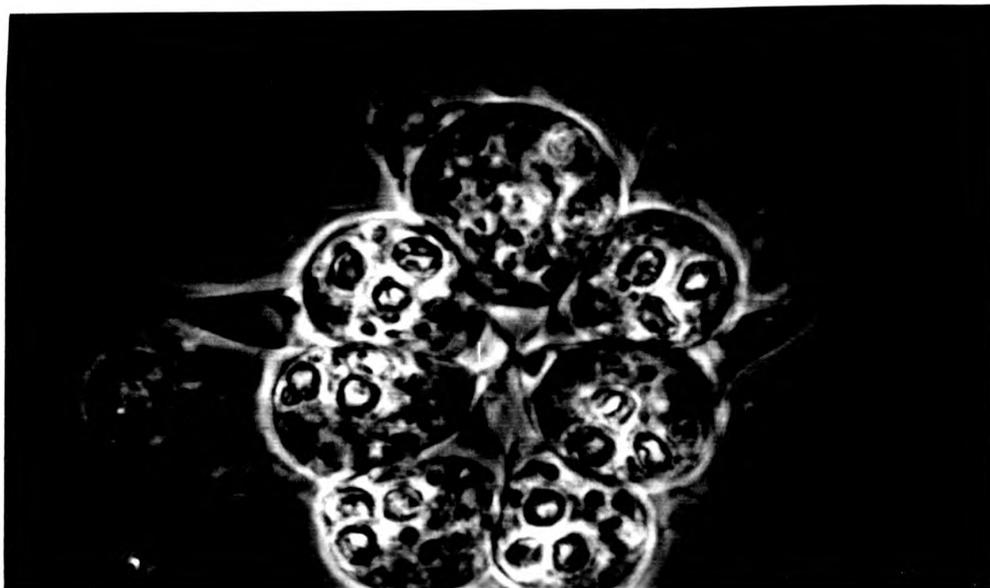

fig.6.9 Lone *Synactinomyxon* sp. *I* spore. The two long epispore processes are visible in different planes but the short conical one is not. (bar=10μm)

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fig 6.10 Spores of *Synactinomyxon* sp. 2 under bright field microscopy (bar=10μm).
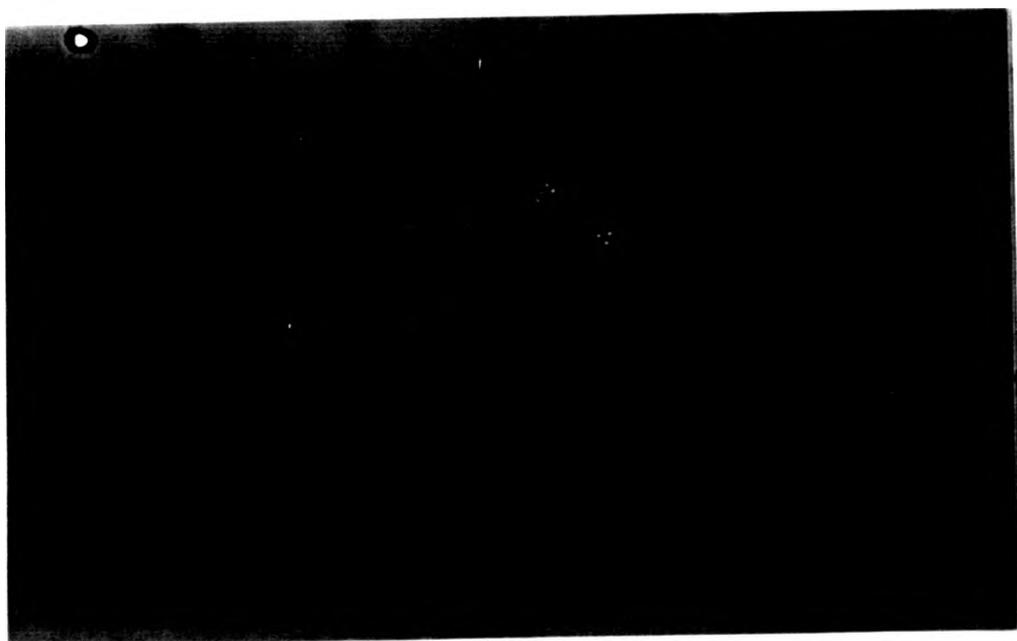
fig 6.11 *Synactinomyxon* sp. 2. note the resemblence of individual spores to those of the genus *Echinactinomyxon* (see Fig). Endospore cavity is goblet-shaped. All spores joined by the tip of one of their epispore processes (arrow) each of which are of equal length. (bar=10μm)


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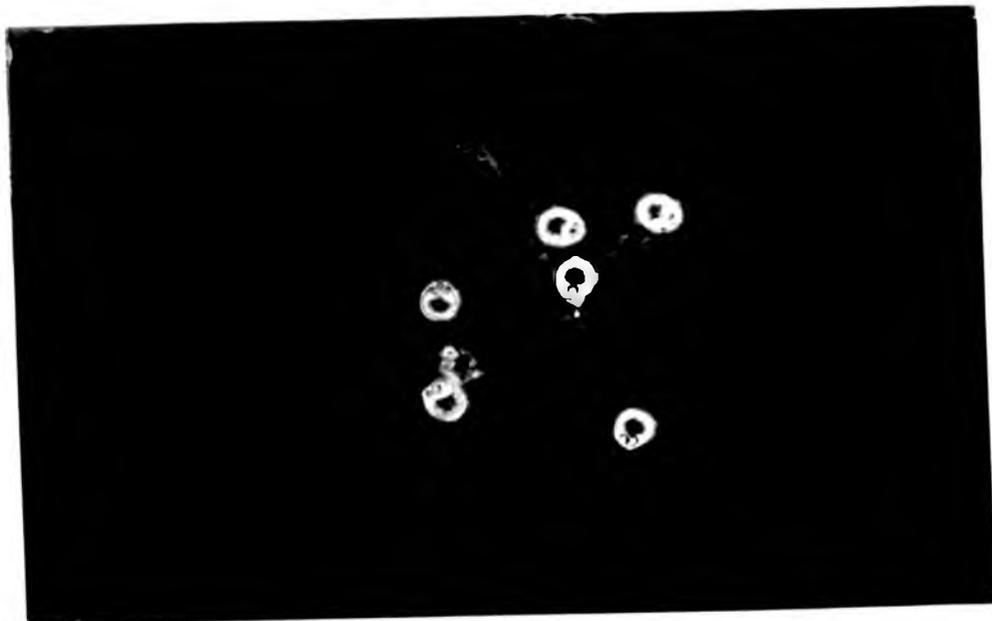
fig.6.12 Phase contrast photomicrograph of *Synactinomyxon* sp. 2 to illustrate the round appearance of the endospore cavity when viewed apically (arrows). The central point where the epispore tips of individual spores join is clearly visible (*) (bar=10μm)
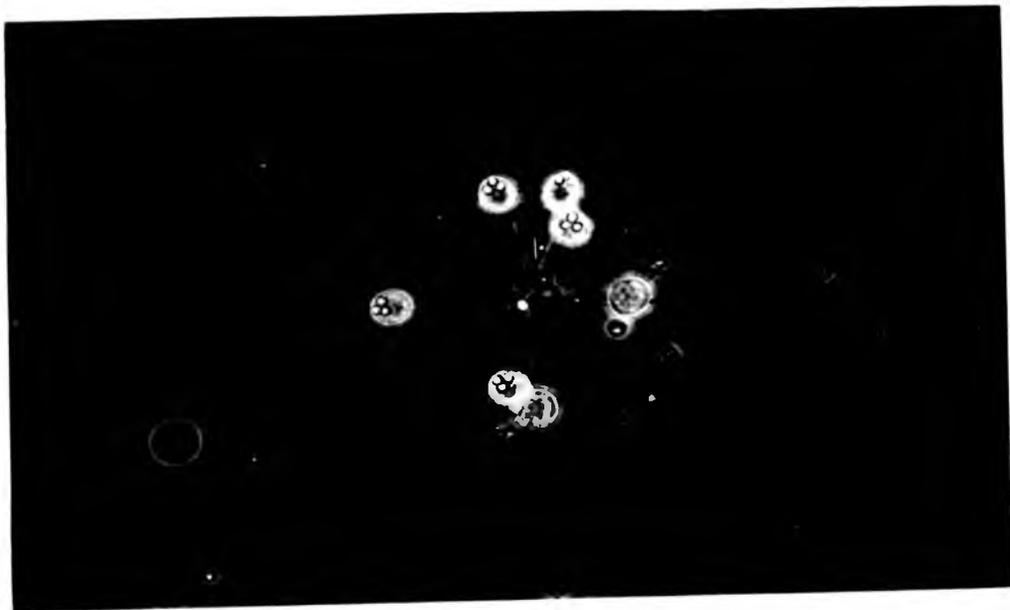

fig.6.13 High power view of the endospore of three *Synactinomyxon* sp. 2 spores to show the subspherical polar capsules, each containing a polar filament with six coils. (bar=10μm)
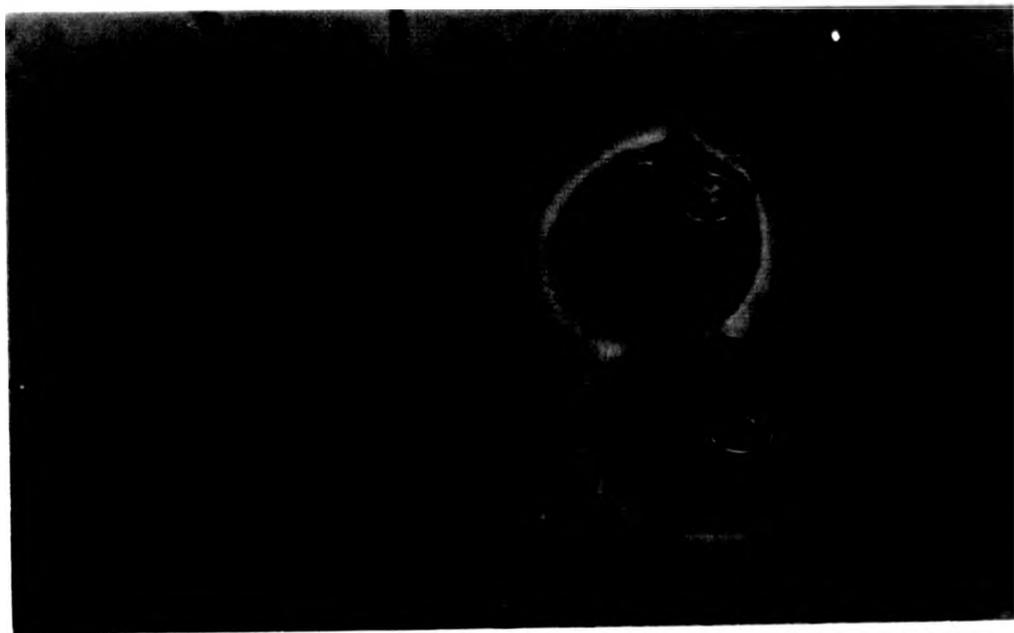
fig.6.14 *Aurantiactinomyxon* spore, under bright field microscopy. Epispore processes barely visible. (bar=10μm)
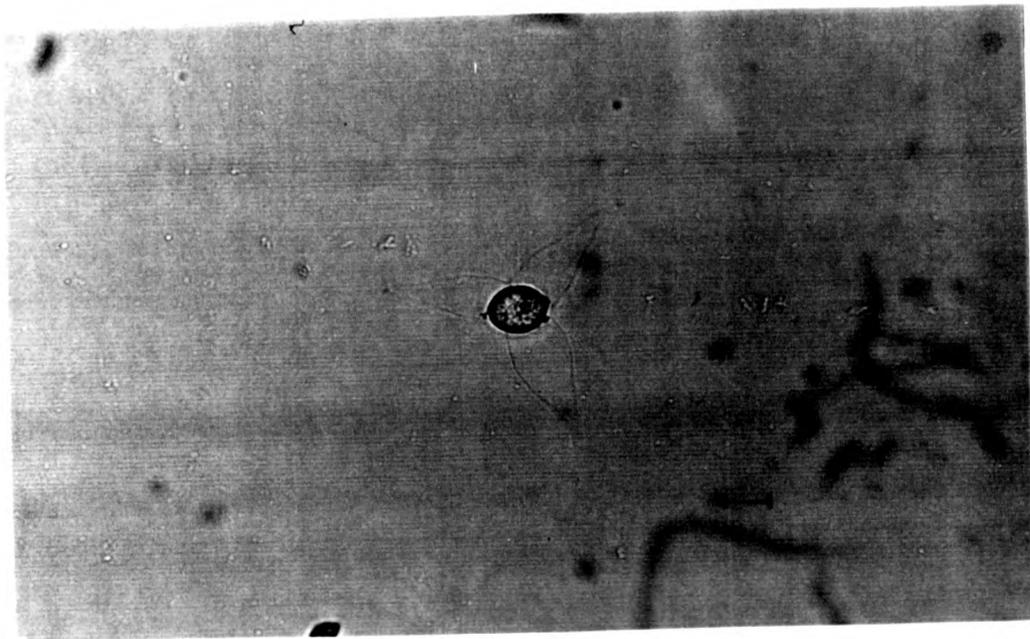

fig.6.15 *Aurantiactinomyxon* sp. spore under phase contrast. Note the leaf-like epispore processes, becoming broader away from the endospore before tapering to a point. (bar=10μm)

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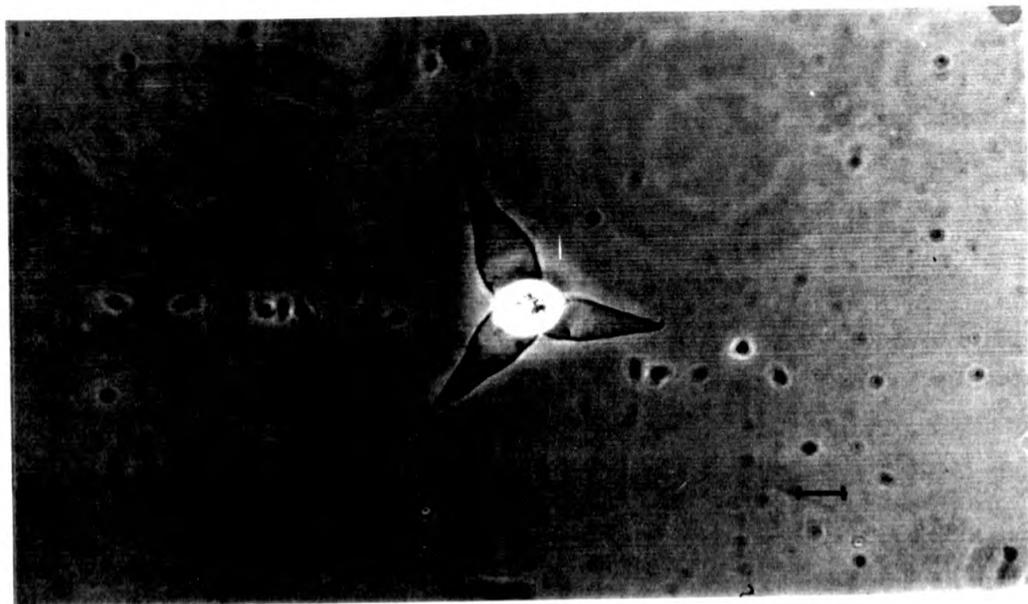
fig.6.16 Empty spore of *Aurantiactinomyxon* sp. with no sporoplasm and only remnants of the polar capsules
(**arrow**). The leaf-like nature of the epispore processes and their downward curve are apparent
(phase contrast) (bar=10μm)
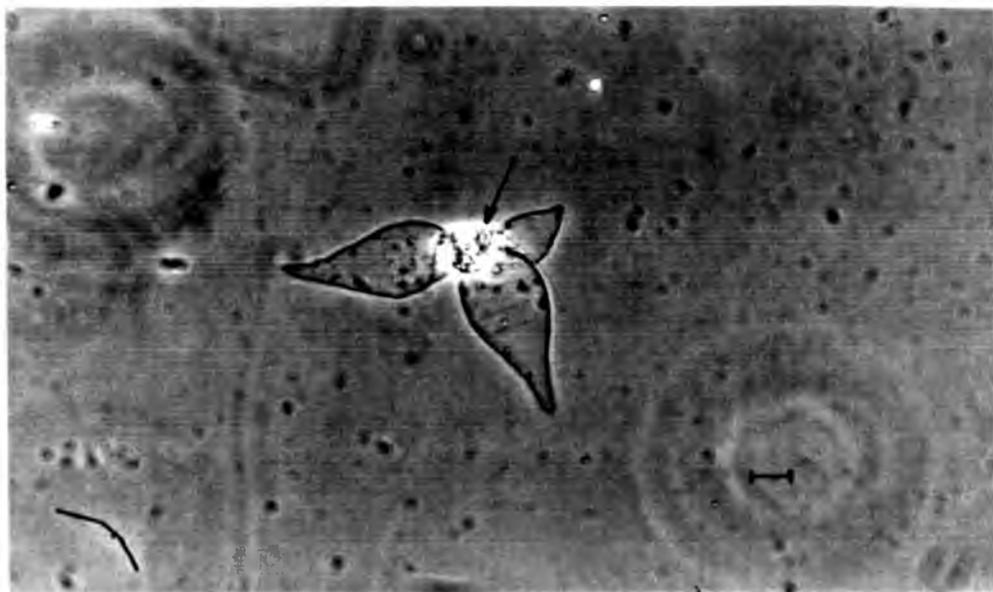
fig.6.17 *Aurantiactinomyxon* spore viewed apically under phase contrast. One epispore process is rounded
due to its incomplete eversion. The tips of the other two are out of focus reflecting the fact that each
process curves downwards in side view. (bar=10μm)

