STUDIES ON THE BIOLOGY OF SOME PARASITES OF THE THREE-SPINED STICKLEBACK, *GASTEROSTEUS ACULEATUS* L. WITH SPECIAL REFERENCE TO THE MYXOSPOREA.

A Thesis presented for the degree of
Doctor of Philosophy to the University of Stirling.

by

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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.

.....

DEDICATION

To my son Meshkat Muntaseer Haque.

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Abstract

A population of three-spined stickleback, *Gasterosteus aculeatus* L., was sampled every two weeks for 14 months from Airthrey Loch on the campus of the University of Stirling. A total of 857 fish were collected and three species of parasites were studied.

The monogenean *Gyrodactylus gasterostei* was found on the skin and fins and two myxosporean parasites, *Sphaerospora elegans* and *Myxobilatus gasterostei,* were present in the kidney. All parasite species were present throughout the year but abundance was correlated with the yearly life cycle of the host fish. Lower prevalence and intensity were observed in summer due to the large number of young fish in the samples.

The comparative ultrastructure and development of the two myxosporeans was studied by light and transmission and scanning electron microscopy. Both myxosporeans followed the general pattern of myxosporean development but showed some novel features.

Sphaerospora elegans had two distinct developmental cycles. A proliferative cycle involving extra-sporogonic stages occurred in the blood whereas spore production occurred in the kidney. Light microscopy of Giemsa stained blood smears suggested proliferation of extra-sporogonic stages by external budding or plasmotomy. Structural similarities between extra-sporogonic blood stages and sporogonic kidney stages are discussed. Although *S. elegans* mostly formed disporous plasmodia, monosporous plasmodia were also occasionally observed. Before the appearance of sporogonic cells within the early pseudoplasmodia, certain areas of pseudoplasmodial cytoplasm became electron lucent, eventually acquired cell organelles and later appeared as sporogonic cells. Developing valvogenic cells contained protuberances at the posterolateral side of spores which disappeared in mature spores. Characteristic lipid bodies were seen in developing capsulogenic cells and developing uninucleated sporoplasmic cells contained abundant glycogen granules. The sporoplasmic cells were devoid of sporoplasmosomes.

Plasmodia of *M. gasterostei* were mono, di or polysporous and showed features of both coelozoic and histozoic myxosporeans, including a unit surface membrane, simple pinocytosis aanand presence of a number of vegetative nuclei and generative cells, the latter which formed pansporoblasts before the initiation of sporogenesis. A membrane bound tubular structure and some electron dense fibrillar bundles are previously undiscovered cytoplasmic organelles of the plasmodia. Developing capsulogenic cells contained characteristic membrane bound vacuoles filled with electron dense (glycogen) material.

Myxobilatus gasterostei attached to the epithelial cells by plasmodial surface projections and there were electron dense areas at the point of attachment. *Sphaerospora elegans* showed occasional hair-like processes projecting from the pseudoplasmodial surface to the microvillous brush border of the epithelial cells. There were no electron dense areas at the point of attachment to the epithelial cells of the kidney tubules.

Early undifferentiated pre-sporogonic stages of both parasites were occasionally present intracellularly in the tubular epithelium suggesting this is a route of entry into the tubular lumen. Early stages of *S. elegans* were also seen in the capillary lumen of the glomerulus. Intracellular and intraluminal stages of *S. elegans* and *M. gasterostei* caused pathological changes in different ways. Histopathological changes associated with *S. elegans* included vacuolation and accumulation of electron dense material in the epithelium whereas *M. gasterostei* caused large vacuolation with necrosis of the epithelial layer. Both parasites caused destruction of glomerular tufts in heavy infections and an increased number of rodlet cells in the epithelial layer were common in both cases.

The two myxosporean species were most abundant during the winter and spring. Extra-sporogonic stages of *S. elegans* were found only in January and June in the rete-mirabile of the eye, circulating blood and kidney. In infections with *S. elegans* sex of the host fish was apparently of no significance, whereas significantly lower infestations occurred in male sticklebacks infected with *M. gasterostei* compared with females. Host size was important in determining the prevalence and intensity of both myxosporean species. Older fish were less heavily infected, possibly due to an acquired immunity or pathogenic effects on the host. A high number of mixed infections indicated that there was no interspecific competition between the two parasites. There appeared to be a continuous recruitment of myxosporeans throughout the year.

Studies on myxosporean spore shedding suggested that spore production and shedding was continuous throughout the year and was uninfluenced by temperature or season.

Gyrodactylus gasterostei was generally more abundant in winter and spring than in summer and autumn, reflecting the greater numbers of small young of the year fish at these times. The age of the host fish was a significant factor influencing the prevalence and intensity of infestation with *G. gasterostei*. Sex of the host had no apparent influence on infestation. The parasite was highly over-dispersed within the host population and its distribution was best fitted by the negative binomial.

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CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 Three-Spined Stickleback.

The teleost family Gasterosteidae consists of 5 genera and 7 species of which the three-spined stickleback *Gasterosteus aculeatus* L., has received most attention from biologists (Wootton, 1976). *Gasterosteus aculeatus* is found between about 35^0N and 70^0N in Europe, parts of Asia and North America and is completely absent from Africa. Its habitat includes fresh, brackish, and salt waters, although it is restricted to coastal marine waters. It is most common in still or slow flowing waters, especially in areas of emergent or submerged rooted vegetation.

Three-spined sticklebacks are predominantly carnivorous fish. Hynes (1950) analyzed the feeding habits of sticklebacks and listed a wide range of food items including copepods, cladocerans and other crustaceans, oligochaetes and larvae and pupae of chironomids. The fish showed a distinct seasonal variation in their diet, with less food eaten during the winter and an increased amount during the breeding season. According to Wootton (1976) temperature has an effect on the food consumption of sticklebacks in that at low temperatures the contents of the stomach are evacuated more slowly.

Breeding and Annual Cycles.

Wootton (1976) described the breeding cycle of the stickleback which has been divided into two phases, the breeding season which lasts for two or three months in the spring or summer and a non-reproductive phase that lasts for the rest of the year. During non-reproductive phases there is little morphological difference between male and female fish, but in the

breeding season there is a marked sexual dimorphism. In the non-reproductive season sticklebacks live in schools. Experiments on the schooling behaviour have shown that a single stickleback prefers to join a large rather than a small group. Schools of sticklebacks tend to disperse the longer the fish have been without food. During breeding and non-breeding seasons sticklebacks show an interesting behaviour which involves rubbing the side of the body against a hard surface. After this fish take up a position with their side parallel to the surface and then make a series of strong tail beats so that at each beat the side of the body is pushed against the surface. This may dislodge ectoparasites from the body surface.

Breeding of sticklebacks starts in spring with the migration to the breeding grounds. This spring migration is most obvious in those sticklebacks which have overwintered in the sea but those fish who have spent the winter resident in fresh water also move from deeper water into shallow ditches and back waters. On the breeding ground the females remain in schools but the males defend territories with each territory holding only one male. Territorial behaviour of male sticklebacks is associated with the selection of a nest site and subsequent building of a nest. The male stickleback induces a female to lay eggs in his nest and enters a parental care phase during which it guards and ventilates the eggs and then cares for the newly hatched young for a week or two.

Growth and Longevity.

After fertilization, hatching of stickleback takes place within a week, depending on temperature. For the first approximately 12 days after hatching, the developing fish depends on its yolk reserves for nutrient. Within 30 days they attain a length of about 17mm and start feeding on infusoria, the larval stages of copepods and other small food items. The growth

of stickleback entirely depends on temperature and an adequate supply of food. Experimental studies have shown that with high temperatures (20^0c) and enough food sticklebacks can attain sexual maturity within four months, but in natural populations such rapid growth and maturation does not occur. According to Wootton (1976) variation in the day length and changes of water temperature may influence the growth of sticklebacks in natural populations. Generally sticklebacks have an annual life cycle. Fish hatch out in late spring or early summer, attain maturity by winter and breed in spring. After breeding most die.

1.2 Parasites of Stickleback.

Wootton (1976) stated that "A surprising aspect of the role of stickleback as a host to parasites is the number of parasite species that take advantage of this opportunity". The author listed parasites representing 6 phyla from stickleback. Hoffman (1967) listed 72 species of all the major parasitic phyla from *G. aculeatus* throughout the world. In the former USSR around 50 different parasites were found (Bykhovskaya-Pavlovskaya *et al.*, 1964). Lester (1974) recorded 36 parasite species in North America. In Britain 40 species of parasites representing 6 phyla have been recorded from three-spined stickleback (Chappell and Owen, 1969; Kennedy, 1974). Sticklebacks may act as definitive or intermediate hosts. Because of its rich parasite fauna and its wide range and abundance the parasite fauna of stickleback has been relatively well studied. Chappell (1969) investigated the distribution and abundance of 8 species of parasites from *G.aculeatus* from a pond in Yorkshire, with reference to seasonal occurrence and variation in parasite fauna with age and sex of the fish, whilst Pennycuick (1971a,b,c) made a detailed quantitative study of three species of parasites from sticklebacks in Somerset.

Members of the monogenean genus *Gyrodactylus* are well known and common skin and gill parasites of *G. aculeatus*. The family Gyrodactylidae are viviparous, giving birth to live young directly on the host. Within the Monogenea this family is one of the largest groups making up nearly one quarter of all monogenean species described from freshwater fish in Europe, America and Africa (Harris, 1982; Hoffman, 1967; Paperna, 1979). Many authors have noted that gyrodactylids are significant pathogens of cultured fish (Hoffman and Putz, 1964; Mackenzie, 1970; Rawson and Rogers, 1973; Malmberg, 1972; Johnsen, 1978; Cone and Odense, 1984; Cusack and Cone, 1986; Bakke *et al.*, 1990, 1992a) where their viviparous reproduction may lead to a rapid increase in parasite numbers to epizootic levels in high density host populations. Mortality may be direct, due to the pathogenic effects of gyrodactylids, or indirect as a result of secondary bacterial or fungal infection (Heggberget and Johnsen, 1982; Cone and Odense, 1984; Cusack and Cone, 1985, 1986).

Lester and Adams (1974b) reported mortalities in sticklebacks due to heavy experimental infections of *G. alexanderi*. In infected fish no other parasitic organisms were observed and they assumed that *Gyrodactylus* caused the death of the fish. They considered that heavy infestation caused such damage to the epidermis that normal epidermal functions such as osmoregulation are disrupted causing fish mortalities. Johnsen (1978) described a disease epidemic amongst Atlantic salmon in Lakeselva River, northern Norway, directly due to *Gyrodactylus salaris* infection. Bakke *et al.* (1990, 1992b) reported *G. salaris* as an important pathogen of Norwegian populations of Atlantic salmon in which infection grows unchecked until the host dies. Pathogenicity of *G. salaris* depends on host genotype (Harris *et al.*, 1993). North Atlantic salmon from northern-western and south-eastern Norway and from Scotland are highly susceptible (Bakke *et al.*, 1990; Bakke and Mackenzie, 1993) with

most fish failing to mount an effective immune response. Cone and Odense (1984) considered that the most important damage caused by *Gyrodactylus* is ulcers due to feeding activity.

1.3 *Gyrodactylus* Species from Stickleback.

Thirteen species of *Gyrodactylus* have been listed by Harris (1982) from three-spined stickleback throughout the world (Table.1.1). As the stickleback has a worldwide geographical distribution it is perhaps not surprising that a large number of species of *Gyrodactylus* have been recorded from it. In Britain Harris (1982) made a comprehensive survey of the fauna of three-spined stickleback and recorded 4 species of *Gyrodactylus* including *G. gasterostei*. *Gyrodactylus gasterostei* was first described by Glaser (1974). From Scotland *G. gasterostei* was first reported by Shinn *et al.* (1993). Chappell (1969) described the biology of *G. rarus* from sticklebacks in the U.K., but Harris (1982) stated that this species was in fact *G. gasterostei*, and that many earlier identifications of *Gyrodactylus* spp. from *G. aculeatus* were of this species.

1.4 Myxosporeans in Three-spined Stickleback.

The Myxosporea Bütschli, 1881 is a class of the phylum Myxozoa Grasse, 1970 which mainly infects fish with a few species occurring in other ectoparasitic animals and invertebrates (El-Matbouli *et al.*, 1992). About 1200 species of myxosporeans are recognised from 46 genera (Lom, 1987). Wootton (1976) listed only 7 species of myxosporean parasites from the three-spined stickleback world wide (Table 1.2). The parasites of this group have recently received worldwide attention due to their significant pathogenicity in a number of

Table 1.1: Species of *Gyrodactylus* from three-spined sticklebacks *Gasterosteus aculeatus* (worldwide).

Species	Locality	Authors
G. elegans V. Nordmann, 1832	Gt. Britain	Bradley, 1861 Houghton, 1862 Sproston, 1946* Dawes, 1947* Cobbold, 1962 Treasurer, 1974
G. medius Katheriner, 1895	N. Germany	Wegener, 1909*
G. rarus Wegener, 1909	N. Germany	Wegener, 1909*
	White and Barents Seas, Baltic sea	Bykhovskaya and Polyansky, 1953*
	Amur river	Gussev, 1955
	Caspian and Japanese seas	Bychowskaya-Pavlovskaya, 1963*
	Gt. Britain	Chappell, 1969 Wootten, 1973 Harris, 1982
G. arcuatus Bychowsky, 1933	Karelia, USSR	Bychowsky, 1933*
	White, Barents and Baltic seas	Bychowsky and Polyansky, 1953*
	Sweden, N. Germany	Malmberg, 1970
	Gt. Britain	Powell, 1966 Harris, 1982 Shinn *et al.* 1993
G. bychowskyi Sproston, 1946	Baltic, White & Barents seas	Bychowsky and Polyansky, 1953*
	Amur river	Gussev, 1955
G. pungitii Malmberg, 1956	Gt. Britain	Powell, 1966 Harris, 1980a
G. alexanderi Mizelle & Kritsky, 1967	California	Mizelle and Kritsky, 1967
	Vancouver	Lester, 1974
	N. Germany	Glaser, 1979 Harris, 1982
G. branchicus Malmberg, 1970	Baltic	Malmberg, 1970 Harris, 1982
G. avalonia Hanek & Threlfall, 1969	Newfoundland	Hanek and Threlfall, 1969
G. canadensis Hanek & Threlfall, 1969	Newfoundland	Hanek and Threlfall, 1969
G. lairdii Hanek & Threlfall, 1969	Newfoundland	Hanek and Threlfall, 1969
G. memorialis Hanek & Threlfall, 1969	Newfoundland	Hanek and Threlfall, 1969
G. gasterostei Glaser, 1974	N. Germany	Glaser, 1974 Harris, 1982 Shinn *et al.* 1993

* See Harris, 1982.

Table 1.2: Myxosporeans from three-spined stickleback (worldwide).

Name	Organs Infected	Place	Authors
Myxidium gasterostei Noble, 1943	Gall bladder	North America USSR	Lester, 1974 Bykhovskaya Pavlovskaya *et al.* 1964
Myxobilatus gasterostei (Parisi, 1912) Davis,1944	Kidney tubules & Urinary bladder	USSR North America UK	Bykhovskaya Pavlovskaya *et al.* 1964 Lester, 1974 Feist *et al.* 1992
Myxobilatus medius Thélohan, 1892	Kidney tubules	USSR	Bykhovskaya Pavlovskaya *et al.* 1964
Myxobilatus sp.	Kidney tubules	North America	Lester, 1974
Sphaerospora elegans Thélohan, 1892	Kidney tubules & Urinary bladder	USA UK	Lester, 1974 Campbell, 1974 Feist *et al.* 1992
Henneguya sp.	Gill	North America UK	Lester, 1974 Kennedy, 1974
Ceratomyxa sp.	Intestine	North America	Hoffman, 1967

economically important cultured fish. These include whirling disease of young salmonids (Hoffman *et al.*, 1962; Lom, 1987), proliferative kidney disease (PKD) in salmonids (Hedrick *et al.*, 1984; Hoffman and Dangschat, 1981; Ferguson and Ball, 1979) and swimbladder inflammation (SBI) in carp (Kovács-Gayer, 1983; Dyková and Lom, 1988b). In wild populations myxosporean epizootics are not common (Mitchell, 1977), but a number of reports on marine fish show that myxosporeans may cause significant loss in wild fisheries (Alvarez-Pellitero and Sitja-Bobadilla, 1993b).

Taxonomy.

The exact taxonomic position of the Myxosporea has been in question since the early 19th century. Shulman (1966) gave an account of the history and classification of the group. Subsequent studies (Lom, 1969a,b; Lom and de-Puytorac, 1965) provided evidence of their affinities with members of the metazoa. Through much of their life cycle the myxosporeans show true multicellularity with morphologically differentiated and functionally specialized cells (Lom, 1987, Lom and Dyková, 1992). The spore of myxosporeans is composed of 1 to 7 shell valve cells, 1 to 2 amoeboid infective sporoplasm cells and 2 to 7 polar capsule cells. Trophozoite stages of myxosporeans also show functional differentiation of cells (Lom, 1987). The only unicellular stage is the sporoplasm released after spore hatching, but this soon divides to form multicellular trophozoites. The term "pluricellular" has been used to describe this type of cellular organisation to distinguish it from the multicellularity of metazoans (Grasse and Lavette, 1978). Grasse (1970) proposed the Myxosporidia, along with the closely related Actinomyxidia should be placed into a new phylum, the Myxozoa. Later, in a comprehensive revision of the classification of the protozoa by Levine *et al.* (1980) the Myxozoa was recognised as a separate phylum of the protistans as follows:

KINGDOM	**Protista**
SUB-KINGDOM	**Protozoa**
PHYLUM	**Myxozoa Grasse, 1970**
CLASS	**Myxosporea Bütschli, 1881.**

The class Myxosporea was further updated by Lom and Noble (1984) and characterised as follows:

> A pluricellular trophozoite stage is the main site of proliferation. Trophozoites often amoeboid in shape contain their own vegetative nuclei and generative cells producing multicellular spores. Trophozoites range from uninucleated pseudoplasmodia producing one spore to macroscopic plasmodia containing numerous spores. Spores with 1 or 2 sporoplasms, 2 to 7 (mostly 2) polar capsules and 2 to 7 (mostly 2) shell valves which meet in sutural line (S). Sexual processes in autogamy .....are serious pathogens of fishes.

Identification of myxosporean species is based on spore morphology in terms of the shape and size of spores, the nature and relative position of the suture, the number of polar capsules with numbers of filament coils and angle of the coils, the number of shell valves and any sculpturing and projections of the valves, the number of sporoplasm cells and sporoplasmosomes, and the presence of an iodinophilous vacuole within the sporoplasm (Lom and Dyková, 1992).

General Developmental Cycle.

Myxosporeans can be histozoic, either intra or intercellularly or in blood vessels, or coelozoic, mainly in the gall bladder, swimbladder or lumen of kidney tubules. In both cases early trophozoite stages develop into small or large plasmodia. The developmental cycle of

a number of species of myxosporeans has been well studied (Lom and de-Puytorac, 1965; Lom, 1969a; Current and Janovy, 1976, 1977, 1978; Desser and Paterson, 1978; Yamamoto and Sanders, 1979; Desser *et al.*, 1983a,b; Lom *et al.*, 1982; Molnar, 1988a; Dyková *et al.*, 1990; El-Matbouli *et al.*, 1992, 1994). The basic developmental sequence of myxosporeans shows considerable uniformity. According to Lom and Dyková (1992) spore formation in myxosporea occurs in two ways:

a) by pansporoblast formation or;

b) by direct division of generative cells

a) Pansporoblast formation: In large plasmodia sporogenesis is initiated by the union of two generative cells. The two generative cells come in close contact and one envelops the other forming a pansporoblast. The enveloping cell is known as the pericyte and the enveloped cell as the sporogonic cell. The sporogonic cell produces the necessary numbers of sporoblast cells which undergo a differentiation process into valvogenic, capsulogenic and sporoplasmic cells, which form the complete spore. Pansporoblasts are generally disporous, or sometimes monosporous. Most histozoic myxosporeans are of this type.

b) Direct division of generative cell: In small pseudoplasmodia sporogony simply begins by the production of a number of cells, which differentiate into one or two spores within the pseudoplasmodial cell. Lom *et al.* (1985a) suggested this was analogous with the pansporoblasts of large polysporic plasmodia. This group includes mainly coelozoic myxosporeans.

Life Cycle of Myxosporeans.

There are still many unanswered questions concerning the life history of myxosporeans. The discovery of extrasporogonic stages in some genera and the involvement of alternate oligochaete hosts and actinosporean stages in a few species has caused a revision of many earlier ideas of the biology of myxosporeans.

An extrasporogonic proliferative cycle has been reported only in *Sphaerospora* species (Lom *et al.*, 1983a, 1985b; Lom, 1987; Baska and Molnár, 1988; Supammataya *et al.*, 1993; McGeorge *et al.*, 1994). These were first described in the blood of myxosporean infected cyprinid fish by Csaba (1976) who did not identify them as myxosporean stages. Although a number of authors suggested a link between the parasites described by Csaba and *Sphaerospora* the link was not experimentally established until Molnár (1984, 1988b) showed them to be part of the life cycle of *S. renicola.* It is not known how widespread extrasporogonic stages are in the life cycle of myxosporeans.

Until fairly recently it was believed that the myxosporean life cycle was direct, involving only a fish host. Earlier studies claimed to have confirmed a direct life cycle by oral introduction of myxosporean spores to fish (Shulman, 1966). However, Mitchell (1977) criticized the reliability of the experimental procedure of these early studies. Odening *et al.* (1989) claimed to have successfully transmitted *S. renicola* by feeding the mature spores to carp. On the other hand several workers also failed to establish experimental infections orally (Lom, 1987). Earlier workers (see Hoffman, 1990) hypothesized that *Myxosoma cerebralis* might be carried by invertebrates such as snails, insect larvae or crustaceans. It has been known for many years that *M. cerebralis* spores are not infective to fish when fresh; to be

infective it was established that they must be aged for about three and a half months in mud (Uspenskaya, 1955, 1978 in Mitchell, 1977; Hoffman and Putz, 1969). More recently Wolf and Markiw (1984) and Wolf *et al.* (1986) demonstrated that oligochaete worms (*Tubifex*) are the obligate alternate hosts of *M. cerebralis* in which the parasite develops as an actinosporean sporozoan (*Triactinomyxon*). According to Lom (1987) these surprising findings initially met with considerable misgivings as this is an unparalleled case of an organism alternating two different life cycles. Although Hamilton and Canning (1987) could not confirm the actinosporean involvement in the myxosporean life cycle, El-Matbouli and Hoffmann (1989) also reported actinosporean stage in the life cycle of *Myxobolus* species. Subsequently other authors (Yokoyama *et al.*, 1991; Grossheider and Körting, 1992; Benajiba and Marques, 1993) also established the presence of an actinosporean stages in the life cycle of *Zschokkella, Myxobolus, Thelohanellus, Hofferellus* and *Myxidium* species.

1.5 Objectives.

The objective of the present study was to study in detail the biology of selected parasites of the three-spined stickleback under natural conditions.

The occurrence of two myxosporean species *S. elegans* and *M. gasterostei* in the same organ system of stickleback provided an opportunity to compare their developmental cycle within the fish host. Their simultaneous occurrence also allowed a comparative study of their epidemiology in relation to external environmental factors and the biology of the host. These aspects of myxosporean biology are little studied in natural fish populations which are free of the artificial conditions imposed by culture. The high level of infection of these

myxosporeans enabled an assessment of their pathogenicity. Although the myxosporean spore is the means by which the parasite is transmitted to the next host, very little is known about spore release including the rate and timing of this process. Experimental studies were conducted to investigate these factors in both *S. elegans* and *M. gasterostei*.

There are still very few studies on the epidemiology of gyrodactylids in wild fish populations despite their pathogenic potential. This study aims to provide data on the factors regulating the parasite population and the nature of the host-parasite relationship in natural habitats.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2 GENERAL MATERIALS AND METHODS.

2.1 Sampling Site.

Samples of stickleback were taken from the boat house located at the west end of Airthrey Loch situated on the campus of the University of Stirling (Fig.2.1). It is a shallow (median depth 1.5m, maximum depth 4.0m) man made eutrophic lake, which receives water via two inflows (Campbell, 1989). The water source of the main inflow is derived from the Bridge of Allan Reservoir and approximately 0.045 m^3/sec of water enters the loch from this source. The second inflow carries a minimal amount of water into the loch. Airthrey Loch also obtains direct fresh water from rainfall. The water temperature in the loch reaches an average of approximately 20^0c in summer and during winter the loch freezes for short periods depending on climatic conditions. The boat house and jetty extends approximately 2.5m into the loch and is shaded by surrounding overhanging trees. Three-spined sticklebacks are readily available around the boat house and jetty throughout the year.

2.2 Fish Sampling.

Three-spined sticklebacks were collected every two weeks from Airthrey Loch using a long handled hand net around the boat house and jetty. Fish were kept in a bucket with loch water at ambient temperature and aeration until examination within 12 to 36 hours of collection. Each sample comprised 15 to 60 fish. Throughout the study period January 1993 to February 1994 a total of 857 fish were examined in 26 samples. Some additional samples were also examined sporadically after February 1994.

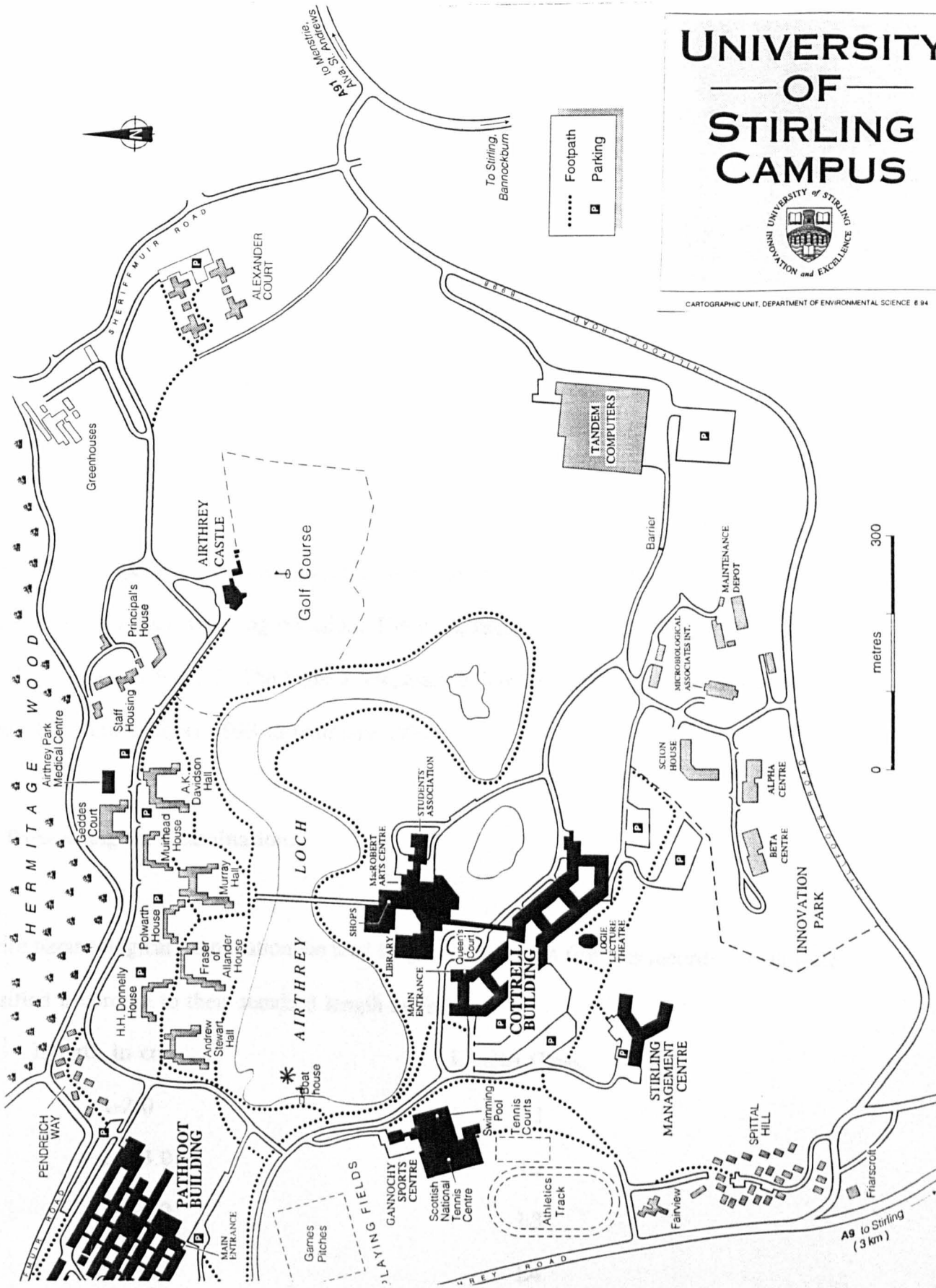


Fig.2.1 Map of Airthrey Loch showing sampling site (*).

The study period was divided into different seasons as follows:

Jan.1993 - Feb.1993 = Winter 1992

Mar.1993 - May.1993 = Spring 1993

Jun.1993 - Aug.1993 = Summer "

Sep.1993 - Nov.1993 = Autumn "

Dec.1993 - Feb.1994 = Winter "

2.3 Temperature.

The water temperature of the sampling site was recorded at a depth of 1m using a thermometer for each sampling occasion. The mean temperature range (2 samples) in each month is shown in Fig.2.2. The highest temperature was over 20^0c in July and August and lowest 0^0c from October 1993 to February 1994.

2.4 Parasitological Examination.

Before parasitological examination the total length (cm) of each fish was recorded. Fish were classified according to their standard length as follows:

Length in cm.	**Length Class.**
1-2.0	L1
2.1-3.0	L2
3.1-4.0	L3
4.1-5.0	L4
5.1-6.0	L5

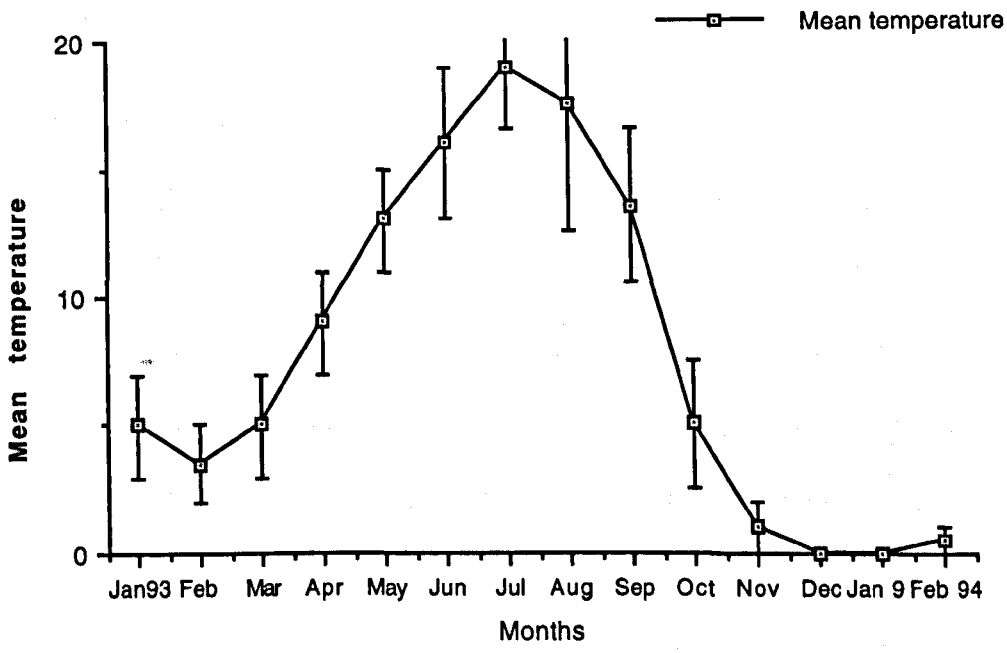


Fig: 2.2 Water temperature at the sampling site during Jan 93 - Feb 94
(2 samples per month, bars represent range of temperature).

Annual Cycle of the Three-spined Stickleback.

The length frequency distribution of sticklebacks from each monthly sample is shown in Fig.2.3. Between January to May 1993 L2-L5 length classes were present in the samples. In June L1 fish, presumably newly recruited, appeared and made up about 30% of the catch. From July to September the population of larger L4 and L5 sticklebacks in the sample decreased, whilst L2 and L3 fish increased in abundance. From October onwards the proportion of L4 fish again increased although L2 fish made up a significant proportion of the catch throughout the autumn and winter.

The pattern of length frequency distribution suggests that the sticklebacks in Airthrey Loch had an annual life cycle where fish hatched during late May to June and possibly also in August and September. Harris (1982) suggested that the variation in the length frequency distribution of sticklebacks is due to their extended breeding season. Fish spawned in early June attain a large size by September whereas those hatched in early August have only a short feeding period before the onset of winter and remain small in size until growth recommences in the following spring. A similar pattern seems to occur in Airthrey Loch. Older fish are apparently lost to the population after breeding. Presumably they die, or possibly they migrate into deeper water. The evidence of this study suggests that *G. aculeatus* has a one year cycle in Airthrey Loch as suggested by Wootton (1976).

2.4.1 Examination of Fish for *Gyrodactylus*.

From each sample 10 to 30 fish were examined for the presence of *Gyrodactylus* and a total of 406 fish were examined. Examination of fish for *Gyrodactylus* was carried out using the

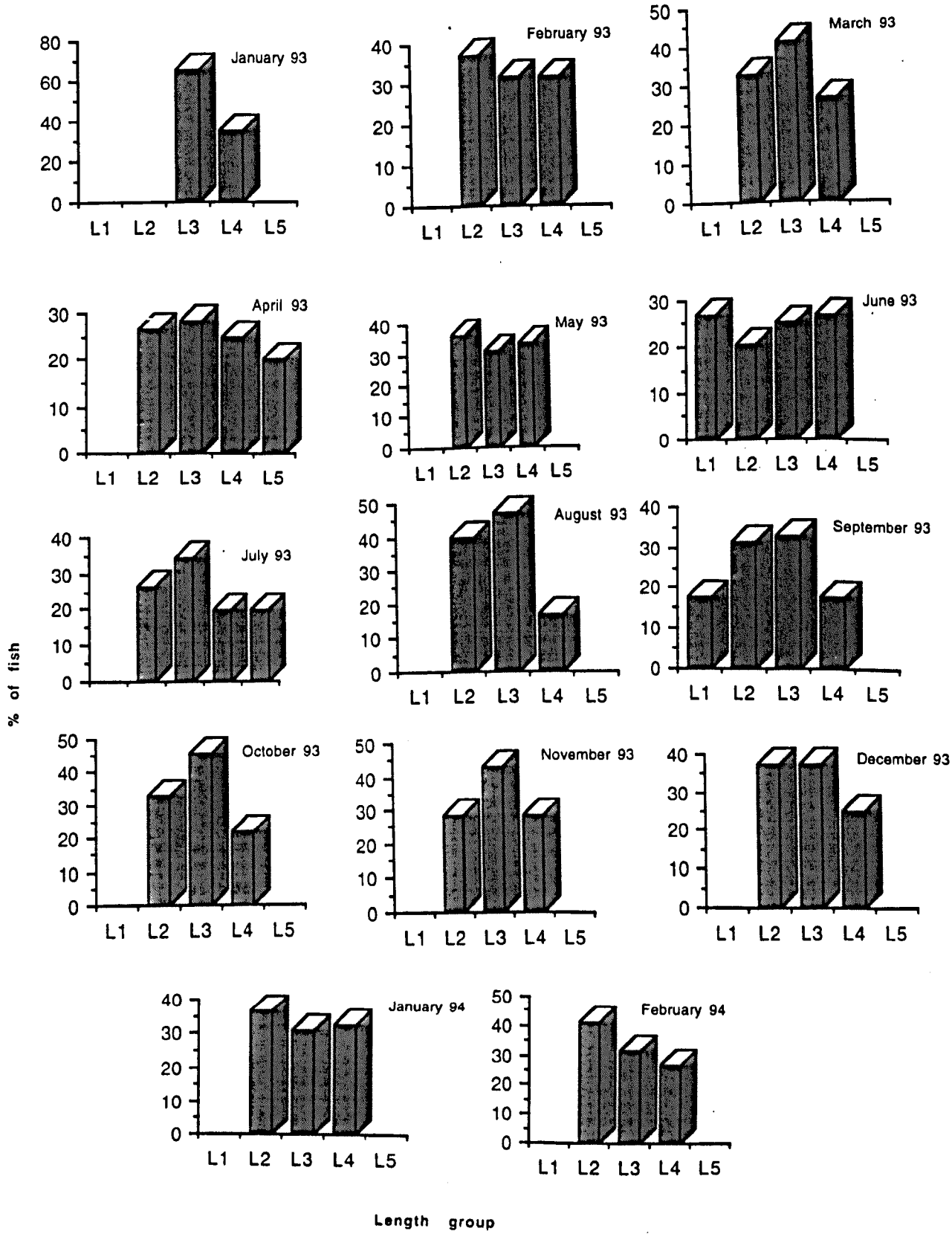


Fig: 2.3 Length frequency distribution of *G. aculeatus* during the sampling period.

method of Malmberg (1970). The fish were killed by insertion of a surgical needle into the brain. Fish were then placed immediately into a plastic Bijoux with 1/4000 formalin (250 ppm) for a minimum of 15 minutes, with occasional shaking. This caused the parasites to drop off the fish. The contents of the pots were then placed in a Petri dish and observed under the dissecting microscope at 1X to 4X magnification. For detailed study of *Gyrodactylus* specimens were placed on a glass microscope slide with a drop of 1/4000 formalin and a cover slip. Excess formalin was absorbed on a piece of filter paper to flatten the parasite. The slide was then examined by phase contrast microscopy at high power (100X + Oil immersion).

2.4.2 Examination of Fish for Myxosporea.

All 857 fish, sampled between January 1993 and February 1994, were examined for myxosporeans. The body cavity of the fish was opened and the various organs dissected out and placed in separate Petri dishes in 0.8% physiological saline prior to individual examination. Organs examined were the heart, liver, kidney, digestive tract, gallbladder, swim bladder, urinary bladder, reproductive organs, brain and the choroidal rete of the eye.

a) Squash preparations.

Organs were examined for myxosporeans using fresh squash preparations. A piece of tissue about 3mm in diameter was compressed between a glass microscope slide and a cover slip. At least three slides from each organ were examined.

Squash preparations were examined using standard light, phase contrast or Nomarski optics

on a Leitz SH Lux compound microscope, a Leitz Wetzlar Orthomat and an Olympus BH2 compound microscope. Histological slides and Giemsa stained smears were examined using standard light optics on a Leitz SH LUX - compound microscope at X40 to X100 magnification under oil immersion.

b) Giemsa staining.

Smears of infected organs were prepared on a microscope slide and after air drying, fixed in absolute methanol for 40 seconds and stained with 10% (10% stock v/v in dis. buffered water) Grunwald Giemsa for 30 to 60 minutes (Drury and Wallington 1980). Slides were then washed in running tap water for 1 minute, blotted dry with paper towels and mounted in Pertex.

c) Detection of parasites in blood.

For examination of fresh fish blood, the caudal region of the fish was severed by a sharp scalpel. A drop of fresh blood from the caudal vein was placed on a microscope slide, covered with a cover slip and examined at x25-x100 magnification under a compound microscope using bright field optics. Dry blood smears were prepared on glass microscope slides. After air drying the smear was fixed in 100% absolute methanol and stained with Giemsa according to the method described in 2.4.2. Blood samples from heart were prepared by the enrichment method of Sövényi and Molnár (1990) for easy detection of myxosporean parasites. A heparinized haematocrit tube was about 40% filled with blood. The other end of the tube was filled with Ficoll-Paque solution leaving a small bubble between the solutions. Subsequently the tube was stoppered from the side of the Ficoll-Paque solution and centrifuged in a haematocrit centrifuge for 1 minute. Centrifugation caused separation of the

lymphocyte layer from the other blood elements, at the density interface of the Ficoll-Paque solution. Then the haematocrit tube was broken and the lymphocyte layer placed on a slide and examined at x25-x40 magnification using bright field optics.

2.5 Histological Techniques.

For histology small pieces of tissue were fixed in 10% buffered formalin for a minimum of 24 hours. Small fish up to 1 - 3 cm in length were fixed whole. The body cavity of these fish was opened before fixation. Fixed tissues were trimmed to a suitable size, placed in a cassette and left in a bowl of water or fixative until loading in the automatic processor (Histokinette 2000). After processing the cassettes were removed from the processor and placed in molten paraffin wax until ready to block out. Blocks were prepared by embedding tissue in Paraplast. Sections were cut at 4-5μm. Sections were floated on a water bath, picked up on a clean glass slide and the slide placed face down on a hot plate. Slides were then dried in an oven at 60^{0}C for a minimum of one hour before staining. Sections were stained with haematoxylin and eosin, periodic acid-Schiff and Grunwald Giemsa stains (Drury and Wallington, 1980)

2.6 Transmission Electron Microscopy (TEM).

A detailed description of processing of tissue for TEM is given in Chapter 3. Briefly, infected tissues were excised and cut into very small pieces while in Karnovsky's fixative (1.3% paraformaldehyde, 1.65% glutaraldehyde). Tissues were fixed for 4 to 6 hours at 4^{0}C. Fixed tissues were then washed briefly in cacodylate rinse, placed in fresh rinse and left

overnight at 4°C. Tissues were post-fixed in 0.5% osmic acid in cacodylate buffer for one hour. The fixed specimens were dehydrated in graded ethanol and embedded in epoxy resin. Semithin sections (0.8nm) were stained in toluidine blue.

Ultrathin sections (0.8μm) were cut using glass knives fitted in an ultra microtome (Leica Ultracut-E). Sections were picked up on naked copper grids, dried and double stained with uranyl acetate and lead citrate. These were then viewed and photographed with a Phillips 301 transmission electron microscope at 60 to 80 KV.

2.7 Scanning Electron Microscopy (SEM).

A detailed description of processing of tissue for SEM is given in Chapter 3. Briefly, a suspension of infected kidney was filtered using acetone resistant Surtolon (Polyamid) Millipore filters (pore size 0.45μm). The filter was then fixed in 4% (v/v) glutaraldehyde in phosphate buffer (pH 7.14) for one week at 4°C. A further secondary fixation followed after washing the filter in phosphate buffer for 4 hours. The filter was then dehydrated in a graded ethanol series and dried in Polaron E 300 in a critical point drying apparatus. Filters were then mounted on stubs coated with gold and examined in a Phillips - 500 scanning electron microscope.

2.8 Measurement of Myxosporeans.

Measurements of myxosporean spores and developing stages were taken from fresh squash preparations of the infected organs. A standard eye piece graticule calibrated by a stage

micrometer slide was used for the measurements. A minimum number of 35 spores and 10 developing stages were measured in each month according to the methods described by Lom and Arthur (1989). All measurements were expressed in μm. Some measurements of pre-sporogonic and sporogonic were taken from enlarged photomicrographs of known scale.

2.9 Photography and Drawing.

Micrographs were taken from fresh and fixed preparations of infected organs. To prevent Brownian movement and to prepare a monolayer of spores, a squash of infected tissue was embedded in 1.5% agar, according to the method described by Lom (1969b). Light and phase contrast micrographs were taken with an Olympus BH-2 research microscope fitted with a automatic photomicrographic (C.35AD) camera. Nomarski interference contrast micrographs were taken on a Leitz Wetzlar Orthomat fitted with a 35mm photomicrographic camera. Drawings were made by using a drawing tube on an Leitz Wetzlar SH Lux compound microscope. Some free hand drawings were also made.

CHAPTER 3

DEVELOPMENT AND PATHOGENECITY OF *SPHAEROSPORA ELEGANS* THÉLOHAN, 1892.

3 DEVELOPMENT AND PATHOGENICITY OF *SPHAEROSPORA ELEGANS* THÉLOHAN, 1892.

3.1 Introduction

Members of the genus *Sphaerospora* are coelozoic myxosporean parasites, commonly found in the lumen of the kidney tubules or in the urinary bladder of freshwater and marine fish. At least 48 species of *Sphaerospora* have been described to date, mostly from freshwater fish (Lom and Dyková, 1992). There are also a few reports of their occurrence in amphibians and reptiles (Desser *et al.*, 1986). The genus has recently received special attention because of the pathogenic effects of a number of those species infecting fish.

3.1.1 Taxonomy.

Since the revised classification of the Myxosporea by Lom and Noble (1984) the genus *Sphaerospora* has been further defined (Lom and Dyková, 1992). The latter authors gave the characteristics of the genus *Sphaerospora* as:

> Spherical or subspherical spores with valvular diameter not significantly exceeding sutural ridges often with lateral protuberances or bumps. The sutural ridge often prominent. Polar capsules subspherical or pyriform. Two uninucleate sporoplasms. Mono or disporous trophozoites coeolozoic in the urinary system of freshwater and marine fish, some are histozoic. Often with intercellular stages and with presporogonic developmental cycle in various body organs. Coeolozoic species from the urinary system (renal tubules and collecting ducts) seem usually to be host specific and have extrasporogonic stages in blood and also some other tissues.

These authors described *Sphaerospora elegans* as the type species of the genus. The classification of *S. elegans* remains as given by Lom and Noble (1984):

Class	**MYXOSPOREA Bütschli, 1881**
Order	**BIVALVULIDA Shulman, 1959**
Sub-order	**VARIISPORINA Lom and Noble, 1984**
Family	**SPHAEROSPORIDAE Davis, 1917**
Genus	***Sphaerospora* Thélohan, 1892**
Species	***Sphaerospora elegans* Thélohan, 1892**

Sphaerospora elegans was first described by Thélohan (1892) from the kidney and ovaries of three-spined stickleback and the nine-spined stickleback, *Pungitius pungitius* L., in France. Subsequently light microscopic descriptions of spores and developing stages were made by a number of authors (Kudo, 1920; Shulman and Shtein, 1964; Shulman 1984). Apart from these descriptions *S. elegans* did not particularly feature in the literature, with only occasional references, usually in relation to other myxosporean infections (Feist, 1988; Hedrick *et al.*, 1988). However, recent ultrastructural studies of this parasite are reported by Feist *et al.* (1991) and Lom *et al.* (1991).

3.1.2 Hosts of *Sphaerospora*.

A number of authors agree that the genus *Sphaerospora* shows a strict host specificity with many species described from only a single fish species (Arthur and Lom, 1985; El-Matbouli and Hoffmann, 1992). In addition *Sphaerospora* spp. are restricted to specific host organs. Most reports on sporogonic stages of *Sphaerospora* show that sporogony occurs in the kidney (Lom *et al.*, 1982, 1985b, 1991; Hedrick *et al.*, 1988, 1990; Desser *et al.*, 1983a; El-Matbouli and Hoffmann, 1992). However, in some species spore formation occurs in other

organs. Molnár (1979a, 1980b) reported gill and cutaneous sphaerosporosis in carp, *Cyprinus carpio L.*, fingerlings in Hungary and Lom *et al.* (1983b) described *S. molnari* sporulating in the gill of common carp in Europe. Sitja-Bobadilla and Alvarez-Pellitero (1992) reported *S. dicentrarchi* spores from the gonads, swimbladder, trunk, head kidney, gut, liver and pancreas of sea bass, *Dicentrarchus labrax.*

3.1.3 Basic Development.

Light and electron microscope studies on a number of *Sphaerospora* species have shown that the basic development of the parasite is similar in all cases (Dyková and Lom, 1982; Desser *et al.*, 1983a; El-Matbouli *et al.*, 1994; Feist *et al.*, 1991; Hamilton, 1980; Hedrick *et al.*, 1990; Lom *et al.*, 1983b, 1985a,b; McGeorge *et al.*, 1994; Sitja-Bobadilla and Alvarez-Pellitero., 1992; Supamattaya *et al.*, 1990, 1991, 1993). Lom and Dyková (1992) noted that *Sphaerospora* produces mono and disporous trophozoites which form one or two spores. They termed them as "pseudoplasmodia" because of their single vegetative nucleus. Pseudoplasmodia are formed by uninucleate primary cells and contain one to two sporogonic cells. Sporogonic cells divide endogenously and give rise to sporoblastic cells of which there are 5 to 6 in monosporous and 10 to 12 in disporous species. Early pseudoplasmodia, before sporoblast formation, may divide by plasmotomy to increase the number of trophozoites.

3.1.4 Extrasporogonic Development.

The genus *Sphaerospora* has attracted particular attention because of its early extrasporogonic developmental stages. These were first recognised in *S. renicola* infecting carp renal tubules

(Csaba, 1976; Lom *et al.*, 1983a). Extrasporogonic stages have now been described in the developmental cycle of a number of *Sphaerospora* species. Csaba (1976) described them as an "unidentified blood organism" found in the blood of carp fingerlings. Later the simultaneous occurrence of these blood organisms with renal *Sphaerospora* species led to the conclusion that these forms were early developmental stages of the latter parasite (Lom and Dyková, 1982; Lom *et al.*, 1983a, 1985b; Csaba *et al.*, 1984; Grupcheva *et al.*, 1985). This connection was confirmed experimentally by Molnár (1984, 1988b). Kovács-Gayer *et al.* (1982) and Körting (1982) also reported on the occurrence of myxosporean extrasporogonic stages in the swimbladder of common carp. Later Baska and Molnár (1988) recommended that blood stages of *Sphaerospora* should be called C-stage and swimbladder stage as K-stage (according to the initial of the authors Csaba and Kovács-Gayer and Körting). Extrasporogonic blood stages have since been reported from several other fish species harbouring *Sphaerospora* (Lom *et al.*, 1985b; Baska and Molnár, 1988; Hedrick *et al.*, 1990; Supamattaya *et al.*, 1991). The basic structure of extrasporogonic stages is similar in all *Sphaerospora* species so far described. The earliest stage is a uninucleate primary cell which encloses one secondary cell. This secondary cell divides endogenously into a number of cells which may develop tertiary cells within them. The number of secondary cells and their inner tertiary cells may vary according to species (Lom *et al.*, 1985b; Baska and Molnár, 1988). At the end of the extrasporogonic cycle the primary cell disintegrates and releases the secondary cells which recommence the cycle (Lom *et al.*, 1983a; Dyková *et al.*, 1990). Extrasporogonic stages of *Sphaerospora* have also been reported from tissues other than blood, including the swimbladder of carp fingerlings (Kovács-Gayer *et al.*, 1982; Körting, 1982; Molnár and Kovács-Gayer, 1985b) and the choroidal rete mirabile of the eye and the interstitial haemopoietic tissue of the kidney of stickleback in the case of *S. elegans* (Lom

et al., 1991). Very recently McGeorge *et al.* (1994) reported *Sphaerospora* extrasporogonic stages from blood, kidney interstitium, liver and occasionally other organs of Atlantic salmon, *Salmo salar*.

3.1.5 Pathogenicity of *Sphaerospora* species.

Although most known pathogenic myxosporeans are histozoic some coelozoic genera may also cause severe damage and the most significant of the latter is the genus *Sphaerospora* (El-Matbouli *et al.*, 1992). The extrasporogonic stages (K-stages) of *S.renicola* cause an extensive proliferative inflammation of the swimbladder wall which becomes cloudy and haemorrhagic with distended blood vessels (Dyková and Lom, 1988b; El-Matbouli *et al.*, 1992). Fish with swimbladder inflammation (SBI) show locomotory disorders due to a marked loss of hydrostatic function. Extrasporogonic stages of the same parasite in the blood (C-stages) of carp fingerlings can cause changes in blood parameters (Kudryashova and Naumova,1978, in Dyková and Lom, 1988b). The most severe pathogenic effects due to a possible *Sphaerospora* species occur in proliferative kidney disease (PKD) caused by the extrasporogonic PKX parasite in rainbow trout (Clifton-Hadley *et al.*, 1984; El-Matbouli *et al.*, 1992). However, not all extrasporogonic stages cause pathogenesis. Extrasporogonic stages of *S. elegans* in the choroidal rete and renal interstitium provoke little or no cellular response (Feist *et al.*, 1991). *Sphaerospora* species can also be pathogenic at the sporogonic stage. Development of pseudoplasmodia and spores of *S. renicola* in the tubular lumen of common carp is associated with dystrophic changes in the tubular epithelium. Heavy infection causes distension of the tubular lumen, and atrophy and necrosis of the epithelium. Dyková and Lom (1988b), after reviewing the pathology caused by *S. renicola*, pointed out that

sporogonic phases might seriously impair the excretory and haemopoietic functions of the kidney. *Sphaerospora tincae* causes mass mortality of tench fry due to enlargement of the head kidney and displacement of other organs (Hermanns and Körting,1985). *S. epinepheli* produced heavy infections in cage cultured grouper causing necrosis of renal tubules and glomeruli (Supamattaya *et al.*, 1993).

Sphaerospora species from organs other than the kidney may also have significant pathogenic potential. A number of authors have reported that *S. molnari,* which infects the epithelium of gill filaments, the branchial cavity and the gill arches of common carp causes hyperplasia and fusion of the secondary lamellae and associated secondary infection by fungi, bacteria and other ectoparasites (Molnár, 1979a; Molnár, 1980b; Lom *et al.*, 1983b; Dyková and Lom, 1988b). *S. testicularis* affecting the testes of male seabass can cause parasitic castration (Sitja-Bobadilla and Alvarez-Pellitero, 1992).

Not all kidney dwelling species of *Sphaerospora* cause significant fish losses. *Sphaerospora cristata* affecting burbot, *Lota lota*, caused enlarged renal corpuscles with severely damaged glomeruli, but even with extensive glomerular lesions, no clinical signs or losses were detected (El-Matbouli *et al.*, 1992). *Sphaerospora dicentrarchi,* a histozoic parasite affecting the connective and muscular tissue of the gall bladder and intestine of sea bass seems relatively harmless unless massive infection occurs (Sitja-Bobadilla and Alvarez-Pellitero, 1992). Feist *et al.* (1991) described only a reduction in the height of the kidney tubule epithelial cells in sticklebacks affected with *S.elegans.*

3.1.6 Objectives.

Knowledge of the biology of *S. elegans* is still far from complete. In particular there is no complete description of the development of the species in the stickleback. The objective of this study was to describe the development and pathogenicity of *S. elegans* at the light and electron microscope level and to compare this with other members of the genus and with other myxosporeans.

3.2 Materials and Methods.

Sticklebacks were collected from Airthrey Loch and maintained in the laboratory until examination as described in Chapter 2.

3.2.1 Light Microscopy.

The presence of *S. elegans* was ascertained by :

(1) Fresh kidney squashes: Kidney squashes were prepared in a drop of 8% physiological saline on a microscope slide and observed using normal phase contrast optics at x40 to x100 magnification (Chapter 2). Measurements of the parasite as shown in Fig.3.1 were taken from fresh squash preparations. An average of 35 spores and 10 to 20 developing stages of the parasite were measured from 5-10 fish from each monthly sample according to the method described by Lom and Arthur (1989).

(2) Air-dried kidney smears: Dried fresh kidney smears were fixed with absolute methanol for 40 seconds and stained by Grünwald Giemsa for 30 minutes (Chapter 2).

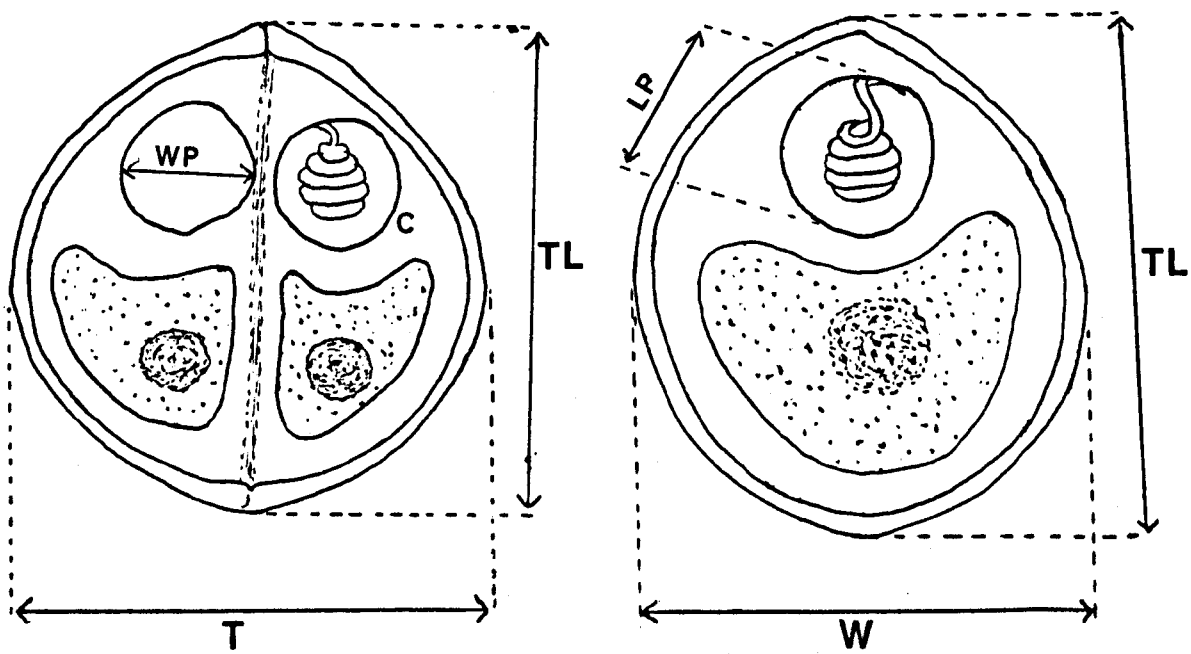


Fig 3.1 Measurements of *S. elegans* spores (after Lom and Arthur, 1989).
TL=total length of the spore
W=width of the spore;
T=thickness of the spore;
LP=length of polar capsule;
WP=width of polar capsule;
C=no. of polar filament coils.

(3) Histology: Kidneys were routinely fixed immediately in 10% buffered formalin and processed according to standard procedures; 6μm sections were stained with haematoxylin and eosin and May-grünwald Giemsa (Chapter 2).

(4). Photography and drawing of specimens under light microscopy were as described in Chapter 2.

3.2.2 Transmission Electron Microscopy (TEM)

For transmission electron microscopy, infected kidneys were excised from the fish and processed according to the methods of Glauert (1977).

Fixation

1. Fresh kidneys were cut into very small (1 mm^3) pieces while immersed in Karnovsky's fixative (1.3% paraformaldehyde, 1.6% glutaraldehyde).
2. Tissues were fixed for 4 to 6 hours at 4^0c.
3. Tissues were then washed briefly in cacodylate rinse, placed in fresh rinse and left overnight at 4^0c.
4. Tissues were transferred to 0.5% osmic acid in cacodylate buffer for 1 hour.

Dehydration and Impregnation

The fixed specimens were dehydrated in 30%, 60% and 90% acetone for 10 minutes each, followed 100% acetone twice for 15 minutes each time. Specimens were impregnated with 100% acetone+ resin mix (1:1) for 30 minutes, followed by another 100% acetone +resin mix (1:3) for a further 30 minutes. Final impregnation was in resin for at least 1 hour.

Embedding

Fresh araldite mix was used for embedding. To ensure the complete mixing of the components of the epoxy resin embedding media, the araldite resin and DDSA hardener (dodecanyl succinic anhydride) were warmed at 60°c before mixing in disposable plastic beakers. BDMA (benzyldimethylamine, 0.4ml for every 10ml of araldite and 10ml of DDSA) was added by a disposable plastic syringe. The mixture was mixed with a wooden stirrer and the whole preparation kept covered with Parafilm until use. Specimens were embedded in Beem capsules warmed in the oven for 1 hour prior to use. The blocks were then polymerised in an oven at 60°c for 48 hours.

Semithin sections of 0.8nm thickness were cut from resin blocks using glass knives fitted in an ultramicrotome (Leica Ultracut- E). Sections were stained for 1 minute with toluidine blue or haematoxylin and eosin and then examined under the light microscope. After the identification of areas of interest, the blocks were trimmed down to leave the relevant area for ultrathin sectioning.

Ultrathin sections of 0.8μm thickness were picked up on naked copper grids and dried and double stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965).

Staining Schedule

1. Grids were submerged face upwards in drops of uranyl acetate for 2 minutes.
2. Grids were rinsed in fresh distilled water and blotted on filter paper.
3. Grids were floated face down on a small drop of lead citrate on a wax block for 2 minutes.

4. Grids were again rinsed in fresh distilled water.

The grids were then viewed and photographed with a Phillips 301, transmission electron microscope at 80 KV.

3.2.3 Scanning Electron Microscopy (SEM)

For scanning electron microscopy infected kidneys were macerated in a mortar and pestle with a few drops of saline. Saline water (10ml) was then added and the suspension filtered using acetone resistant Surtolon (Polyamid) millipore filters with pore size .45μm. These preparations were immediately fixed in 4% (v/v) glutaraldehyde in phosphate buffer (PH 7.4) and left for one week at 4^0c. They were then washed in phosphate buffer for 4 hours followed by secondary fixation in 1% osmium in the same buffer for 2-4 hours. After dehydration in a graded ethanol series they were dried in a Polaron E 3000 critical point drying apparatus. Filters were then mounted on stubs coated with gold and examined in a Phillips-500 scanning electron microscope.

3.3 Results.

3.3.1 Light Microscopy

3.3.1.1 Fresh Squash of Infected Organs.

The kidney was the only organ containing pre-sporogonic and sporogonic stages of *S. elegans*. Apart from the head kidney all areas of the kidney were parasitised by mature spores and pre-sporogonic and sporogonic stages. In many cases the lumen of the renal

tubules was completely filled with mature spores and developing parasites (Fig.3.2). Bowman's spaces of the glomeruli were also parasitised by spores and developing parasites. Spores were rare in the kidney interstitium, but early pre-sporogonic stages were quite common. Occasionally mature spores were seen in the ureters and urinary bladders.

Pre-sporogonic and Sporogonic Stages

In fresh kidney squash preparations presporogonic stages appeared as round or oval shaped bodies up to 26.5μm in diameter. The outer surface of these stages was smooth, their cytoplasm containing only one nucleus and some refractile granules. Several inner cells were present within the presporogonic stage.

Sporogonic stages were mostly disporous pseudoplasmodia. Occasionally monosporous plasmodia were found (Fig.3.3). They were up to 17μm in size containing a single spore and many refractile granules and vacuoles. Disporous plasmodia reached 25.2μmx9.45μm in size, contained two spores and rather fewer granules and vacuoles than monosporous stages. Spore development within disporous plasmodia was mostly synchronous. It was frequently observed that spores in pseudoplasmodia contained a large vacuole at the posterior end (Fig.3.3a).

Spore

In fresh kidney smears mature spores appeared spherical in shape when viewed perpendicularly to the suture line (Fig.3.4a). Spore dimensions did not vary significantly between monthly samples. Mean spore size based on 480 spores was: length 11.0μm (7.7μm-11.5μm) width 10.6μm (7.0μm-10.8μm). In valvular view the spore was almost round in shape (Fig.3.4c). Anteriorly there was a prominent thickening of the shell valves. The two

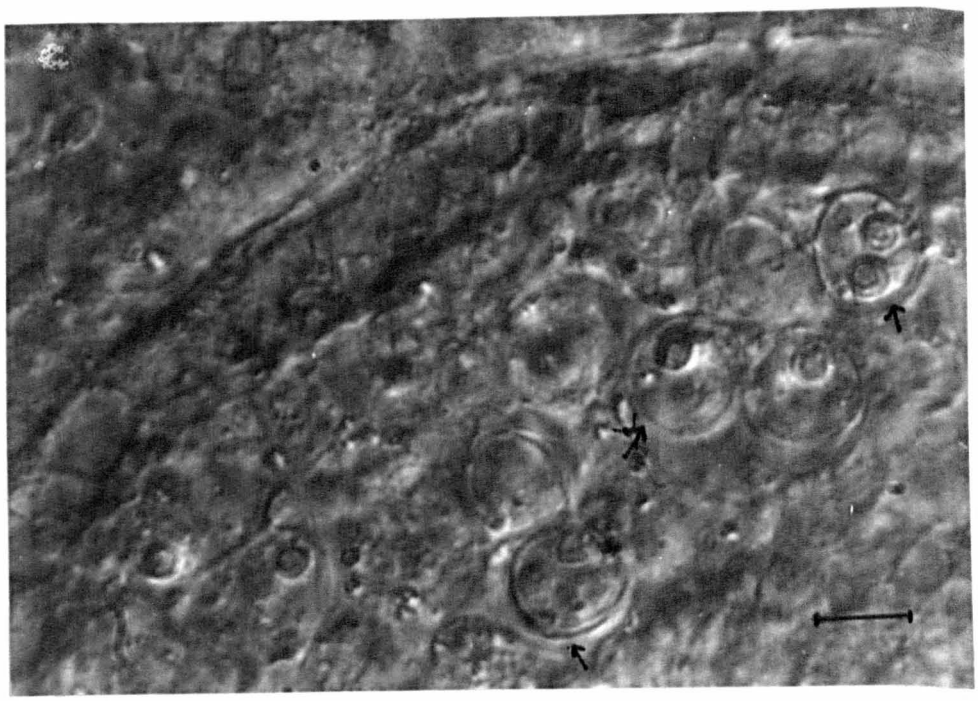


Fig 3.2 Fresh squash preparation of a kidney tubule heavily infected by sporogonic stages (arrows) of *S. elegans*. Nomarski interference contrast (bar=10μm).