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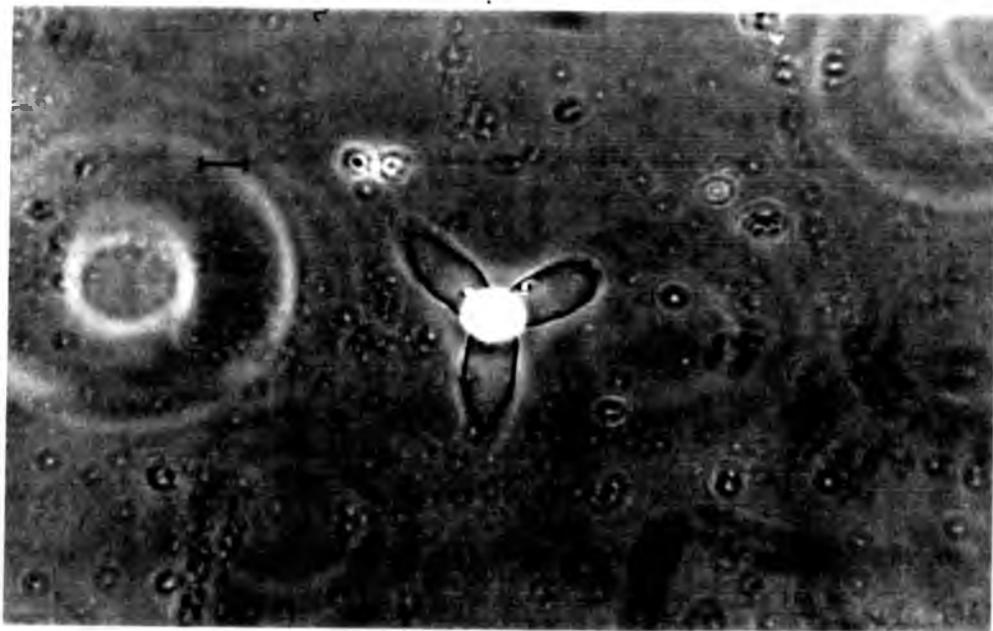
fig.6.18 *Triactinomyxon mrazeki* spore from well containing dead worms. Note prominent style (**arrow**) and numerous sporozoites in the sporoplasm (bar=10μm)
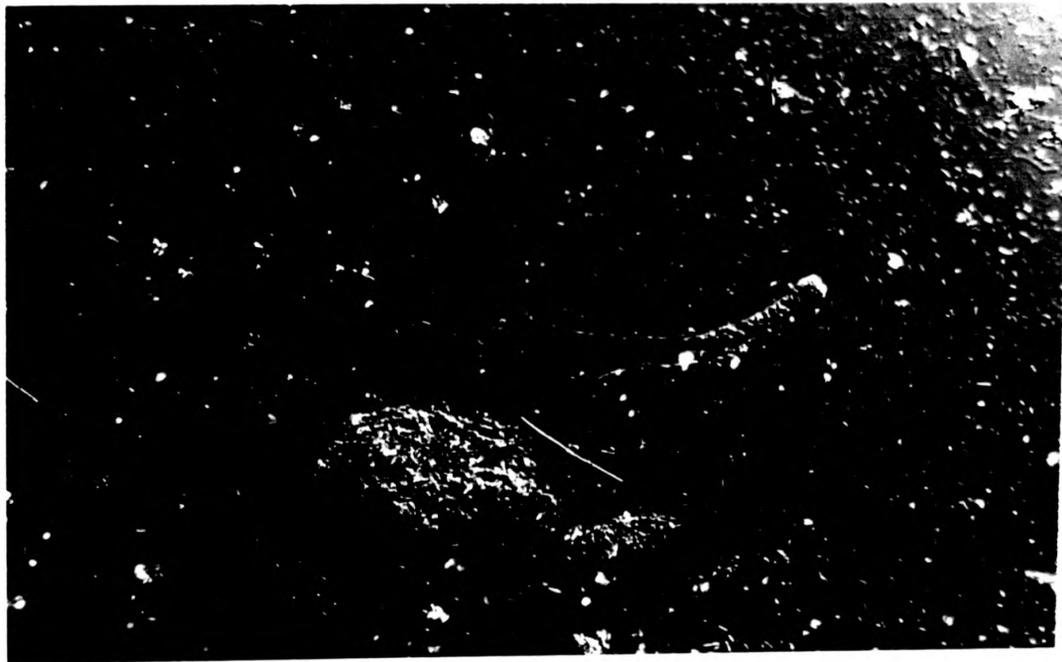
fig.6.19 *Raabeia* sp. spore with fully everted epispore processes (phase contrast, bar=10μm)
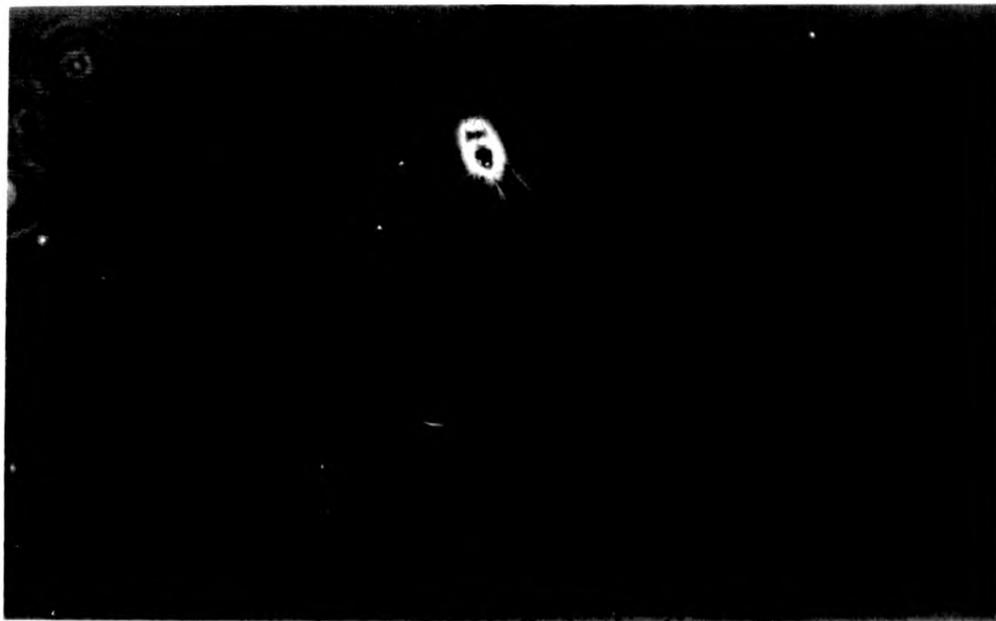
fig.6.20 Recently disrupted pabsporoblast of *Raabeia* sp. Endospores are clearly visible as are polasr capsules. Many epispore processes are incompletely everted (arrows) (phase contrast, bar=10μm)
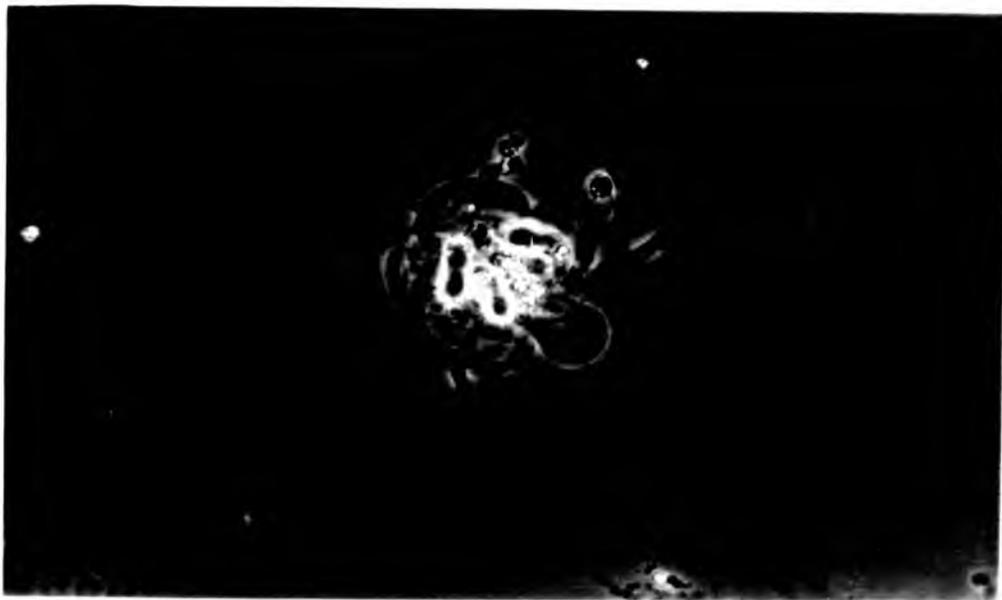
fig.6.21 Later photomicrograph of *Raabeia* pansporoblast. The pansporoblast membrane is visible (arrowhead). Epispore processes of spores contained within are beginning to swell and evert in response to contact with water (arrow) (phase contrast, bar=10μm)

fig 6.22   Recently released *Raabeia* spore with all three epispore processes yet to become fully swollen (phase contrast, bar=10μm)

fig.6.23   Close up of the endospore cavity of *Raabeia* sp. to show the enclosed sporoplasm (S), apical, pear-shaped polar capsules (arrows) and the 'clear space' described in the text (*). Epispore processes arise from the equator of the endospore cavity. (bright field, bar=10μm)

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fig.6.24 *Raabeia* sp. spore of Fig 6.23 under phase contrast. The 'clear space' is more obviously visible. (bar=10μm)

fig.6.25 Aberrant spore of *Raabeia* sp. with six, rather than three epispore processes (phase contrast, bar=10μm)

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fig.6.26   Daily *Aurantiactinomyxon* sp. spore release: Worm one
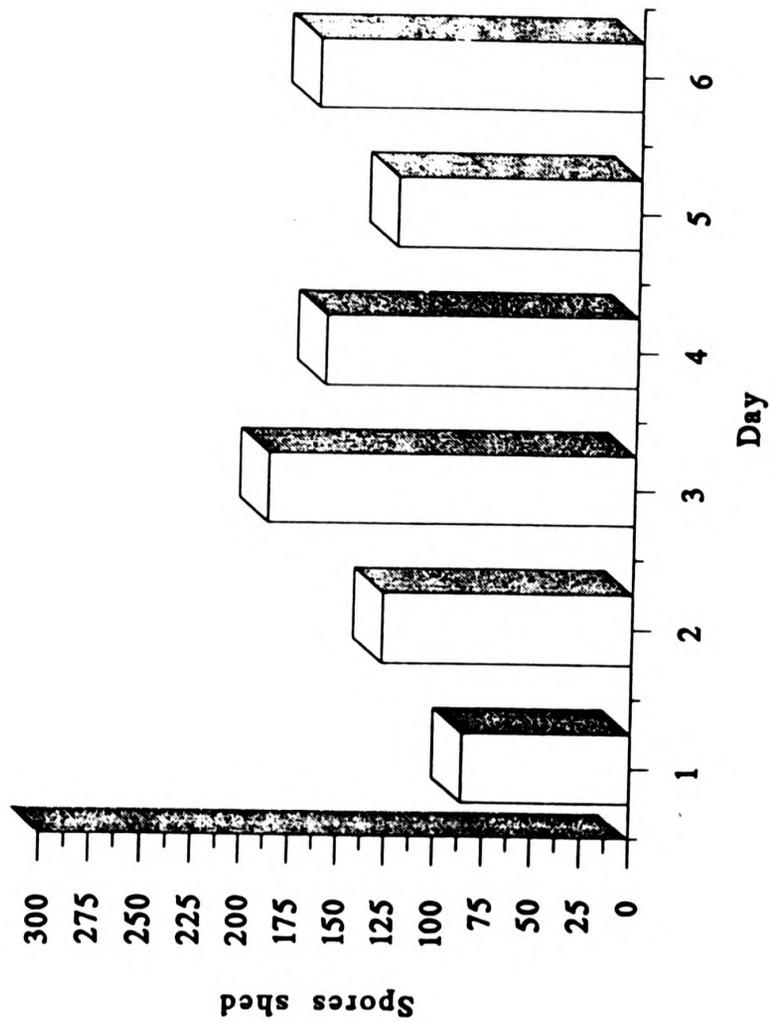
fig.6.27   Daily *Aurantiactinomyxon* sp. spore release: Worm two

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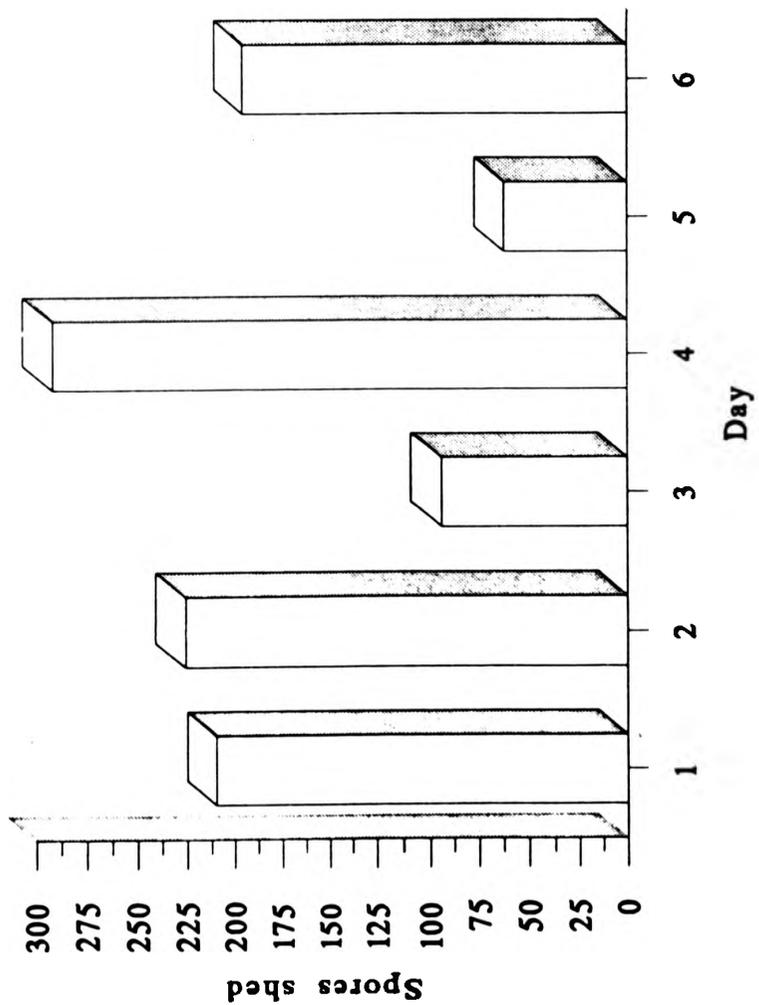
fig.6.28 *Aurantiactinomyxon* sp. spores release from worm one over two days
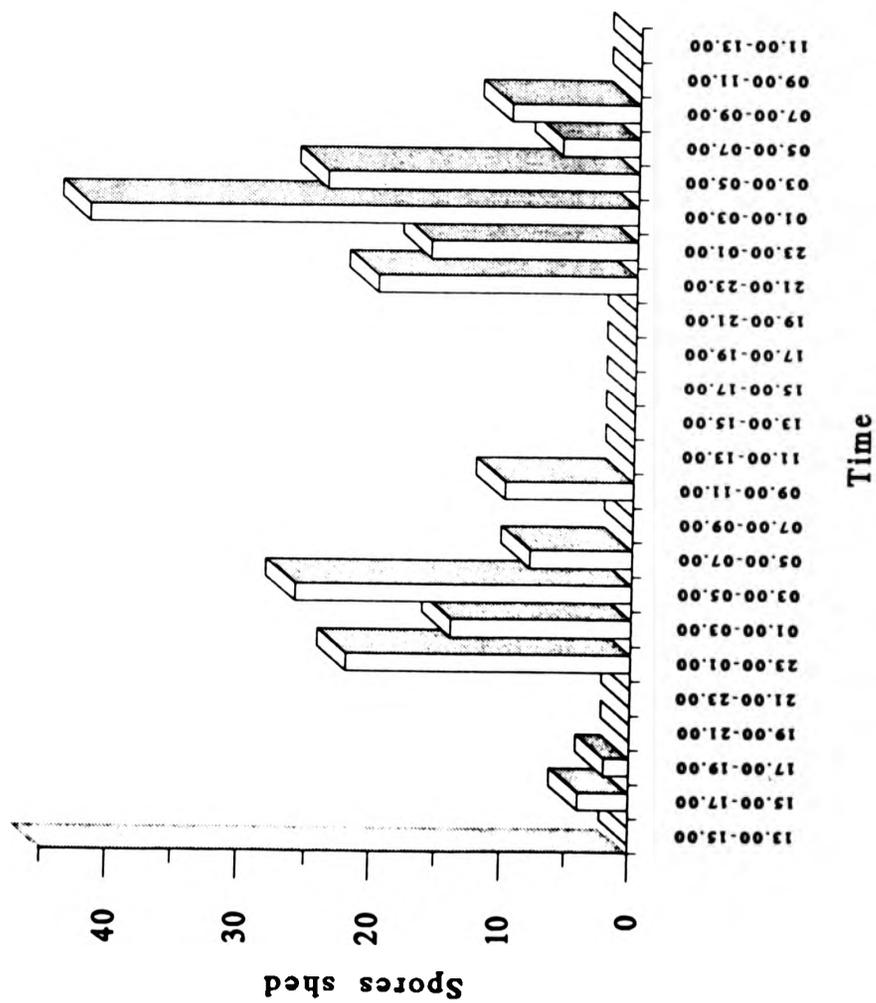
fig.6.29 *Synactinomyxon* sp. *1* polar filament release in response to the mucus of brown trout. Note the spiralling of the everted filament (**arrows**) (phase contrast, bar=10μm)
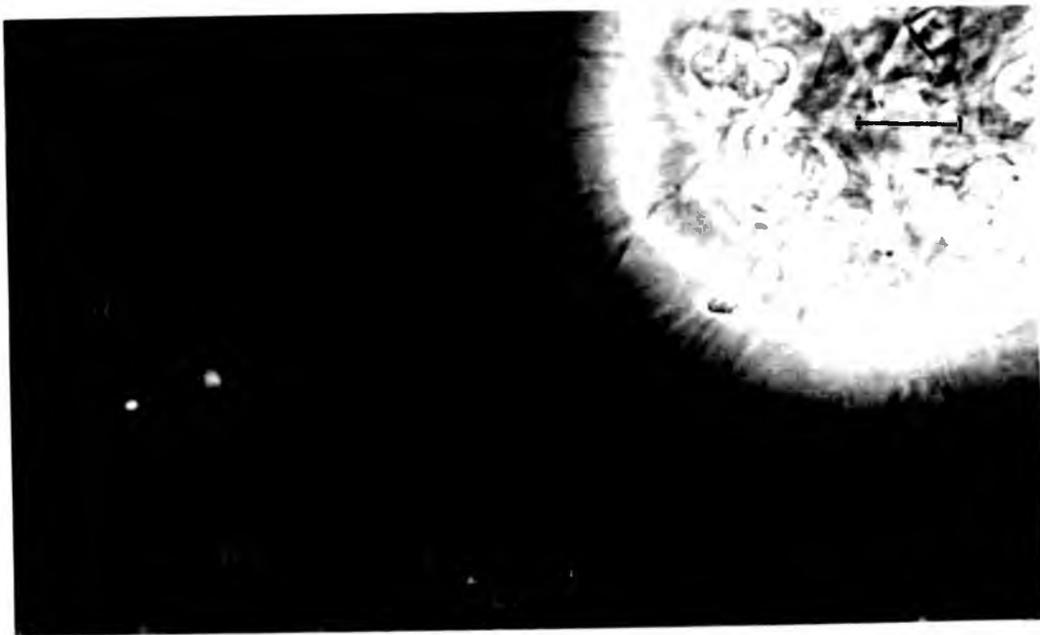
fig.6.30 *Raabeia* sp. filament eversion in response to Atlantic salmon mucus (**arrows**). note the comparatively low filament length to spore size ratio compared to that of *Synactinomyxon* sp. *1* (phase contrast, bar=10μm)