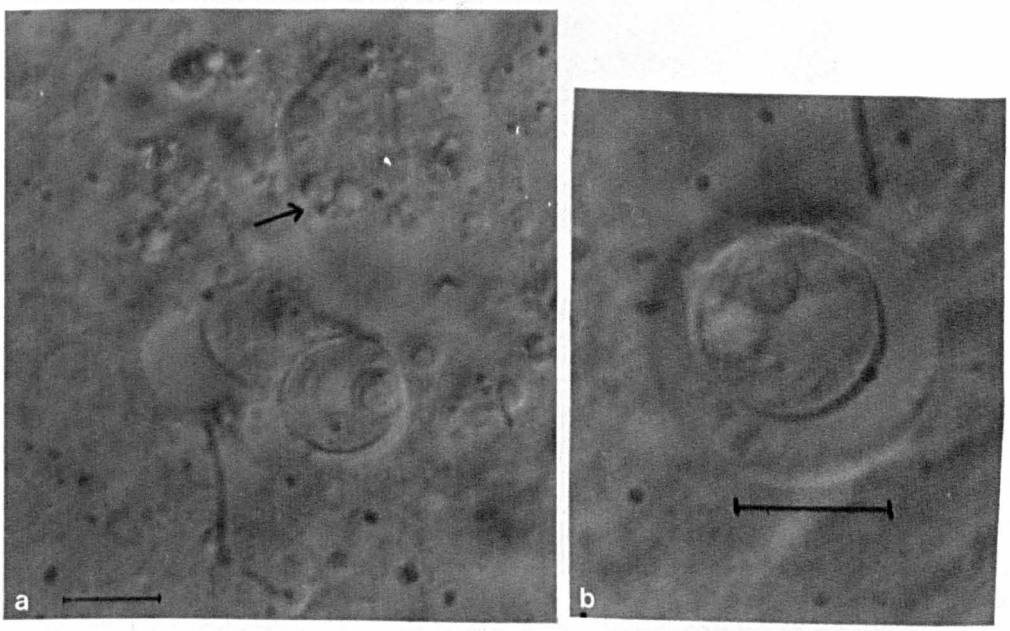


Fig 3.3 Fresh squash preparation of kidney tubule. Nomarski interference contrast (bar=10μm). (a) Presporogonic stage (arrow) and disporous plasmodia; (b). monosporous plasmodia.

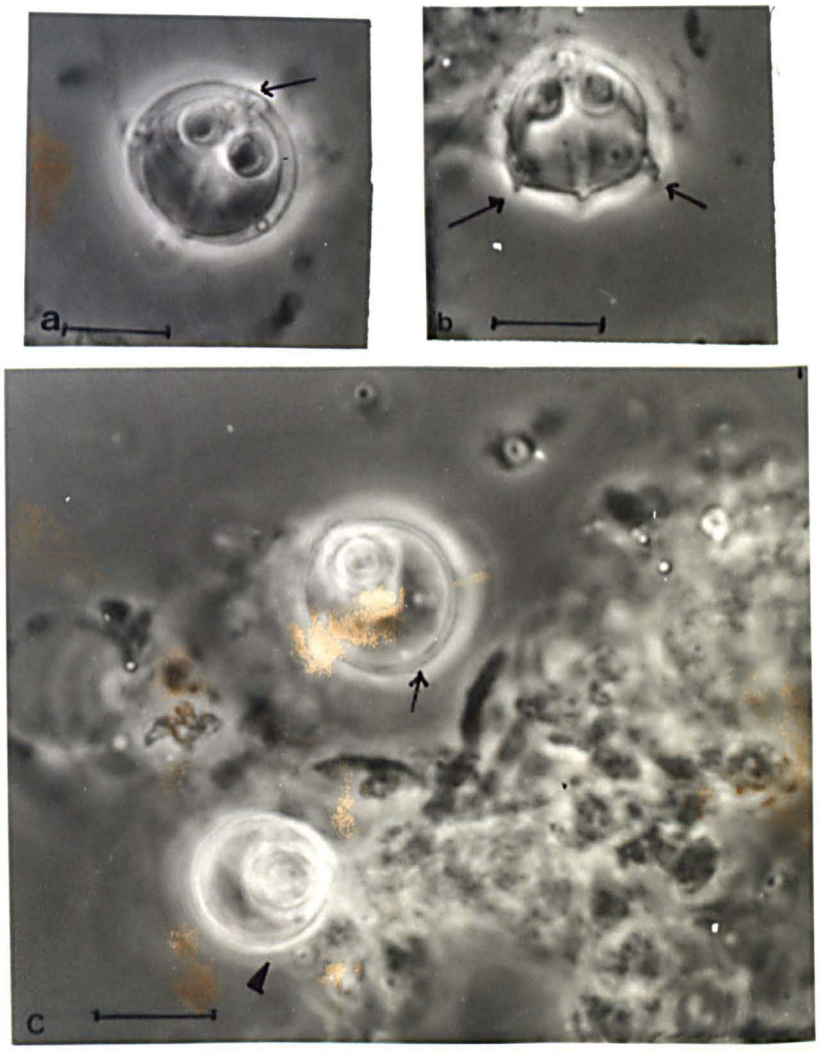


Fig 3.4 Fresh squash preparation of mature and immature spores of *S. elegans*. Phase contrast (bar=10μm). (a) Sutural view of mature spores. Note anterior thickness (arrow) of valvogenic cells. (b) Sutural view of immature spore. Note lateral protuberances (arrows) of valvogenic cells. (c) Valvular view of mature (arrow) and immature (arrow heads) spores.

polar capsules were spherical with a mean diameter of 4μm (3.4μm-4.4μm, n=440). Occasionally a spore with unequal polar capsules was found. The polar filament made 5 to 6 coils at an approximately 45^0 angle to the sutural line. Two sporoplasm cells occupied most of the remaining posterior part of the spore. Along the suture the two valves formed a smooth, prominent and very thick sutural line. In immature spores there were always two conspicuous protuberances on the posterior of the shell valves equi-distant from the sutural line (Fig.3.4b). The valvogenic cell nucleus was observed within these protuberances. With the maturation of the spore these protuberances became less prominent and completely disappeared in the fully mature spore. Sculpturing on the surface of the valves was not very clear under light microscopy.

3.3.1.2 Giemsa Stained Smears.

In Giemsa stained kidney smears early pre-sporogonic stages were observed as pseudoplasmodia enclosing two to three inner cells (Fig.3.5a,b). Sporogonic stages were found as disporous pseudoplasmodia enclosing 12 inner cells (Fig.3.5c).

Extrasporogonic Stages

Extrasporogonic stages of *S. elegans* were found in dry smears of circulating blood, choroidal rete of the eye and kidney.

Extrasporogonic infections in June 1993 were very light with only one organism per four or five fields in Giemsa stained blood smears at x100 magnification. These stages were oval to pleomorphic in shape (n=10, $\bar{X}$=13.95μm, 7μm-35μm) with a slightly thickened external

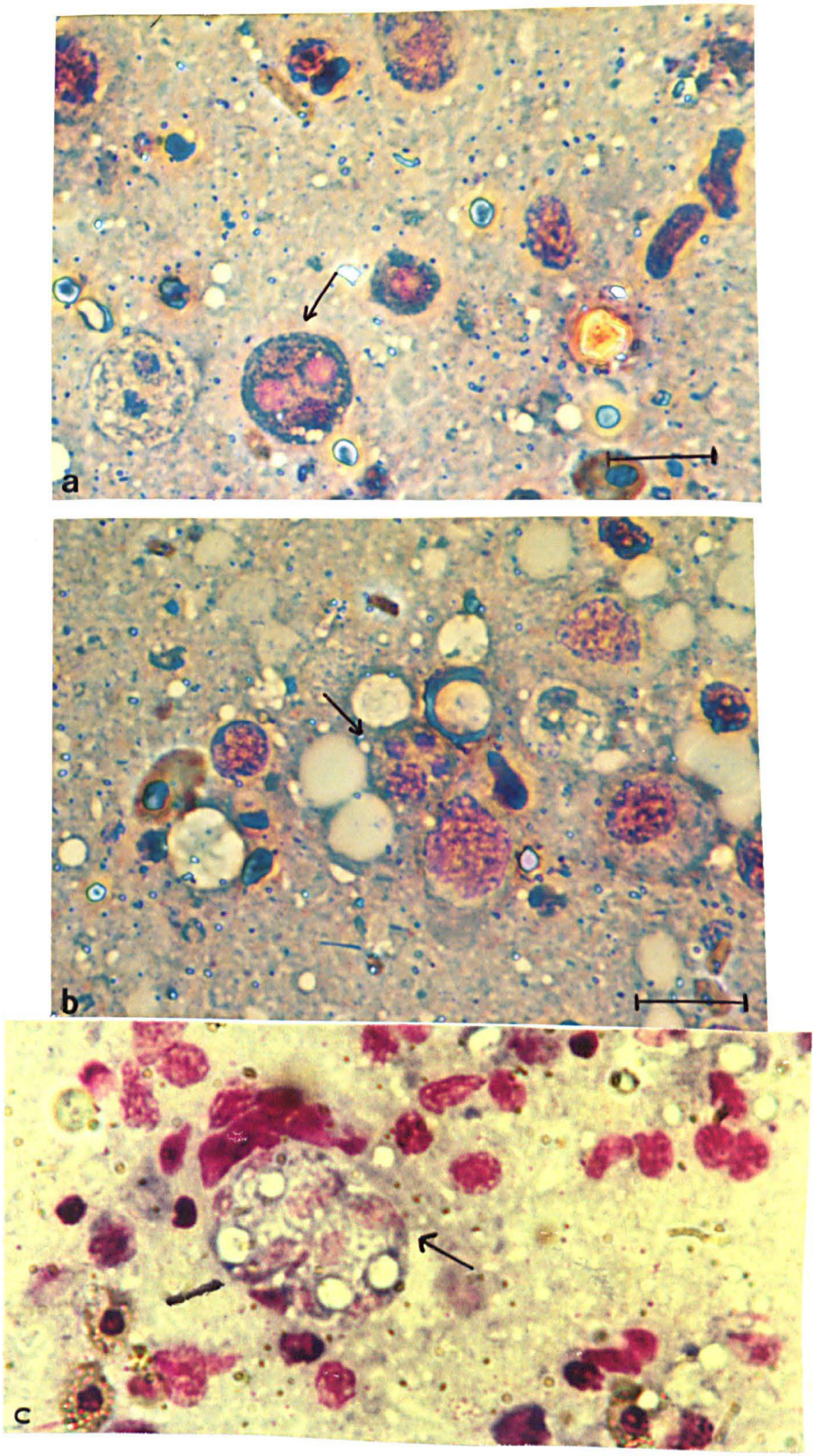


Fig 3.5 Giemsa stained impression smears of kidney showing pre-sporogonic and sporogonic stages of *S. elegans* (bar=10μm).

a. Pre-sporogonic stage (arrow) with 2 inner cells.

b. Pre-sporogonic stage (arrow) with 3 inner cells.

c. Sporogonic stage (arrow) with 12 cells.

membrane which stained deeply with Giemsa. The developmental sequence of extrasporogonic stages as observed in blood smears is shown in Figs.3.6 and 3.7. The earliest recognisable stages were 7-10μm in size with a large eosinophilic nucleus surrounded by dark blue cytoplasm, probably representing a primary cell (Fig.3.6a_1,a_2). This earliest stage had a few cytoplasmic processes extending from the surface (Fig.3.6a_1) which were sometimes lacking (Fig.3.6a_2). Primary cells with a single secondary cell were very rare (Fig.3.6b_1-b_2), but primary cells with two secondary cells (n=15, 14μm-24.42μm) were frequently found (Fig.3.6c_1-c_7). Primary cells could contain secondary cells of different types. Occasionally one or both of the secondary cells were crescent or spindle shaped (Fig.3.6c_1,c_2). In some cases two secondary cells were found in a single vacuole within the primary cell (Fig.3.6c_3). The cytoplasm of primary cells with secondary cells stained pale blue, with a large nucleus and many vacuoles. The cytoplasm of early secondary cells was barely detectable (Fig.3.6c_4). Primary cells were frequently observed with macrophages attached to them or closely associated with them. As the secondary cell increased in size its basophilic cytoplasm became more obvious (Fig.3.6c_5). Primary cells with 2 secondary cells sometimes had surface processes of varying thickness (Fig.3.6c_3,c_4,c_6&c_7). Stages with 3 and 4 secondary cells were found less frequently (Fig.3.6d_1d_2 &e_1e_2). In some cases where there were 3 secondary cells within the primary cell the former were very large occupying the whole cytoplasm of the primary cell (Fig.3.6d_1), but in other cases they were very small and situated closely around the nucleus of the primary cell (Fig.3.6d_2). Four secondary cells within the primary cell were sometimes equal in size and closely attached to each other indicating synchronous division of the two secondary cells (Fig.3.6e_2). Residual bodies appeared in primary cells with 6 or more secondary cells (Fig.3.6f). These were located within a round vacuole of 2.8μm to 4.7μm in size. Large primary cells containing many

Fig 3.6 Extrasporogonic stages of *S. elegans* from Giemsa stained blood and kidney impression smears (bar=10μm).

a_1. The earliest stage found was a primary cell without any secondary cells. Note few cytoplasmic projections of this stage.

a_2. Primary cell with many vacuoles in the cytoplasm.

b_1&b_2. A primary cell with a single secondary cell (arrow) was very rarely observed.

c_1&c_2. Primary cells with crescent and spindle shaped secondary cells (arrows) indicates recent division of a single secondary cell.

c_3. Endogenous division of a secondary cell is indicated by two secondary cells within a single vacuole (arrow). Note primary cell cytoplasmic projection and vacuoles.

c_4. Primary cells with two secondary cells were most frequently observed. Note surface projections and vacuoles (arrows) of primary cell.

c_5. The cytoplasm of the secondary cell stains clearly with Giemsa.

c_6&c_7. Primary cells with two secondary cells can grow to a large size. Note cytoplasmic vacuoles.

d_1. Primary cell with 3 large secondary cells.

d_2. Primary cell with 3 small secondary cells surrounding the nucleus of the primary cell.

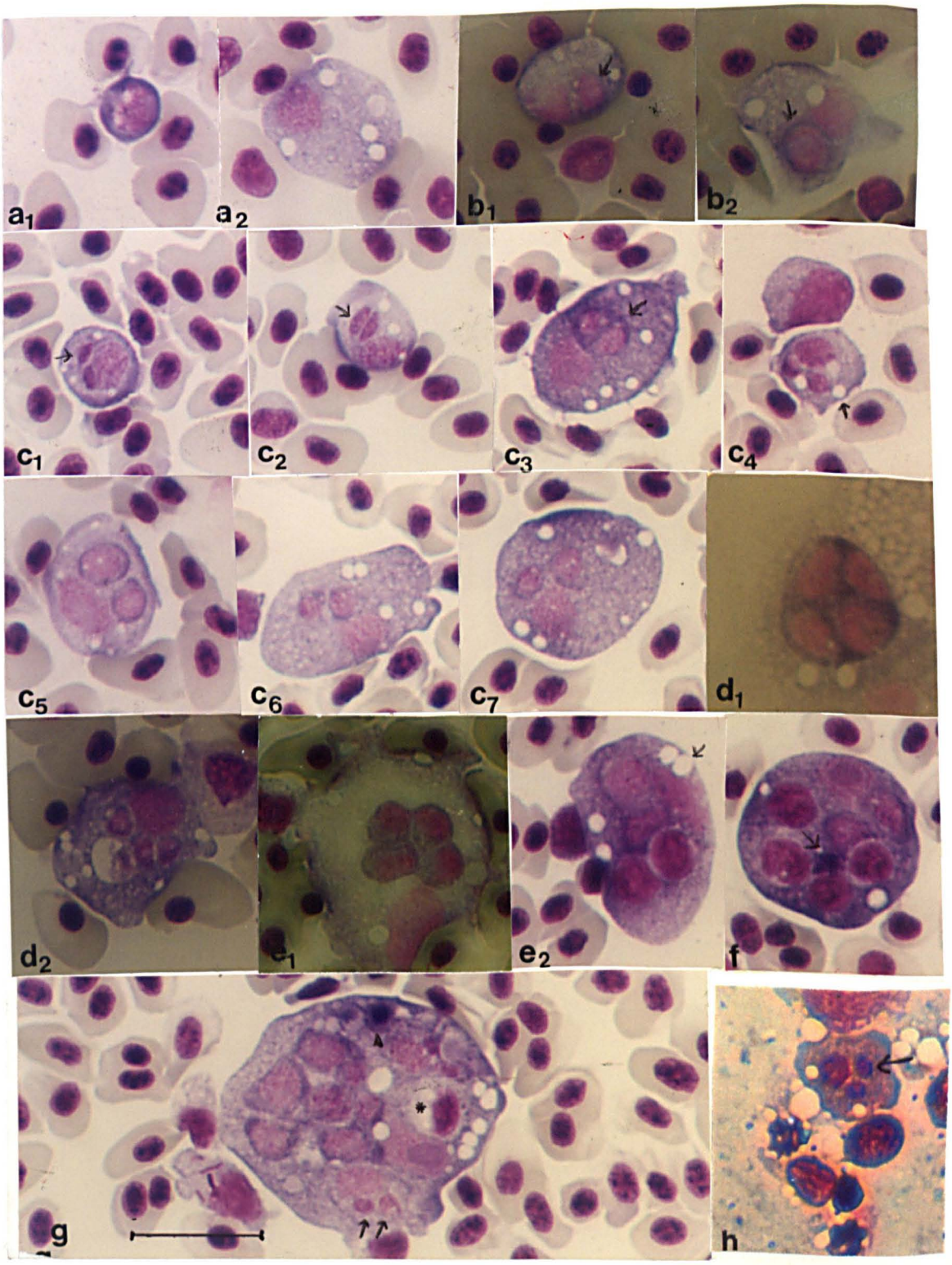
e_1. Primary cell with 4 equal sized closely attached secondary cells indicating recent synchronous division of 2 primary cells.

e_2. Primary cell with 4 secondary cells. Note cytoplasmic vacuole (arrow).

f. Primary cell with 6 secondary cells. Note residual body (arrow) in the cytoplasm of primary cell.

g. Primary cell with 9 secondary cells. Note two tertiary cells within two of the secondary cells (arrows); residual body (arrow head) and engulfment of host red blood cell within the cytoplasm of primary cell (*).

h. Primary cell with two secondary cells (arrow) from a kidney smear.


a1
a2
b1
b2
c1
c2
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c4
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c6
c7
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e1
e2
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g
h

secondary cells occasionally contained phagocytosed blood cells in the cytoplasm (Fig.3.6g). Extrasporogonic blood stages reappeared in the circulating blood in January 1994. Most of them were frequently seen with macrophages attached (Fig.3.7a), a characteristic occasionally seen in the June 1993 specimens. Most of the primary cells with 2 to many secondary cells had 1 to 3 small cells attached at the surface (Fig.3.7b_1-b_3,c,d,f&g). These small cells had a distinct nucleus and vacuolated cytoplasm. Occasionally they had their own secondary cells (Fig.3.7g). These small cells may indicate proliferation of extrasporogonic stages by external budding or plasmotomy. Primary cells with two secondary cells reached 24.42μm in size. Residual bodies were found within the primary cell containing 4 secondary cells. They were situated in separate vacuoles of 2.8μm to 4.7μm in size (Fig.3.7c). Primary cells reached a maximum size of 35.28μm x 25.48μm, and contained up to 15 secondary cells (Fig.3.7g). The size of the secondary cells varied from 1.8μmx1.8μm to 7.5μmx5.6μm. Rarely secondary cells contained up to 3 tertiary cells (Fig.3.7h). Tertiary cells were approximately 1.4μm to 2.3μm in diameter and round in shape. Many large empty vacuoles were present in the cytoplasm of some larger primary cells which contained secondary cells of different sizes, the larger ones of which had dark blue staining cytoplasm (Fig.3.7d,e). Cytoplasmic processes of these primary cells were easily detectable. A few large primary cells were observed with extensively vacuolated cytoplasm and a few residual bodies, but with no secondary cells (Fig.3.7f). These primary cells had a small extrasporogonic cell attached to their surface. Such vacuolated primary cells have possibly released their secondary cells. Occasionally secondary cells with tertiary cells were found in the disintegrated cytoplasm of a primary cell (Fig.3.7i &j). The nucleus and cytoplasm of the disintegrated primary cell were faintly stained. This presumably represents release of the secondary cells by disintegration of the primary cell. Unlike blood stages, extrasporogonic

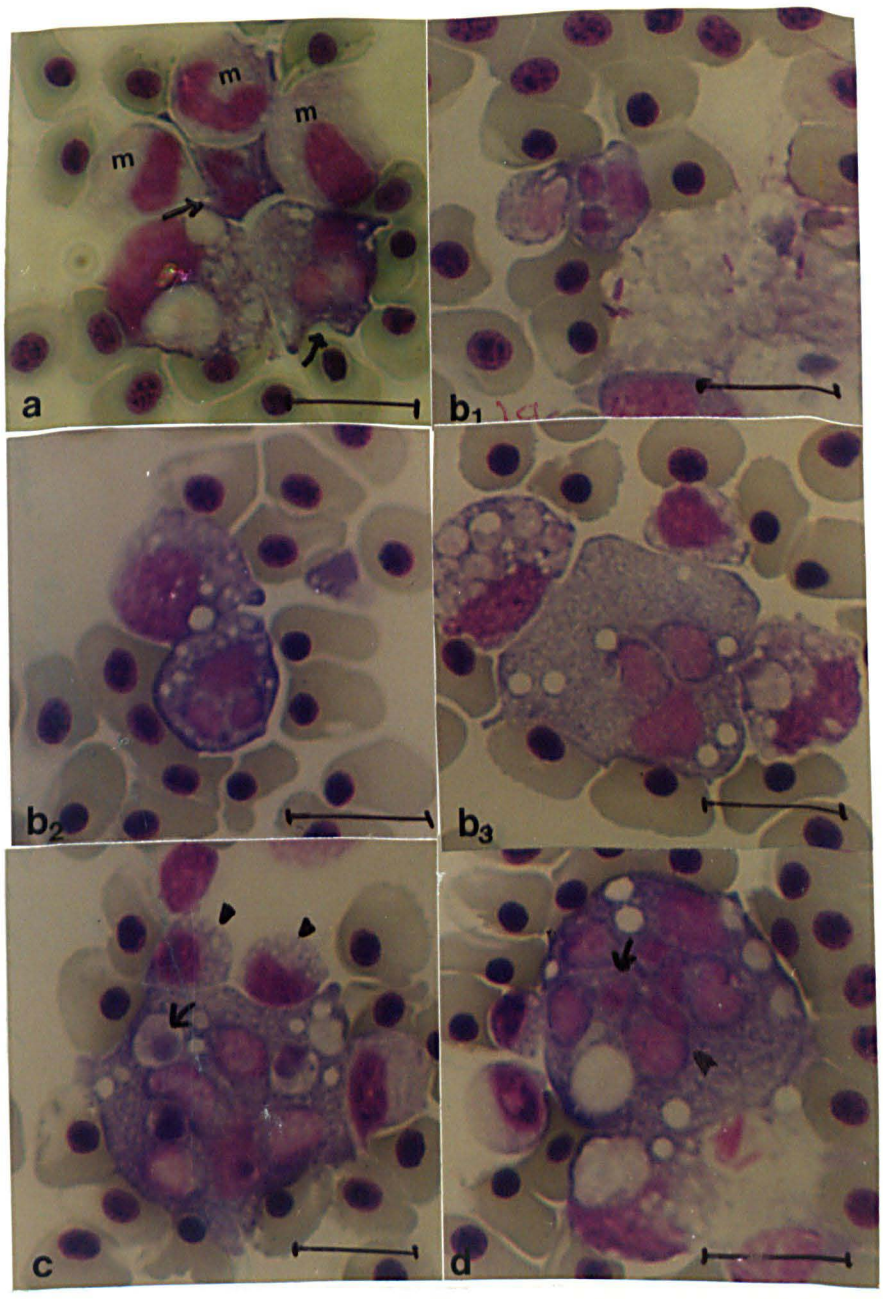

Fig 3.7 Features of extrasporogonic blood stages of *S. elegans* found in January 94 (bar = 10μm).

a. Early blood stages (arrows) often found in close association with macrophages (m).

b_1-b_3. Primary cells with two secondary cells attached to nucleated cell at the surface indicating external budding. Note this stage can become large (b_3).

c. Primary cell with 4 secondary cells; residual bodies (arrow). Note external budding (arrow heads).

d. Primary cell with 6 secondary cells, 2 of which each contain one tertiary cell (arrow head), two secondary cells in the centre are very small in size (arrow) and may be transforming into residual bodies.

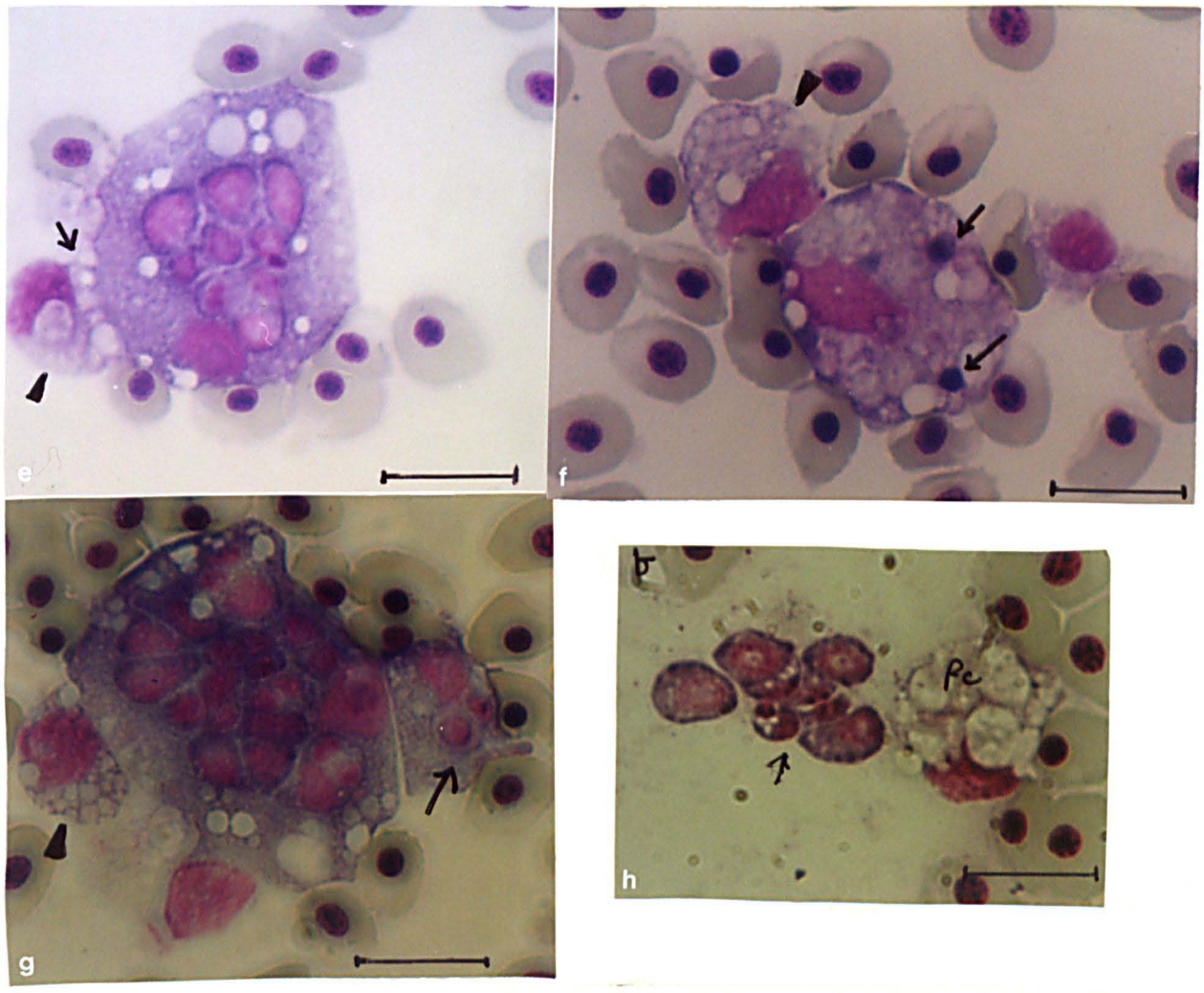

Fig 3.7 (contd.)

e. Primary cell with a number of secondary cells. Note cytoplasmic projections of primary cells (arrows). Note external budding (arrow head).

f. Vacuolated primary cells with residual bodies (arrows). Note there are no secondary cells which have probably been released. Note external budding (arrow head).

g. Primary cell with 5 secondary cells. Note external budding (arrow heads) contains 2 secondary cells (arrow).

h. Primary cell (Pc) with vacuoles and a number of secondary cells (arrows) close to it, probably just released from the primary cell or released during processing of the slide.

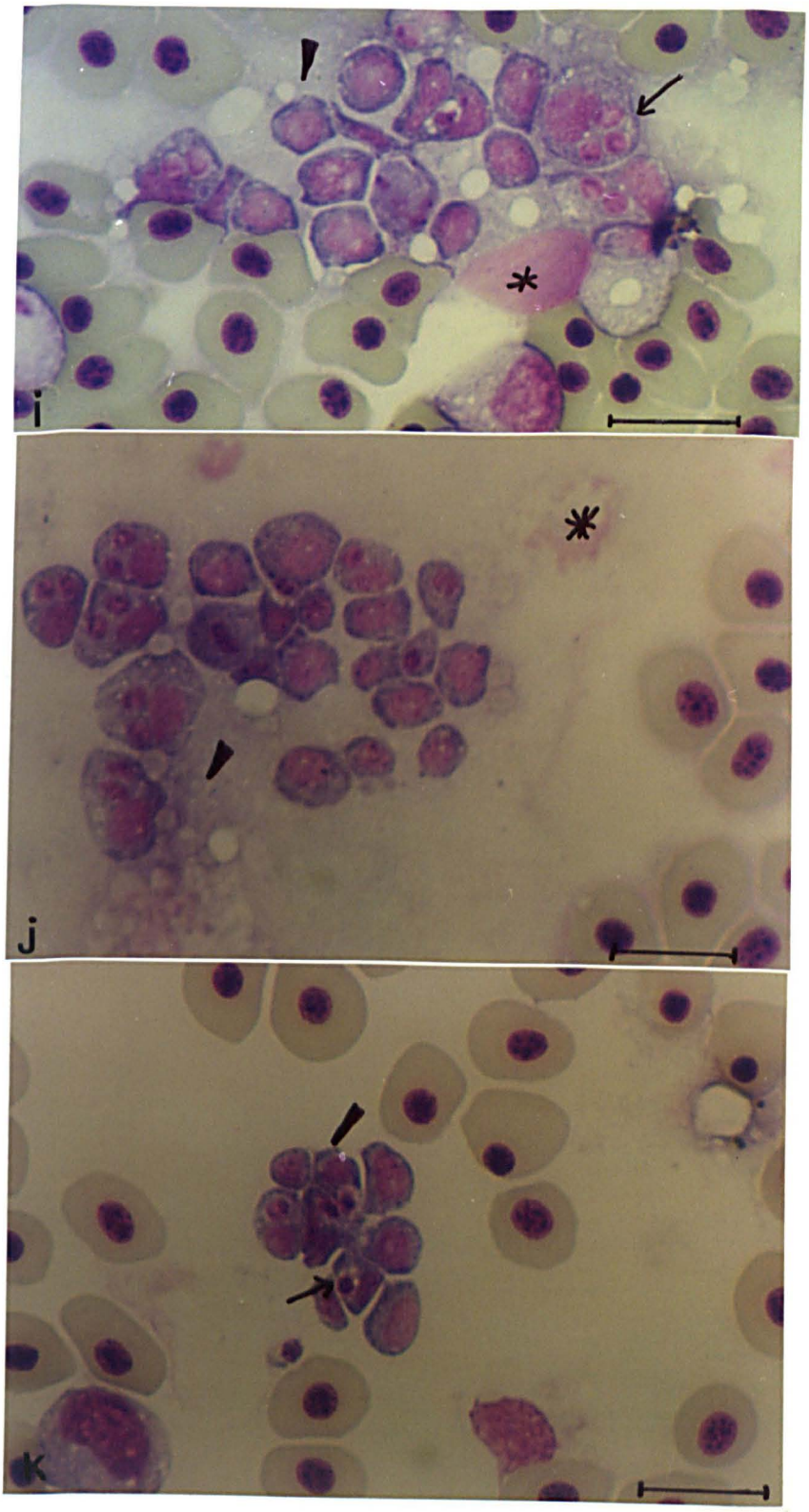

Fig 3.7 (contd.)

i&j. Disintegration of primary cell and release of secondary cells. Note one of the secondary cells contains 3 tertiary cells (arrow), (*) faintly stained nucleus and cytoplasm (arrow heads) of the disintegrated primary cell.

k. Probably newly released secondary cells. Note some of the secondary cells contain single (arrow) or 2 (arrow heads) tertiary cells whereas some lack a tertiary cell.

stages in the rete mirabile of the eye and interstitial spaces of the kidney were rarely found and could not be studied in detail. In fresh preparations the rete mirabile stages were mostly round and were packed tightly inside the capillary lumen. They ranged from 10.5μm to 25μm (n=12, $\bar{X}$=4.48) in size and contained only a few inner cells, which could not counted precisely, and a small number of refractile granules. They were not detected in histology and TEM samples. Extrasporogonic stages in the interstitial spaces of the kidney tubules were not clearly identified in fresh preparations or in histological sections and were only detected in Giemsa stained kidney smears. The only extrasporogonic stage seen in kidney smears was a primary cell with two secondary cells showing the same characteristics as those in the blood (Fig.3.6h).

3.3.1.3 Histology.

Mature spores, pre-sporogonic and sporogonic stages were found mainly in the tubular lumen and Bowman's space of the renal corpuscles. The spores are mostly found in the anterior part of the kidney, but the lumen of the proximal and distal tubules were sometimes equally parasitised (Fig.3.8).

3.3.2 Transmission Electron Microscopy.

3.3.2.1 Ultrastructure of Pre-sporogonic Stages

The earliest stages observed were spherical pseudoplasmodia with a smooth external surface. The pseudoplasmodia had dense, but undifferentiated cytoplasm, very few mitochondria and one or two electron dense areas (Fig.3.9a). The mitochondria were round with plate-like

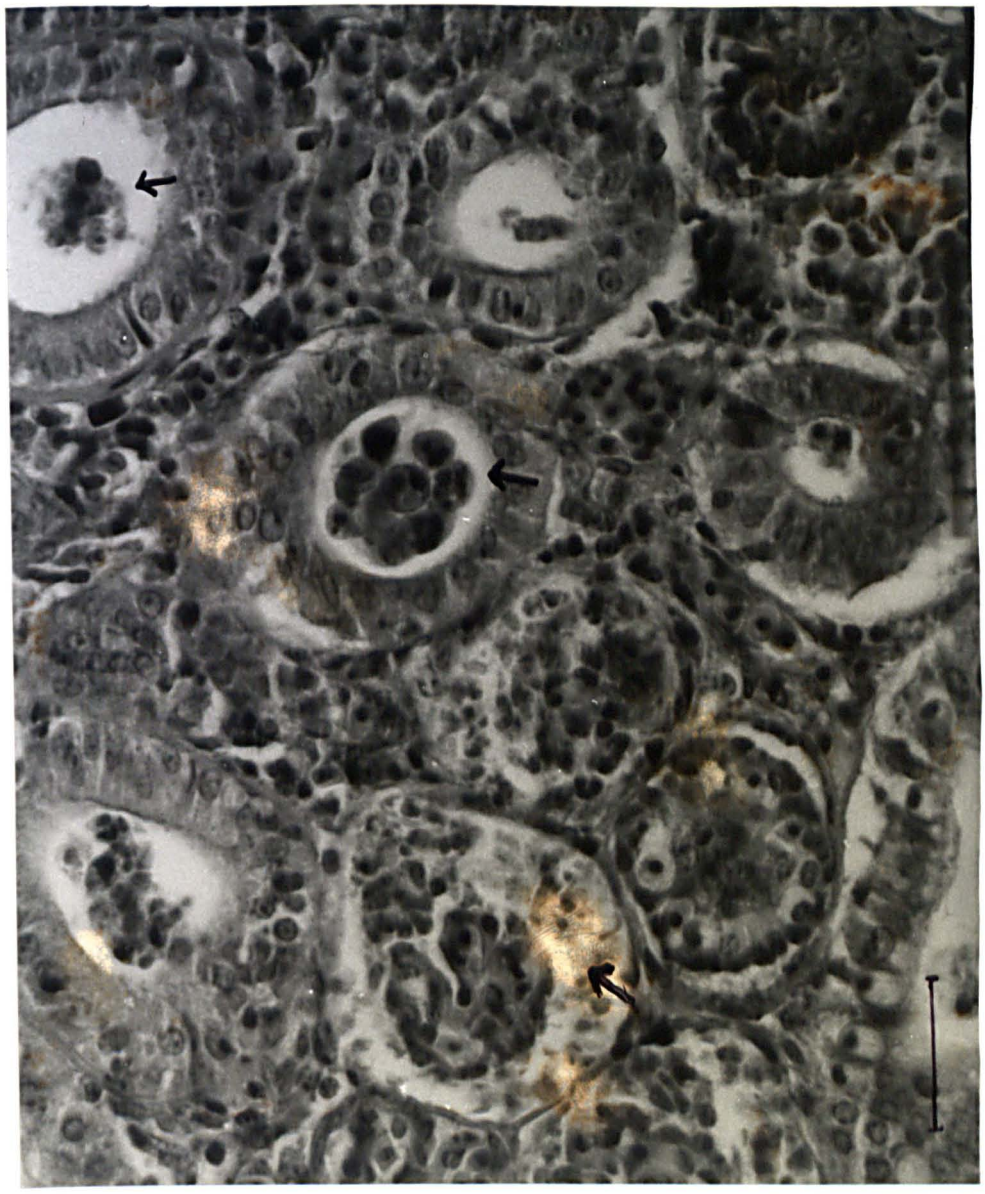

Fig 3.8 Histological section of kidney showing stages of *S. elegans* (arrows) in the renal corpuscles and lumen of proximal tubules (H & E bar=10μm).

cristae. The nucleus was not detectable. In larger pseudoplasmodia the nucleus was apparent and mitochondria were increased in number and some were more elongated. There was no evidence of sporogonic cell development at this stage (Fig.3.9b).

The next developmental stages recognized were pseudoplasmodia containing one or two sporogonic cells (Fig.3.10a,b). The nucleus of the pseudoplasmodium lacked a nucleolus. Sporogonic cells were contained in a vacuole and bounded by a double unit membrane. The sporogonic cell contained a large, round, centrally located nucleus in which a nucleolus was not observed. Mitochondria were mostly round and aggregated in one area of the cytoplasm of the sporogonic cell. The origin of the sporogonic cells was not clear, but they were not apparently derived by the endogenous division of the pseudoplasmodial nucleus. In some pseudoplasmodia areas of cytoplasm were less dense, with fewer mitochondria. Subsequently a nucleus appeared in this area bounded by the endoplasmic reticulum of the pseudoplasmodial cytoplasm. Finally a cell membrane developed around the cytoplasm forming a sporogonic cell within the pseudoplasmodium (Fig.3.11).

3.3.2.2 Ultrastructure of Sporogonic Stages.

As the sporogonic cell increases in size its cytoplasm contains more free ribosomes, mitochondria, smooth and rough endoplasmic reticulum and numerous microtubules. Ultimately 12 sporoblastic cells were formed within the pseudoplasmodium and these differentiated to form two complete spores, each with 2 valvogenic, 2 capsulogenic and 2 sporoplasmic cells (Fig.3.13). In ultrathin sections it was difficult to follow the exact order of the differentiation of the sporoblast cells, but the presence of capsular primordia in early

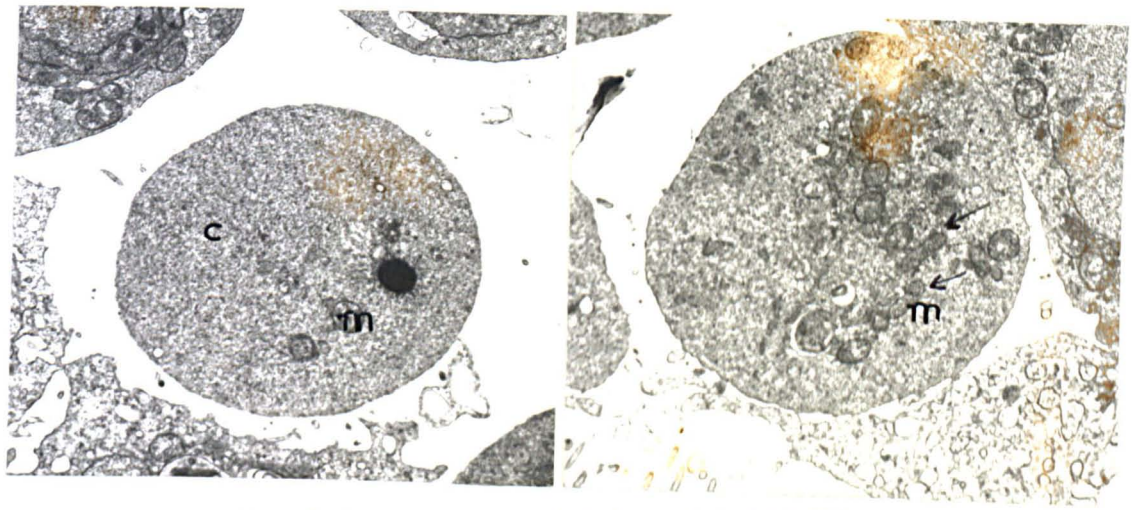

Fig 3.9 The earliest stages of pseudoplasmodia observed in kidney tubule (x11,200).
a. Note smooth external surface, dense cytoplasm (c) and few mitochondria (m).
b. Note mitochondria (m) are increased in number and are elongated (arrows) in slightly older pseudoplasmodium.

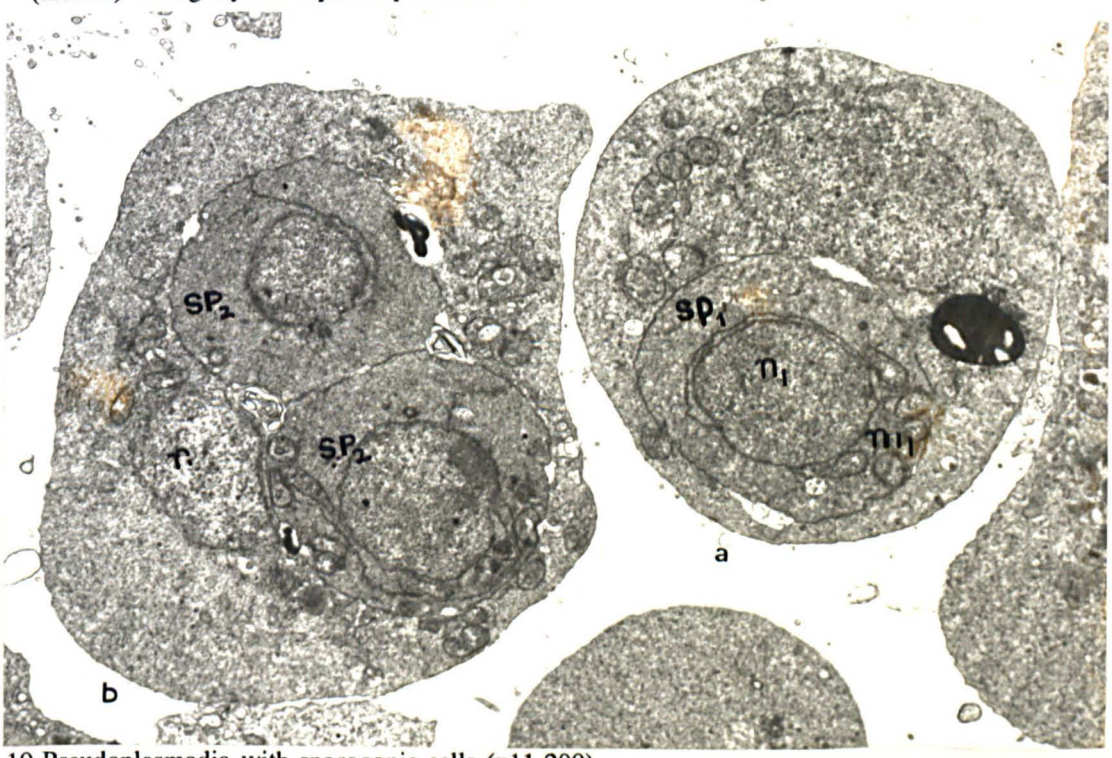

Fig 3.10 Pseudoplasmodia with sporogonic cells (x11,200).
a. Pseudoplasmodium with single sporogonic cell (SP_1); n_1-nucleus and m_1-mitochondria.
b. Pseudoplasmodium with two sporogonic cells (SP_2); n-nucleus of pseudoplasmodial cell.

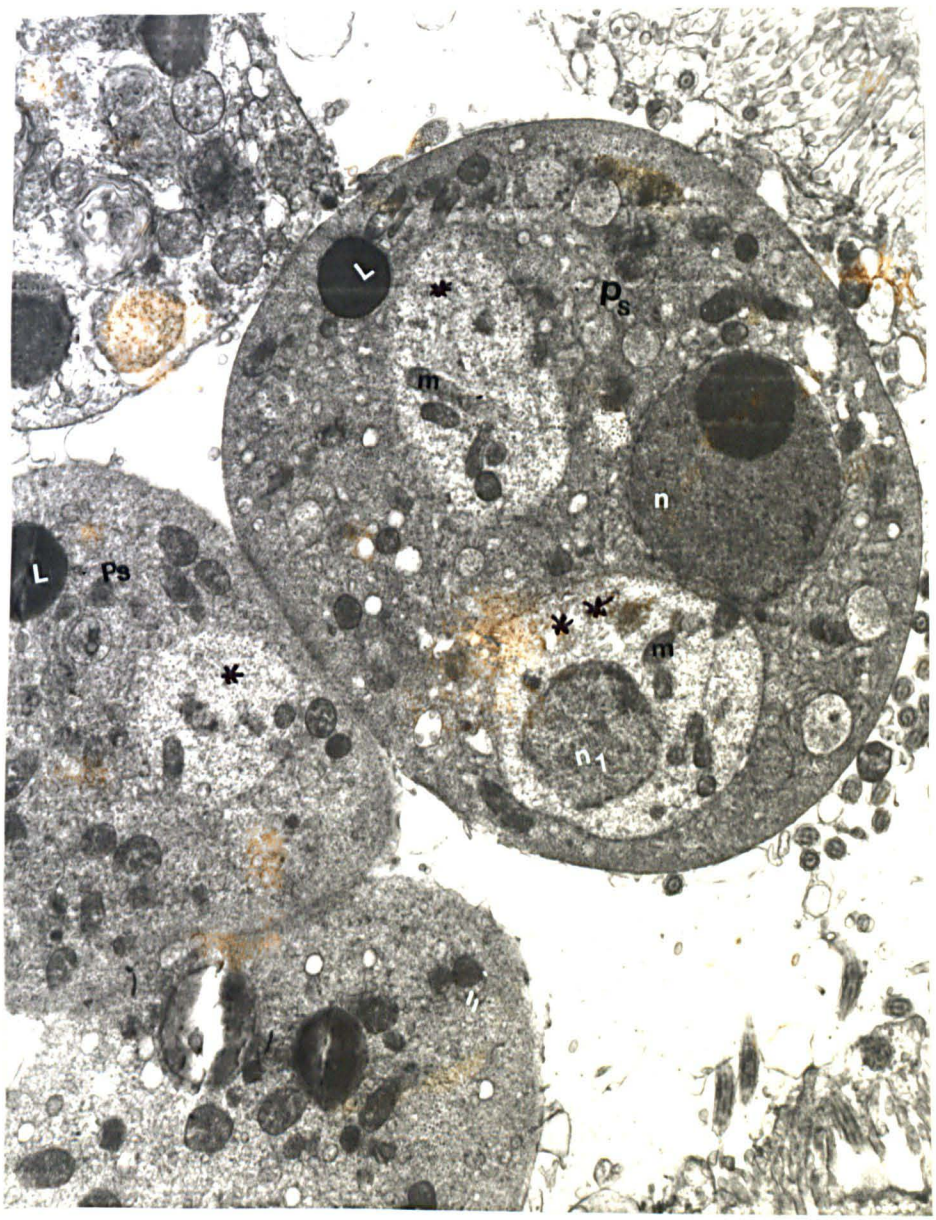

Fig 3.11 Early pseudoplasmodia of *S. elegans* showing formation of sporogonic cells. An electron lucent cytoplasmic area was frequently observed in the early pseudoplasmodial cytoplasm (*). This area acquired mitochondria (m), subsequently a nucleus appeared within it and the area became bounded by ER of the pseudoplasmodial cytoplasm (**). Ps-pseudoplasmodium; n-nucleus of pseudoplasmodium; n_1-nucleus of sporogonic cell; L-lipid body surrounded by endoplasmic reticulum (x90,000).

sporoblast cells suggests that the capsulogenic cell is the first to become differentiated (Fig.3.12).

Development of Capsulogenic Cells

Early capsulogenic cells can be easily recognised by the presence of capsular primordia within them (Fig.3.13). The cytoplasm of capsulogenic cells contained a large amount of RER around the nucleus, a few large mitochondria which were round or elongated with a small number of cristae, many free ribosomes, and a Golgi apparatus. Electron dense glycogen particles were also scattered in the cytoplasm. The nucleus was large, round and less dense than the cytoplasm of the capsulogenic cell. Electron dense heterochromatin was present beneath the nuclear envelope. There was a distinct central nucleolus. Occasionally myelenic formations were observed in the cytoplasm of the capsulogenic cell (Fig.3.13).

In later developmental stages of capsulogenic cells, a bulb like capsular primordium and a long external tube were seen(Fig.3.14). The capsular primordium was bound by a narrow electron dense layer covered by a thin membrane. Beneath this there was an electron lucent layer. The central area (core) of the capsular primordium contained dense, homogeneous granular material which increased in electron density with maturation of the spore. This core material was also present in the external tubule. The external tubule formed a maximum of 4 loops in the cytoplasm of the capsulogenic cell.

In the final stage of capsulogenic cell development, the external tube disappeared from the cytoplasm, which also coincided with the appearance of the polar filament within the capsule (Fig.3.15). In transversely sectioned capsules the polar filament appeared as "S" shaped with

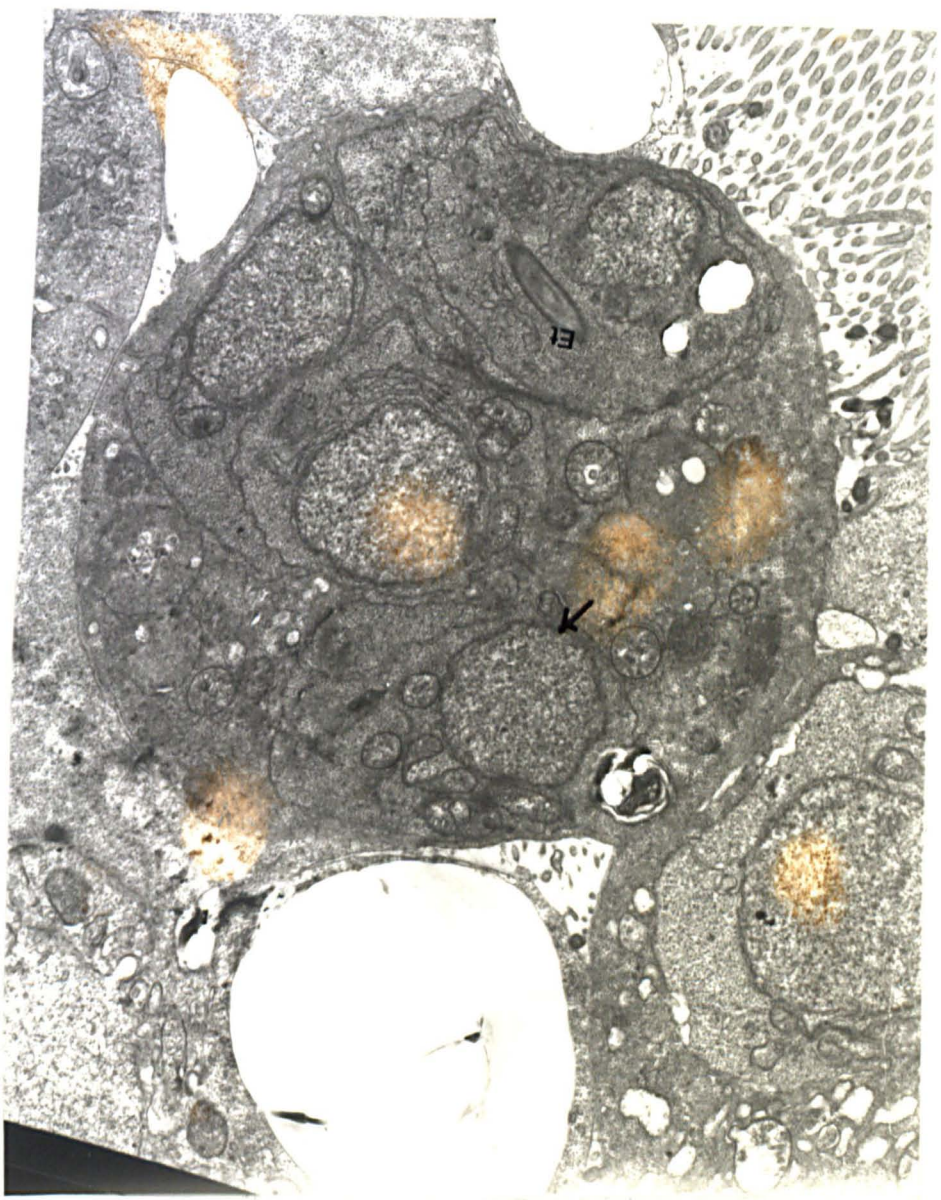

Fig 3.12 Pseudoplasmodium of *S. elegans* with 4 sporoblast cells. Note appearance of capsular primordia as the external tube (Et). Nuclei of sporoblast cells are very large with evenly distributed heterochromatin (x15000).

a thin lucent band around an electron dense area. With the maturation of the capsulogenic cells mitochondria decreased in number, RER became scattered throughout the cytoplasm and the nuclei became small and positioned laterally near the polar capsule. Large electron dense bodies, presumably lipid,were found adjacent to the polar capsule.

Development of Sporoplasmic Cell.

The cytoplasm of the uninucleated sporoplasm cells was denser than that of the capsulogenic cell and contained numerous free ribosomes, many mitochondria which were round or elongated, small amounts of RER scattered throughout the cytoplasm and a less dense nucleus in which a nucleolus was not apparent in the early stages of development (Fig.3.13).

As development proceeded a small nucleolus appeared in the nucleus, mitochondria reduced in number and the cytoplasm became denser, probably due to the presence of glycogen granules. No electron dense sporoplasmosomes were seen (Fig.3.13).

Development of Valvogenic Cell.

The cytoplasm of the two valvogenic cells was less dense than that of the capsulogenic and sporoplasmic cells (Fig.3.13). It contained a few round or elongated mitochondria and a distinct but not dense nucleus with a distinct nucleolus. Valvogenic cells were conspicuous before they began to encircle the other sporoblastic cells. An electron dense cell junction formed at the attachment point of the two valvogenic cells, which later formed the suture of the developing spore. At the apex of the developing spore valvogenic cell cytoplasm formed a thickened electron dense area around the opening of the filament discharge canal (Fig.3.16). At the ultrastructural level the filament discharge canal appeared as an electron

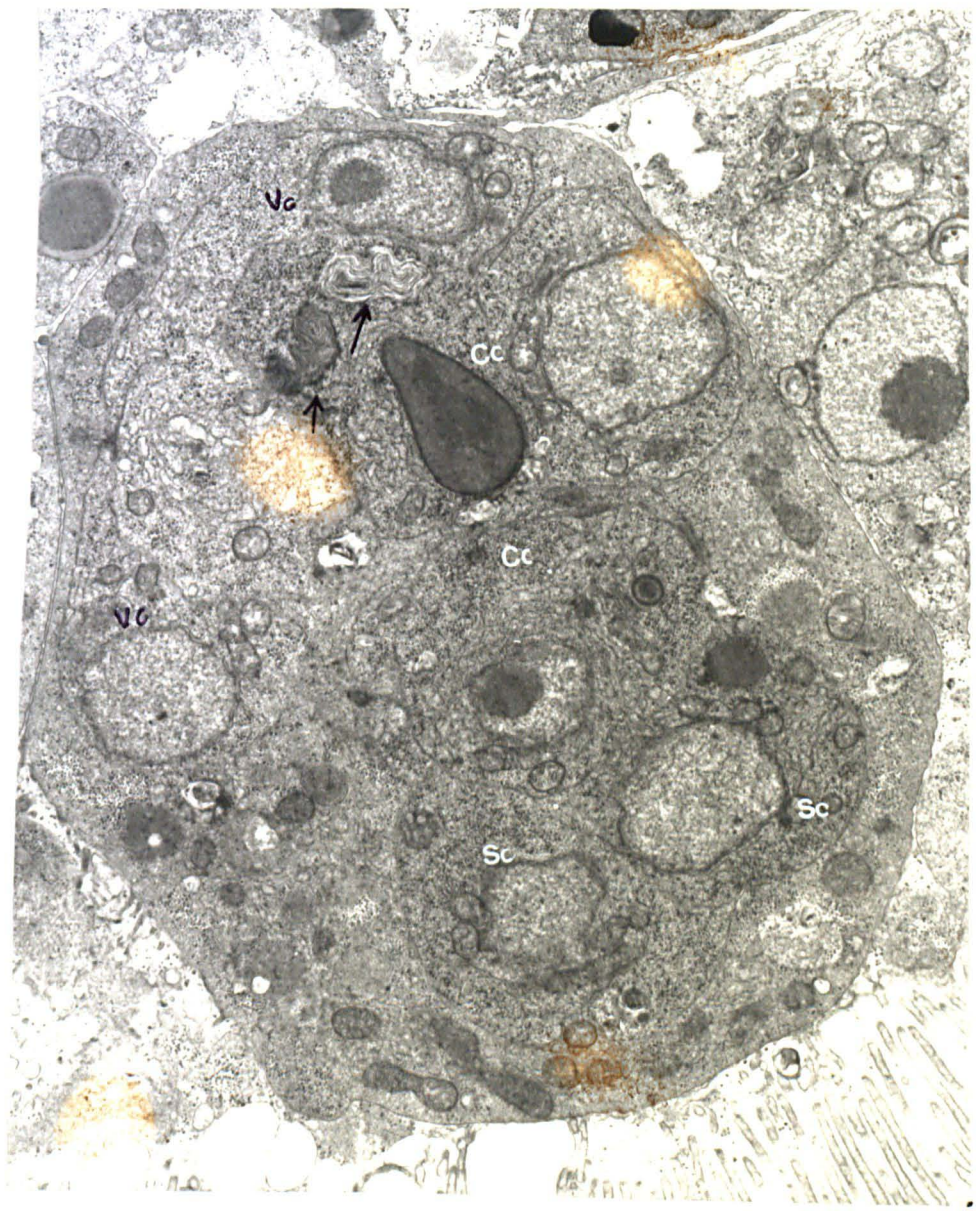

Fig 3.13 Differentiation of sporogonic cell into 2 capsulogenic cells (Cc), 2 sporoplasmic cells (Sc) and 2 valvogenic cells (Vc). Arrows indicate myelenic formations in the capsulogenic cells (x12,980).

dense area surrounded by a thin electron lucent layer. The opening of the polar capsule was not continuous with the filament discharge canal and was stoppered by a thin electron dense band which was continuous with the wall of the developing polar capsule (Fig.3.15).

Two protuberances were formed by each of the valvogenic cells at the posterior end of the immature spore (Fig.3.17). The valvogenic cell nucleus was lodged in the bulge of the protuberance. The anterior part of the protuberance contained only the cytoplasm of the valvogenic cell.

With the maturation of the spore the protuberances became shorter and ultimately disappeared completely in the mature spore. In the mature spores the valvogenic cells were inconspicuous and thinly spread around the spore. Cell organelles were not detectable (Fig.3.18).

3.3.2.3 Ultrastructure of Pseudoplasmodia

In section early pseudoplasmodia had a round or slightly elongate shape. The wall of the pseudoplasmodia was delimited by a single unit membrane. The cytoplasm contained a single nucleus, many ribosomes, RER, mitochondria and some RER bounded lipid inclusions, Golgi and a few vacuoles. There were also electron dense and electron lucent globular inclusions. Mitochondria were round and elongated with plate like cristae.

The cytoplasm of pseudoplasmodia usually contained one or two developing sporogonic cells which were round to slightly elongated. The cytoplasm of the latter contained few ribosomes which gave it a less dense appearance than the cytoplasm of pseudoplasmodia. The cytoplasm

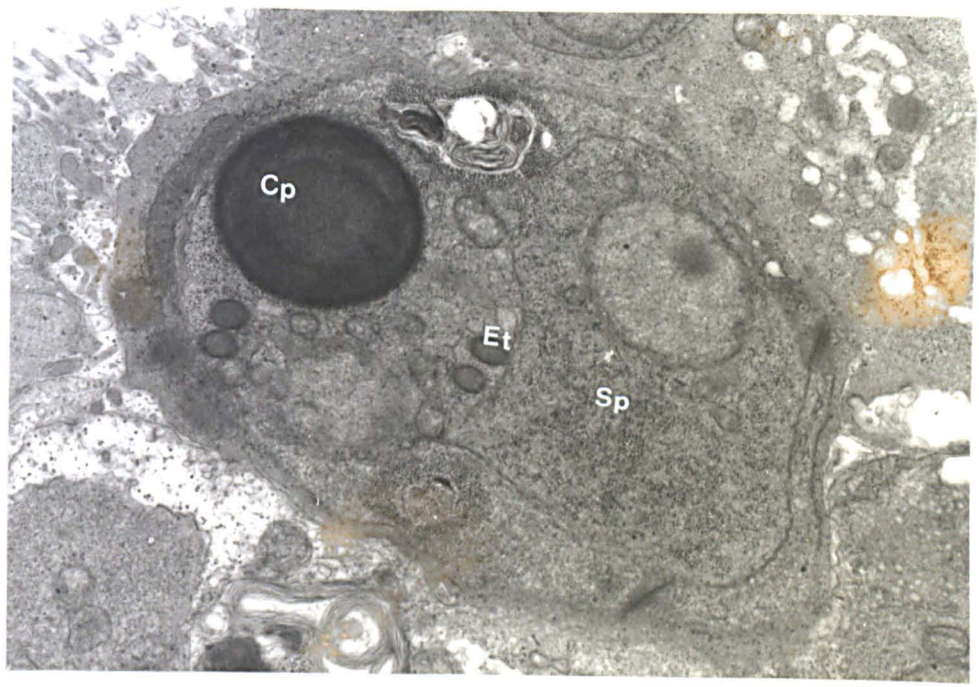

Fig 3.14 Section of a developing spore of *S. elegans* showing capsulogenic cell with bulb shaped capsular primordium (Cp) and external tube (Et) and sporoplasmic cells (Sp)(x12,000).

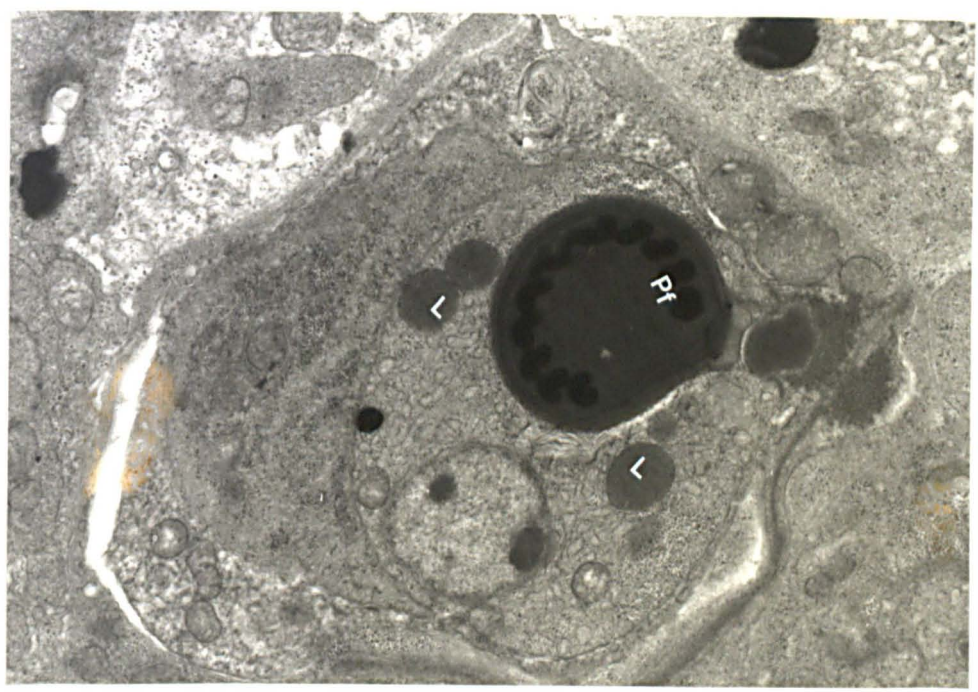

Fig 3.15 Development of polar capsule (x12,750).
Note with the appearance of the polar filament (pf) in the bulb-shaped capsular primordium, the external tube can no longer be seen in the cytoplasm of the capsulogenic cell. L-lipid body in the capsulogenic cell cytoplasm.

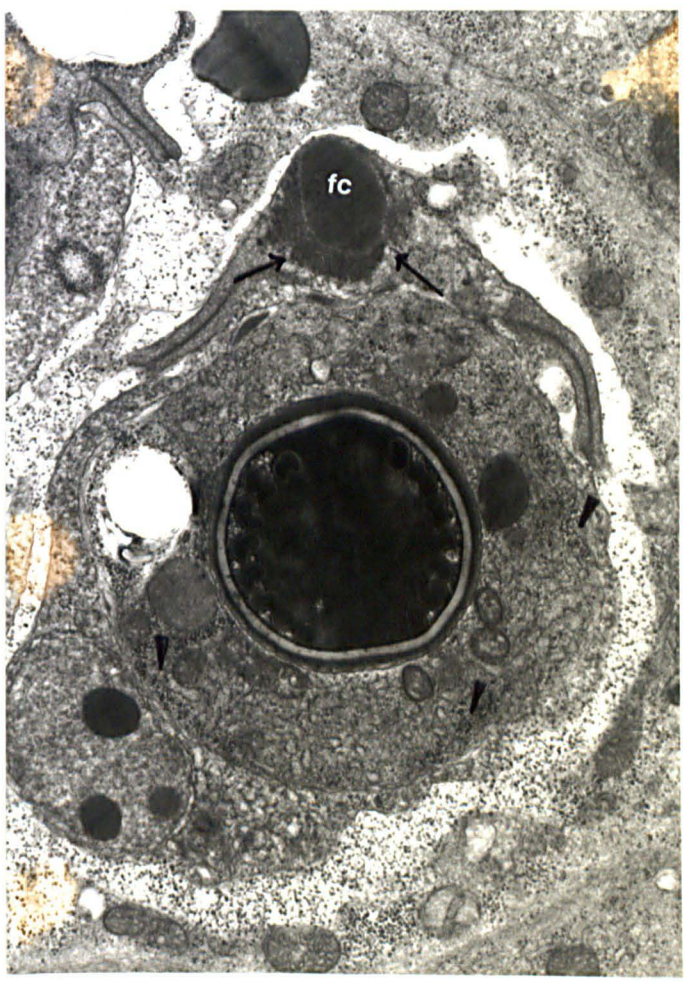

Fig 3.16 Development of the valvogenic cell. Note at the apex of the developing spore valvogenic cell cytoplasm forms a thickened electron dense area (arrows), filament discharge canal (fc). Arrow heads indicate scattered electron dense glycogen granules in the cytoplasm of capsulogenic cell. (x12,000).