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fig.6.31 *Synactinomyxon* sp. *2* polar filament release in response to Atlantic salmon mucus (**arrows**) Note the variability in length to which polar filaments are everted. (phase contrast, bar=10μm)
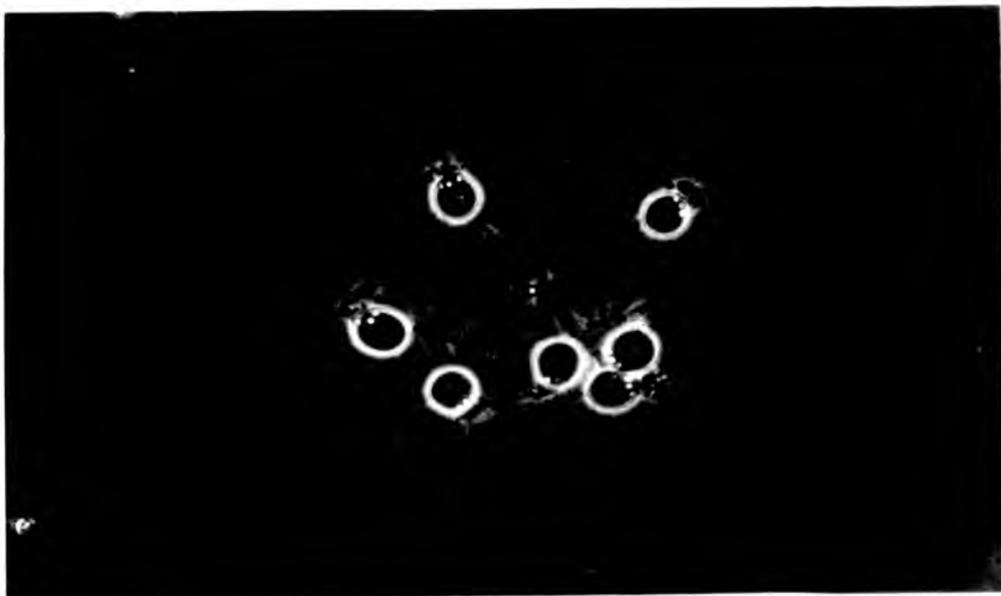
fig.6.32 Motility of two sporoplasms (1, 2) hatched from *Synactinomyxon* sp. 2 spores which had everted their polar filaments in response to mucus from Atlantic salmon. Note the empty shell (arrowhead) left behind and the pseudopodia responsible for movement. (phase contrast; photos taken at 7 second intervals; bar=10μm)
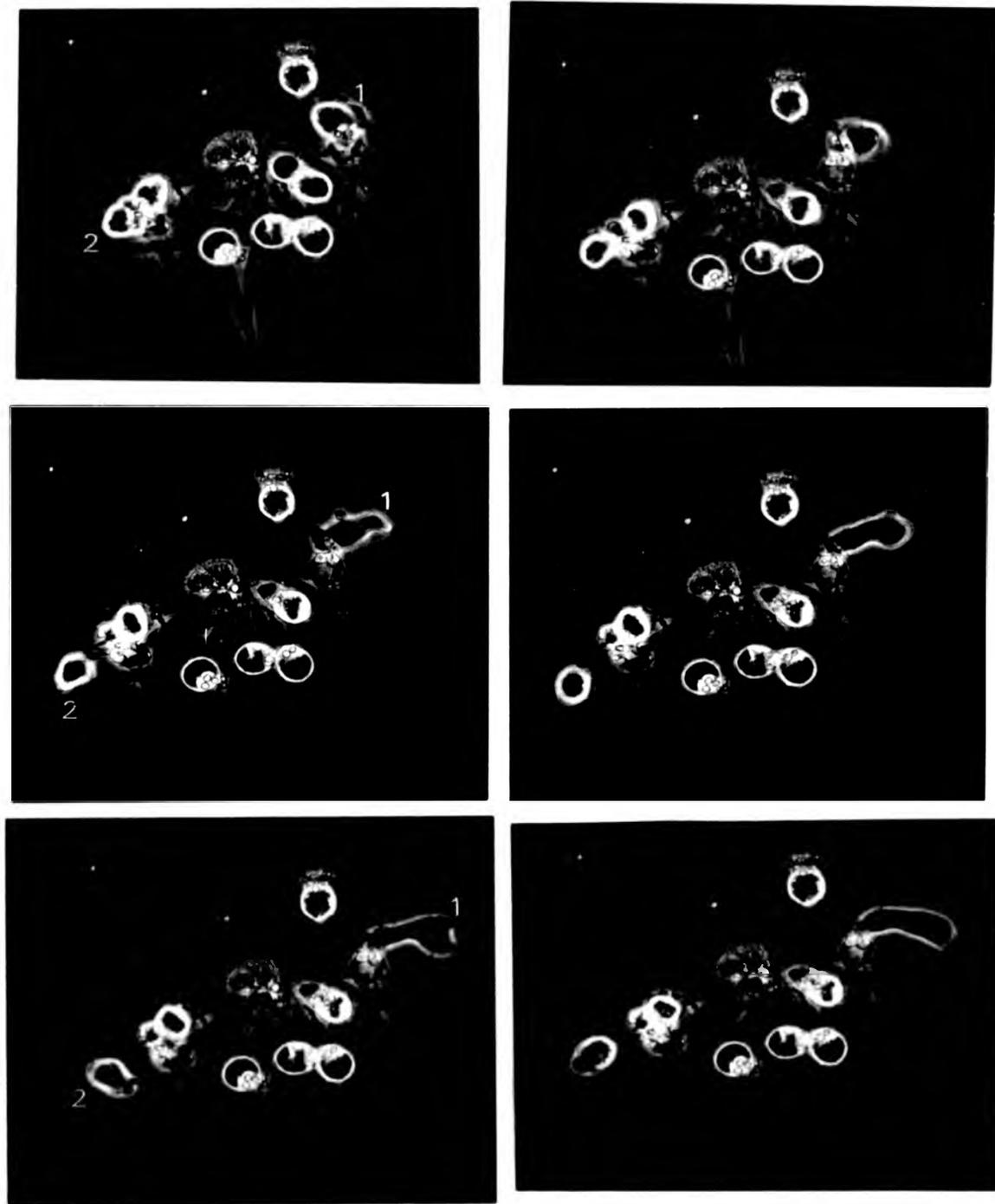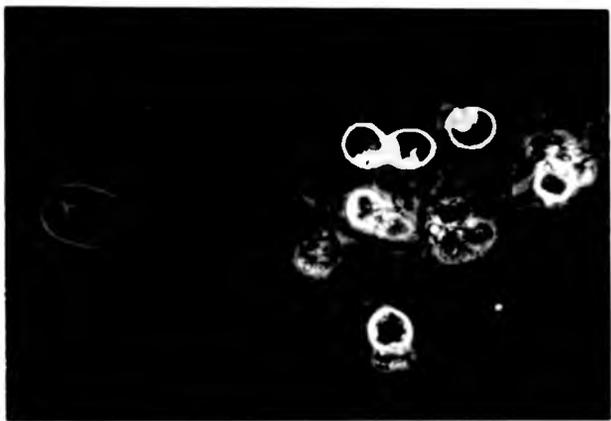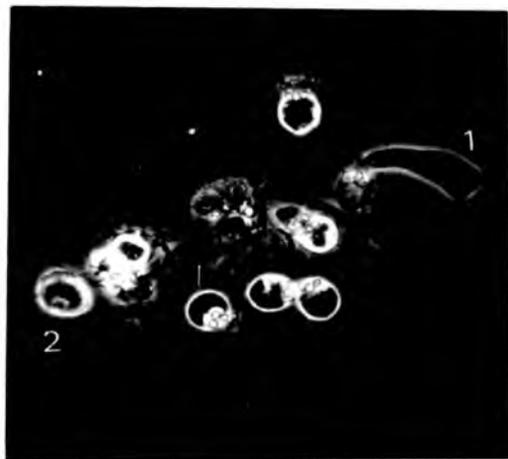
morphologically and were probably dead; the large numbers of bacteria present in the cell well water from which they were obtained were a reflection of the death of the worms. Some changes in spore morphology may therefore have occured not characteristic of living spores.

The endospore measured 52μm (45-60μm) by 12.4μm (12-14μm), contained at least 60 sporozoites, and was situated apically beneath the three polar capsules at the anterior of the style. Polar capsules were oval, 7μm long by 4μm wide. The style of the epispore was 130μm (110-150μm) in length and broadened from a width of $\sim15\mu$m anteriorly to $\sim25\mu$m at the base where the anchor-like projections arose. These epispore processes were curved, 129μm (107-153μm) in length, but frequently were still not fully everted (fig.6.18). (N=10)

(e)   *Raabeia* sp

This species was identified from the worm population of the settlement tank again in a single sample. It was particularly notable for the large size of its epispore processes. The host oligochaete species was not identified. The overall prevalence of *Raabeia* infection in all worms was 0.04%, but in the sole sample in which it was present this figure was 0.09%.

Spores of the genus *Raabeia* are characterised by their three long attenuated epispore processes which are usually curved. No style is present, the epispore processes diverging from the equator of the endospore. The sporoplasm of the endospore is flask or barrel-shaped and contains many sporozoites (fig.6.19).

As well as large numbers of free actinospores, numerous released but intact

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pansporoblasts, each containing eight spores were found in well water containing infected oligochaetes (fig.6.20). Upon manual rupturing of the envelope, the spores escaped and their epispore processes rapidly swelled, telescoping out from inside themselves (fig.6.21). Thus numerous spores had incompletely everted processes (fig.6.22).

The epispore processes were very long, curved, and tapered to a point when fully swollen. They were 219μm (170-290μm) by ~10μm wide. The endospore cavity measured 18.2μm (12-25μm) in length and was 12.8μm (10-20μm) in width. Polar capsules were pear-shaped and on average 7μm x 5μm. However, between the sporoplasms in the endospore cavity and the polar capsules lay a large clear area perhaps containing the cytoplasm of the capsulogenic cells (fig.6.23 & 6.24). This space was 6.4μm (5-7μm) in length, making the capsule to base of endospore length 24.6μm (19-30μm). (N=16)

Occasionally aberrant spores with six epispore processes arising from a single endospore were seen rather than the normal three (fig.6.25).

Patterns of actinosporean release

Aurantiactinomyxon sp. spores were chosen for many of the following studies, due to the higher prevalence of this species in the worms studied (see discussion).

The number of *Aurantiactinomyxon* sp spores released per day for two infected worms over a six day period is shown in fig. 6.26 & 6.27. Worm one yielded a total of 852 spores over the six days, averaging 142 spores per day; worm two yielded 1,080 spores at an average of 180 per day. Despite this average figure, no regular fixed

pattern of release in terms of numbers per day was evident.

Worm one, yielding 86 and 128 spores in day one and two of the daily release study was used in the study of circadian release when exposed to normal photoperiod. Release per two hour period for those two days showed marked variations (fig.6.28). Mean release per two hours was ~7 spores for day one, ~11 for day two. However there was a marked tendency towards higher levels of release between 9pm and 9am, with some periods during the daylight hours showing little or no release. The vast majority of actinosporean spores, around 72% on day one, and 64% on day 2, were released between 23.00h and 05.00h.

Stimuli for actinosporean polar filament release and sporoplasm hatching

Four species of actinosporean, *Synactinomyxon* sp *1*, *Synactinomyxon* sp *2* *Aurantiactinomyxon* sp and *Raabeia* sp all discharged their polar filaments in response to the mucus of brown trout, Atlantic salmon, stickleback and bream (fig.6.29, 6.30 & 6.31). *Triactinomyxon* sp was not tested due to the fact that the spores found appeared to be dead (see above). In all cases eversion was instantaneous, and thus rarely directly observed as it occurred before the slide could be transferred under the objective. Not every spore in a sample was seen to evert its filaments however. Following filament release, many *Synactinomyxon* sp *2* spores hatched along the suture line around the endospore cavity, releasing their sporoplasms into the surrounding medium. Sporoplasms were motile, moving by means of clear pseudopodia with undulating outer membranes. The progress of two sporoplasms from hatched spores was followed by time lapse photography, with pictures being taken every 6 seconds (fig.6.32). Not all

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spores, even those with everted filaments, hatched to release their sporoplasms. Spores from control preparations occasionally everted their polar filaments, but this was much delayed in comparison with experimental preparations. Eversion could be artificially stimulated mechanically by exerting pressure on the coverslip of a spore preparation.

Ultrastructure of *Aurantiactinomyxon* sp

Semi-thin sections showed that numerous pansporoblasts of *Aurantiactinomyxon* sp caused a hypertrophy of the gut epithelium of the host oligochaete. All epithelial cells in a particular section could be seen to be infected by parasites at different stages of development (fig.6.33 & 6.34). Released pansporoblasts each containing eight spores could be seen in the gut lumen along with some parasites free in the coelomic cavity. Mature spores could be seen within pansporoblasts with their telescopically folded epispore processes evident.

At the TEM, ultrastructural level, in addition to stages in gut epithelial cells, released pansporocysts could be found in the lumen of the gut and also the coelomic cavity.

The earliest stages seen consisted of cells containing two closely opposed nuclei in a diplokaryon arrangement (fig.6.35). The cytoplasm of such cells was bounded by a single membrane which, although irregular in contour, possessed no folds or projections. Few cytoplasmic organelles were present other than mitochondria and ribosomes. Small electron dense bodies were irregularly spaced through the cytoplasm, along with membrane bound vesicles containing particulate matter. These latter

fig.6.33 Low power magnification of semi-thin section to demonstrate pansporoblasts of *Aurantiactinomyxon* sp. in the gut epithelial cells (arrows) surrounding the lumen of the gut. (bar=10μm)
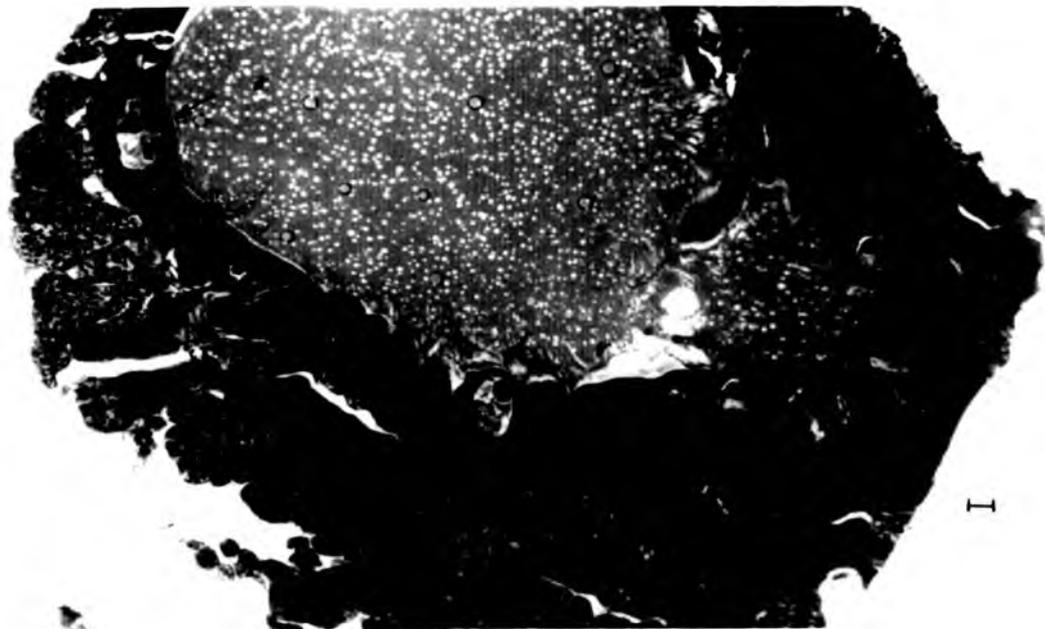
fig.6.34 Higher magnification to show pansporoblasts containing developing spores of *Aurantiactinomyxon* sp. occupying adjacent epithelial cells of the host gut. (bar=10μm)

fig.6.35 Earliest *Aurantiactinomyxon* stage seen. Single binucleate cell with nuclei (N_1, N_2) very closely apposed. Cytoplasm contains ribosomes and mitochondria (M). Note electron dense bodies (arrows) and phagosomes (Pg). (uranyl acetate/lead citrate, mag x7,400)
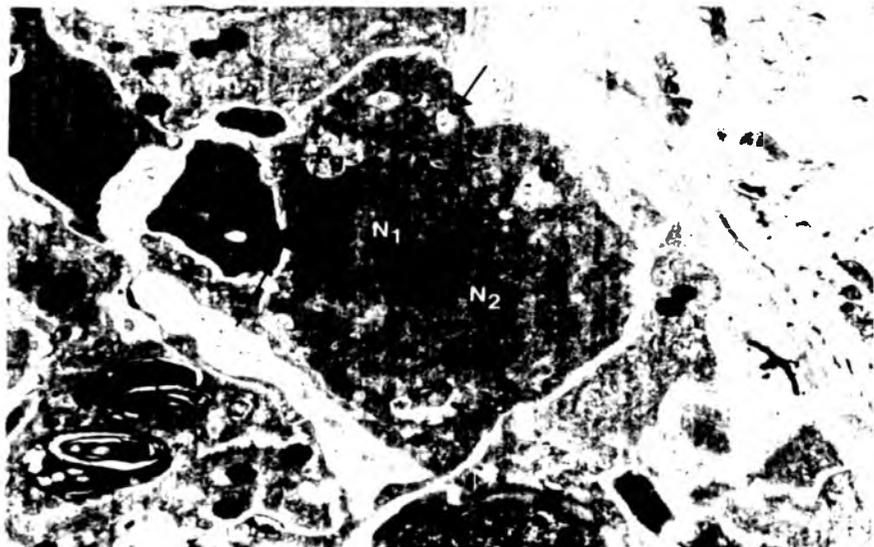
fig.6.36 Early encircled *Aurantiactinomyxon* stage. The internal cell possesses a nucleus with eccentric nucleolus. Dense bodies and phagosomes, some of which are very large (Pg), are restricted to the cytoplasm of the encircling cells (arrows). Encircling cell nucleus (En) evident. (uranyl acetate/lead citrate, mag.x9,200)

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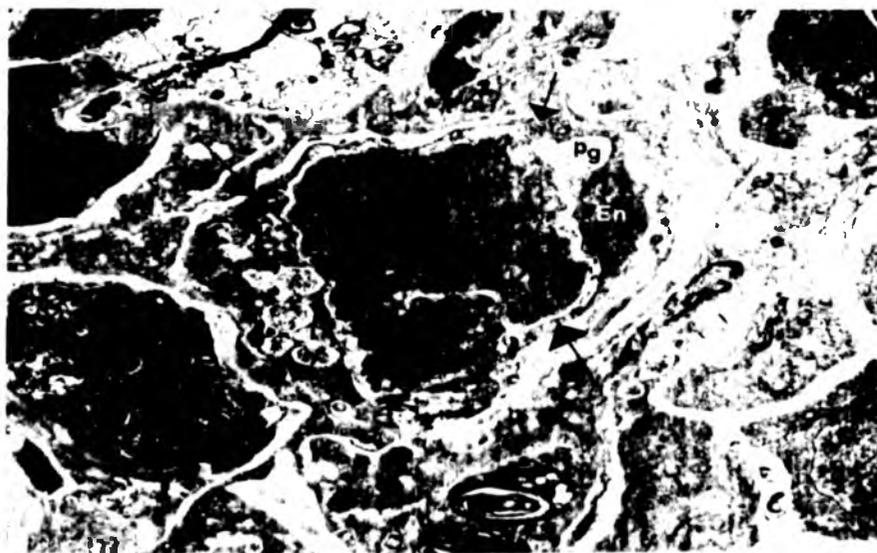
structures were present in large numbers in the cytoplasm, often in groups, and were described as phagosomes by Lom & Dykova (1992c). The two nuclei were similar in appearance and contained prominent nucleoli.

In the more developed stages seen, some inner cells were encircled by two others (fig.6.36, 6.37 & 6.38). The two encircling cells overlapped each other, with their end tips remaining free; they were connected to each other by cell junctions between opposed overlapped portions of cytoplasm. The cytoplasm of the encircling cells retained the dense bodies and phagosomes characteristic of the cytoplasm of binucleate stages, but these were absent from internal cells. The nuclei of encircling cells lacked a nucleolus. The space between internal cells was filled with an amorphous substance. Internal cells had numerous cytoplasmic mitochondria and nuclei with a dark staining eccentric nucleolus.

There then appeared to follow a period during which the number of internal cells increased. In a single section, two to ten internal cells could be seen, which appeared to be of two differentiable types, large and small. Large cells possessed tubular structures in their cytoplasm and very large nuclei which lacked nucleoli. Small cells had nuclei with prominent nucleoli and many cytoplasmic mitochondria (fig.6.38, 6.39 & 6.40).

During this period, the encircling cells formed only a very thin layer at certain points but still fully enclosed the internal cells. Nuclear and cytoplasmic structure remained intact as did the junctions joining the cells to each other. They continued to contain numerous phagosomes but the electron dense bodies of earlier encircling cells were rare.

fig.6.37 Developing stage of *Aurantiactinomyxon*, close to the coelomic cavity (*) of the host. Encircling cells contain cytoplasmic phagosomes (Pg) and dense bodies (arrows). Internal cells contain many mitochondria (M), and occasional phagosomes. Nucleus of an encircling cell (En) and an internal cell (In) visible. (uranyl acetate/lead citrate, mag.x11,800)
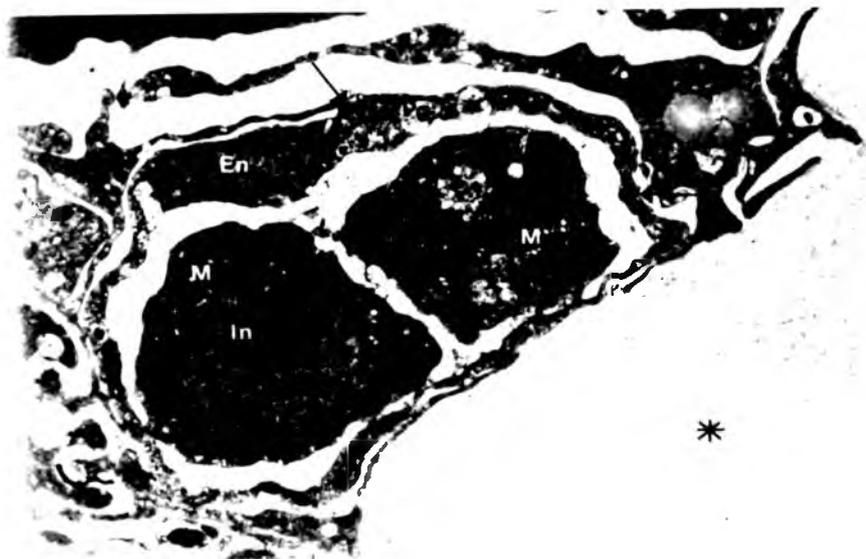
fig.6.38 More developed *Aurantiactinomyxon* stage. The overlapping sutures joining the envelope cells together can be seen (arrows). Encircling cell nuclei (En) visible. The cytoplasm of one envelope cell contains a very large phagosome (Pg). (uranyl acetate/lead citrate, mag.x11,800)

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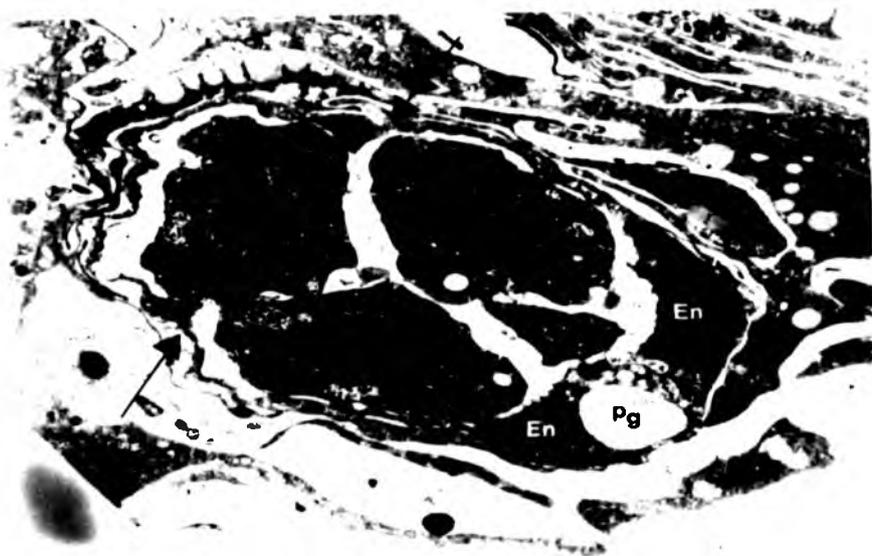
The sporoplasm of maturing spores was a syncytium containing a number of nuclei along with many germ cells (fig.6.45 & 6.46). Germ cells possessed few organelles and in some cases appeared to be dividing. The nuclei of the sporoplasm were often encircled by endoplasmic reticulum and appeared to lack nucleoli. The cytoplasm of the sporoplasm contained mitochondria, small vacuole-like inclusions and large numbers of dark staining, round to oval, sporoplasmosome-like bodies (fig.6.43-6.46).

Three valvogenic cells encircled the capsulogenic and sporoplasm cells of the spore as it matured. The cells joined at thickened desmosome-like junctions which often formed a noticeable projection or process at the apex of the spore above the mid point between the capsulogenic cells (fig.6.49). Valvogenic cells formed a thin layer around the capsulogenic and sporoplasm cells anteriorly (fig.6.47-6.51), but posteriorly formed a huge mass of concentrically folded membranes (fig.6.41 & 6.42). Initially these membranes contained cytoplasmic contents and valvogenic nuclei in which large eccentric nucleoli were present (fig.6.41). As the spore matured, the cytoplasm disappeared completely leaving the two valvogenic cell outer membranes in close apposition concentrically stacked in a telescopic fashion. A mass of this elongated membrane could be found posteriorly and around mature spores (fig.6.42).

The earliest capsulogenic stage seen was that of a primordium made up of a number of layers (fig.6.44). The outer membrane encircled an electron lucent layer, internal to which was an inner darker matrix. External tube formation was not seen. Adjacent capsulogenic cells were joined along a straight boundary which in places was strengthened by cell junctions. Such junctions also occurred between the valvogenic and

fig.6.39 *Aurantiactinomyxon* internal cells appear to lie in an amorphous matrix (*) but this may be artefactual. The cytoplasm of the encircling cells remains intact. Some internal cells are large and posses a large nucleus which lacks a nucleolus (In₁. Others are smaller with nuclei possessing eccentric nucleoli (In₂). The cytoplasm of both features mitochondria (M), but areas of tubular structures (**arrows**) are only present in large cells. (uranyl acetate/lead citrate, mag.x6,000)
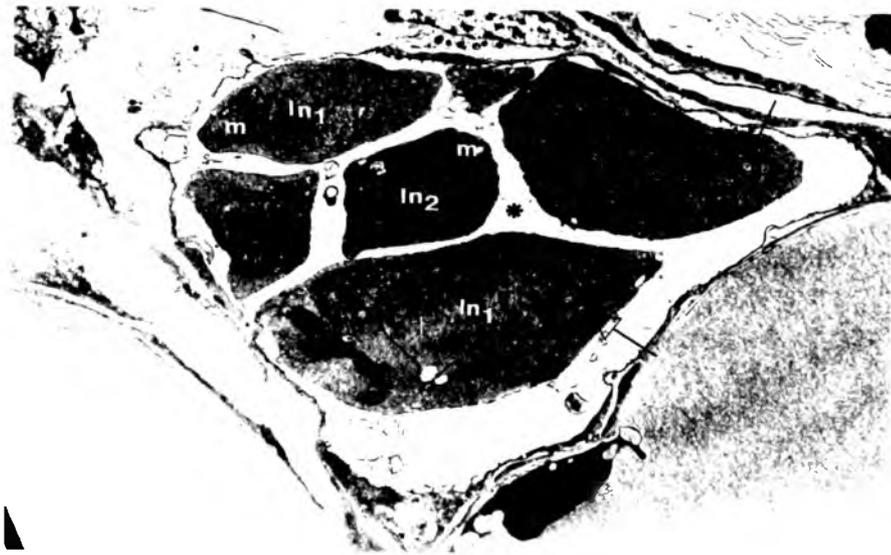
fig.6.40 *Aurantiactinomyxon* stage containing nine internal cells in the plane of the section. (uranyl acetate/lead citrate, mag.x7,800)

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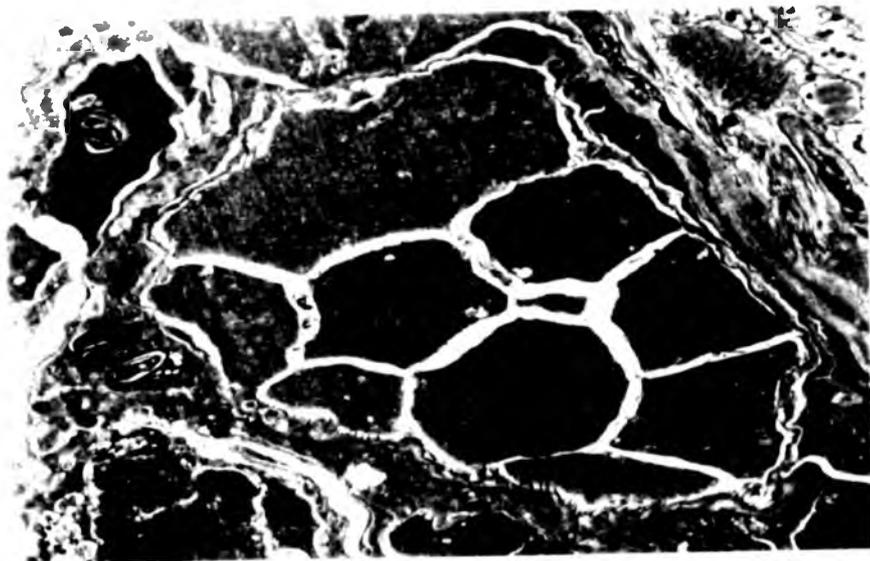
fig.6.41 Valvogenesis in *Aurantiactinomyxon*. Posterior to the developing spore the valve cells are forming a mass of concentrically folded membranes (*). At the stage illustrated the nuclei (N) and cytoplasm (C) of the valvogenic cells remain present at points, but at others the valvogenic cell outer membranes lie together containing no intervening cytoplasm (arrowhead). The encircling cells around the developing spore remains intact (arrows). (uranyl acetate/lead citrate, mag.x13,500)
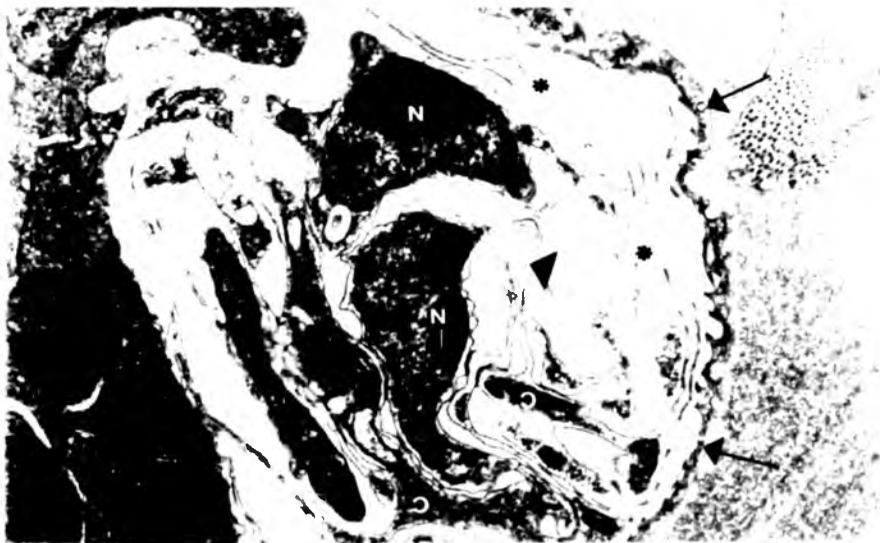
fig.6.42 Mature *Aurantiactinomyxon* spore in the gut lumen (L) still enclosed by the encircling pansporoblast cells (arrowhead). Phagosomes and dense bodies are still present in large numbers in the encircling cell cytoplasm. The sporoplasm lies centrally (*) and is surrounded by fold upon fold of telescopically stacked valvogenic cell membranes containing little or no intervening cytoplasm (arrows). These will form the three epispore floats of the spore on its release into water. (uranyl acetate/lead citrate, mag.x6,000)