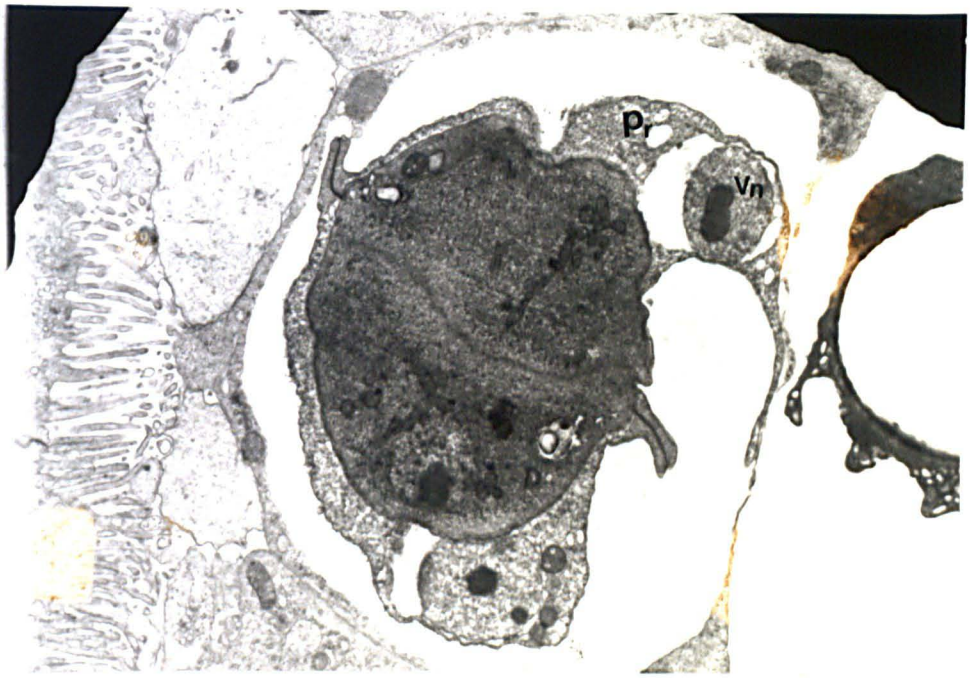

Fig 3.17 Section of immature spore (x9,440).
Note lateral protuberances (Pr) of valvogenic cell, Vn-valvogenic cell nucleus.

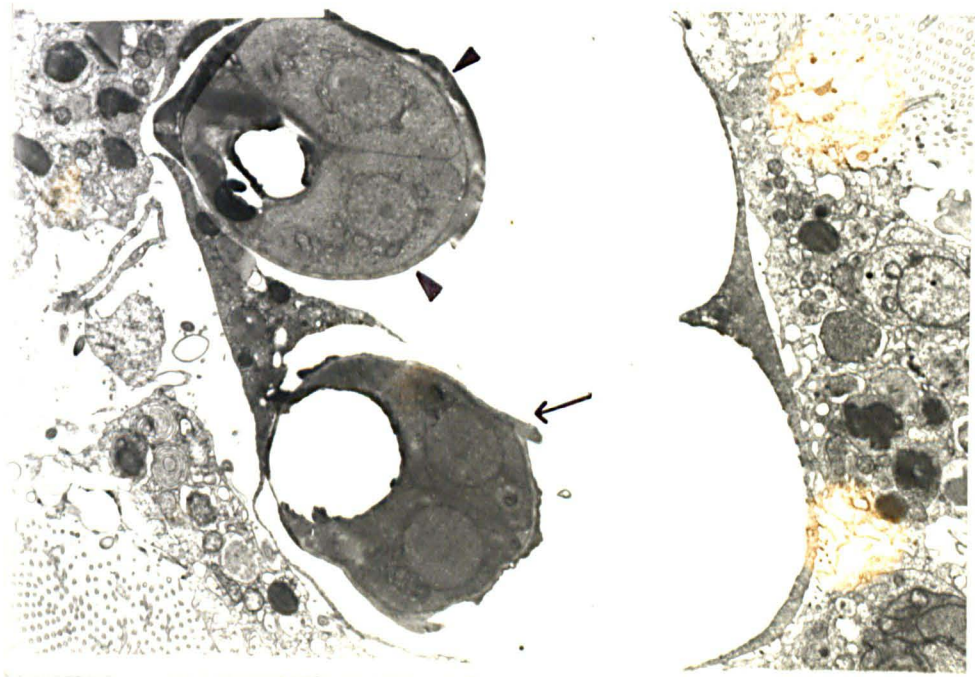

Fig 3.18 Section of immature and mature spore within the pseudoplasmodium of *S. elegans* (x6,160). Note protuberances (arrow) in immature spore and disappearance of the protuberances in mature spore (arrow heads). No sporoplasmosomes are seen in mature spores.

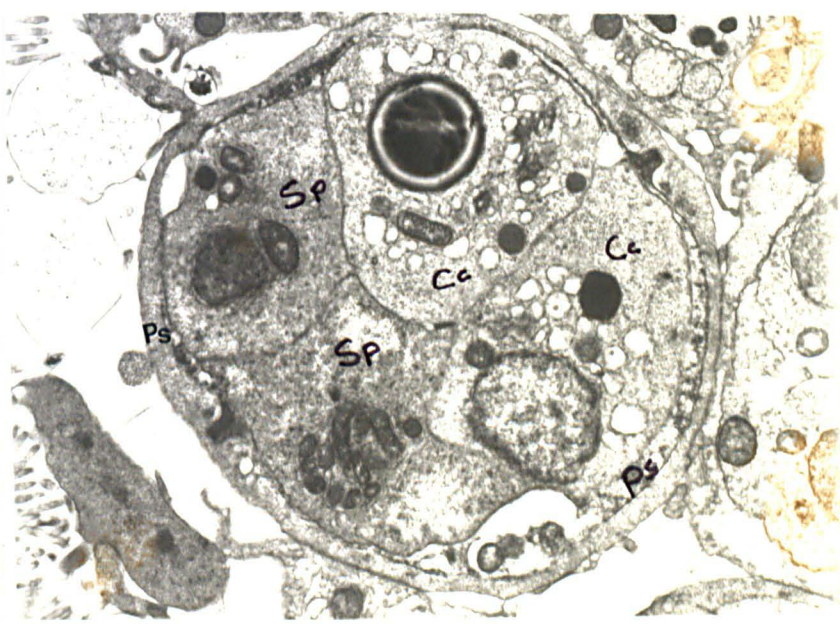

Fig 3.19 Section of a developing spore showing arrangement of capsulogenic (Cc), sporoplasmic (Sp) and valvogenic cells (Vc). Note pseudoplasmodia (Ps) thinly covering the spore (x8,250).

of the sporogonic cell also contained microtubules and mitochondria similar in structure to those of the pseudoplasmodia.

With the development of the spore, pseudoplasmodia lost most of their organelles and became more vacuolated and irregular in shape. The pseudoplasmodia eventually formed only a thin covering around the spores (Fig.3.19).

3.3.3 Scanning Electron Microscopy.

Scanning micrographs of immature pseudoplasmodia showed that they had an outer surface sculptured with many small depressions (Fig.3.20a). With the growth of the pseudoplasmodium, these depressions apparently increased in number but were smaller in size and became less prominent (Fig.3.20b). Depressions were not observed in pseudoplasmodia containing mature spores (Fig.3.20c).

In mature spores the two shell valves were equal in size and joined at the middle in a thick smooth sutural line. At the anterior tip of the spore the shell material was thickened to form the opening of the filament discharge canal (Fig.3.21a). The two openings were situated just at the anterior tip of the spore, one in each shell valve. The suture passed between the two openings. The surface of the spore was covered with a reticulate pattern of ridges, not uniformly distributed and less prominent on the posterior side of the spore. The ridges were more prominent in immature spores (Fig.3.21b).

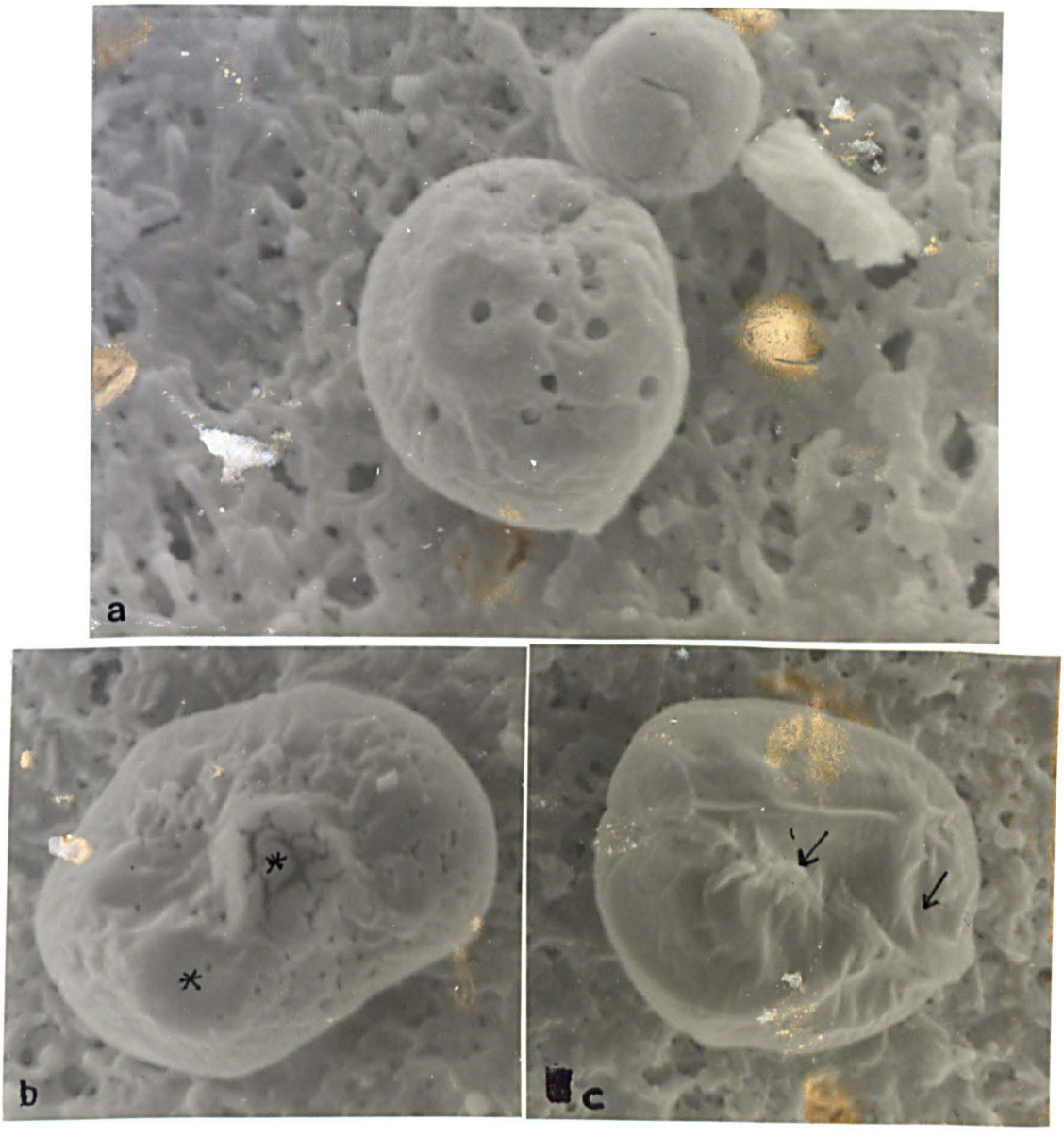

Fig 3.20 Scanning electron micrographs of pseudoplasmodia.

a. Early stage. Note external surface with surface depressions (x8,500).

b. Late stage. Note surface depressions less obvious, (*) indicating anterior protuberances of developing spore (x8,500).

c. Late stage. Note surface depressions cannot be seen. Arrows indicate ridges of valvular sculpturing (x6,500).

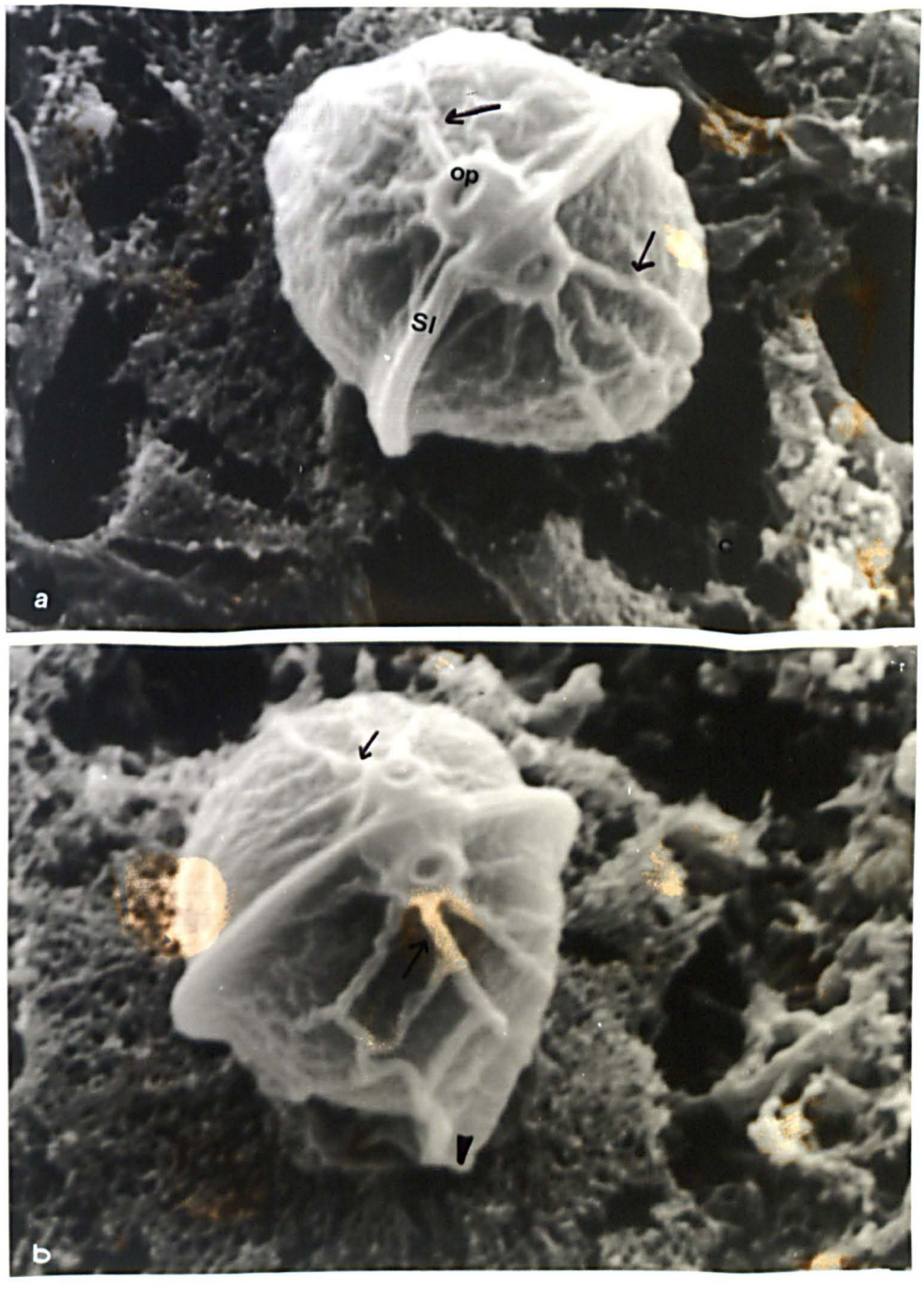

Fig 3.21 Scanning electron micrographs of spores (x16,500).
a. Mature spore. Note sutural line (Sl), surface sculpturing (arrows) and openings of polar capsules (Op).
b. Immature spore. Note prominent surface sculpture (arrows), protuberance (arrow heads).

3.3.4 Entrance of Parasites into Tubular Lumen

In fresh kidney smear preparations spores and developmental stages were frequently seen in the glomerular Bowman's space and the neck segment of the kidney tubules, apparently passing towards the lumen of the proximal convoluted tubule. This was also observed in some TEM sections (Fig.3.22). The parasite was not normally found in the epithelial layer of the kidney tubule, but in a few cases presporogonic stages were seen apparently migrating through the epithelial layer into the tubule lumen (Fig.3.23). Pre-sporogonic stages were also found in the interstitial haemopoietic tissue of the kidney (Fig.3.24).

3.3.5 Host Attachment and Pathogenicity.

Early pseudoplasmodia were seen freely floating in the tubular lumen (Fig.3.28a) whereas late pseudoplasmodia had hair-like surface projections which wedged between the brush border of kidney tubule epithelium (Fig.3.28b).

No pathological change was observed in light infections, but in heavy infections pathological changes were sometimes detected in the tubular epithelium and glomerulus of the kidney by light and electron microscopy.

Renal tubules with parasites in the lumen exhibited severe vacuolation of the epithelium and pyknotic nuclei (Fig.3.25). An increase in the number of of rodlet cells was also apparent (Fig.3.29).

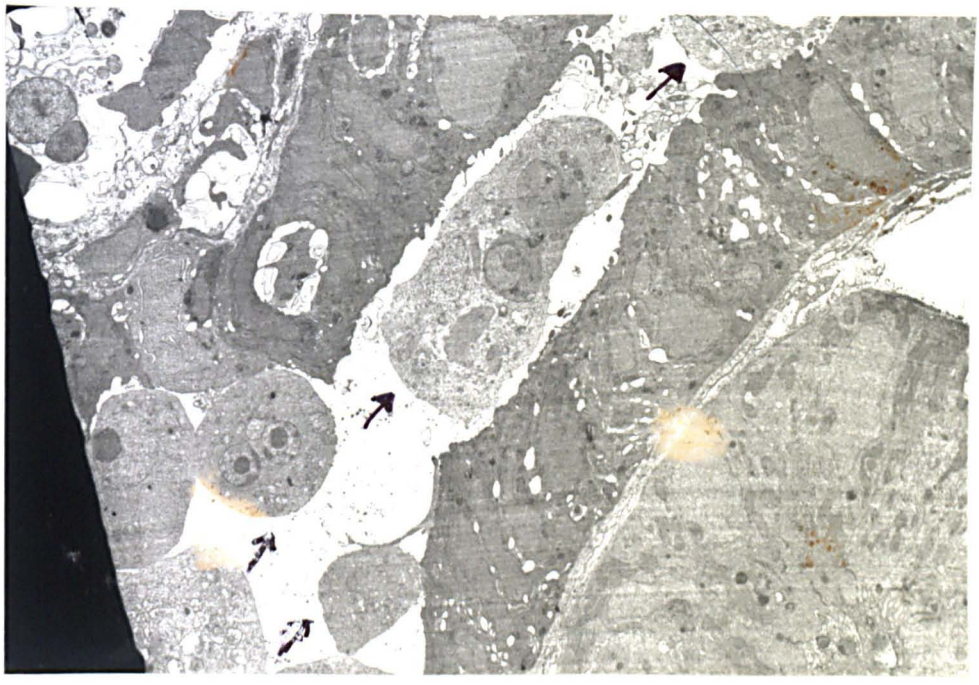

Fig 3.22 Pre-sporogonic stages in the Bowman's space of the glomerulus (arrows) and in the neck segment of the proximal tubule (arrow heads) (x3,300).

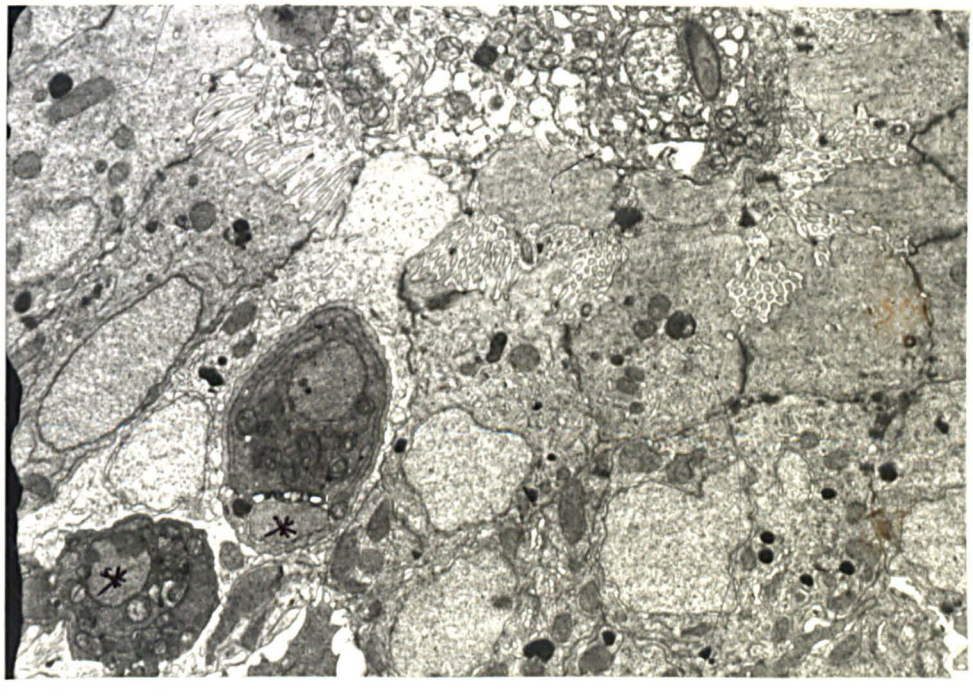

Fig 3.23 Intra-cellular pre-sporogonic stages (*) were frequently observed in the epithelial layer of kidney tubules suggesting a possible route of entry to the tubular lumen (x5,760).

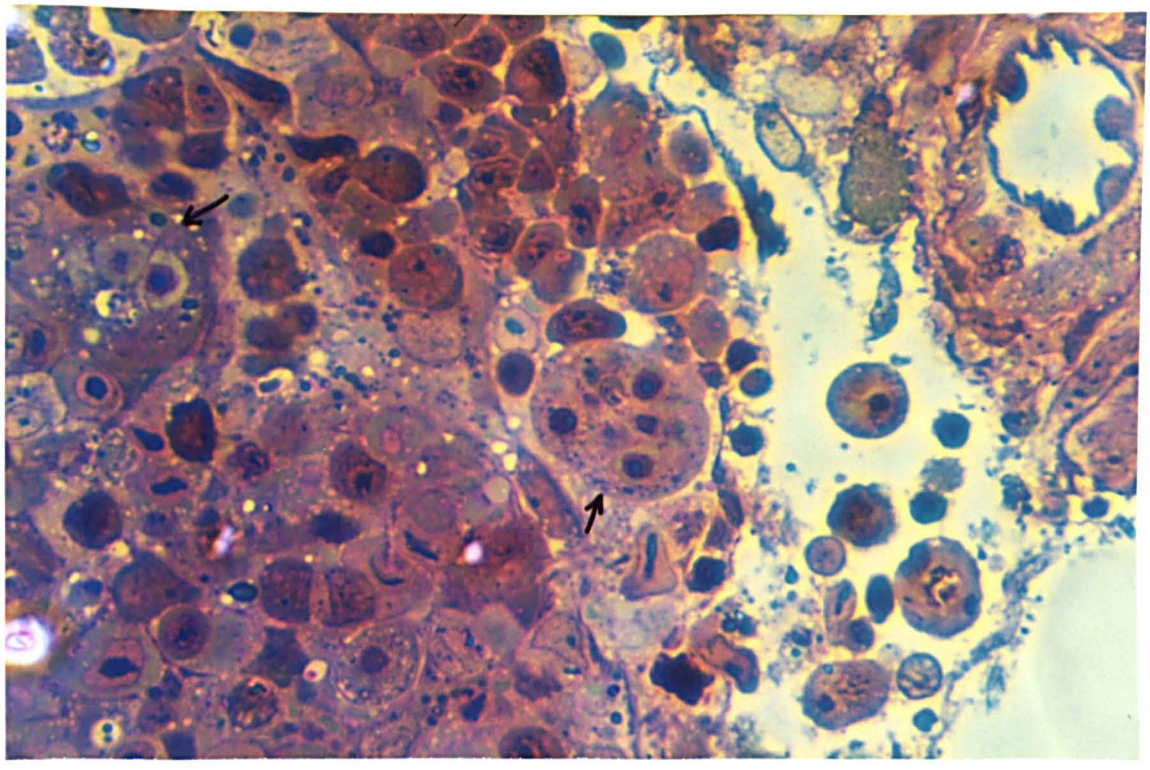

Fig 3.24 Semi-thin section of pre-sporogonic stages (arrows) in the interstitial space of the kidney (bar=10μm).

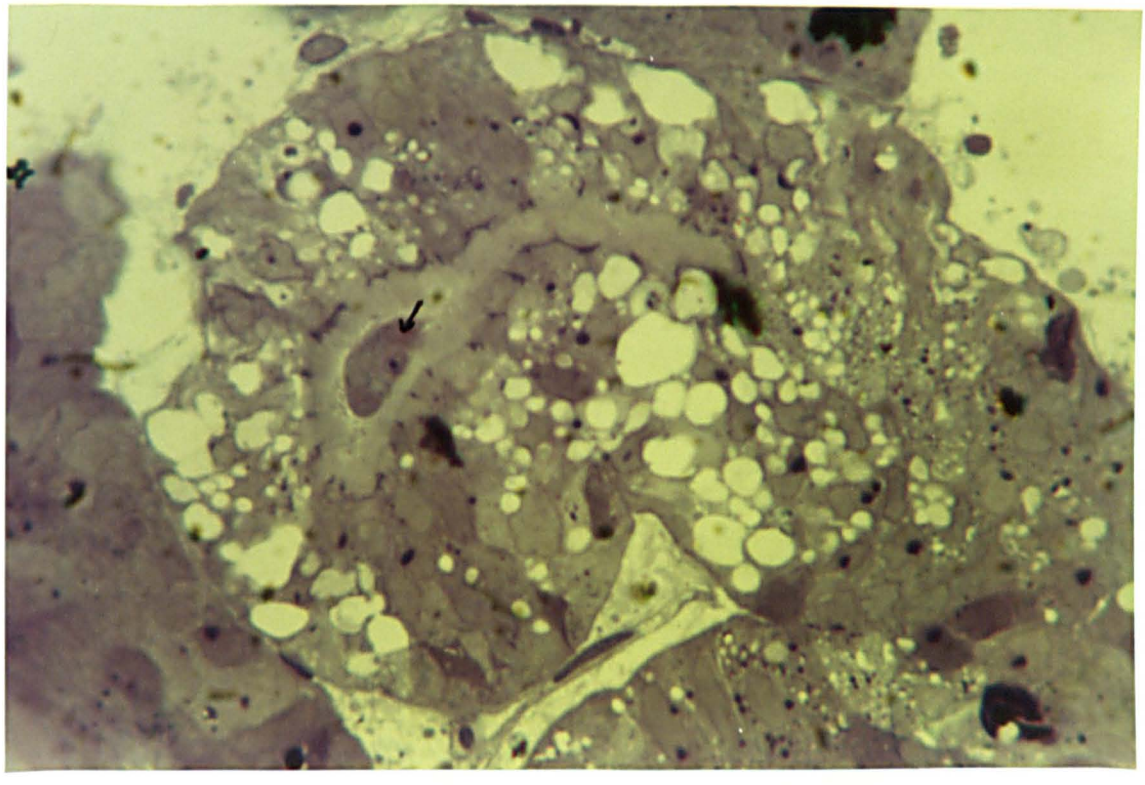

Fig 3.25 Semi-thin section of tubular epithelium showed extensive vacuolation associated with parasitic stages (arrow) in the lumen (bar=10μm).

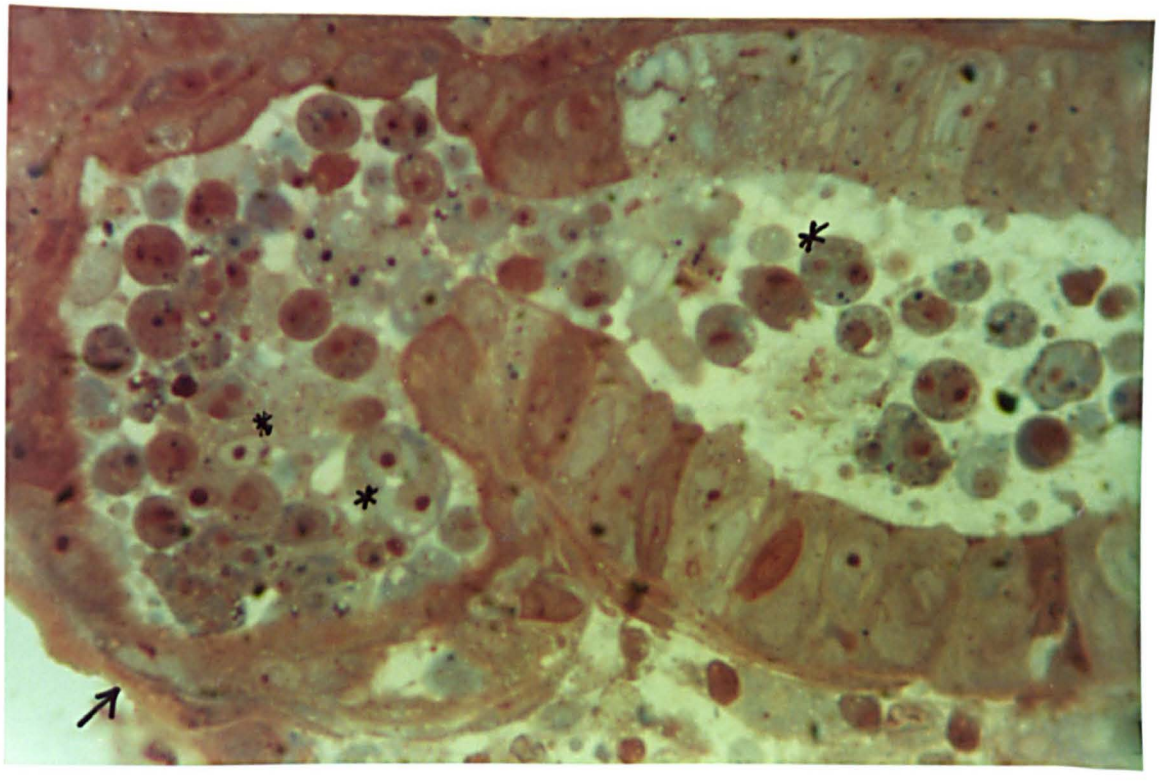

Fig 3.26 Developmental stages of *S. elegans* (*) caused complete destruction of glomerular tufts Wall of Bowman's capsule became thickened (arrow), parasites are also seen in the lumen of neck segment (semithin section, bar=10μm).

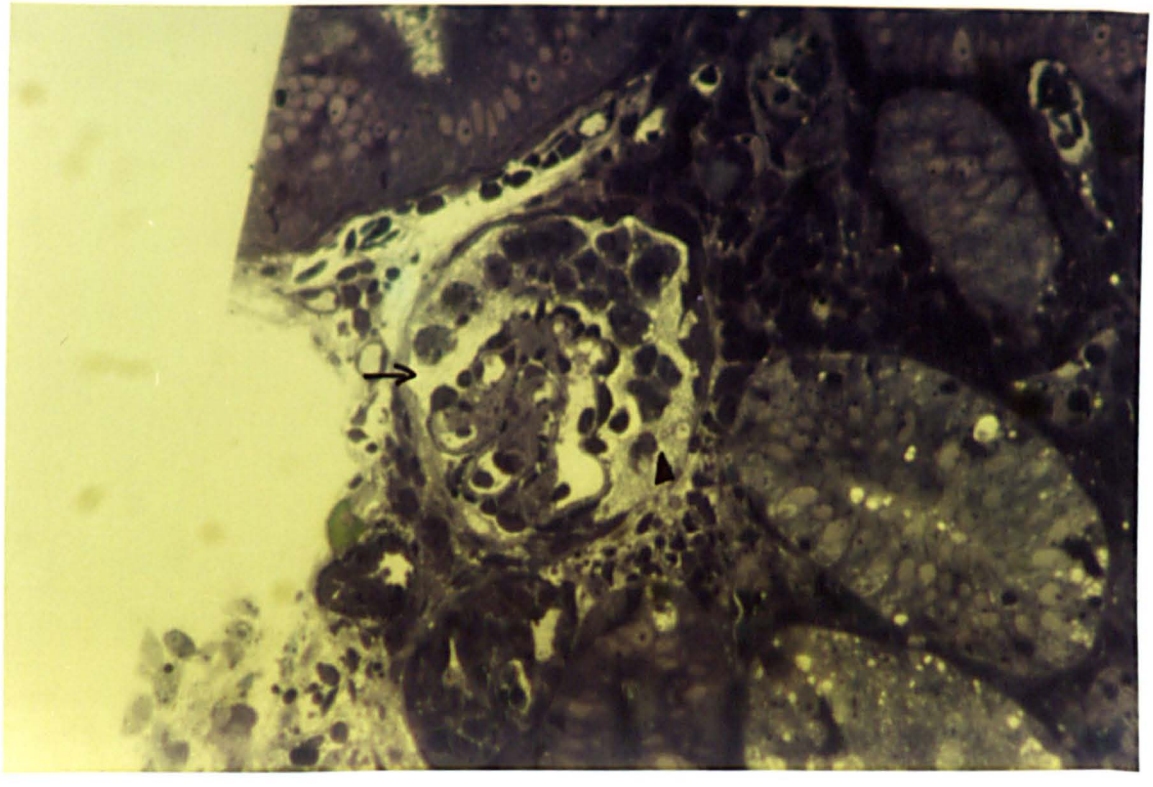

Fig 3.27 Semithin section of kidney showing pre-sporogonic stages (arrow) in the Bowman's space (arrow head)(bar=10μm).