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fig.6.43 Differentiating spore of *Aurantiactinomyxon* within the pansporoblast. Epispore floats formed by valvogenic cells can be seen in the centre and at the lower right (*). The capsulogenic cell contains an almost mature polar capsule (**arrow**). The developing sporoplasm contains many sporoplasmosome-like structures (**S**). Some phagosomes (**Pg**) are present. (uranyl acetate/lead citrate, mag.x4,500)
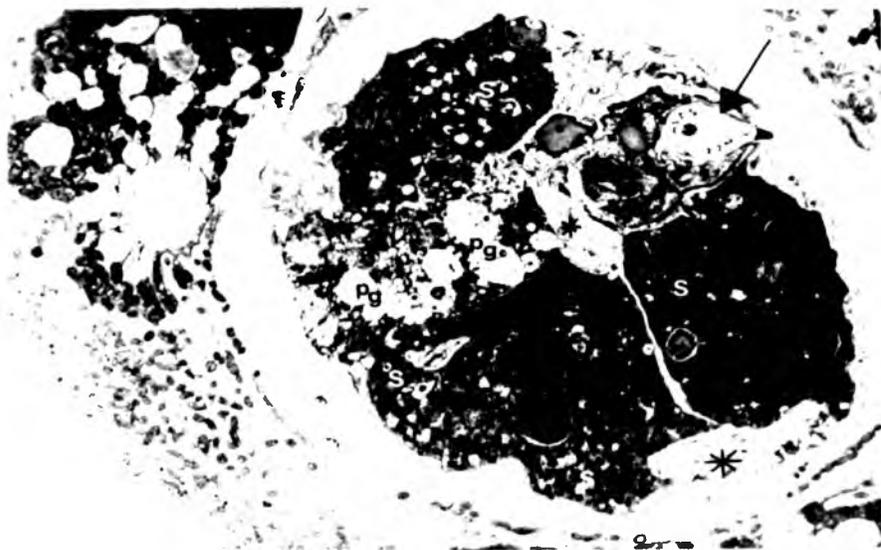
fig.6.44 Developing spores of *Aurantiactinomyxon* within the pansporoblast. Sutures joining the pansporoblast cells are still evident (**arrows**). An early capsulogenic cell contains a capsular primordium but no external tube is evident (*). (uranyl acetate/lead citrate, mag.x8,500)

fig.6.45 Sporoplasm of *Aurantiactinomyxon* containing large numbers of sporoplasmosome-like bodies in the cytoplasm (arrows). The sporoplasm appears to be a syncytium containing many nuclei (N) and germ cells (Gc). (uranyl acetate/lead citrate, mag.x11,500)
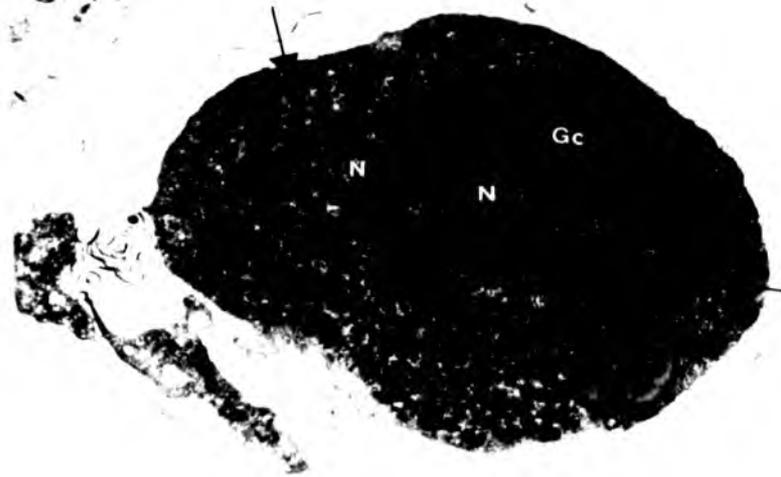
fig.6.46 Detail of sporoplasm of *Aurantiactinomyxon* with germ cells and nuclei. Many sporoplasmosomes are present (arrows) along with light, membrane-bound vesicles or inclusions which may be degenerate mitochondria (M). (uranyl acetate/lead citrate, mag.x17,000)

fig.6.47 & 6.48   Polar capsule formation in *Aurantiactinomyxon*. The lucent and dark layers typical of myxosporean capsular primordia are evident, but in the *Aurantiactinomyxon* appear bowl shaped and do.not form a complete sphere. Thus the layers do not subtend the entire primordium anteriorly (**arrowheads**).   Filament coils are already present in these 'incomplete primordia' (**arrows**).   Note the thickened junctions (**J**) between the capsulogenic and enveloping valvogenic cells, leaving a gap through which the stopper of the mature capsule will eventually protrude.   Mitochondria (**M**) are present in the capsulogenic cell cytoplasm (**Cc**) (uranyl acetate/lead citrate, mag.x32,000 (fig.6.47); mag.x25,500 (fig.6.48)

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fig.6.49 More mature *Aurantiactinomyxon* polar capsule, with an incomplete capsule wall posteriorly (arrow). The confluency of the lucent layer of the capsule wall with the outer wall of the polar filament is evident. Note the prominent raised and thickened valvogenic to valvogenic cell junction suture (Vj), formed by the valvogenic cells (Vc) comprising the endospore; these represent the surface ridges of the endospore seen in SEM. (uranyl acetate/lead citrate, mag.x3,000

fig 6.50 Maturing capsule of *Auranttactinomyxon*. A prominent stopper (S) is visible at the anterior of the capsule. This protrudes between the two valvogenic cells (Vc). Junctions between the valve and valve cells (Vj), capsulogenic and capsulogenic cells (Cj) and capsulogenic and valve cells (arrows) are all thickened and dense in places. The ridges formed on the surface of the endospore by the valvogenic cells are evident (arrowhead). The coils of the polar filament appear in a figure of eight shape in cross section. The interior of the capsule appears speckled and contains a darker area. (uranyl acetate/lead citrate, mag.x20,500)

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fig 6.51 Mature capsule of *Aurantiactinomyxon*. The confluency of the lucent layer of the capsule wall and the outer layer of the filament is evident, as is the prominent stopper (S) protruding between the gap left by the valve cells (Vc) at their thickened junctions (**arrowhead**) with the capsulogenic cell (Cc). This gap is seen to be a circular aperture in SEM. (uranyl acetate/lead citrate, mag.x30,000)

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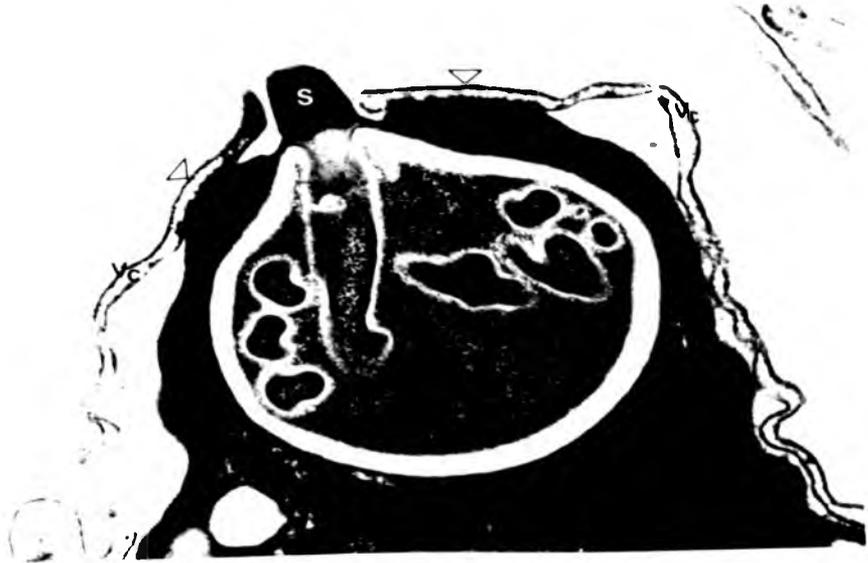
fig.6.52 Scanning electron micrograph of *Aurantiactinomyxon* spore. Note the three apertures for polar filament release (arrows) and the prominent sutural ridges (arrowheads)

capsulogenic cells either side of the future opening canal of the polar capsule (fig.6.47-6.51).

In many sections, polar capsule formation was seen to be unusual. Rather than the capsular primordium being a complete round or club shaped structure, it was often semi-circular or bowl shaped (fig.6.47, 6.48 & 6.49). This was wholly membrane bound. The bottom curve of the bowl possessed the outer dark layer and inner very lucent layer characteristic of the capsule wall of mature spores. Both of these layers were present as a crescent, only partially encircling the primordium. The remainder of the primordium was enclosed solely by a single membrane, and its interior was of a density intermediate to that of the lucent and dark layers. Invariably the polar filament was already present, becoming coiled within the incompletely walled primordium.

In mature capsules, the outer dark and lucent layers wholly enclosed the capsule forming its outer wall up to the stopper (fig.6.50 & 6.51). The lucent layer of the capsule wall was confluent with the wall of the filament itself. The filament contained an electron dense substance, was figure-of-eight, or S-shaped in cross section and became helically coiled within the capsule. In some capsules the inner matrix was 'speckled' and contained a single dark region (fig.6.50) whilst in others it was more evenly staining (fig.6.51).

The mouth of the mature capsule was covered by a prominent stopper structure (fig.6.51) which stained darkly and was covered by the capsulogenic cell membrane. The stopper protruded through a preformed canal (fig.6.48) left by the valve cells, between the cell junctions described above which connected the valve and capsulogenic cells.

Scanning electron micrographs of mature spores of *Aurantiactinomyxon* demonstrated the prominent raised sutural ridges formed by the valvogenic cells above the capsulogenic cells that were seen by TEM. The apertures created by the valvogenic cells for the discharge of polar filaments were also evident (fig.6.52), and were spaced between the sutural ridges. No further ornamentation was present on the spore surface.

6.3.4   Experimental infection of Atlantic salmon with *Aurantiactinomyxon* sp.

All experimental fish for this trial died within 28 days from an unknown cause. No extrasporogonic or sporogonic *Sphaerospora* stages were found in any material from dead and moribund fish either fresh or in histology.

6.4   DISCUSSION

6.4.1   Farm tanks and their sediments

Although they were few in number, and none were found to harbour actinosporean infection, it was evident that some oligochaetes were able to enter the tanks at all farms concerned. This is suprising because the farms concerned have filters which run constantly at 60-100μm in all but spate conditions. When sediments were filtered from tanks, it was clear that management practices of weekly cleaning and the avoidance of overfeeding meant that no build up of benthic fauna occurred. These were in daytime samples. Invertebrate drift, which occurs in the rhithral habitat during the night, might lead to larger numbers of worms entering the tanks and during spate conditions filters are generally removed as they clog too quickly. Under such conditions

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a large number of invertebrates disturbed by fast flowing spate water might be swept into tanks. It is therefore not possible to rule out that fish may become infected simply by the ingestion of whole, live alternate hosts harbouring infections.

No actinosporean spores were found in any tank or water supplies during the filtration study, even during the period which the epidemiological study had given to expect them to be present. It was evident from the present study that actinosporean spores were very delicate, particularly their epispore processes, and could thus easily be destroyed or broken by the speed at which the water passed into the filter. Yokoyama *et al.* (1991) found that fish exposed even to very high numbers of actinospores ($2.7x10^3$-$1.5x10^5$) harvested by very gentle filtration methods did not become infected with myxosporeans, suggesting that even this process damaged actinosporeans. Secondly, from the diurnal release study, as well as the data of Yokoyama *et al.* (1992), it is evident that the release of actinospores may be diminished at certain times of the day, but enhanced during the night. All filtered samples in the present study were taken during the day. Future studies should therefore attempt to sample on a 24hour basis.

The failure to establish experimental infections by seeding oligochaetes taken from the site free of fish with *Sphaerospora* spores could be explained by a number of factors. The worm host (*Tubifex tubifex*) may not have been the correct species for the parasite, or it is possible that some environmental cue or trigger requirement necessary to initiate actinosporean development was not fulfilled in the experimental system.

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The need for the use of phase contrast optics to scan for released actinosporeans in water is to be emphasised. Although ordinary bright field microscopy proved more suitable for distinguishing occasional released whole intact pansporoblasts and pansporoblasts within worms, epispore processes were barely visible. These were easily seen under phase, so that rapid scanning of large numbers of samples could be confidently undertaken. In addition, under phase the endospore is more clearly visible, often being surrounded by a 'glowing' fringe.

The cell well method for obtaining actinosporeans and maintaining live worms, was found to be an excellent way to scan for the presence of infection. The technique was modified in the present study due to the unavailability of an inverted phase contrast microscope. Instead of scanning each well on a plate *in situ*, the water in each well had to be sub-sampled onto a microscope slide and scanned. Although this meant that scanning was less rapid it did not appear to be detrimental in other ways.

Yokoyama *et al* (1991) advocated the retention of single individual worms in each well, perhaps because the oligochaete studied in their experiment was of a large size. In the present study this would have generated many hundreds of wells and thus too many samples to be screened daily. The technique was therefore modified, with five to ten worms isolated in each well. This figure did not diminish worm survival rates to any greater extent than that exhibited by single worms. Worms in wells found to be positive for released actinospores were then isolated and studied further to identify the infected individual.

However the technique has some limitations which may be of importance and which should not be overlooked. Some wells screened were negative for infection on

one occasion, but at a later date showed actinosporean release. As was shown in the experiment on daily release, an infected individual may shed few, if any, actinospores on particular days. This indicates a bias in the screening method, in that infected but non releasing worms may pass unnoticed. It could also however be attributed to a recent onset of release. Worms should therefore be scanned on a number of occasions before being rejected as negative.

A related aspect of actinosporean biology not mentioned by Yokoyama *et al* (1991; 1992) was the problem of actinosporean species which inhabit a coelomic location. Such species remained undetected using the cell well methods. Since localisation as a coelomic or gut-epithelial species is '...well defined and constant...' according to genus and species (Janiszewska, 1955), the technique is selectively biased towards species of actinosporeans inhabiting the gut epithelium. If coelozoic actinosporean species are the alternate life cycle stage of a myxosporean they must await the death of the worm host, or perhaps its ingestion by the next host, to ensure transmission. This should be borne in mind in future studies as many actinosporean infections and thus potential second hosts/actinosporean stages may pass unnoticed, even if at relatively high prevalence in the oligochaete population. *T. mrazeki* in the present study was found only on a single occasion, and then amongst dead hosts, and Granata (1912) found intact spores of *Sphaeractinomyxon* sp in putrefying *Limnodrilus*. A modification to the method is therefore proposed for future studies: Following the screening of apparently uninfected worms on a number of occasions, they should be cut into segments and left in wells for a period of time prior to a further screening. In such a way, coelomic infections would be detectable. Without such a postscript to the

method, prevalences determined for each species may not be truly representative. For species which are released from their hosts, the cell well method provides large numbers of free, released, undamaged actinospores for infection studies.

6.4.2 Specific identity of actinosporeans found

<u>Problems in making comparisons with published reports</u>

An acknowledged problem concerning the proposed two host life cycle was that of the relative numbers of species of actinosporeans and myxosporeans described. Lom & Dykova (1992a) suggested that this could be explained by very large phenotypic variation in fish hosts. That many myxosporean species are synonymous has been hypothesised by Lom & Arthur (1989). However, if we assume that all myxosporeans have a corresponding actinosporean stage the difference in relative number would seem too great, even allowing for large numbers of synonyms. Much of the difference can perhaps be accounted for by the historic difference in level of scientific interest in the two groups. Until identified as myxosporean life cycle stages, actinosporean parasites were a poorly studied group of little economic relevance. The actinosporean literature is sparse in comparison to that of myxosporeans; descriptions of species and their distribution reflective of the interest of a small number of individual workers. Marques (1984) listed actinosporeans as reported from just 29 sites in 10 countries. Apart from recent myxosporean related reports, almost all papers on these parasites since 1958 have been from French workers. As study levels increase as a result of the renewed rise in scientific interest in, and economic relevance of, these parasites to fish diseases we can expect a rise in the number of species described.

The taxonomy of the two groups will, in the near future, require radical and substantial revision. Although a number of authors, particularly the group of Hoffmann in Germany, have repeatedly reiterated this fact, none has as yet proposed a means of doing so. Problems are arising in the manner in which experimentally proven two-host life cycles are presented. In reporting experimental evidence for two-host life cycles authors are failing to describe actinosporean stages in sufficient detail. In the future this will create taxonomic and identification problems, and preclude workers making adequate comparisons. Progress is therefore likely to be held up in determining the specificity, variability and geographical distribution of the Myxozoa. In an extreme hypothesis a single actinosporean species might be responsible for a range of phenotypic myxosporean spore forms in different hosts, organs or seasonal period, yet if not initially described to a degree whereby its future safe recognition is guaranteed, this fact could go unnoticed.

Großheider & Körting (1992) failed to provide the generic identity of the actinosporean stage of *Hoferellus cyprini* giving simply a photograph in which it appears to perhaps belong to *Guyenotia* or *Aurantiactinomyxon*.

Other authors (El-Matbouli *et al*. 1992a; Benajiba & Marques, 1993), although giving the generic identity provide only a drawing or photograph of the relevant actinosporean, and failed to detail measurements and their range. Such data is vital if comparisons are to be made. In the present study it was impossible to compare the Scottish *Aurantiactinomyxon* sp with that claimed to be responsible for *M. giardi* infections in eels by Benajiba & Marques (1993) or with that causing *H. carassi* infections of goldfish (El-Matbouli *et al*. 1992a). Yet measurements taken using the

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scale bar, from the single spore photograph provided in the latter paper indicate that the *Aurantiactinomyxon* sp concerned is remarkably similar morphologically and in dimensions to that herein described.

A further group of authors including Wolf & Markiw (1984), El-Matbouli & Hoffmann (in press) and Kent, Whitaker & Margolis (1991; 1993a) provide measurements, descriptions and photographs of actinosporean stages, but neglect to compare them with species in the literature. The *Triactinomyxon 'gyrosalmo'* of Wolf & Markiw (1984) was later identified as *T. dubium* Granata, 1924, by Hamilton & Canning (1987). The onus must therefore be upon authors to do their utmost to achieve an identification.

Authors presenting such information would not consider describing a myxosporean species in such poor terms. Indeed, poor quality descriptions of myxosporeans in the past created a situation whereby at present many very similar and hard to distinguish species exist, especially in some genera, resulting in subsequent arbitrary reidentification and recognition (Lom & Arthur, 1989). This resulted in the guidelines for species definitions within the myxosporeans produced by the latter authors. Similar such guidelines need to be urgently established for the Actinosporea, without which the complex problem of solving the classification of the Myxozoa could become more difficult, if not impossible.

The comparatively low numbers of oligochaete worms obtained from rivers supplying farms sampled in the present study reflected their clean, clear, highland nature. In such rivers the oligochaete fauna can be expected to be restricted (Clyde

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River Board, pers. comm.). Combined with the probable low prevalence of actinosporean infection in the worm population, this made the search for wild infections unrewarding. It would have been desirable in the present study to have sampled the settlement tank at other farms with salmon *Sphaerospora*. However, of the other sites, two discharge directly into sea lochs, whilst the third farm had recently cleaned out its settlement tank. It is also important to recognise that the actinosporean fauna of the settlement tank may be unrepresentative of that in the wild, and that it is not a source of infection for salmon on the farm. Certainly host oligochaete families are in different proportions between the settlement pond and the river especially with respect to the absence of naiids from the settlement tank. Despite this, the pond provided an ideal site of study due to the fact that its environmental conditions of low oxygen and very fine particulate sediment are those under which certain genera of oligochaete thrive. A large number of potential hosts were thus present in a confined area which may have been exposed to very large numbers of salmon *Sphaerospora* spores and may thus have carried actinosporean stage infections of this parasite. In addition, the fact that one actinosporean, *Synactinomyxon* sp 2, was found in two different classes of oligochaete suggests that some actinosporeans may have a wide host specificity.

Specific identity of actinosporeans

The study found five actinosporean species to be present in the sediments of the settlement tank at farm C. Some of these appear to be new species. Because of the developments in the myxozoan life cycle outlined in the introduction to this chapter it is clear that all five species almost certainly have an alternating myxosporean stage in

their life cycle, presumably in a fish in the river supplying farm C. This presents a dilemma in that if named now as a new species it is likely that the actinosporean concerned will eventually be found to have a long-known myxosporean stage. The actinosporean name will thus be reduced to synonymy by the rules of priority. However, by not naming a species and publishing its description comparison with future reports will be made impossible.

(a)   *Synactinomyxon* sp *1*

Only two species of this genus have been described. *S. tubificis* Stolc, 1899 and *S. longicauda* Marques, 1984 are found in the gut epithelium of *T. tubifex* in Czechoslovakia and France respectively (Janiszewska, 1955). Marques (1984) found both parasites to be relatively common (Marques, 1984). In most respects *Synactinomyxon* sp *1* resembles *S. tubificis.* Janiszewska (1955) failed to include measurements for this species or to provide scale bars on his drawings. From the drawings however, the long epispore processes of *S. tubificis* appear to be around three times longer than the diameter of its endospore. In the settlement tank species, epispore processes were only fractionally longer than the equivalent diameter. Marques (1984) gave a figure of 30μm for the long epispore processes of *S. tubificis*, significantly longer than the mean 18μm of the Scottish species. His value of $12\text{-}15\mu$m for the endospore cavity is slightly smaller than the $14\text{-}19\mu$m of the Scottish *Synactinomyxon* sp *1.* It therefore seems unlikely that the Scottish parasite is *S. tubificis.* *S tubificis* was described from the UK by Hamilton & Canning (1987) from three sites in England. The authors noted only its presence, providing no measurement details or information

on its identification. It is suggested that *Synactinomyxon* sp *1* from the present study is a new species.

(b)   *Synactinomyxon* sp *2*

The individual spores of this species were identical to those of the actinosporean genus *Echinactinomyxon*, and the parasite was initially identified as such. However, Marques (1984) reported a *Synactinomyxon* sp. as a common infection of *T. tubifex* in France. He described the spores of this species as close to *Echinactinomyxon*, but classified them *Synactinomyxon* by virtue of the fact that all eight spores from a single pansporoblast were joined by means of the tip of one of their epispore processes. In this species, later named *Synacinomyxon longicauda* by Marques & Ormieres (1982) all epispore processes are of equal length. The spore has a truncated ellipsoid endospore body of 22-25μm, 80μm x 8μm epispore floats and 7μm polar capsules (Marques, 1984). The Scottish species had very slightly smaller capsules and slightly shorter epispore processes but is otherwise indistinguishable. It is therefore tentatively identified as *S. longicauda*.

Since there is a possibility of confusion between *S. longicauda* and *Echinactinomyxon* spp, a comparison with described species of this genus is also pertinent. The type species, *E. radiatum* was described by Janisweska (1957) from the gut epithelium of *Tubifex tubifex* and was recorded from five sites in the UK by Hamilton & Canning (1987).

However the endospore cavity of *S longicauda* spores is of a completely different shape to that of *E radiatum* and is shorter (20-23μm compared to 27-30μm). In addition

the epispore processes of *E. radiatum*, at around 125μm in length compared to the 64.4μm of the species under study are very much longer. Styer *et al* (1992b) recorded two species of this genus, *E. 'major'* and *E. 'minor'* from *Dero digitata* in the USA. *E. 'major'* has much longer epispore processes (138μm) than *S longicauda* (55-80μm). *E. 'minor'* has a similar endospore dimension (19x23μm) to the Scottish species, although in the latter it is of a more rounded shape. Epispore processes of *E. 'minor'* are also longer than those of the species under study.

No members of the genera *Echinactinomyxon* and *Synactinomyxon* have as yet been reported as life cycle stages of myxosporean parasites.

(c)   *Aurantiactinomyxon* sp

The *Aurantiactinomyxon* sp described in the present study differs from the type species *A. raabeiniuoris*. Although the epispore processes of *A. raabeiniuoris* are of similar length to those of the Scottish species, they are less broad. Its endospore diameter is also larger (17μm compared to 12-15μm). *A. trifolium* (Marques, 1984) has a much larger endospore (20-25μm) than the Scottish species under study (12-15μm) and very much larger epispore processes (40-50μm compared to 19-31μm). *A. stellans* from tubificid sp (Marques, 1984) is also very much larger than the *Aurantiactinomyxon* from the present study, having epispore floats of 70-90μm (compared to 19-31μm). Ormieres (1968 -cited by Marques, 1984) described two forms, large and small, of *Aurantiactinomyxon pavinsis* from *Stylaria herigianus*. Both these forms have markedly shorter epispore processes than the Scottish species. It is suggested therefore that *Aurantiactinomyxon* sp. described in the present study is a new species.

Aurantiactinomyxon was of particular interest due to its high prevalence at farm C, where the salmon harbour *Sphaerospora* infections of high prevalence and intensity. In addition at least four, possibly five myxosporean species have been shown to have *Aurantiactinomyxon* as their actinosporean stage.

The *Aurantiactinomyxon 'major'* responsible for proliferative gill disease (PGD) of channel catfish in the southern USA (Burtle *et al.*, 1991) may be the alternate stage of a *Sphaerospora* sp (*S. ictaluri* has been proposed as the aetiologic agent of PGD). Two species of the genus *Aurantiactinomyxon* were described from the oligochaete *Dero digitata* in ponds harbouring PGD infections by Styer, Harrison & Burtle (1992b). *A. 'major'* has a larger endospore at 18-22μm and longer, thinner epispore floats at 11x36μm than the equivalent figures of 12-15μm and 10-14μmx19-31μm compared to the Scottish species. It also differs in having rounded tips to its epispore processes compared to the distinctly pointed ends to the floats of the species under study. The second species, *A. 'minor'*, also possessed rounded epispore tips. Its endospore is of a very similar diameter to that of the Scottish *Aurantiactinomyxon* (13-16μm) but at 36μm it has longer epispore processes.

Comparisons with the *Aurantiactinomyxon* spp of Benajiba & Marques (1993), El-Matbouli *et al* (1992a) and the actinosporean of Grosheider & Korting (1992) associated with myxosporean transmissions, are not possible since all provide only a single photograph of the species concerned and no measurement data. As outlined above, the *Aurantiactinomyxon* sp responsible for *H. carassi* infections (El-Matbouli *et al*, 1992a) appears to be very similar to that from Scotland. The *Aurantiactinomyxon* sp implicated in the *C shasta* life cycle by Bartholomew *et al*, (1992) cannot be

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compared as the material was presented at a conference. An unidentified *Aurantiactinomyxon* was reported but not described by Hamilton and Canning (1987) from a single UK site, making its comparison with the species from farm C impossible. The *Aurantiactinomyxon* sp. under study therefore appears to be a new species.

(d)   *Triactinomyxon* sp

Marques (1984) listed 9 species of this genus: *T. ignotum; T. magnum; T. legeri; T. mrazeki; T. dubium; T. ohridiensis; T. petri; T. naidanum;* and *T. robustrum.* *T. ignotum* and *T. dubium* were recorded in the UK by Hamilton & Canning (1987) who pointed out that the *T 'gyrosalmo'* of Markiw & Wolf (1984) was probably *T. dubium.* *T. ignotum, T. legeri* and *T. mrazeki* were described in some detail by Mackinnon & Adam (1924) from the Thames in London. *Triactinomyxon mrazeki* has a style of ~150μm, and endospore of 25-65μm and contains over 50 sporozoites. These measurements are virtually identical to those of the *Triactinomyxon* sp from the settlement pond at farm C. The farm species has a style of 110-150μm, endospore 45-60μm and 60+ sporozoites. In addition to the very close measurement similarities, the latter figure, that of the number of sporozoites, is of particular significance since *T. ignotum, T. ohridensis, T. naidanum, T. magnum, T legeri* and *T. dubium* have only 8, 8, 12, 16, 24, and 32 sporozoites respectively.

It is therefore considered that the actinosporean from the salmon farm is *Triactinomyxon mrazeki* Mackinnon & Adam, 1924. This differs in size and sporozoite number from the agent of whirling disease, *T. dubium* (El-Matbouli & Hoffmann, 1989); and from the *Triactinomyxon* of Kent *et al* (1993a) responsible for *Myxobolus arcticus*

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infections. The latter species has a shorter style (73.5-82.0μm compared to 110-150μm) but much longer epispore processes (294-360μm compared to 107-153μm) than *T. mrazeki*. It also differs from the *T. 'myxobolus cotti'* of El-Matbouli & Hoffmann (1989) since this species has just 16 sporozoites and a total style + epispore length of just 88.64μm on average. Two species of this genus were reported in Styer *et al* (1992b), *T 'major'* and *T. 'minor'*. Neither possessed a style according to the authors, leading to doubt as to whether their generic identification was correct.

(e)   *Raabeia* sp

Both *Raabeia* spp described by Janiszewska (1957) were identified from the coelomic body cavity of oligochaetes; *Raabeia magna* from *Limnodrilus hoffmeisteri* and *Raabeia gorlicensis* from *T. tubifex*. The endospore of *R. magna* is 51-58μm, much larger than the 24.6μm of the Scottish species. The barrel shaped endospore of *R. gorlicensis* is 35μm long, again larger than the Scottish species, and it also has longer epispore processes. *R. furciligera* (Jan & Krzton, 1973 -cited by Marques, 1984) differs from the Scottish species by virtue of the 1-5 ramified furcae formed at the tips of its epispore processes. *Raabeia* sp of Yokoyama *et al* (1991), the alternate life cycle stage of *Myxobolus* infections of goldfish, has a similar epispore length (200μm) to that of the Scottish *Raabeia* (mean 219μm, range 170-290μm), but has an endospore of just 9μm diameter and polar capsules of 3.3μm, much smaller than their equivalents in our species. The *Raabeia* sp. in the present study therefore appears to be a new species.

The clear space between the base of the polar capsules and the top of the endospore cavity described in the species from Scotland is also apparent in the

photographs of Yokoyama *et al.* (1991), but to a lesser extent. However no reports have previously mentioned the aberrant *Raabeia* spores, with six rather than three epispore proceses seen in the present study. Aberrant spores are a common feature of myxosporean development (Shulman, 1966), but appear not to have been described from actinosporeans.

6.4.3 Relationship to salmon *Sphaerospora*

If the role of actinosporeans as alternating life cycle stages of myxosporeans is generally applicable to all myxosporeans, we must assume that all five actinosporeans described from the settlement pond in this study have a corresponding myxosporean stage parasitising one or more fish species in the water supplying the farm, or in the farm itself. Fish species in the river at farm C include wild Atlantic salmon, brown trout, sea trout, eels, and three-spined stickleback. The latter species can be found swimming in the settlement pond itself and individuals harbour both *Sphaerospora elegans* and *Myxobilatus gasterostei* infections (personal observations).

Aurantiactinomyxon was chosen for much of the further study during the investigation. This was primarily due to its higher prevalence in total worms studied, compared to other actinosporeans found during the survey and thus more material was available for study of this genus; also it is not unreasonable to suppose that its high prevalence indicates a possible role in the life cycle of the salmon *Sphaerospora*. The settlement pond receives all water from tanks on site and the high levels of infection of fish at the farm with *Sphaerospora* sp, might result in the deposition of many hundreds of thousands of sphaerospores into the sediments of the pond. Thus the 'correct'

alternate actinosporean stage of the salmon *Sphaerospora* life cycle might be present at a much higher level than such a stage of another non-salmonid myxosporean. Worms harbouring infections of actinosporeans which were life cycle stages of any other myxosporean would have been infected by myxospores released from wild fish. However the need for caution in this hypothesis is reflected by the fact that Hamilton & Canning (1987) found no changes in the prevalence of the correct actinosporean *Triactinomyxon* stage of *M. cerebralis* upon the addition of spores to a worm population in large numbers (however Markiw (1986) showed a limit to the dose dependency of actinosporean production- see Introduction). In an experimental whirling disease system Hamilton & Canning (1987) also found two other species of actinosporean at a higher density than the 'correct' *Triactinomyxon* sp.

A second reason for the selection of *Aurantiactinomyxon* for further study was that, of the five genera reported from the settlement pond, only three have been recorded as having a role in myxosporean life cycles: *Raabeia*, *Triactinomyxon*, and *Aurantiactinomyxon*. This does not, of course, rule out that the other species found are likely to have myxosporean stages. *Raabeia* and *Triactinomyxon* have, in all reports to date, been associated with *Myxobolus* sp infections in fish (sub-order Platysporina: family Myxobolidae). *Aurantiactinomyxon* spp were however implicated in PGD (*Sphaerospora ictaluri*), *Hoferellus cyprini*, *H. carassi*, *Ceratomyxa shasta* and *Myxidium giardi* infections. All of the latter species are of the sub-order Variisporina. The first three are all of the family Sphaerosporidae to which the salmon *Sphaerospora* belongs. *C. shasta*, along with *Leptotheca* spp, is a member of the Ceratomyxidae; the close relations of this group to the salmon *Sphaerospora* and other *Sphaerospora* spp. has

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already been mentioned (chapter 3). Therefore there appears to be a possible pattern that indicates that platysporine myxosporeans may have triactinomyxid actinosporean stages, whilst variisporine myxosporeans such as *Sphaerospora* have aurantiactinomyxid stages.

It was unfortunate that the fish experimentally infected with *Aurantiactinomyxon* sp died within three weeks post-exposure. This was probably attributable to the difficulties of maintaining salmon over long periods in small tanks, especially since many control fish held under the same conditions died also. El-Matbouli *et al.* (1992c) could find no sign of *M. cerebralis* infections in experimentally infected fish from 20 hours post actinosporean exposure until day 40 onwards. Therefore the apparent absence of any developmental *Sphaerospora* stages in the fish that died following exposure is not conclusive in showing that the *Aurantiactinomyxon* was not infective to salmon, and thus not responsible for salmon *Sphaerospora*. Early stages of salmon *Sphaerospora* might have been expected to be detectable earlier in an infection than whirling disease stages, due to the proliferative nature of the *Sphaerospora* extrasporogonic stage, however the epidemiological study (chapter 4) indicated that a prepatent period of a number of weeks existed in natural (presumably actinosporean-induced) infections. A further problem lay in the low dose rate to which fish were subjected. As was shown by the daily release experiment, the number of *Aurantiactinomyxon* spores shed was variable from day to day, and for the two worms studied was quite low. Markiw (1992a) illustrated that infections with *M. cerebralis* were not produced by dose rates of less than 100 spores per fish, and that even at 100 spores per fish, resulting infections

were very light. Perhaps had the fish survived, an attempted transmission with even the correct actinosporean stage would have failed due to insufficient supplies of spores. It is pertinent at this point to contrast the background to the current study and the search during it for an alternate host and spore, with that undertaken by successful workers in the literature (see below).