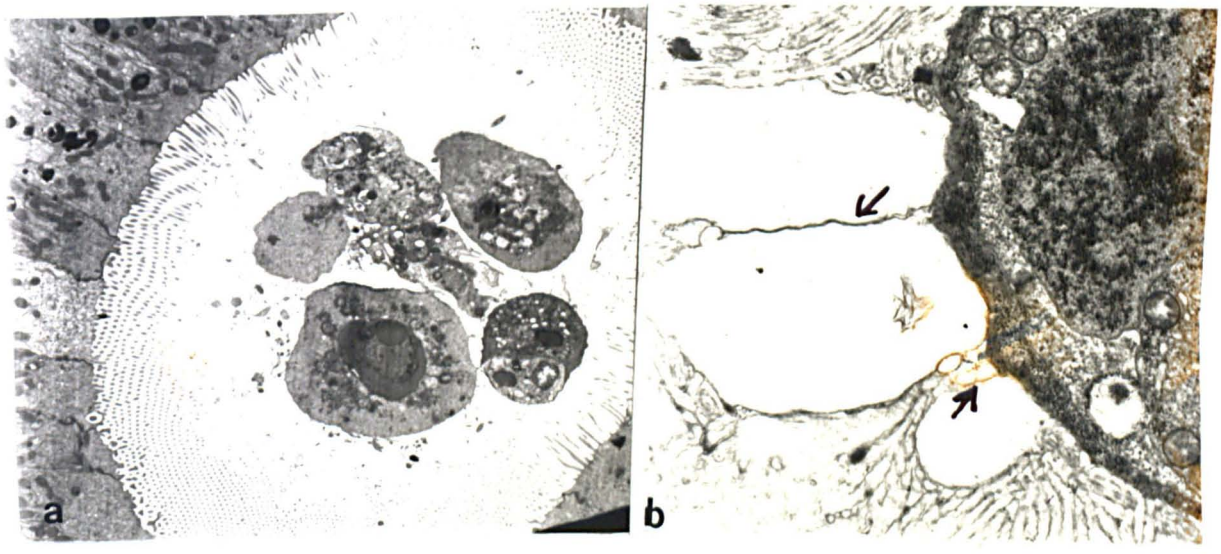

Fig 3.28 Attachment to host tubular epithelial cell.
a. Early pseudoplasmodium freely floating in the tubular lumen (x12,390).
b. Late pseudoplasmodium attached to the host cells by thin hair-like cytoplasmic projections (arrow) (x36,550).

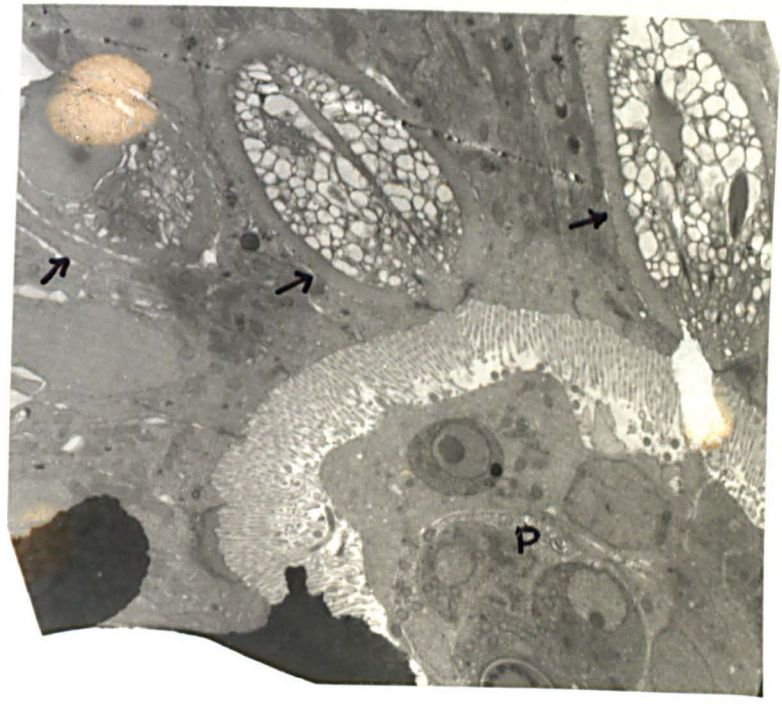

Fig 3.29 Increased number of rodlet cells (arrows) in the epithelium of kidney tubules. Parasitic stages (P) in the tubular lumen (x3,960).

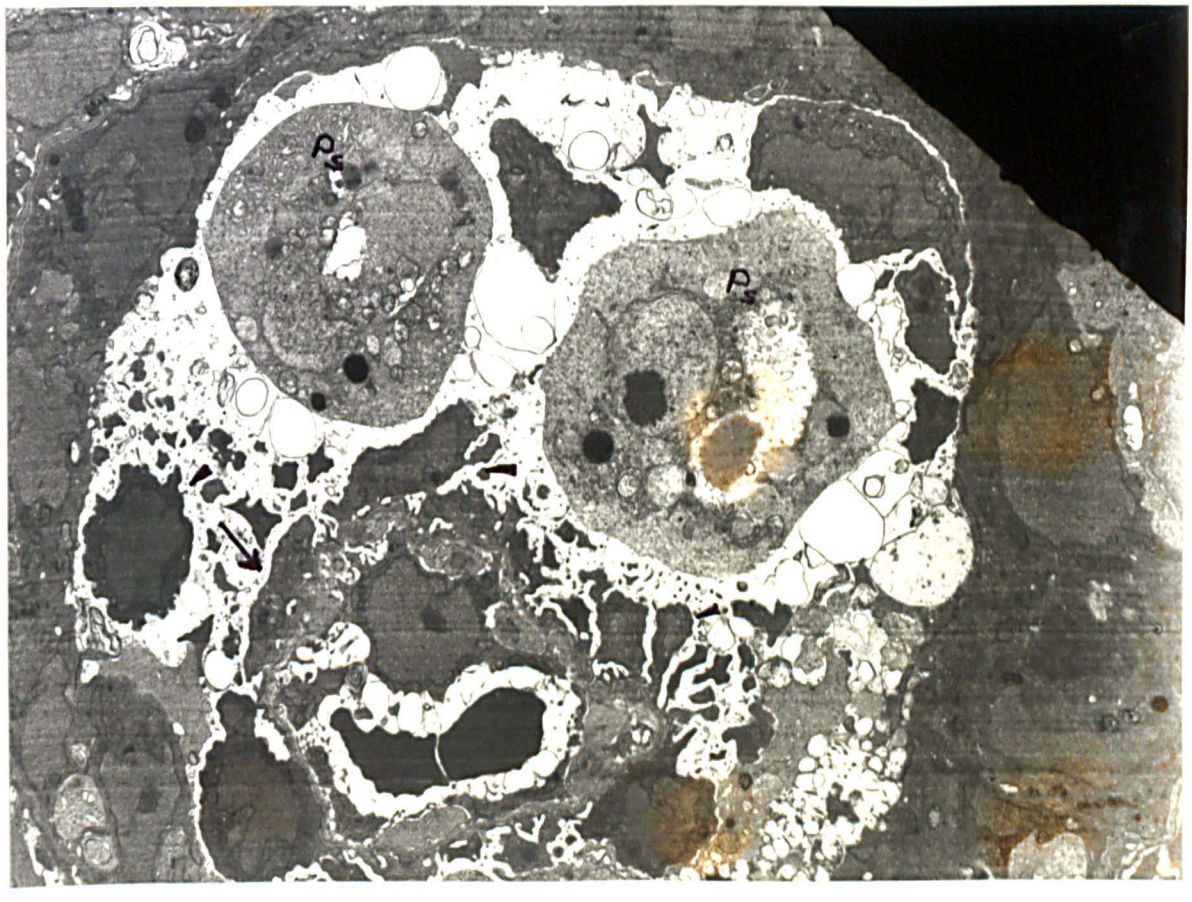

Fig 3.30 Early pseudoplasmodia (Ps) of *S. elegans* in the Bowman's space associated with shrinkage of glomerular tufts (arrow) and disorganisation of mesangial cells (arrow heads) (x5,600).

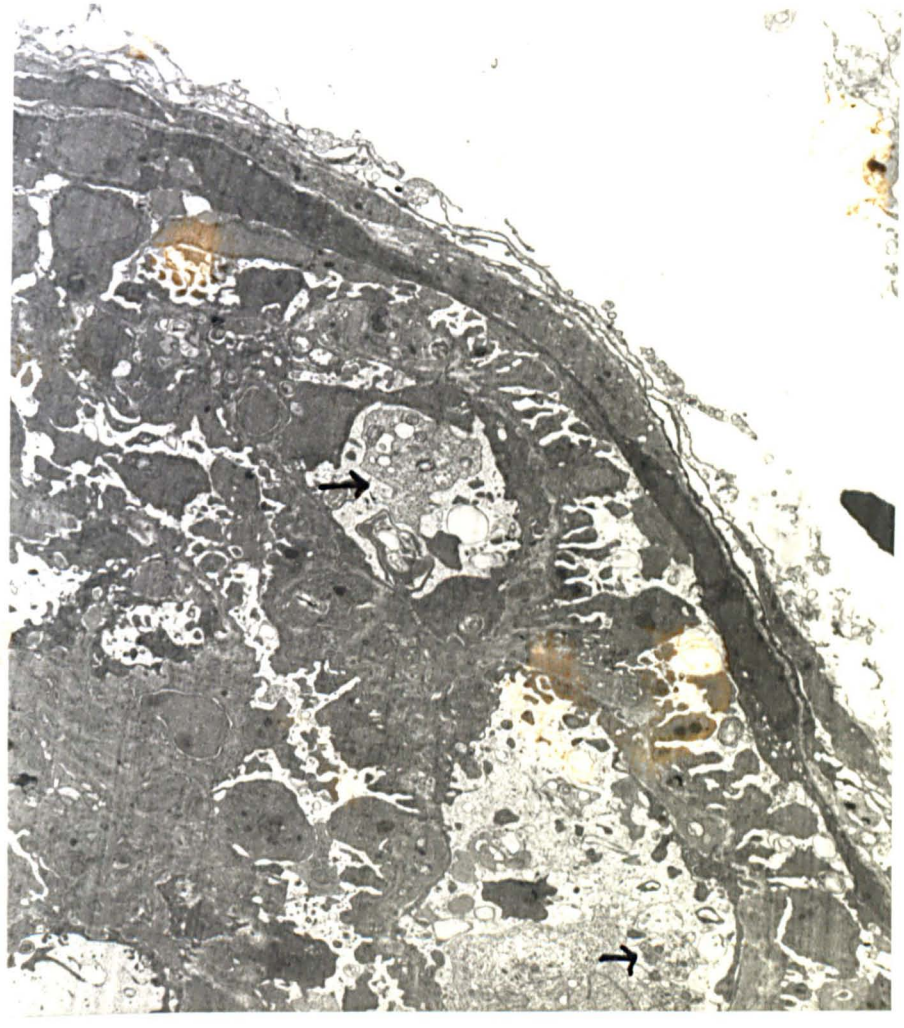

Fig 3.31 Early pre-sporogonic stages in the capillary lumen (arrow) of renal corpuscles suggesting extrasporogonic stages transfer to the kidney via blood before starting sporogenesis (x5,060).

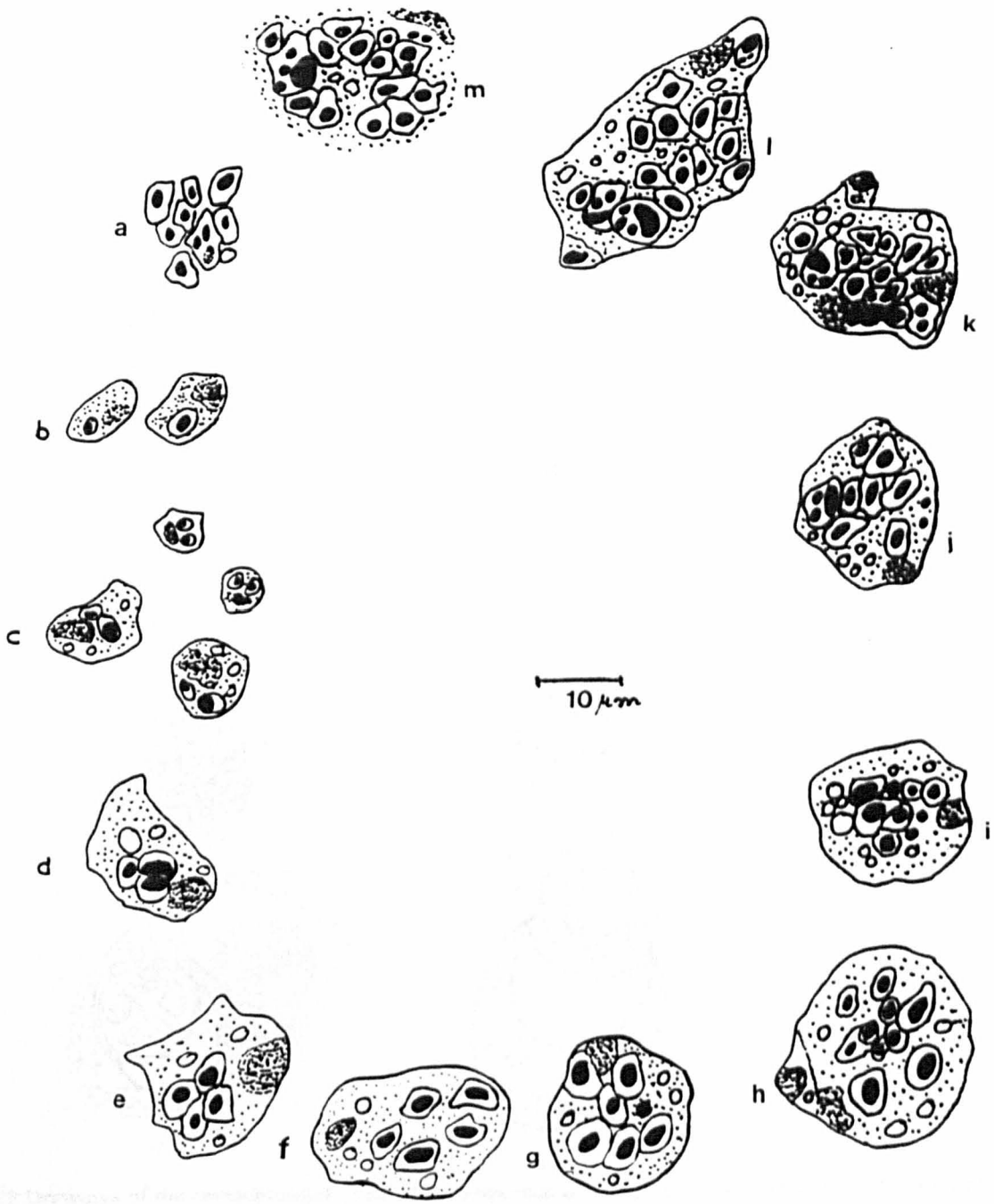

Fig 3.32 Drawings of extrasporogonic blood stages as studied from Giemsa stained dry blood smears by light microscopy (bar = 10μm).
a. Uninucleated primary cell.
b. Primary cells with single secondary cell.
c. Primary cells with 2 secondary cells.
d. Primary cell with 3 secondary cells were very rare.
e. Primary cell with 4 secondary cells.
f-m. Primary cells with 5 to 15 secondary cells. Note residual bodies in 6 secondary cell stage (g). Plasmotomy can be seen in larger primary cells with many secondary cells (h,k,l).

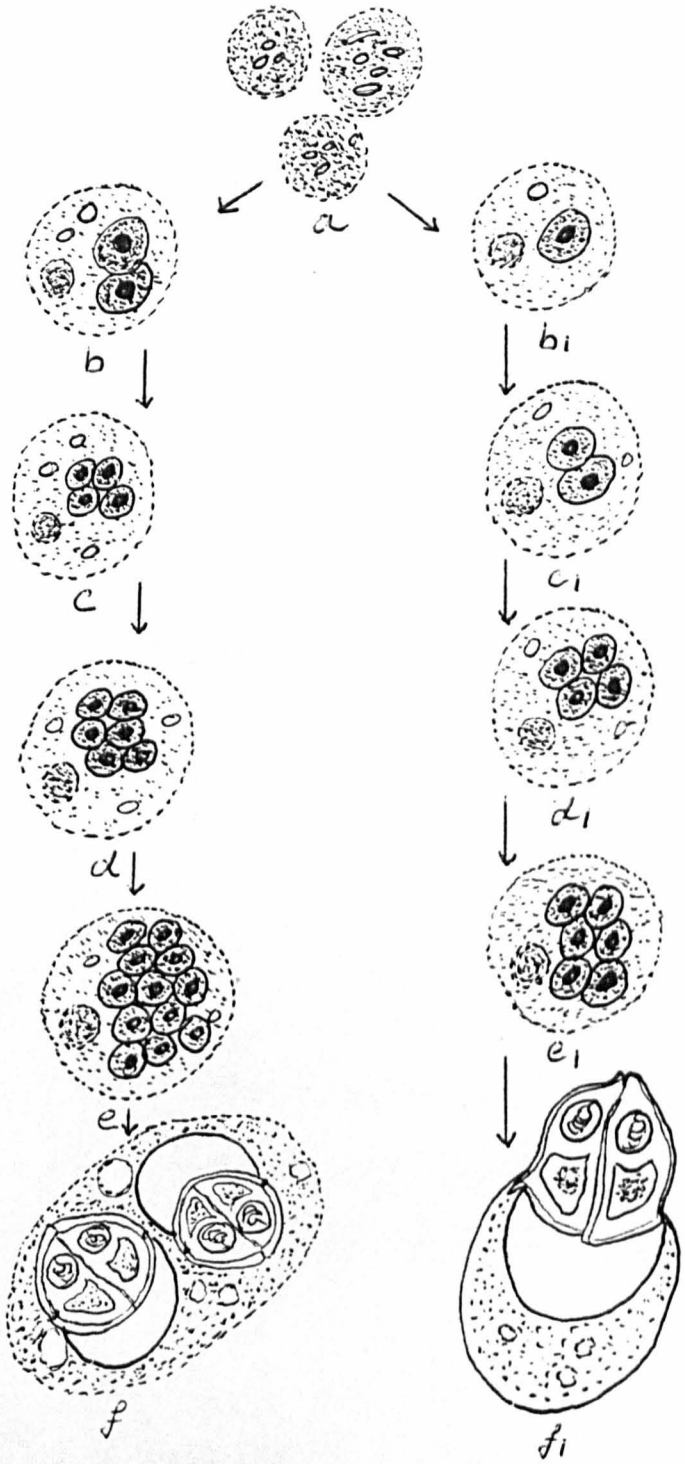

Fig 3.33 Drawings of the developmental cycle of monosporous and disporous plasmodia of *S. elegans* within the kidney lumen as elucidated by light and electron microscopy.

a. Earliest stage with dense cytoplasm and few mitochondria.

b. Two sporogonic cells within a disporous plasmodium.

c. Formation of 4 sporoblast cells from 2 sporogonic cells.

d&e. Further endogenous divisions resulting up to 12 sporoblast cells.

f. Differentiation and arrangement of sporoblast cells resulting in two mature spores in disporous plasmodia.

b_1. Single sporogonic cell within a monosporous plasmodium.

c_1. Formation of 2 sporoblast cells from a single sporogonic cell.

d_1&e_1. Endogenous division resulting in up to 6 sporoblast cells.

f_1. After differentiation and arrangement of sporoblast cells a single spore forms in a monosporous plasmodium.

In the glomerulus, parasites were mainly observed in the Bowman's spaces (Fig.3.26 & 3.27). The infected renal corpuscle was characterised by hypertrophy associated with atrophy of the surrounding renal parenchyma. The mesangial matrix was vacuolated and the glomerular tufts shrunken. Some infected glomeruli showed disorganisation of mesangial cells, due to mesangial proliferation, with different degrees of destruction of renal corpuscles (Fig.3.30). The heavily parasitised glomeruli were not only displaced but also underwent atrophy (Fig.3.26) and were replaced by masses of developing parasites. Parasites were occasionally found inside the capillary lumen of the glomerular tuft (Fig.3.31). In these cases destruction and degeneration of endothelial cells was noticed. The capillary lumen became dilated.

3.4 Discussion.

The basic developmental sequence and structure of *S.elegans* from *G. aculeatus* is very similar to that of other *Sphaerospora* species described (Lom *et al.*, 1982, 1985a,b; Molnár, 1988a; Hedrick *et al.*, 1990; Supamattaya *et al.*, 1991, 1993), but there are several novel features.

3.4.1 Site of Parasite Development.

Sphaerospora infections have been mostly described from the renal system of fish, but recently sphaerospores have been reported from other organs including swimbladder, gill, liver, gonads, gall bladder, intestine, stomach and spleen (Molnár, 1979a; Körting, 1982; Csaba *et al.*, 1984; Lom *et al.*, 1983b; Sitja-Bobadilla and Alvarez-Pellitero, 1992;

Supamattaya *et al.*, 1991, 1993). Hermanns and Körting (1985) found large masses of *S. tincae* in the head kidney of tench, although the parasite was absent from the excretory part of the kidney. Recently *S.dicentrarchi* was reported (Sitja-Bobadilla and Alvarez-Pellitero, 1992) to occur in various organs of the fish, including the head kidney. *Sphaerospora elegans* was found only in the excretory part of the kidney within the Bowman's space and lumen of the kidney tubules of the stickleback. It must be noted that the head kidney of the stickleback has no excretory function and thus contains no tubules (Wootton, 1976).

Feist *et al.* (1991) also reported that the pre-sporogonic and sporogonic stages of *S. elegans* in stickleback were confined to the excretory part of the kidney with occasional occurrence of mature spores in the ureters and urinary bladders as found in the present study. Sporogony of *S. elegans* is exclusively coelozoic in the kidney of the three-spined stickleback.

3.4.2 Extrasporogonic Stage.

Myxosporean extrasporogonic stages have been defined as stages which proliferate without forming spores, before or at same time as the sporogonic phase takes place, and at a site other than where sporogony occurs (Lom, 1987; Lom and Dyková, 1986). The extrasporogonic stages of *S. elegans* were found in the circulating blood, capillary lumen of the choroidal rete mirabile of the eye and interstitial spaces of the kidney of three spined stickleback. Hedrick *et al.* (1988) found extrasporogonic stages of *S. elegans* mainly in the kidney interstitium of three-spined stickleback. Lom *et al.* (1991) described extrasporogonic stages of *S. elegans* in the choroidal rete and occasionally in the swimbladder rete and renal interstitial tissue of *G. aculeatus*. However, these authors did not find stages in the

circulating blood. Feist *et al.* (1991) indicated that extrasporogonic blood stages may be absent in *S. elegans* or occurred for only a very short period of time which did not coincide with their sample collections. In the present study choroidal rete stages were rarely observed and only over a limited period. Renal interstitial extrasporogonic stages were also very rare. Extrasporogonic stages were not found in the swimbladder rete in the present study. Previous reports on sphaerospore extrasporogonic stages show that they are most common in the circulating blood, swimbladder, kidney interstitium and liver (Csaba *et al.*, 1984; Lom *et al.*, 1983a; Supamattaya *et al.*, 1992), although El-Matbouli and Hoffmann (1992) described extrasporogonic stages in the lumen of the ureter of rudd, *Scardinius erythrophthalmus*, infected with *S. scardinii*. The present study suggests that the main site of extrasporogonic proliferation of *S. elegans* is the circulatory blood because most of the extrasporogonic developing stages were found there. Some of the stages presumably become trapped in the rete mirabile of the eye and in the kidney. The rapid disappearance of these stages from the blood might be related to the host's physiological conditions, an environmental stimulus or be genetically determined. In histozoic myxosporean species the extrasporogonic stages (if any) are not known, but a number of species show evidence of a proliferative cycle with plasmodia enclosing a number of secondary cells (e.g. *Myxidium* sp., Lom *et al.*, 1989a; *Myxobolus* spp. in the brain of cyprinid fish, (Ferguson *et al.*, 1985, Lom *et al.*, 1989b).

Light microscopic examination showed that the general morphology and developmental sequence of the blood forms was similar to that recorded by other authors for *Sphaerospora* spp. (Csaba, 1976; Lom *et al.*, 1983a; Baska and Molnár, 1988; Hedrick *et al.*, 1990; Supamattaya *et al.*, 1991; Lom *et al.*, 1991; McGeorge *et al.*, 1994).

The earliest extrasporogonic stage seen was a primary cell without any secondary cells (Fig.3.6a). Lom *et al.* (1983a) observed single cells without any secondary cells only in the blood of carp infected with *S. renicola* and suggested that they were secondary cells released from disintegrated primary cells. In the present study these stages were frequently observed in Giemsa stained blood smears. Primary cells were observed with up to 15 secondary cells (Fig.3.7). On release from the primary cell these cells may act as new primary cells and produce further secondary cells as suggested by Lom *et al.* (1983a). If so, extrasporogonic stages are a proliferative stage in the lifecycle of *S. elegans*. It is not certain whether the single extrasporogonic cells seen in this study were primary cells resulting from a new infection or were newly released secondary cells.

Primary cells with single secondary cells were very rarely found, whereas primary cells with two secondary cells were very common (Fig.3.6b,c). These may actually represent released secondary cells containing two tertiary cells. Thus after release from a mature primary cell a secondary cell with two tertiary cells acts as a primary cell with two secondary cells. Two secondary cells in a single vacuole or two spindle shaped secondary cells indicated recent division of a single secondary cell (Lom *et al.*, 1985b). A primary cell with two secondary cells was the earliest stage found in swimbladder infections of *S. renicola* (Dyková *et al.*, 1990). Primary cells with three secondary cells were very rare in *S. elegans*. Similarly, Lom *et al.* (1983a) found them to be extremely rare in carp blood and suggested that this was because of the asynchronous development of two secondary cells which meant that 4 secondary cells were more common, as also occurred in *S. elegans*. Some *Sphaerospora* species are known to have more than one nucleus in the extrasporogonic primary cell. Baska and Molnár (1988) described 2 to 4 nuclei in parasites from bleak and Lom *et al.* (1985b)

found 2 nuclei in some primary cells of *S. gobionis*. PKX has also been reported as having a multinucleated primary cell (Kent and Hedrick, 1986). However, extrasporogonic stages of *S. elegans* had only a single nucleus in the primary cell.

Secondary cells of *S. elegans* blood stages usually contained two tertiary cells (Fig.3.6k, 3.7j,k) and rarely three (Fig.3.7). Lom *et al.* (1991) found one or two tertiary cells in choroidal rete stages of *S. elegans*. The number of tertiary cells seems to vary in different *Sphaerospora* species, e.g the secondary cell of *S. renicola* C-stages has one tertiary cell (Csaba, 1976; Lom *et al.*, 1983a). Single tertiary cells have also been reported from sphaerospore extrasporogonic stages of blue bream, white bream and asp (Baska and Molnár, 1988) and gudgeon, roach and tench (Lom *et al.*, 1985b).

As in K-stages of *S. renicola* (Csaba *et al.*, 1984; Dyková *et al.*, 1990) the final product of the *S. elegans* extrasporogonic stages is a secondary cell containing two tertiary cells. Baska and Molnár (1988) and McGeorge *et al.* (1994) termed these as "triple forms" described from the blood of a range of cyprinid fish and Atlantic salmon. McGeorge *et al.* (1994) suggested that salmon "triple formations" when released from primary cells were identical to the early pseudoplasmodium with two sporogonic cells found in the lumen of the kidney tubule.

Residual Body.

A residual body appeared in primary cells with four to six secondary cells (Fig.3.6j&3.7e) and was also found in subsequent stages. Csaba (1976) observed the residual body at the 6,7 and 8 secondary cell stages in *S. renicola*. Residual bodies in extrasporogonic stages have

also been reported (Lom *et al.*, 1983a, 1985b; Baska and Molnár, 1988) from other *Sphaerospora* species. In the present study it was frequently observed that within the same primary cell some secondary cells did not grow while others increased in size and became clearly identifiable with their surrounding cytoplasm (Fig.3.7c,d). These small and non-developing secondary cells possibly later transform into residual bodies as suggested by Lom *et al.* (1985b). These authors also suggested that residual bodies are the division products of the secondary cells and released within the secondary cell they constitute a tertiary cell, but if released outside the secondary cell they appear as the residual body.

Size of Blood Stages.

The size of the blood stages in *S. elegans* is comparatively large (7μm to 35.28μm x 25.48μm). Hedrick *et al.* (1988) reported that blood stages of *S. elegans* in the kidney of stickleback ranged in size from 6.3μm to 12.5μm with up to nine secondary cells. Baska and Molnár (1988) in a study of *Sphaerospora* blood stages in cyprinid fish reported primary cells of maximum size 20μm to 60μm and primary cells with 16 to 40 secondary cells in white bream, *Blicca bjoerkna*, and asp, *Aspius aspius*. However, to date the largest *Sphaerospora* blood stage described is that from Atlantic salmon which are up to 70μm in size and contain up to 120 secondary cells (McGeorge *et al.*, 1994).

Macrophages with Blood Stages.

Primary cells of *S. elegans* were frequently observed with macrophages adjacent or attached to them, as reported by other authors (Lom *et al.*, 1985b; Hedrick *et al.*, 1988; Supamattaya *et al.*, 1991). Phagocytosed blood cells were also seen in the primary cell cytoplasm (Fig.3.6a) of *S. elegans*. This has been recorded in *Sphaerospora* sp. from tench, where the

presence of the blood cell gives the appearance of a binucleated primary cell (Lom *et al.*, 1985b). Blood cells in the cytoplasm of primary cells are also reported in asp infected with *Sphaerospora* sp. (Baska and Molnár, 1988) Phagocytic activity of myxosporeans seems not uncommon as Uspenskaya (1981, in Lom *et al.*, 1985b) reported ingestion of chondrocytes by trophozoites of *Myxosoma cerebralis*.

Lom *et al.* (1991) described the ultrastructure of extrasporogonic stages of *S. elegans* in the choroidal rete mirabile and also reported their occasional presence in the swimbladder rete mirabile of stickleback. These cells were 8μm to 22μm in diameter and contained up to 18 secondary cells, although more usually only 1-5 were found. Tertiary cells were found in secondary cells and primary cells were extensively vacuolated. Residual bodies were not described by these authors. Lom *et al.* (1991) suggested that these stages represent part of the life cycle of *S. elegans*. Their similarity with the blood stages described in the present study supports this hypothesis.

In general extrasporogonic blood stages of *S. elegans* showed different morphologies. The morphological diversity of *Sphaerospora* stages in the blood of common carp has also been mentioned by Csaba (1976), Lom *et al.* (1985b) and Baska and Molnár (1988). Although the general structure of the extrasporogonic stages described in this study agrees with that of other *Sphaerospora* species they showed some distinctive features including, (i) a highly vacuolated primary cell, (ii) engulfment of host blood cells and (iii) presence of small uninucleated extrasporogonic cells attached to the primary cell, which are possibly the products of plasmotomy or budding of the primary cell. Mitchell (1977) stated that coelozoic myxosporean trophozoites may divide by plasmotomy or budding. However, this process has

not previously been reported in extrasporogonic stages of *Sphaerospora* species, although Kent and Hedrick (1986) assumed PKX might multiply by endogenous binary fission or plasmotomy (external budding) in the blood and kidney interstitium of infected hosts. A number of authors have suggested that all *Sphaerospora* spp. have distinctively different extrasporogonic stages, which are host specific, and the morphology of which might become a valuable criterion for taxonomic identification (Lom *et al.*, 1985b; Baska and Molnár, 1988; Keper and Trsova, 1989). Ultrastructural studies of *Sphaerospora elegans* extrasporogonic blood stages are needed to better determine differences between them, but this will not be easy because of their short duration and low intensity in the blood.

3.4.3 Presporogonic Stage.

The light microscopic structure of pre-sporogonic and sporogonic stages of *S. elegans* was similar to that described by Feist *et al.* (1991) and of other studies of *Sphaerospora* spp. including PKX (Clifton-Hadley and Feist, 1989; Dyková and Lom, 1982; El-Matbouli and Hoffmann, 1992; Feist *et al.*, 1991; Lom *et al.*, 1985b; Supamattaya *et al.*, 1991).

Much less is known about the pre-sporogonic stages of myxosporeans compared with their sporogonic stages. The origin of the sporogonic cells within the pseudoplasmodia is not clear. Lom *et al.*(1982) assumed that in *S.renicola* production of an interior daughter cell (i.e sporogonic cell) within the mother cell (i.e pseudoplasmodium) occurred by internal cleavage. It is believed that in polysporous plasmodia generative cells arise from vegetative nuclei which, together with some cytoplasm, become surrounded by membranes to form generative cells, although the actual process of cell membrane formation has not been

followed (Current and Janovy, 1977; Lom de Puytorac, 1965; Mitchell, 1977). Early pseudoplasmodia of *S. elegans* have dense cytoplasm, few mitochondria and one or two electron dense areas (Fig.3.9). Sporogonic cells do not arise by the endogenous division of the pseudoplasmodial nucleus, rather the latter, together with some cytoplasm and associated organelles, become surrounded by endoplasmic reticulum which forms a cell membrane. The sporogonic cell thus formed then undergoes further division resulting in 6-12 sporoblast cells. Formation of sporoblast cells from sporogonic cells is by endogenous cleavage. Molnár (1988a) described multiple internal cleavage before sporogony of *S. renicola* in the swimbladder and assumed this process might occur in other myxosporea.

Most previous studies support the idea of endogenous division of sporogonic cells in *Sphaerospora* as suggested here. Some species of *Sphaerospora* are reported to have tertiary cells within sporogonic cells (Lom *et al.*, 1982, 1985b), but no tertiary cells were observed in the sporogonic cells of *S. elegans*.

3.4.4 Sporogonic Stage.

The differentiation of sporoblast cells to capsulogenic, sporoplasmic and valvogenic cells occurs very quickly, probably just after their formation. Capsulogenic, sporoplasmic and valvogenic cells can be easily identified before they assume their final arrangement in the developing spore (Fig.3.10). Development of capsulogenic cells and the formation of spore capsules of *S. elegans* is similar to the general process described for myxosporeans (Lom and de Puytorac, 1965). However, there are some unusual features in sporogenesis of *S. elegans*. The external tube and bulb like structure of the capsular primordium seem to appear

simultaneously in the early capsulogenic cell (Fig.3.10). This is not recorded for most myxosporeans. Desser *et al.* (1983b) reported the appearance of both parts of the capsular primordium at the same time in *Thelohanellus nikolsky*. The presence of glycogen granules in the cytoplasm of sporoblast cells is a common feature among myxosporeans (Lom *et al.*, 1982). However, their concentration and quantity might be variable in different species (Current and Janovy, 1978, 1979; Desser *et al.*, 1983; El-Matbouli *et al.*, 1990; Sitja-Bobadilla and Alvarez-Pellitero, 1992). Lipid inclusions were observed in the early stage of polar capsule development in *T. nikolsky* and these were not uncommon in *S. elegans* (Fig.3.12). Dense lipid inclusions have also been reported for other *Sphaerospora* species (Lom *et al.*, 1985b; Hedrick *et al.*, 1990). The presence of 4 coils of the external tube of *S.elegans* indicates that it is quite long, whereas in *S.gallinae* the external tube is very short, making only 2 coils in the cytoplasm of the capsulogenic cell (Lom *et al.*, 1985a).

Two uninucleate sporoplasm cells are a typical characteristic of the genus *Sphaerospora* (Lom and Noble, 1985; Lom *et al.*, 1985b), although recently a binucleated single sporoplasm cell has been observed in *S. dicentrarchi* (Sitja-Bobadilla and Alvarez-Pellitero, 1992). Sporoplasm cells of *S. elegans* are typicaly uninucleated but the absence of sporoplasmosomes is a distinguishing feature from other *Sphaerospora* species.

A characteristic feature of the valvogenic cell in *S. elegans* is the formation of an electron dense anterior thickening around the filament discharge canal, which is derived from the cytoplasm of the valvogenic cell (Fig.3.13). The protuberances formed at the posterior side of the immature spore by the valvogenic cells are also reported by Lom *et al.* (1983b) for *S. renicola* but have not been reported by other authors. Their function remains unclear.

3.4.5 Pseudoplasmodia.

Most of the pseudoplasmodia of the Sphaerosporidae are disporous (Desser *et al.*, 1983a; Lom *et al.*, 1985a), although some *Sphaerospora* species are reported as having only monosporous plasmodia (Lom *et al.*, 1983b, 1985b; Hedrick *et al.*, 1990). Feist *et al.*(1991) reported only disporous plasmodia in *S. elegans*. In the present study, although disporous pseudoplasmodia were usual, monosporous plasmodia were observed with both light and electron microscopy.

Early pseudoplasmodia of *S. elegans* were found freely floating in the lumen of the kidney tubule and subsequently they become attached to the microvillous border of the epithelial cells. Hairlike extensions from the pseudoplasmodia were wedged among the microvilli and were in contact with the epithelial cells at the junction between them (Fig.3.26). Desser *et al.* (1986) observed similar very thin elongated cytoplasmic processes of *S.ohlmacheri* pseudoplasmodia attached to the inner wall of kidney epithelial cells in bull frog tadpoles. *Sphaerospora* pseudoplasmodia are usually in close contact with the microvilli of epithelial cells and have cytoplasmic projections wedged deeply among the microvilli (*S. renicola, S. gallinae*, Lom *et al.*, 1982, 1985a), but these were not seen in the present study. In *S. gobinis* pseudoplasmodia may adhere to the microvilli or may float in the lumen of the tubules (Lom *et al.*, 1985b). *S. renicola* has electron dense cell junctions at the point of contact between pseudoplasmodial projections and epithelial cells (Lom, 1982) which were not found in *S. elegans*.

3.4.6 Spore structure

The spore dimensions of *S. elegans* from this study are similar to previously published data for this species (Table 3.1). The horn like protruberances in the immature spore of *S. elegans*, which became less prominent with the maturation of the spore, were also reported by Feist *et al.*, (1991). Ultrastructural observations revealed that the posterior projection of the immature spore contained the nucleus of valvogenic cells at its base (Fig.3.14) as suggested by Feist *et al.* (1991).

According to Feist *et al.* (1991) spores of *S. elegans* from *Pungitius pungitius* have ridges adjacent to the sutural ridge under light microscopy. They suggested that these ridges were the edge of numerous shallow depressions or pits, which were prominent at the posterior of the spore. Ultrastructurally they appeared to arise from valvogenic cell cytoplasm. However, in this study no posterior depressions of the spores were observed and this may represent a variation in spore morphology influenced by the host fish species.

SEM study of the spore revealed surface sculpturing which was most prominent at the anterior near the sutural ridge (Fig.3.16,17), as recorded by Feist *et al.* (1991). Listebarger and Mitchell (1980) suggested that in *Chloromyxum catostomie*, valvular ridges and pits of the spore coat may enhance the flotation of the spore in the water, thus aiding its dispersion.

3.4.7 Intracellular Stages.

Intracellular presporogonic stages of *S. elegans* were found migrating through the tubular

Table 3.1: Comparison of spore dimensions of *S. elegans* from different authors.

Length (μm) x±1SD(range)	**Width (μm)**	**Capsule length**	**Capsule width (μm)**	**Reference**	**Place**
9μm	9μm	_	_	Lester, 1974	Canada
8.3μm	7.4μm	3.3	3.3	Hedrick *et al.* 1988	U.S.A
10.2(7.8-11.6)	10.1(7.8-11.6)	3.9(3.6-4.1)	3.9(3.6-4.1)	Feist *et al.* 1991	U.K and France
11.0(7.7-11.5)	10.6(7.0-10.8)	4.0(3.4-4.4)	4.0(3.4-4.4)	Present study	Scotland

epithelium and this suggests a mode of entry into the tubular lumen (Fig.3.19). Molnár (1980a) and Lom *et al.* (1983b) described migration of early stages of *S.renicola* into the tubular lumen through the glomerulus or between the epithelial cells of the kidney tubule. Epithelial migration into the tubular lumen has also been suggested as a route of entry of PKX myxosporeans in salmonids (Kent and Hedrick, 1985, 1986; Hedrick *et al.*, 1988).

3.4.8 Pathogenicity

Pathological changes seemed minimal in light to medium infections of *S. elegans* but were occasionally severe in heavy infections. No pathological effects could be determined due to the presence of extrasporogonic stages, perhaps because of the very low intensity of infection. The only host response observed was macrophages closely attached to the extrasporogonic stages. Macrophages might destroy the extrasporogonic stages by their phagocytic action as suggested by a number of authors (Lom and Dyková, 1992; McGeorge *et al.*, 1994). Feist *et al.* (1991) did not observe any pathological effect due to extrasporogonic stages of *S. elegans* in the rete mirabile and kidney interstitium. In the present study electron microscopy revealed that development of pseudoplasmodia and spores of *S. elegans* in heavily infected kidneys is associated with increased vacuolation and the presence of dark non-homogeneous inclusions and an increase in the number of rodlet cells in the epithelium. Renal corpuscles were compressed and became atrophied due to large numbers of pre-sporogonic stages in the Bowman's space (Fig.3.26; 3.27 & 3.30).

The pathological changes described here are similar to those reported in renal sphaerosporosis of other fish species (Molnár, 1980a; Desser *et al.*, 1983a; Fischer-Scherl

et al., 1986; Hermans and Körting, 1985; Dyková and Lom, 1988; Supamattaya *et al.*, 1991, 1993). However, the pathological damage observed in this study was not so severe, except in heavy infections of *S.elegans*, as that described by some other authors for *Sphaerospora* species. Supamattaya *et al.* (1990) mentioned that in general *Sphaerospora* species do not affect wild fish severely. This is in contrast to cultured fish where severe outbreaks of disease have been reported. Presumably this is because features of the culture system, such as environmental stress, overcrowding or poor water quality, increases the susceptibility of fish to infection by myxosporeans.

Dyková and Lom (1988) reviewed the pathogenicity of myxosporeans in intensive carp culture in Europe. *Sphaerospora renicola* in the tubular lumen is associated with dystrophic changes to the tubular epithelium. Heavy infection by large masses of parasites distended the tubular lumen causing atrophy and necrosis of the epithelium. They concluded that these histopathological changes may seriously impair the excretory as well as the haemopoietic function of the kidney. Severely damaged glomeruli with developing stages located in the Bowman's spaces have been found in carp (Molnár, 1980a) and brown trout (Fischer-Scherl *et al.*, 1986) with kidney sphaerosporosis. El-Matbouli *et al.* (1992) described *S. cristata* in Bowman's space and capillary loops. Affected renal corpuscles were clearly enlarged and glomeruli were severely damaged, but they could not detect clinical signs or losses although extensive glomerular lesions were present.

Fischer-Scherl *et al.* (1986) observed *S. truttae* infection in kidney tubule lumen and renal corpuscles of brown trout (*Salmo tratta*) and suggested impaired function of excretory kidney in heavily infected fish.

Supamattaya *et al.* (1990) described epithelial vacuolation with an increased number of rodlet cells and glomerular destruction of the kidney due to the presence of *S. epinephali* in grouper. Corbel (1975) described in a comprehensive review of immune responses of fish that in kidney disease of salmonids various cell types including giant cells were observed as a cellular response by a number of authors. The presence of large multinucleated giant cells containing phagocytosed debris is reported in PKD granulomatous reactions (Ellis *et al.*, 1985). They speculated that the giant cells might perform an effective defence against the PKD organisms in brown trout and Atlantic salmon. Giant cells were not found in sticklebacks. The function of rodlet cells in fish is obscure and therefore the reason for their increased numbers in some *Sphaerospora* infections is unknown, but may represent a form of host reaction.

CHAPTER 4

DEVELOPMENT AND PATHOGENECITY OF *MYXOBILATUS GASTEROSTEI* (PARISI, 1912) DAVIS, 1944.

4 DEVELOPMENT AND PATHOGENICITY OF *MYXOBILATUS GASTEROSTEI* (PARISI, 1912) DAVIS, 1944.

4.1 Introduction.

The genus *Myxobilatus* is a relatively poorly known coelozoic myxosporean parasite found in the kidney and urinary bladder of freshwater and marine fish. Currently 24 species of *Myxobilatus* are known (Table 4.1), most of which are not well studied. *Myxobilatus gasterostei* is a common parasite of the three-spined stickleback, found in the kidney and urinary bladder.

4.1.1 Taxonomy.

Davis (1944) defined the genus *Myxobilatus* under the family Myxobolidae. Lom and Noble (1984) in a revised classification of the class Myxosporea redefined the genus within the family Sphaerosporidae. Presently the genus *Myxobilatus* is characterised as (Lom and Dyková, 1992):

> Spores elongated, anteriorly pointed; shell valves often with fine ridges, extended posteriorly in two caudal appendages. Polar capsules pyriform. Binucleated sporoplasm may contain the iodinophilous vacuole. Trophozoites small to large, disporic to polysporic, coelozoic in the excretory system, from kidney tubules down to urinary bladders, rarely histozoic.In freshwater and marine fish.

Myxobilatus gasterostei is the type species of the genus. According to Lom and Noble (1984), the taxonomic position of the parasite is:

Table 4.1: Known species of *Myxobilatus*.

Species	Host	Organs Infected	Reference
Asymmetrical Spores.			
1. *M. gasterostei* (Parisi, 1912) Davis, 1944.	*Gasterosteus aculeatus*	Kidney tubules and urinary bladder.	Arthur and Margolis, 1975 Lester, 1975; Lom, 1992
2. *M. platessae* Basikalowa, 1932	*Platessa platessa*	Urinary bladder.	Schulman, 1984; Lom, 1992
3. *M. baicalensis* Dogiel, 1957	*Asprocottus herzensteini*	Urinary bladder.	Schulman, 1984
4. *M. mictosporus* Kudo, 1920	*Lepomis cyanellus* *L. humilis* *Micropterus salmoides*	Urinary bladder.	Davis, 1944 Booker and Current, 1981
5. *M. ohisensis* Hedrick, 1941 (Davis, 1944)	*Aplodinotus grunniens*	Urinary bladder	Davis, 1944
6. *M. caudalis* Davis, 1944 (Davis, 1944)	*Aplodinotus grunniens*	Urinary bladder	Davis, 1944
Symmetrical Spores.			
7. *M. medius* Thélohan, 1892	*G. aculeatus* *Pungitius pungitius* *Pungitius platygaster*	Uriniferous tubules.	Arthur and Margolis, 1975
8. *M. legeri* Cepede, 1905	*Abramis brama, Abramis ballerus* *Alburnus alburnus, Blicca bjoerkna* *Cobitis barbatulus, Leuciscus idus* *Nemacheilus barbatulus, Rutilus rutilus*	Uriniferous tubules and urinary bladder, kidney tubules.	Arthur and Margolis, 1975; Lom, 1986, 1992 Molnár, 1988
9. *M. wisconsinensis* Mavor and Strasser, 1916	*Perca flavescens*	Urinary bladder	Arthur and Margolis, 1975
10. *M. sinipercae* Dogiel and Akhmerov, 1960	*Siniperca chua-tsi*	Urinary bladder	Arthur and Margolis, 1975

Species	Host	Organs Infected	Reference
11. *M. pseudorasborae* Shulman, 1962	*Pseudorasbora parva*	Urinary bladder	Arthur and Margolis, 1975
12. *M. varicorhini* Kandilov, 1963	*Varicorhinus capoeta*	Urinary bladder	Arthur and Margolis, 1975
13. *M. paragasterostei* Zaika, 1965	*Asprocottus megalops* *Batrachocottus baicalensis* *B. multiradiatus* *Leuciscus leuciscus baicalensis* *Paracottus kessleri* *Rutilus rutilus lacustris*	Uriniferous tubules Urinary bladder	Arthur and Margolis, 1975
14. *M. naturi* Guilford, 1965	*Noturus gyrinus*	Urinary bladder	Guilford, 1965
15. *M. cotti* Guilford, 1965	*Cottus bairdi*	Urinary bladder	Guilford, 1965
16. *M. mastacembeli* Qadri & Lalitha Kumari, 1965	*Mastacembelus armatus*	Cyst on intestinal serosa	Quadri and Lalitha Kumari, 1965
17. *M. synodontis* Siau, 1971	*Synodontis ansorgii*	Cyst on gill	Arthur and Margolis, 1975; Lom, 1992
18. *M. yukonensis* Arthur and Margolis, 1965	*Cottus cognatus*	Kidney tubules and urinary bladder	Arthur and Margolis, 1975; Lom, 1992
19. *M. asymmetricus* Davis, 1944	*Stizostedion vitreum*	Urinary bladder	Davis, 1944
20. *M. fragilicaudus* Shulman, 1969	*Pomoxis anularis*	Urinary bladder	Davis, 1944
21. *M. rupestris* Herrick, 1941	*P. sparoides*	Urinary bladder	Davis, 1944
22. *M. schulmani* Mytenev, 1975	*Pungitius pungitius*	Kidney	Schulman, 1984
23. *M. gobii* Evlanov, 1981	*Gobio gobio*	Urinary tubules of kidney	Schulman, 1984; Lom, 1986, 1992
24. *M. nostalgicus* Lom, 1986	*Tinca tinca*	Kidney tubules	Lom, 1986

Class	**MYXOSPOREA Bütschli, 1881**
Order	**BIVALVULIDA Schulman, 1959**
Sub-order	**VARIISPORINA Lom and Noble, 1984**
Family	**SPHAEROSPORIDAE Davis, 1917**
Genus	***Myxobilatus* Davis, 1944**
Species	***Myxobilatus gasterostei* (Parisi, 1912) Davis,1944**

4.1.2 Hosts of *Myxobilatus* Species.

The genus *Myxobilatus* is found in only a few fish species which include the large mouth bass, *Micropterus salmoides*, slimy sculpins, *Cottus cognatus*, and some other members of the family Cottidae and several cyprinid species including roach, rudd, ide, bleak, white bream, common bream and blue bream. *Myxobilatus* species were originally described as members of the genus *Henneguya* (Parisi, 1912; in Arthur and Margolis, 1975). Davis (1944) pointed out that *Myxobilatus* differed from *Henneguya* on the basis of fundamental differences in spore structure and established the genus *Myxobilatus* within the family Myxobolidae. He described two new species and transferred five others from *Henneguya* to *Myxobilatus*. Most of the *Myxobilatus* species described are coelozoic, although Quadri and Lalitha Kumari (1965) published an inadequate description of a histozoic species, *M. mastacembeli*, from the intestine of the freshwater fish, *Mastacembelus armatus*, in India.

4.1.3 Morphology.

Davis (1944) described spores of *Myxobilatus* as bilaterally symmetrical with two polar

capsules at the anterior end. The body of the spore is flattened at one side and strongly convex on the opposite side. Flattening occurs at right angles to the sutural plane. The two shell valves are prolonged separately at the posterior end to form tail processes. An iodinophilous vacuole is present in the sporoplasm. The spore valves have several ridges running parallel to the sutural ridges. Trophozoites (pre-sporogonic and sporogonic stages) are irregular in shape or greatly elongated and mono, di and polysporous.

Davis (1944) also described the fundamental differences between the spores of *Myxobilatus*, *Henneguya* and *Myxobolus*. In both *Henneguya* and *Myxobolus* spores are flattened parallel to the sutural plane which gives the spore a biconvex shape in side view with sutures extending around the margin (Fig.4.1a). In *Myxobilatus* the flattening is at right angles to the sutural plane and is much more pronounced on one side than the other. The sutural plane forms the median plane of the bilaterally symmetrical spore. The flattened side of the spore is the ventral side (Fig.4.1b).

Arthur and Margolis (1975) pointed out that the flattening of the spore in side view sometimes produces an asymmetrical spore and on the basis of this they divided *Myxobilatus* spores into two categories, i.e species with symmetrical spores and those with asymmetrical spores (Table 4.1). These authors described the spore of *M. gasterostei* as asymmetrical with a lenticular shape in sutural view and with one side strongly flattened in side view. The anterior end of the spore is tapered and the caudal processes usually slightly divergent distally. The valves are of uniform thickness with a straight sutural line and 6 to 7 surface striations. The polar capsules are pyriform and equal in size with 5 to 6 filament coils in each capsule. The iodinophilous vacuole was not positive for glycogen with Lugol's iodine

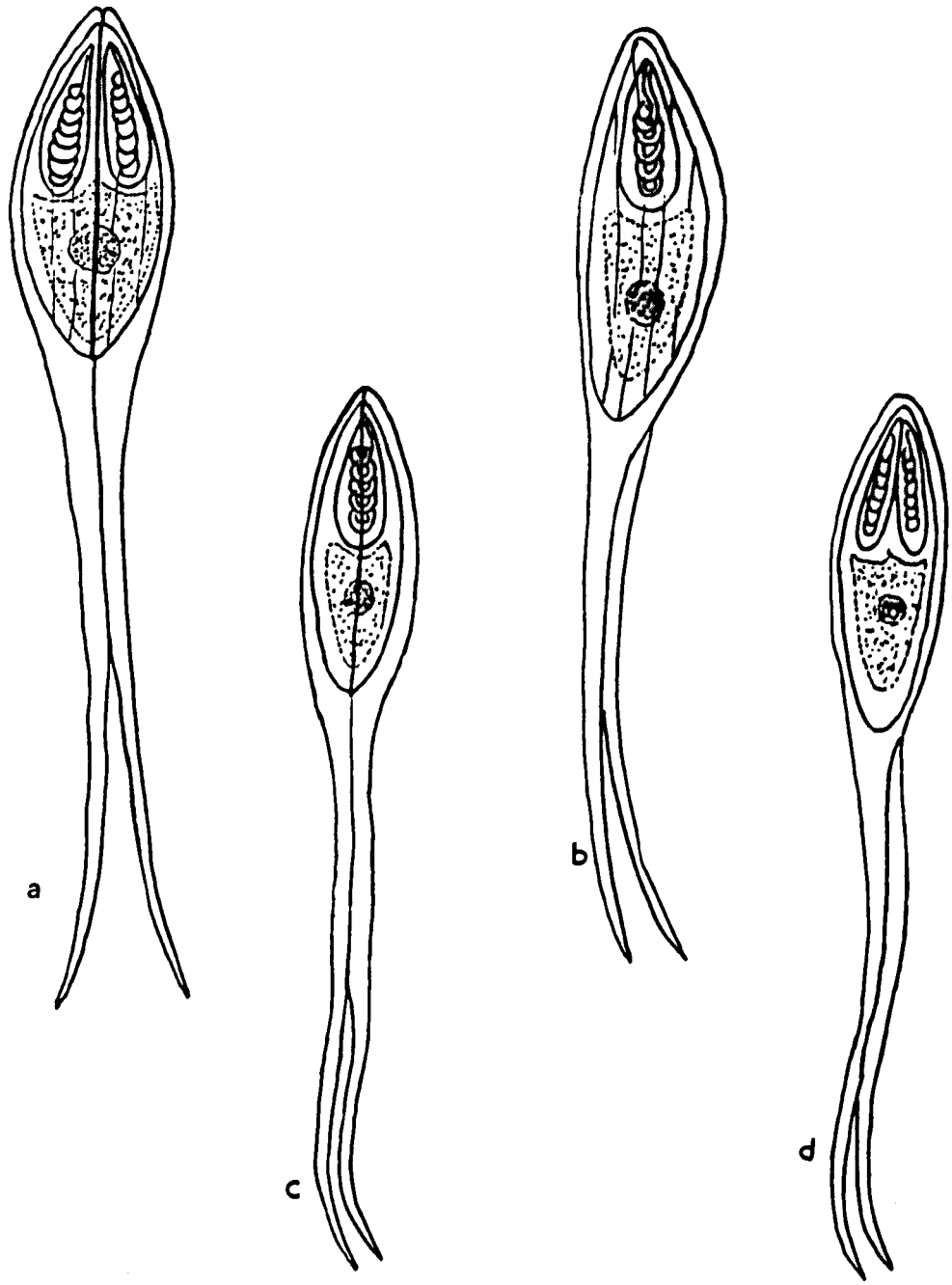

Fig 4.1 Drawings of *Myxobilatus* and *Henneguya* species showing structural differences of the spores. *Myxobilatus* in sutural (a) and valvular view (b); *Henneguya* in sutural (c) and valvular view (d).

solution. A mucous envelope (Lom and Vavra, 1963) is lacking.

Our present knowledge of the genus *Myxobilatus*, including *M. gasterostei*, is mainly based on light microscopic examinations. The most comprehensive descriptions are those by Davis (1944), Guilford (1965) and Lom (1969b).

The most recent light microscopic study was of *Myxobilatus legeri* (Molnár, 1988c), from the urinary tracts of cyprinid fish in Hungary. Molnár (1988c) found development of the parasites comprised two stages. One is a vegetative proliferation that takes place intracellularly in epithelial cells of the collecting tubules of the kidney. The second is plasmodial sporogony which occurs in the lumen of renal tubules, ureters and urinary bladder. The entire development of the parasite is strictly seasonal.

4.1.4 Ultrastructure.

The only ultrastructural study of a species of *Myxobilatus* is by Booker and Current (1981) who described the plasmodium morphology of *Myxobilatus mitraspora* within the urinary bladder of largemouth bass and reported the formation of three distinct forms of plasmodia influenced by seasonal changes of temperature. In winter they observed only one type of plasmodia consisting of flat sheet-like structures convering large areas of urinary bladder mucosa. They were attached firmly to the host tissue and their surface contained numerous fine microvilli. These winter plasmodia contained very few pansporoblasts and spores. During spring and summer the parasite also formed elongated fingerlike plasmodia from which spherical plasmodia containing mature spores were released by a budding process.

Most spore production occurred in free-floating spherical and oval plasmodia. Booker and Current (1981) did not describe the detailed sporogenesis of the parasite within these plasmodia.

Myxobilatus gasterostei was first described from the kidney tubules of the three-spined stickleback in Italy by Parisi (1912). Davis (1944) described the plasmodia and spores of *M. gasterostei* using the light microscope along with other 5 species of the genus. Shulman and Shtein (1964) reported this parasite from the USSR in the urinary bladder of *G.aculeatus* and *Pungitius pungitius*. Lester (1974) mentioned the occurrence of *M. gasterostei* in three spined sticklebacks from North America whilst Arthur and Margolis (1975) reported *M. gasterostei* from the urinary bladder of the three-spined stickleback from Vancouver Island, British Columbia. These latter authors illustrated and described the spore based on light microscopic observations and compared the dimensions of formalin preserved spores with previously published measurements.

There is very little information on the pathological effects of *Myxobilatus* species. Lester (1974) described narrowing of infected kidney tubules and loss of the brush border of the kidney tubular epithelium in *M. gasterostei* infections.

4.1.5 Objectives.

The present study describes the development of *M. gasterostei* from the kidney tubules of the three-spined stickleback, by light and electron microscopy. The light microscopic structure of the parasite is compared with previous studies whilst the detailed ultrastructure of the

parasite is compared with that of other myxosporeans. The study also includes SEM descriptions of the mature spores and plasmodia. The pathological effects of the parasite were also studied by light microscopy and TEM to determine the extent of pathogenicity and host reaction.

4.2 Materials and Methods.

After collection from Airthrey Loch, fish were maintained and examined in the laboratory as described in Chapter 2.

For light microscope study fresh and air dried kidney smears of different organs including gills, liver, kidney, gall bladder, reproductive organs, swimbladder, brain and blood were prepared and examined as described in Chapter 2. Measurements of the spores were taken in the fresh state as shown in Fig.4.2.

For histology and electron microscopy tissues were processed routinely as described in Chapter 3.2.2 , 3.2.3 and 3.3.4.

Fresh spores and developmental stages of the parasite were studied with phase contrast optics under oil immersion at x100 magnification. An average of 35 spores and 10 to 20 developing stages were measured from 5 to 10 fish in each month according to the methods described by Lom and Arthur (1989).

In the case of spores with unequal polar capsules measurements were taken for both capsules.

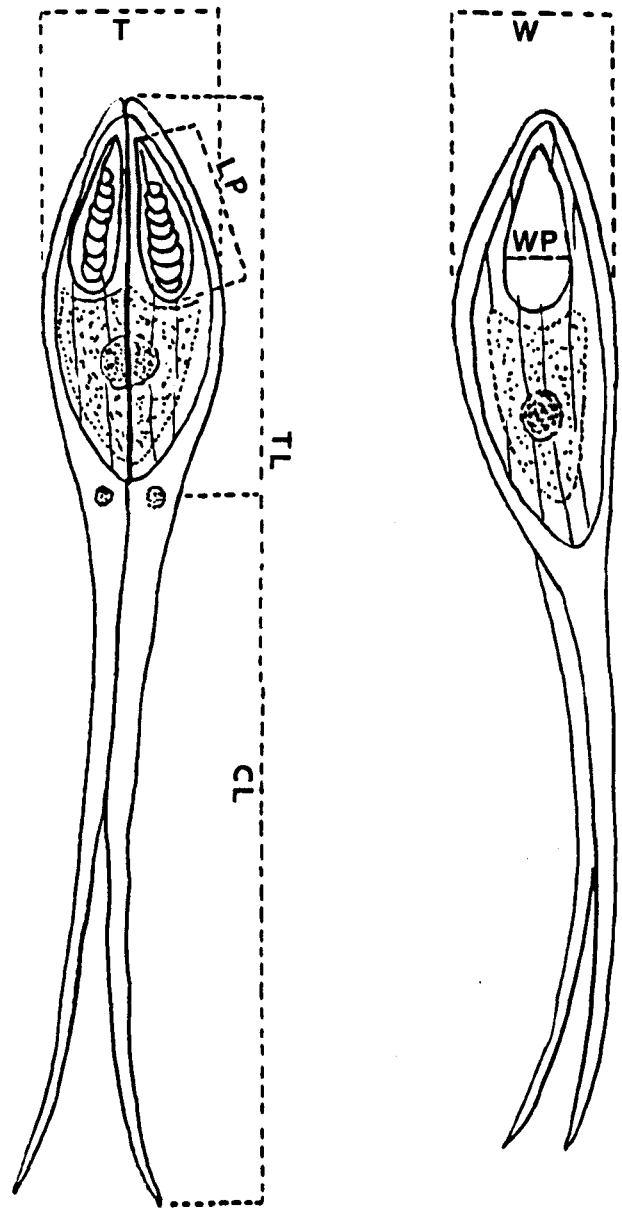

Fig 4.2 Measurements of *M. gasterostei* spores (after Lom and Arthur, 1989)
TL = Total length of the spore
W = Width of the spore
T = Thickness of the spore
CL = Length of caudal processes.
LP = Length of polar capsule
WP = Width of polar capsule.

To detect the iodinophilous vacuole (glycogen vacuole) fresh spores were stained with Lugol's iodine (1g-I, 10g KI and 100ml H_2O) and parasitology iodine (5g I, 10g KI and 100ml H_2O); control spores were incubated in saliva for a maximum of 1 hour at 37^0c before staining with Lugol's iodine. Methods for the photography and drawing of the parasite and developmental stages are as described in Chapter 2.

4.3 Results.

4.3.1 Light Microscopy

4.3.1.1 Fresh Squash of Infected Organs.

Spores and developing stages of *M. gasterostei* were found only in the kidney of stickleback. The main site of their development was the glomerulus and lumen of the proximal and distal tubules. Occasionally spores and developing stages were seen in the interstitial tissue of the kidney. In heavy infections the tubular lumen and Bowman's spaces of the glomeruli were found packed with the mature spores and developing stages of the parasite (Fig.4.3 & 4.4). Spores were occasionally found in the ureter and urinary bladder.

Pre-sporogonic and Sporogonic stages.

In fresh kidney squash preparations, pre-sporogonic stages appeared as round to elongated to irregular in shape. Very early stages were mostly round in shape and up to 16.8μm in diameter. The outer surface of these stages appeared to have numerous globular bodies attached. Their cytoplasm contained one to many nuclei, some refractile granules and a few vacuoles of different sizes.

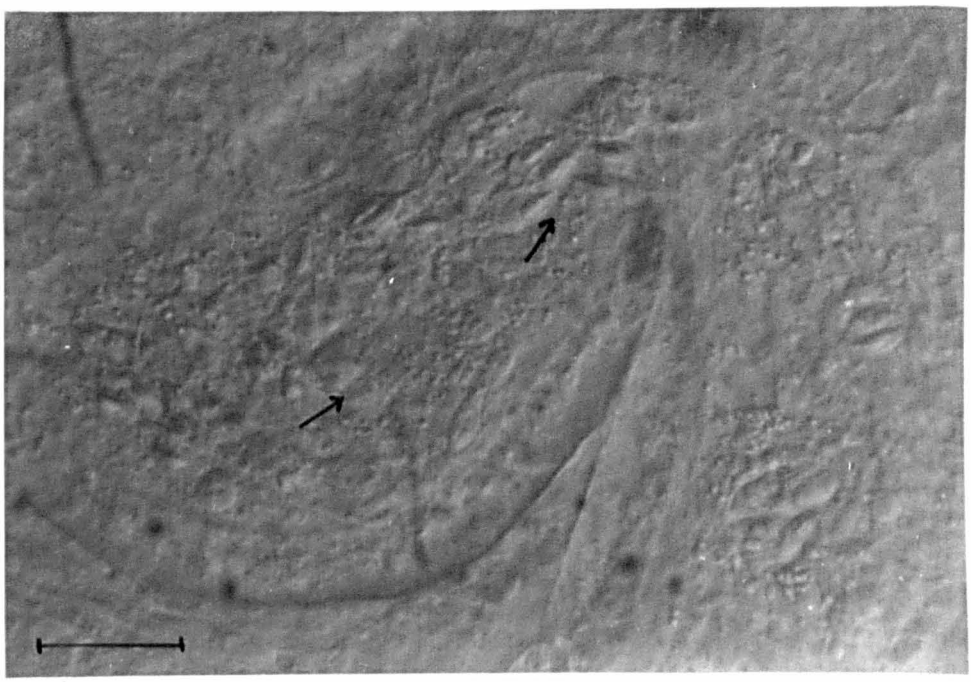

Fig 4.3 Fresh squash preparation of a kidney tubule showing tubular lumen packed with spores and developing stages of *M. gasterostei* (arrows)(bar=10μm).