A future experiment might hold sediment from the settlement pond in the laboratory and expose naive Atlantic salmon to it. This would establish whether the material present in the settlement tank was definitely infective for the salmon *Sphaerospora*, but would still have not establish which of the worm and actinosporean species present were the correct host and actinospore respectively. It seems unlikely that a sample of sediment, of the size that could be held in the lab, could possibly contain a different or wider range of oligochaete hosts and actinosporean species than the much larger areas of the settlement tank that were sampled in the field resulting in the discovery of the five species of four genera reported here.

Markiw (1986) showed prominent peaks of release of *Triactinomyxon* sp from *Tubifex tubifex* for about twenty days in three experimentally infected populations. Her figures of mean release per worm for two of the studies were 3420 and 1790 from a worm population of 15,000 over 60 days. However only 20% (3,000) of these worms became infected. Therefore a mean of 285 and 149 spores were shed per infected worm per day in each case. This figure compares well to the mean daily release of 142 and 180 spores for two *Aurantiactinomyxon* infected worms in the present study. However Markiw (1986) showed that daily release varied markedly over the 60 day period; during

a 10-20 day peak period of release the 3000 infected worms in the two samples released 2090 and 1190 spores per minute respectively, giving mean peak daily release figures per worm of 1003.2 and 571.2. This is considerably more than the figures of the present study. It seems likely from the epidemiology of the salmon *Sphaerospora* that if *Aurantiactinomyxon* sp. were the correct actinosporean stage of the parasite, its release into the environment would be at a peak at the time that oligochaetes were sampled for the present study. A higher peak release for Markiw's study is hardly suprising, even if we assume that a presporogonic proliferative phase occurs in the worm, since she seeded her two populations of tubificids with 380 and 38 spores per worm. It seems unlikely that worms in the settlement tank could ingest anywhere near so many spores as were provided in the experimental system, even allowing for the fact that so many *Sphaerospora* infected fish are present on site.

The daily variation in release numbers in the present study was particularly notable. Markiw's mean release figures give no impression of how release patterns may vary from day to day between individual worms within the same worm host population. Further study into the dynamics of actinosporean infection, especially in the wild, is warranted and could aid understanding of the epidemiology of myxosporean parasites. This may perhaps provide insights which could establish specific times when waters are particularly infective and help in the devising of control and treatment strategies.

There are large differences in the numbers of actinospores released between this study and that of Yokoyama *et al* (1991). Average daily release of *Raabeia* sp was 1-2×10^6 spores, a figure ~100x greater than that of *Aurantiactinomyxon* infected worms, and 20x the peak release figures of Markiw (1986) for experimentally infected tubificids.

The worms for the Japanese study were from infected farm pond sediments, but the host concerned, *Branchiura sowerbyi*, is a particularly large oligochaete (10cm+ long). The heavy infections and high daily release figures can be possibly attributed to this. Because the Japanese worms were obtained from a non experimental system there was no way of knowing what stage of release they had reached. Peak release for this population might have been even higher. Comparison does however show that marked day to day variation in numbers of spores released is common to both the *Raabeia* sp from Japan, and the *Aurantiactinomyxon* under study here.

Yokoyama *et al* (1992; 1993a) presented information on the circadian patterns of actinosporean release. Release peaks occurred during the night; by experimentally manipulating the photoperiod to which the worms were exposed, release time could be influenced. The present study showed similar nightly peaks for an individual worm. There was much variation between each two-hourly period even during peak release time, and a limited daytime release. Since quite low numbers of actinospores are shed during the day, their chances of detection in the subsample taken for counting may be diminished, and low levels of release missed. Since only one worm individual was studied it is impossible to generalise as to whether similar patterns are shown by all worms infected by *Aurantiactinomyxon*, or indeed whether release time might vary according to whether worms are at peak release or near the onset or end of release. In addition, in a cell-well a worm is subject to non-natural environmental conditions, having no available substrate, food, or water flow, and a less stable temperature; all factors which might have an affect upon the host-parasite relationship and thus influence

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patterns of release. It cannot be assumed that such findings are necessarily an indication of what happens in relation to farm based infections. A more elaborate experimental system is really required to provide sufficient replicates and material for study to rule out variation.

Polar filaments of a range of actinosporean spores were seen to evert in response to the mucus of salmon, brown trout, stickleback and bream. This indicated that the process may be less specific than would be assumed from Hoffmann *et al.* (1992c). They reported that the actinosporean stage of the *M. cerebralis* life cycle was able to discriminate between naive host fish and those which had previously been infected with whirling disease, and that actinosporeans introduced to the latter fish failed even to evert their filaments. It seems difficult to conceive that a recognition system in a group such as the myxozoa is so sensitive as to not only distinguish a salmonid host from a non-salmonid one, but also a naive from a previously infected individual. Such a system would need to occur at the point of contact between the fish mucus and the polar capsule stopper of the spore, be rapid since the host is fast moving, and be remarkably discriminating. Perhaps our two *Synactinomyxon* spp, *Aurantiactinomyxon* and *Raabeia* sp, which evert their polar filaments to a range of fish species' mucus are the alternate life cycle stages of myxosporeans with broad host specificity, and thus do not have to be so discriminating. Alternatively they could be species in which host recognition and suitability is not established at the mucus level or is not so precise. Yokoyama *et al*, (1992; 1993a) found a similar reaction, with the mucus of several fish species able to stimulate filament eversion in their actinosporean species. However, they found that

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actinospores of the genera studied would not release their filaments in response to mucus from certain fish species (see introduction), indicating that a definite chemosensory response is present. In the present study care was taken to use fresh material and preparations, since spores in old, control preparations, as they dried out, spontaneously everted their filaments. In addition, a percentage of all released actinosporeans when harvested from cell wells were found to have spontaneously lost their filaments and sporoplasms, perhaps due to the fact that the earliest released spores may have been in the well for 24 hours.

Yokoyama *et al* (1992; 1993a) and Benajiba & Marques (1993) showed that the sporoplasm of actinosporeans of genera such as *Aurantiactinomyxon*, *Raabeia*, and *Echinactinomyxon* was released from spores in response to fish mucus, as occurred in the present study. A proportion of *Aurantiactinomyxon* spores spontaneously lost their sporoplasms, as did those of the other genera studied. Similar observations were made for *Synactinomyxon* sp., *Echinactinomyxon* sp. and *Aurantiactinomyxon* sp. by Marques & Ormieres (1982), with the sporoplasm of some species remaining motile for up to 1 hour. However, in the present study spontaneously lost sporoplasms often exited not via the hatching of the spore along the suture of its shell valves, but by moving out of the endospore cavity into the arms of the epispore.

The movement of the released sporoplasm by means of pseudopodial projections is reminiscent of the manner in which the trophozoite of the myxosporean *Chloromyxum leydigi* moves (Lom *et al.*, 1983a). Sporoplasms presumably require a motile ability if they are to actively establish in the epithelium of the fish following spore hatching on the epithelial surface, and move to deeper layers to reach the circulatory system.

Ultrastructure of *Aurantiactinomyxon*

Lom & Dykova (1992c) and Marques (1984, 1987) identified the earliest stages of actinosporeans as binucleate cells, with nuclei arranged as in a diplokaryon. This stage was also seen in the *Aurantiactinomyxon* sp under study. Marques (1984, 1987) attributed such stages to a fusing of two cells produced by an earlier shizogony. In this hypothesis, this cell later divided, each nucleus having a predetermined fate. One produced an encircling cell and the alpha line of gametocytes, the other an encircling cell and the beta gametocytes (fig.6.2).

Lom & Dykova (1992c) attributed the presence of early non-developed stages when mature spores were apparent to their representing the cells of a proliferative mechanism. Such a mechanism would seem necessary in actinosporeans to explain why, for example, the *Aurantiactinomyxon* sp under study is present at a prevalence of less than 1% in the population of oligochaetes, yet each infected worm contains hundreds of spores. It seems very unlikely that a single oligochaete could take in a large number of myxosporeans, whilst neighbouring worms did not ingest any. Another answer could be autoinfection, but experimentally this has failed on a number of occasions.

Lom & Dykova (1992c) regarded the four cell unit of two enveloped and two encircling cells as the first definite non-proliferative and therefore sporogonic, pansporoblast stage.

Triactinomyxon legeri early stages closely resembled the *Aurantiactinomyxon* sp in this study in nuclear appearance and also in the presence of dense cytoplasmic inclusions and phagosomes. Lom & Dykova (1992c) suggested that phagosomes

represented feeding by the invagination of folds of plasmalemma and that they were evidence of a high metabolic rate within the cell. Phagosomes were not seen in *Neoactinomyxon eiseniellae* (Marques, 1987). This seems a reasonable hypothesis, with the encircling cells acting as 'nurse' cells for the sporogonic cells

Two differentiable cell types were seen in *Aurantiactinomyxon* in the present study, one containing phagosomes and inclusions and forming the encircling cells, the other lacking them and forming the inner cells. Lom & Dykova (1992c) described similar cell types in *T. legeri*, but the folds and projections of the cytoplasm of early stages of *T. legeri* were not seen in the present study.

The junctions between *T. legeri* envelope cells did not overlap in the way shown for the *Aurantiactinomyxon* sp, but rather were formed by the edges of each cell.

Lom & Dykova (*op cit*) did not describe the possible alpha and beta gametocyte differential characteristics seen in the present study. However, the sexual stages of actinosporeans require further investigation. They did note that encircling cells retained their intact structure in *T. legeri* in much the same manner as they do in the *Aurantiactinomyxon* sp.

The same structural similarities exist between the *Aurantiactinomyxon* sp. sporoplasm and that of *T. legeri*. The sporoplasm represents a plasmodium of germ cells and nuclei. However, the dense bodies seen in the early germ cell cytoplasm of *T. legeri* were not seen in this study. The *Aurantiactinomyxon* sp, *T. legeri* (Lom & Dykova, 1992c) and *N. eiseniellae* (Marques, 1987) all contain sporoplasmosomes in the sporoplasmic cytoplasm, though they differ in size between species. The manner in which the valve cells of *Aurantiactinomyxon* sp surround the other cells comprising the

spore is similar to that described for *T. legeri* and *N. eiseniellae*. However, the valve-valve cell junctions of *T. legeri*, described as inconspicuous by Lom & Dykova (1992c) were prominent raised and thickened ridges forming the valve-valve cell suture in *Aurantiactinomyxon*. These are reminiscent of the septate junctions which join myxosporean shell valves. These prominent ridges are present on the surface of mature spores and form three ridges between the polar filament discharge holes when viewed under SEM.

The telescopically folded membranes of the valve cells of the *Aurantiactinomyxon* sp are seen in both *N. eiseniellae* and *T. legeri*. These represent the epispore processes seen in released mature spores and inflate on entry into water. That they retain their nuclei, but lose all trace of their cytoplasmic contents during development was shown by Marques (1987) and Lom & Dykova (1992c). This also occurs in the *Aurantiactinomyxon* sp; that nuclei are retained can be further evidenced by their clear presence in the epispore processes of mature spores seen under phase and Nomarski interference light microscopy.

Important differences are apparent between the capsulogenic process in the *Aurantiactinomyxon* sp being studied and that of *T. legeri* and *Aurantiactinomyxon eiseniellae*. The dense capsular primordium stage and the sinuous join between adjacent capsulogenic cells in *T. legeri* was not seen in *Aurantiactinomyxon* sp. The dense primordial cap was also not present in *N. eiseniellae* (Marques, 1987).

Marques (1984) demonstrated the unusual manner in which the capsules are formed in *Aurantiactinomyxon raabeiunioris* and *S. tubificis*. In these two species the capsule was formed by a cup-shaped, thick structure with a fibrous layer gradually

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subtending the vesicle wall. Filament coils become deposited within the developing primordium irrespective of whether the whole capsule had been formed. These findings mirror those of *Aurantiactinomyxon* sp described in the present study and contrast markedly with those of Lom & Dykova (1992c) from *T. legeri* and Marques & Ormieres (1982) from *Aurantiactinomyxon eiseniellae*.

However, the cell junctions seen between both capsulogenic and capsulogenic cells and those between capsulogenic and valvogenic cells in *Aurantiactinomyxon* sp were seen by Lom & Dykova (1992c) in *T. legeri*. They seem to act in the same manner as the directly comparable structures described on rare occasions in Myxosporeans (Lom & dePuytorac, 1965; Desportes & Theodorides, 1982) and present in the sporogonic stage of the salmon *Sphaerospora* sp (see Chapter 2). The dark mass within the centre of the matrix of the maturing capsules was seen in both *T. legeri* (Lom & Dykova, 1992c) and the *Aurantiactinomyxon* sp under study, but the "speckled" matrix of the latter was not described in *T. legeri*, it being uniformly dense in the latter species.

The "empty space" around the hind part of the capsule was attributed to artefact by Lom & Dykova (1992c). This view is supported by the present study in which no such spaces were seen. Almost mature myxosporean polar capsules are difficult to section and sometimes pull out or away from surrounding tissues (personal observations).

Stopper appearance and structure and its covering by capsulogenic cell membrane at the point at which it protrudes the valve cells are in agreement with other authors, as are the grooves along the length of the polar filament itself, and its confluency with the

lucent layer of the capsule wall.

Lom & Dykova (1992c) described the similarities and differences between actinosporean and myxosporean development. They pointed out that the very different gross appearance of mature spores of the two groups is misleading since cell structure at both the light and electron microscope level is more or less identical. Similarities include multicellular spores; desmosome-like junctions; pansporoblast formation; the presence of cells within cells; the absence of centrioles in cell division; the presence of sporoplasmosomes; polar capsule formation, filament structure and morphogenesis; intercellular capsulogenic to capsulogenic and capsulogenic to valvogenic cell junctions within spores; and the similarity between early actinosporean cells and myxosporean secondary cells. To these can be added the presence of pre- or extra-sporogonic proliferative cycles.

Differences are few. The sporoplasm of actinosporeans contains large numbers of cells, whilst, as a rule, that of myxosporeans comprises a single, binucleate cell or two uninucleate cells. Only in some *Kudoa* spp are enveloped sporoplasm cells present (Stehr, 1986 found two morphologically distinct sporoplasm cells in *Kudoa thyrsitis*, one of which enveloped the other). In all actinosporeans eight spores are formed within each pansporoblast, and the pansporoblast is composed of two enveloping cells which retain viability throughout sporogony. In myxosporean pansporoblasts, only one or two spores form, and the single pansporoblast envelope cell usually becomes degenerate. In actinosporeans the shell valves produced are hollow, having lost all cytoplasmic contents, and have inconspicuous cell junctions, whilst in myxosporeans the valves are

solid, degrading into dense structures which are joined by distinct septate junctions. The only other difference lies in the capsule release system. In actinosporeans the capsule is stoppered by a granular cone which projects through an aperture made by the valve cells into the external medium. In myxosporeans this structure lies above the tip of the capsule and fills a hollow canal made by the valve cell.

In comparison to the similarities between the two groups ultrastructurally and in their development, their differences are few and appear relatively less important. Indeed the present study has shown that one of the differences mentioned by Lom & Dykova (1992c), that actinosporeans have inconspicuous valve cell junctions, can be discounted by their presence in *Aurantiactinomyxon* at the TEM and SEM level. Possibly as the number of actinosporeans ultrastructural descriptions increases, the range of actinosporean groups and genera studied will widen and many gaps will be filled.

The majority of the differences between the two groups can surely be attributed to their ecology, adaptation to the different hosts involved and the significance and role of the two stages. The actinosporean phase is both short lived and delicate (Markiw, 1992b; Yokoyama *et al*, 1991) and is planktonic (Janiszewska, 1955). The hollow valve cells of actinosporeans, with no cytoplasmic contents, form the floats which render the spores planktonic and therefore represent an adaptation to this existence and thus to host-finding. Released actinospores must attain a height in the water column at which they are likely to encounter the correct fish host. Myxosporeans by contrast have a thickened resistant set of shell valves, are very long lived and sink to the sediments of their habitat. The latter again can be considered to represent a host-finding mechanism,

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this time to seek an oligochaete.   Myxospores are equipped to withstand harsh environmental conditions including freezing and passage through the intestine of a range of piscivorous birds and fish, and may thus represent a disseminatory phase. Differences in polar capsule release, with actinosporean stoppers directly in contact with the environment whilst those of myxosporeans plug a canal in the shell valve may again be attributed to host aspects, since polar filament release would appear to require a more rapid response in the case of actinosporeans to ensure contact with a potentially fast moving host.

Chapter 7
General discussion

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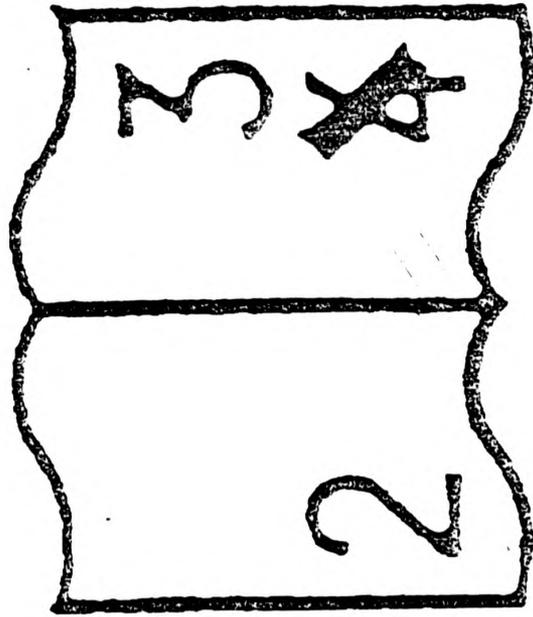
GENERAL DISCUSSION

This study has attempted, by a variety of approaches, to investigate the life cycle of a single myxosporean species parasitising Atlantic salmon.

A long term, regular sampling programme over a three year period and encompassing three cohorts of fish at two farms has provided epidemiologiacl data on two distinct life cycle stages of the parasite. Detailed light and electron microcope studies on these two stages, extrasporogonic and sporogonic have described their morphological features, from which the parasite's developmental sequence was determined. Apart from reports of PKX, which does not appear to sporulate in salmonids, the study presents the first record of extrasporogonic stages of a myxosporean from Atlantic salmon, and only the second from a salmonid host. The extrasporogonic stages were particularly notable for their large size and for the very high numbers of secondary cells seen in the most developed forms compared to other reports. Sporogonic development fitted a similar pattern to that reported by other ultrastructural studies, but details such as the capsulogenic-valvogenic cell junctions seen in both the salmon *Sphaerospora* and the actinosporean of the genus *Aurantiactinomyxon*, proposed to have a role in the organisation of capsulogenesis have been less often described and merit further attention. Clearly myxosporean and actinosporean sporogenesis involve complex cellular interactions between functionally and structurally differentiated cell types.

Attempts to identify the parasite highlighted the problems associated with the identification of the myxosporeans as a group, with spores of the species under study being morphologically similar to both the genus *Leptotheca* and the genus *Sphaerospora*. Although the parasite was eventually assigned to *Sphaerospora*, the reasons for doing

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so were primarily based on a lack of information regarding the existence of extrasporogonic cycles in *Leptotheca*. The relationship between these two genera warrant further investigation. The most likely candidate, based on reports of *Sphaerospora* from salmonids was *S. truttae*. The salmon parasite was transmitted successfully to naive brown trout as well as Atlantic salmon so strengthening the link with *S. truttae*. However, it was not transmitted to rainbow trout, indicating that it might have a restricted host range even amongst salmonids. The importance of studying spore variation was highlighted by the fact that spore morphology changed markedly as spores matured, and by small variations between spores found in Atlantic salmon and those from experimentally infected brown trout. It was not possible to identify the parasite to species level due to a lack of comparable information on *S. truttae*, particularly on extrasporogonic stages and details of ultrastructure. This further illustrates the problems of myxosporean taxonomy, which is reliant upon spore measurements and morphometrics to distinguish species. There are a growing number of reports of *Sphaerospora/ Leptotheca* spores from salmonid urinary systems, some of which have been poorly described and very few of which can confidently be recognised in new hosts or locations. It was therefore very difficult to draw together these reports to find common features. Until cross infection experiments involving the laboratory exposure of salmonids to actinosporean infective agents for the species concerned are possible, the inter-relationships of these species will remain uncertain.

The epidemiological study demonstrated that the parasite exhibited a very similar picture each year. The evolution of a narrow window during which waters are infective, timed to coincide with the presence of large numbers of newly susceptible fry, would

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appear to be a very effective strategy. This is especially so, since the study has shown that fish mount an effective defence against the parasite and are subsequently refractory to reinfection. Fish were found to retain spores and sporogonic stages for at least 18 months in freshwater. The fact that fish may not release spores, or release them over a long period of time could serve to disseminate the species, and may mean that older fish are important reservoirs of infection. The variation in infection intensity shown between individual fish and individual farms, indicated that the degree to which extrasporogonic stages could proliferate might be limited, and so infection intensity, despite the presence of proliferative stages in the life cycle, may still be directly related to the number of infective agents establishing in the fish. This is important for control; if the extrasporogonic stage had no such limitation, then any strategy which reduced but did not eliminate the degree of exposure to infective agents would be of little or no value. By demonstrating a limit, the present study has proposed that such a strategy could result in low intensity infections, which induce little or no pathology (chapter 5).

The study of the pathology of the salmon *Sphaerospora* indicated that the parasite can be placed alongside other members of the genus such as *Sphaerospora renicola* and PKX, whose extrasporogonic stages of the life cycle are pathogenic. Since the salmon *Sphaerospora* is considered to be responsible for morbidity and mortality at certain farms and may also render fish more susceptible to secondary invaders (chapter 5), it is worth considering possibilities for control. The present study has accumulated ecological information on the host-parasite relationships of salmon *Sphaerospora*, in terms of its epidemiology, pathology and development. In addition it has identified a number of aspects of actinosporean biology, such as circadian patterns of spore release. These may help to focus on certain points in the life cycle, or short times of the year

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when interventions by whatever means might be most effective. Burtle, Harrison & Styer (1992c) considered that the use of such data could potentially help to avoid undesirable and drastic large scale, wholesale chemical treatments.

The realisation of the presence of alternate hosts in at least some myxosporean life cycles has provided a whole new area for investigative research into control measures. It is notable than many of the long-known, effective control measures applied to limit *M. cerebralis* spread and infection, such as the application of UV irradiation to rearing water and the liming of ponds (Hoffman, 1975), were unknowingly effective against the build up of alternate hosts and/or destroyed incoming actinosporean stages, as well as myxosporean spores. Thus many such measures worked without a requisite understanding of the reasons for their success. These measures could now be refined and further improved, concentrating upon the elimination of potential alternate hosts in earthen pond culture systems, the destruction of actinosporean stages in these worms, or by the killing of released actinosporean stages before they reach the fish, by chemical or other means. This would potentially minimise fish infections, and reduce a parasite's ability to cycle within farms. By concentrating on intervention at the alternate host/actinospore stage in the life cycle, the use of chemotherapeutants on fish, and the associated risks of toxicity, cost and residues might be avoided.

In the case of the salmon *Sphaerospora*, no build up of infection exists; rather, farm infections recur annually by the influx with the water supply of infective, presumably actinosporean, stages generated by natural infections of wild fish populations. Here though there still exist opportunities for control, some of which the present study has identified. The elimination of wild fish and/or the elimination of oligochaetes from natural water systems is neither possible nor environmentally

acceptable, and so other strategies must be pursued. Life cycle and epidemiological studies have shown that extrasporogonic stages of the salmon *Sphaerospora* are present in a fish population for a limited period of time, and are the most pathogenic stage. A chemical treatment would therefore only have to be delivered to the fish for a restricted period to be effective in eliminating or reducing infection levels. This could perhaps make the use of compounds such as Malachite green, or Fumagillin feasible despite the problems associated with their long term usage (Hedrick, Groff & McDowell, 1988; Sitja-Bobadilla & Alvarez-Pellitero, 1992). The testing of treatments was beyond the scope of the present study, but their investigation is warranted, especially due the fact that the period for which they would need to be used might be quite restricted. The identification of a pre-patent period in salmon *Sphaerospora* infections is important for control strategies. Care would need to be taken in the application of treatments, especially those such as Fumagillin which are most efficaceous as prophylactics to begin administration early, prior to the parasite's first detection.

Since the study indicated a synchronous but limited time scale of release of infective stages, expensive or logistically difficult techniques such as the use of UV irradiation or the implementation of fine filters to lower infection levels by the removal of infective stages would only have to be imposed for a relatively short period of time. The UV irradiation of infective waters appears a very effective means of reducing myxosporean infections (Sanders *et al*, 1972; Hoffman, 1975; Wyatt, 1978). However the current economic situation within the salmon farming industry is unlikely to justify the installation of such expensive equipment, despite its benefits to fish health in general. Should the diurnal release pattern characteristic of the *Aurantiactinomyxon* sp from Scotland (chapter 6) prove applicable to the actinosporean responsible for salmon

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Sphaerospora, this period may be further restricted to just a few hours per night to significantly decrease the levels of infection. Simple strategies such as not using water from the river for a few hours and recirculating or using bore-hole hatchery water for that period may therefore be effective. A later transfer of fish from uninfected sites, or uninfective bore-hole hatchery water onto infective waters might also be effective where possible, in reducing the levels of infection in the population. This has been shown to be successful in reducing *M. cerebralis* and PKD infections (Hoffman, 1990; Ferguson & Ball, 1979; Ellis *et al*, 1985).

Another possible means of rearing healthy fish in infective waters might be the stocking of resistant fish strains at infected farms. From the literature on *C. shasta*, PKX and *M. cerebralis* infections, it is clear that single salmonid species strains may exhibit varying degrees of susceptibility to myxosporean parasites, related to their historic exposure to the parasite concerned (Ellis *et al*, 1985). It seems likely then that wild salmon from the same water body/river that supply the hatcheries may be more resistant to salmon *Sphaerospora*, due to evolutionary exposure to the parasite, than are currently stocked strains of fish bought in from other producers.

It is clear that stresses such as handling, grading and high stocking density should be minimised during the time that the extrasporogonic stages are at their highest levels of prevalence and intensity. This is effective in reducing losses due to PKD also (Ferguson & Ball, 1979; Clifton-Hadley *et al*, 1984). The latter authors considered PKD a chronic disease made acute by stress.

Another means of easing losses due to PKD is the transfer of fish onto partial salt water (Lom & Dykova, 1992d). This is a management practice introduced at farm A where facilities exist for the mixing of fresh water with water from an adjacent sea

loch. It seems to effectively halt losses due to the salmon *Sphaerospora* and, further, aids in fish smoltification. This, combined with the fact that sporogonic stages were found in the present study to be lost within a short period of time in the sea, makes it unlikely that the parasite can cause losses in marine rearing facilities.

The present study identified five actinosporean species, three of which appear to be previously undescribed, from a single habitat at a farm which harbours *Sphaerospora* infections. Very few species of actinosporean have been studied in detail, and so the information presented here on the ultrastructure of actinosporean development and on actinosporean biology are important, not only with respect to control, but also to the scientific problems of the alternate host life cycle and its associated taxonomic difficulties.

Many problems that arose with respect to identifying the correct alternate oligochaete host harbouring an actinosporean life cycle stage of salmon *Sphaerospora* can be related to the nature of the infections at the farms concerned. Almost all reports of two-host life cycles to date have come from experimental systems where the seeding of thousands of mature myxosporean spores to a correct oligochaete population has resulted in very high prevalences of infection and the subsequent release of many thousands of actinosporean spores for infection studies. Other successful studies have been from enclosed recirculating study systems in which a particular myxosporean is known to recur on a seasonal basis (Hedrick *et al*, 1992p) or pond farms with mud bottomed sediments where an infection is endemic (Yokoyama *et al*, 1991).

All the farms studied have high prevalences of infection with *Sphaerospora*, and some have high intensities of infection also. The farming situation obviously modifies

the situation by presenting many hosts in a small enclosed environment, enhancing the chances of an actinosporean reaching a fish if it enters a tank than if it remains in the river. Due to the planktonic nature of actinosporeans it could also be that they accumulate in a tank, remaining at a certain height and thus not being drained away. This could explain the high prevalences and intensities of infection in farmed salmon, and also show why managers have associated high levels of infection with salmon *Sphaerospora* with slower flowing tanks. Altering tank flow by increased input or drainage, or by modifying the tanks flow characteristics have successfully reduced infection intensities at one farm.

The farms of the present study feature modern intensive systems and facilities with painted metal, or fibreglass tanks in which no sediment build up can occur due to efficient management cleaning practices. The source of infection is therefore from the natural environment. It is evident that somewhere in the wild large numbers of actinosporeans are being released. A relatively small proportion of the total river flow is diverted to the farm yet this contains sufficient actinosporeans to generate 100 % prevalence. When one considers that the rest of the water flowing past the farm must contain a similar concentration of actinospores, it is clear that wild fish with comparatively low intensity levels of *Sphaerospora* infection produce enough spores which enter worm hosts to ensure the production of many thousands of actinosporeans, and may thus further evidence that a proliferative cycle must exist in the actinosporean stage. It also dramatically illustrates the way in which a two host life cycle may have contributed to the success of myxosporeans.

Reports of transformative life cycles still remain rare, being proven for only ten

or so species. Lom and Dykova (1992a) therefore raise the possibility that perhaps such life cycles will only be found to be relevant to some species, genera or families of myxosporeans. Although such caution is obviously necessary it should however be emphasised that, at ten (and growing), there are already more than twice the number of successful experimental transmissions in the last ten years via alternate hosts than trustworthy reports in the last one hundred by direct infections using mature spores. We can anticipate an increase in the frequency of such two host reports in the future.

There remains the important question of what happens in the marine environment. All reports to date have shown alternate hosts in only freshwater species of myxosporean. Suggested hosts in the marine environment include sipunculids and marine polychaetes (Odening, 1989; Janiszewika, 1955) The latter author cited Ikeda (1912) as having described *Tetractinomyxon* sp from a marine sipunculid, *Petalostoma minutum* off the coast of Plymouth, UK.

Odening (1991) claimed that since sexual stages were present during actinosporean development, the terminology used in myxosporean life cycle studies must change to reflect the position of oligochaete worms as definitive hosts; fish would then be intermediate hosts in which the myxosporean phase took place, ie:-

PRESENT TERM		NEW TERM
myxospore	=	myxo-gametophore
spore	=	gametophore
sporogony	=	gamegony
pansporoblast	=	pangamoblast
sporoplasm	=	gametoplasm

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However the comparative lack of precise knowledge on sexual processes, especially with respect to the Myxosporea, means that such measures might only confuse matters further, and seem at present too large a step to take. Most authors are currently using the terms alternate host or first/second host rather than definitive host/intermediate host. This appears acceptable, but an alternative arrangement might also be to simply state fish host/oligochaete host, and use the terms myxosporean stage and actinosporean stage.

Another question posed is that of the need for the alternate cycle to be obligate. Is such a strict alternation necessary, or to some degree can either or both the myxosporean and actinosporean phases be capable of cycling independently for one or more generation?

A number of workers have repeatedly failed to reproduce results which prove the two host life cycle (Hamilton and Canning, 1987; Baska, Molnar and Szeckely, 1991, pers comm.) indicating that more detailed studies are necessary and that other factors may be involved. El-Matbouli *et al*. (1992), for example, revealed that although successful in transmitting five species of myxosporean via bisporogy in tubificid spp, they have repeatedly failed to do so with two other species, such as *Henneguya zschokkei* and *Myxidium giardi*. Benajiba and Marques (1993), have however achieved a completed lifecycle of the latter parasite, *M. giardi*, using tubificid spp.

It is obvious now that at least some myxosporean and actinosporean species are actually different life cycle stages of a single group of organisms. Corliss (1985) suggested that many taxa may have to be amalgamated even at high levels as a result.

The problems of developing a new system of taxonomy for these organisms can be illustrated if we look at the table below:

MYXOSPOREAN	ACTINOSPOREAN
Myxobolus (cerebralis)	*Triactinomyxon* sp
Myxobolus (cotti)	*Triactinomyxon* sp
Myxobolus (pavlovskii)	*Hexactinomyxon* sp
Myxobolus (sp)	*Raabeia* sp
PGD *(Spherospora ictaluri?)*	*Aurantiactinomyxon* sp
Hoferellus cyprini	*Aurantiactinomyxon* sp
Hoferellus carassii	*Aurantiactinomyxon* sp
Myxidium giardi	*Aurantiactinomyxon* sp
Ceratomyxa shasta	*Aurantiactinomyxon* sp

Already just with these few successfully transmitted species, it can be seen that five myxosporeans of the genus *Myxobolus* transform into three separate actinosporean genera, whilst four separate myxosporean genera all transform into actinospores of the genus *Aurantiactinomyxon*. Resolution of the taxonomic position looks impossible until clearer patterns emerge, as more and more species' life cycles are completed. It also serves to demonstrate how contrived the current spore-based taxonomy is. One possible explanation could be the hypothesis of Lom and Dykova (1992a), that both the amazing variability in spore forms seen in both actinosporeans and myxosporeans, and perhaps also the closeness of some species, could be explained by the possibility that a single

myxozoan species can infect a range of fish and worms. It may then have a large number of phenotypes, the expression of which is dependent upon the host species, whether it be fish or worms, and the tissue infected, ie, host induced phenotypic variation. Perhaps novel approaches involving molecular techniques, for example, need to be applied to determine the relationships between species.

It seems that in the present situation the rule of priority in classification may cause problems. If a situation arises, for example, whereby a very well known, well described and referenced, important pathogenic myxosporean stage is found to possess a poorly known actinosporean stage, which was, however described prior to the myxosporean stage. To change the name of the whole organism to that accorded to the actinosporean would be very confusing. It could for example, lead to a situation where two species of myxozoan had identical myxosporean stage and actinosporean stage genera, but one was known under the generic name of its actinosporean stage, the other by the generic name of its myxosporean stage. However, as was pointed out by Kent *et al* (1993a) many actinosporean names will probably be suppressed since their species represent alternate life cycle stages of earlier known myxosporean taxa. In terms of level of the interest, number of species described, available literature and commercial importance of host organisms, it would seem that to retain myxosporean names would be preferable. Even now, all workers talk of the actinosporean stages of myxosporean life cycles, and not vice versa. Another possible approach would be to keep the two classes and erect a new classification system with new names into which the proven two host life cycle myxozoans are transferred as they are described.

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