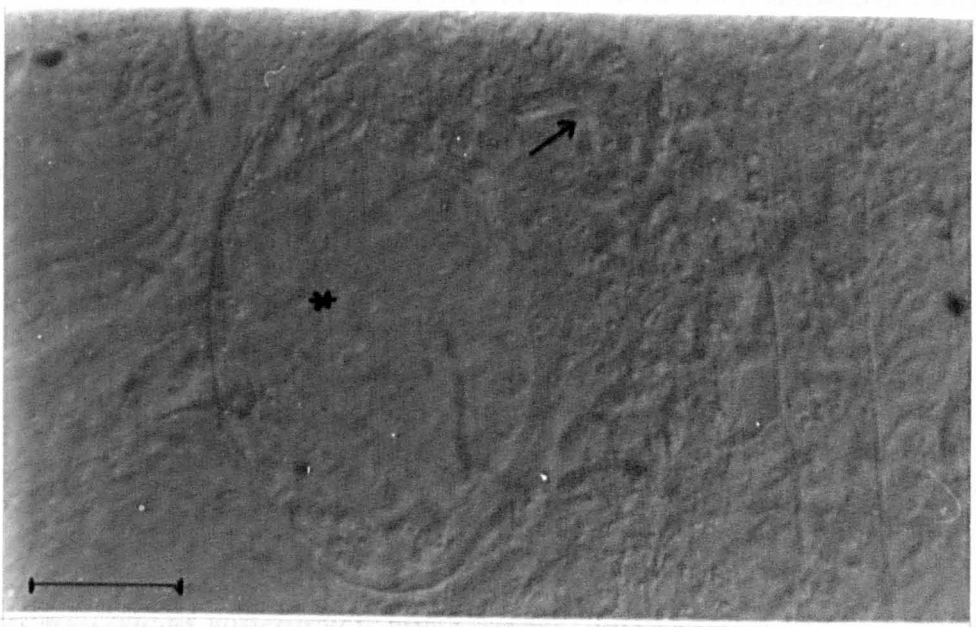

Fig 4.4 Fresh squash preparation of a kidney tubule showing Bowman's space packed with spores and developing stages of *M. gasterostei* (arrows). (*) Indicates glomerulus (bar=10μm).

Monosporous, disporous and polysporous sporogonic plasmodia were seen (Fig.4.5). Most of the monosporous plasmodia were elongated with mean dimensions of 11.6μm x 8.6μm (n=50). Disporous plasmodia were round or elongated to irregular in shape with mean dimensions of 13.6μm x 10.6μm (n=50). Spore development in disporous plasmodia was mostly synchronous. The position of the two spores inside these plasmodia was not uniform. Usually the two spores lay with their ventral sides in apposition in round disporous plasmodia. In some disporous plasmodia which were elongated the two spores lay with their anterior ends opposite to each other.

Polysporous plasmodia had mean dimensions of 17.9μm x 14.8μm (n=30). They were round to elongate to irregular in shape. They contained 3-8 spores whose development was not synchronous. In plasmodia with mature spores the caudal processes of the spores were often seen protruding through the surface. The cytoplasm of all three types of plasmodia contained vacuoles of different types.

Spores.

Measurements of fresh spores were as follows (n=480): total length=21μm-38.85μm ($\bar{X}$=30.28$\pm$8.25), length of the caudal process=10.5μm-26.25μm ($\bar{X}$=20.32$\pm$6.81), length of spore body=10.5μm-12.6 μm ($\bar{X}$=10.20$\pm$1.9), width of spore=4.2μm-6.3μm, ($\bar{X}$=5$\pm$1.5), thickness of spore=4.2μm-6.8μm ($\bar{X}$=5.2$\pm$1.5), length of the polar capsule=4.2μm-7.3μm ($\bar{X}$=5.4$\pm$2), width of the polar capsule=1.5 μm-3.1μm ($\bar{X}$=3$\pm$1.8).

The spore body of *Myxobilatus gasterostei* is asymmetrical, spindle shaped in sutural view and with one side slightly flattened in the valvular view (Fig.4.6a). The anterior end is

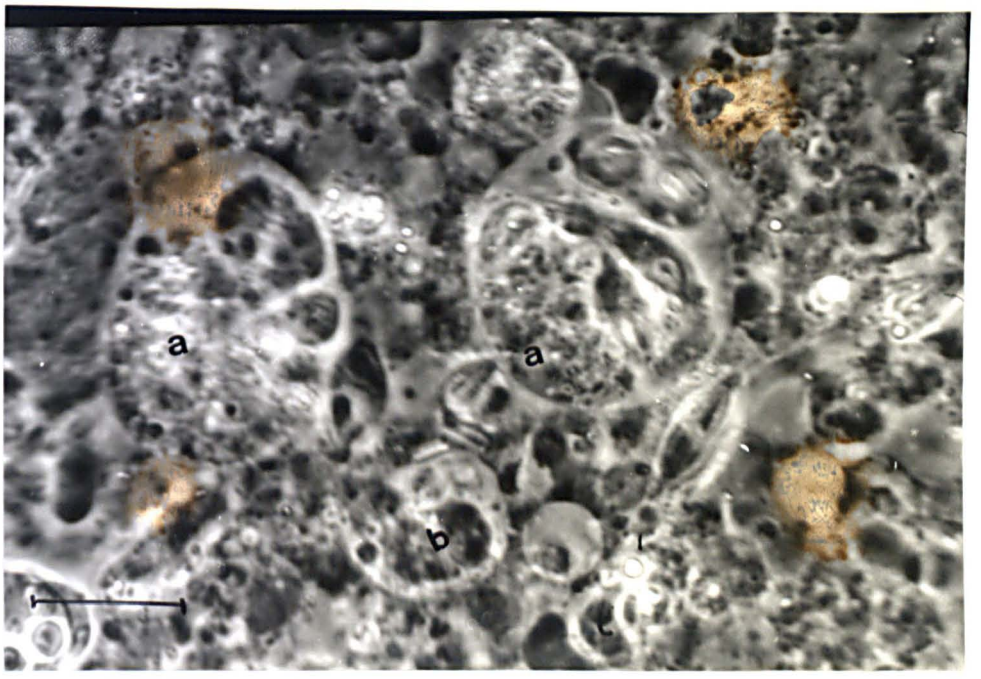

Fig 4.5 Fresh squash preparation of kidney showing different types of plasmodia and early developing stages of *M. gasterostei*. (a) Polysporous plasmodia, (b) disporous plasmodium, (c) monosporous plasmodium (bar=10μm).

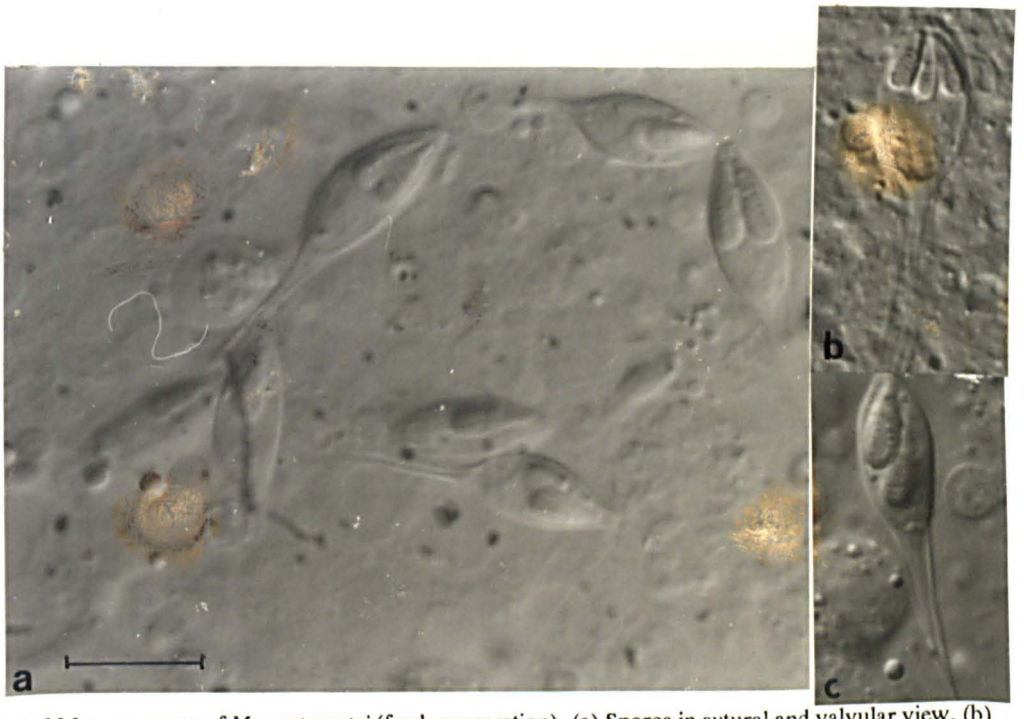

Fig 4.6 Mature spores of *M. gasterostei* (fresh preparation). (a) Spores in sutural and valvular view, (b) spores with three polar capsules, (c) spores with unequal polar capsules (bar=10μm).

tapered and the posterior end has a forked caudal process. The latter exhibits slight divergence distally. The sutural line is thick and straight and lies in between the two polar capsules. The surfaces of the valves have 6 to 9 ridges which run parallel to the sutural ridges and appear as fine longitudinal striations or lines under the light microscope. Polar capsules are pyriform and mostly of equal length. The polar filament forms 5 to 8 coils and the maximum length of the extended polar filament was 47.25μm. The sporoplasm cell appeared binucleated and finely granulated. It did not stain positively with Lugol's iodine.

Spores with unequal polar capsules were frequently observed in every sample (Fig.4.6c). The positions of the two polar capsules were also sometimes abnormal. Spores with unequal caudal processes were rarely seen, but sometimes spores with three polar capsules were observed (Fig.4.6b).

4.3.1.2 Giemsa Stained Dry Smears.

In these preparations, developmental stages and mature spores showed exactly the same characteristics as seen in fresh squash preparations. The polar capsules of the mature spores stained dark blue. In some cases the extended polar filaments were also darkly stained. The sporoplasm appeared as a lightly stained binucleated cell (Fig.4.7a). Developing plasmodia were found to contain 2 to 12 inner cells which stained more darkly than the plasmodia (Fig.4.7b). Mono, di and polysporous plasmodia with a maximum of 8 spores were observed (Fig.4.7a,b).

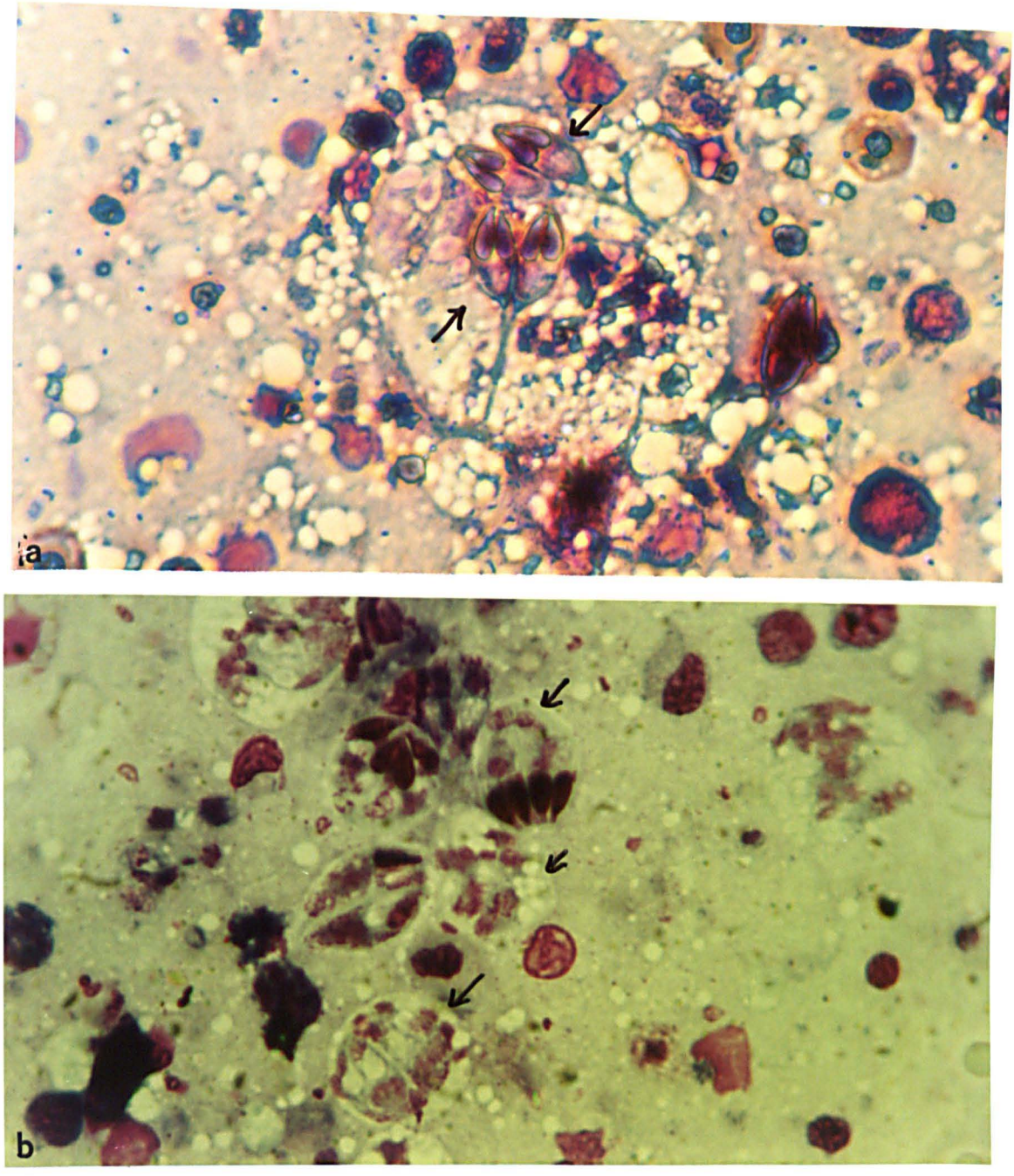

Fig 4.7 Giemsa stained dry smears of kidney showing spores and developing stages of *M. gasterostei* (bar=10μm). (a) Mature spores (arrows), (b) developing plasmodia (arrows).

4.3.1.3 Histology.

Histology confirmed the exact location of the parasites within the different parts of the kidney. Spores and pre-sporogonic and sporogonic stages were predominantly present within the lumen of the tubules. In sections of heavily infected kidney the proximal and distal tubule were found equally filled with the parasites. In a few cases spores and presporogonic and sporogonic stages were observed in the Bowman's space of the renal corpuscles. Spores were rarely found in the interstitial tissue of the kidney. Occasionally pre-sporogonic developing stages were seen within the cells of the epithelial layer of the renal tubules.

4.3.2 Electron Microscopy

4.3.2.1 Ultrastructure of Mono and Disporous Plasmodia.

The earliest mono- and disporous plasmodia observed consisted of a large primary cell enclosing a single sporogonic cell (Fig.4.8), which was round to elongate in shape with an irregular outline. Mitochondria of the primary cell were fewer in number than those of the sporogonic cells. The cytoplasm contained one large dark nucleus, many free ribosomes and very little RER. A few vacuoles and electron dense areas were also observed in the cytoplasm. The cytoplasm of the sporogonic cell was denser or lighter than that of the primary cell with large mitochondria, many free ribosomes and a large nucleus with an electron dense eccentric nucleolus.

Single sporogonic cells were observed to divide endogenously (Fig.4.9). Two sporogonic cells were sometimes found connected by cytoplasmic bridges (Fig.4.10). Early mono and

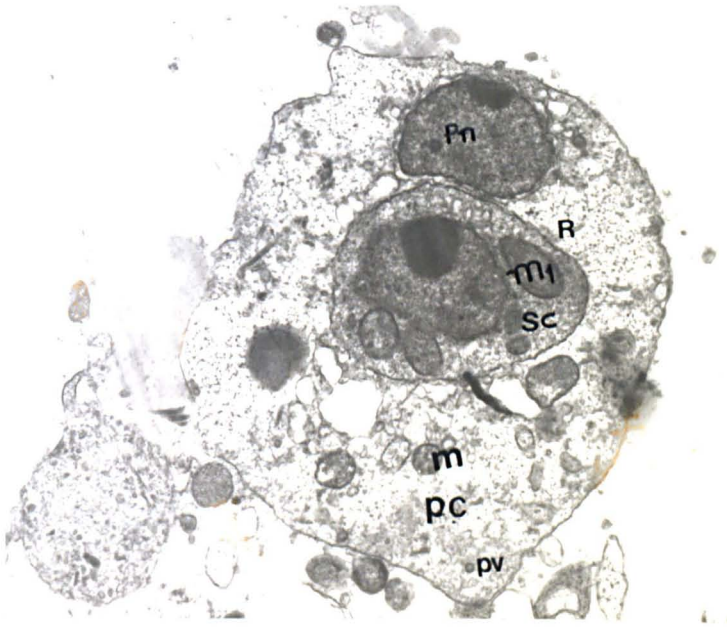

Fig 4.8 Early monosporous plasmodium enclosing a single sporogonic cell in the tubular lumen (x16,500). The cytoplasm of the primary cell (PC) is lighter with few mitochondria (m), many free ribosomes (R), pinocytotic vesicles (PV) and a dark nucleus (Pn). Sporogonic cell (Sc) with dense cytoplasm, large mitochondria (m_1).

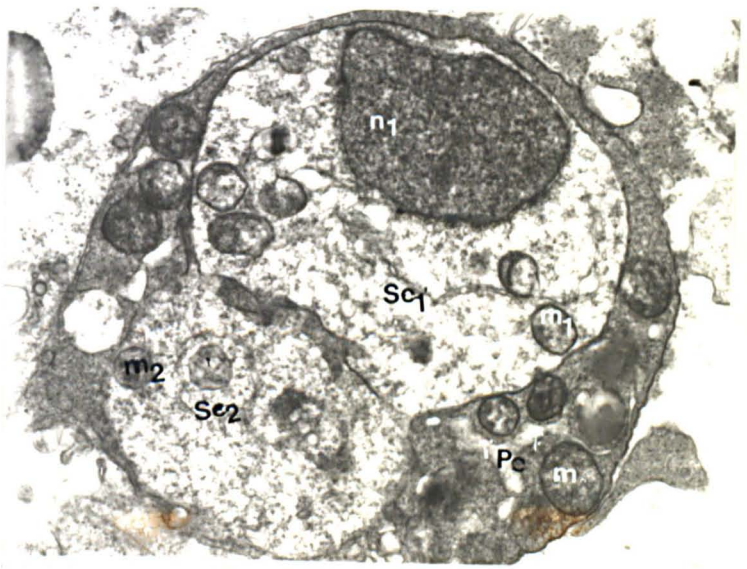

Fig 4.9 Endogenous division of sporogonic cell (Sc_1) within the primary cell(Pc) (x15,600).
n_1=nucleus of sporogonic cell,
m= mitochondria of primary cell,
m_1=mitochondria of sporogonic cell,
Sc_2=new sporogonic cell,
m_2=mitochondria of new sporogonic cell.

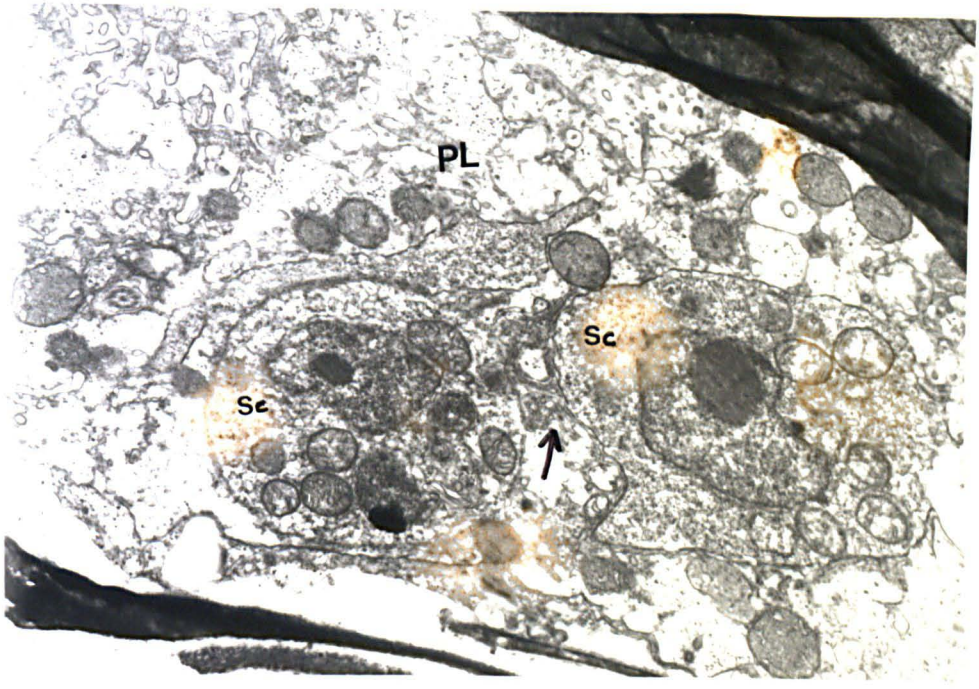

Fig 4.10 Two sporogonic cells (Sc) connected by cytoplasmic bridge (arrow) within the early plasmodium (PL) (x18,620).

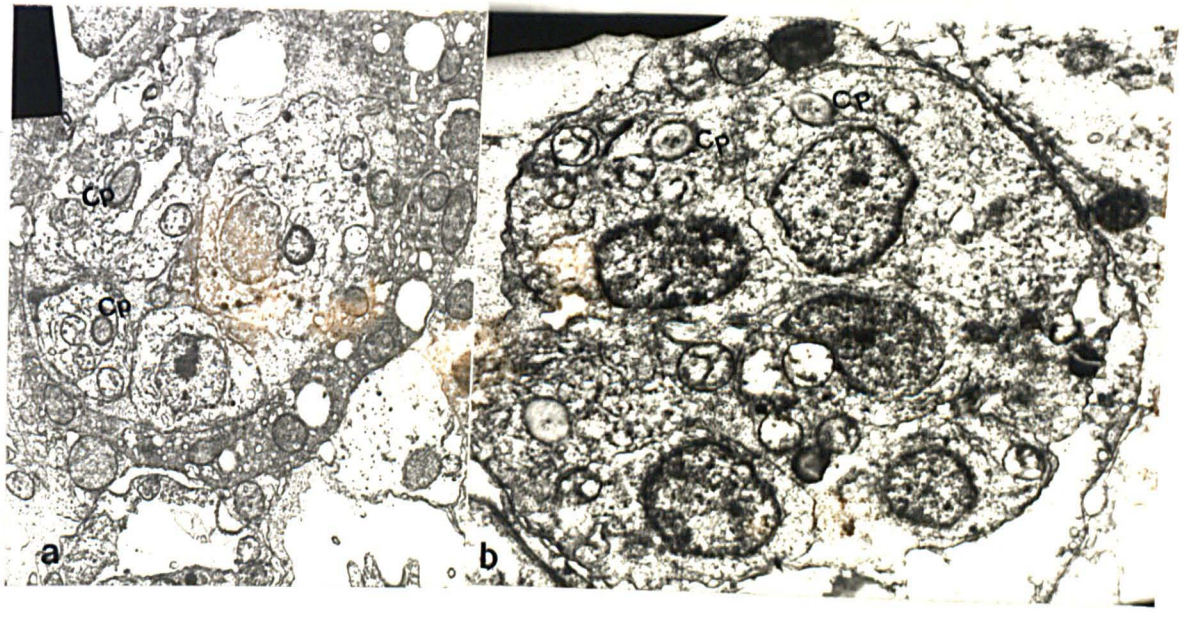

Fig 4.11 Pre-sporogonic stages of *M. gasterostei*; (a) with 4 sporoblast cells, note capsular primordia (Cp) (x12,640), (b) 5 sporoblast cells with capsular primordia (Cp) in capsulogenic cell (x11,760).

disporous plasmodia could be differentiated until the sporogonic cell divided completely. In monosporous plasmodia sporogonic cells produce 5 sporoblast cells and in disporous plasmodia 10 sporoblast cells (Fig.4.12).

Development of Capsulogenic Cell.

Differentiation of capsulogenic cells occurred before the formation of the complete set of sporoblast cells (Fig.4.11a). Capsulogenic cells can be easily recognized at an early stage by the presence of capsular primordia in their cytoplasm. The latter also contains large amounts of RER, some large round and elongated mitochondria with branched tubular cristae, many free ribosomes, a large nucleus, Golgi apparatus and some scattered microtubules (Fig.4.12).

Formation of Polar Capsules.

The earliest stage of the polar capsule appeared in the differentiated capsulogenic cell as a ring like cross section of a cylindrical tube externally surrounded by longitudinally arranged microtubules (Fig.4.11b). When the capsulogenic cell reached its approximately final position in the developing spore the tube became bulb shaped (Fig.4.14). The swollen end had a granular, less electron dense cortical zone. This central zone was surrounded by 4 layers, 2 of which were electron dense and 2 which were electron lucent. The outermost layer was made up of scattered coarse electron dense granules. The second layer beneath it was made up of electron lucent, fine granules. Beneath this was a third, electron dense layer and interior to this was another electron lucent layer, which surrounded the cortical zone. The third electron dense layer became electron lucent during the growth of the capsular primordium.

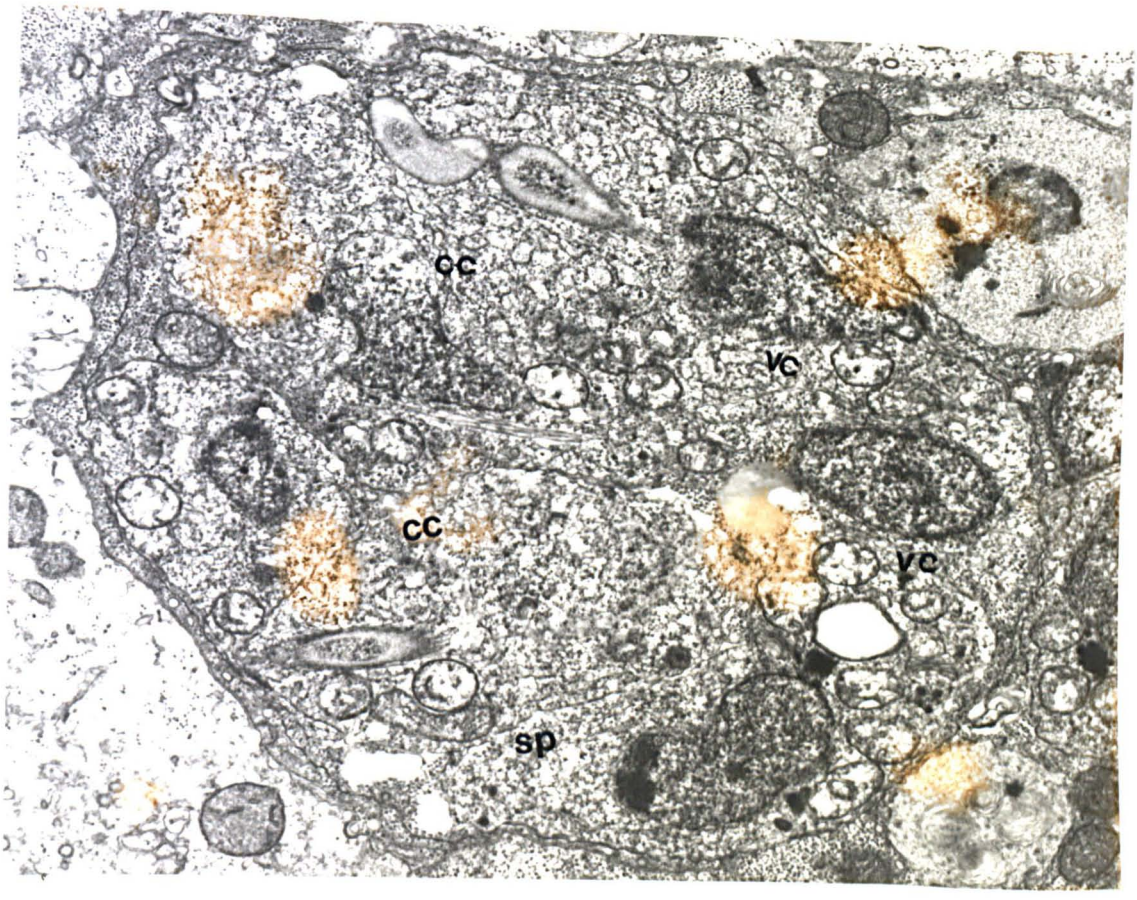

Fig 4.12 Plasmodium with differentiated sporoblast cells: SP-sporoplasmic cells; CC-capsulogenic cells; VC=valvogenic cells (x21,000).

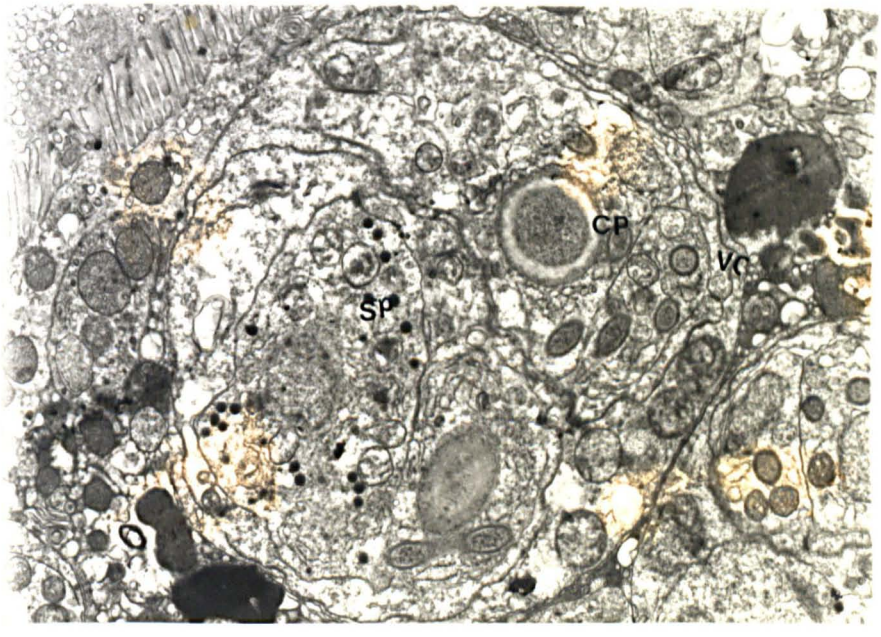

Fig 4.13 Disporous plasmodium with differentiated sporoblast cells (x13,500).
Cp=Capsulogenic cell.
Sp=Sporoplasmic cell.
Vc=Valvogenic cell.

The other end of the capsular primordium remained as a long external tube until the formation of the polar filament within it. In the earliest stages of capsulogenesis the proximal end of the external tube became fixed at the apex of the capsulogenic cell, where it was in proximity with the future polar filament discharge canal (Fig.4.15). Microtubules surrounding the long external tube extended up to the dense tip of the valvogenic cell. Microtubules were not found around the bulb shaped end of the primordium. In cross sections of the external tube, 28 to 32 microtubules could be observed longitudinally arranged around it (Fig.4.15). The external tube had almost the same structure as the bulb shaped primordium, except it lacked the electron lucent layer around its central core. The junction of the external tube and bulb shaped end of the primordium became constricted, and the boundary around this constriction became more electron dense (Fig.4.14). In a cross section of the developing capsulogenic cell, up to 4 sections of external tube were observed. The granular material in the capsular primordium appeared as oblique strands in longitudinal section (Fig.4.16).

Initially the bulb shaped primordium was positioned with its broad end at the anterior side of the developing spore (Fig4.17). As development proceeded the bulb shaped primordium reversed its position and assumed the normal shape of a polar capsule.

After the complete development of the capsular primordium the polar filament appeared as a loose coil in both the external tube and the bulb shaped primordium at the same time (Fig.4.18). With the growth of the polar capsule it became more coiled. The external tube gradually disappeared from the cytoplasm of the capsulogenic cell. In cross section the filament had an "S" shape with a membrane bound hollow central zone. Ultimately up to 6 to 9 very electron dense coils appeared within the elongated polar capsule (Fig.4.19).

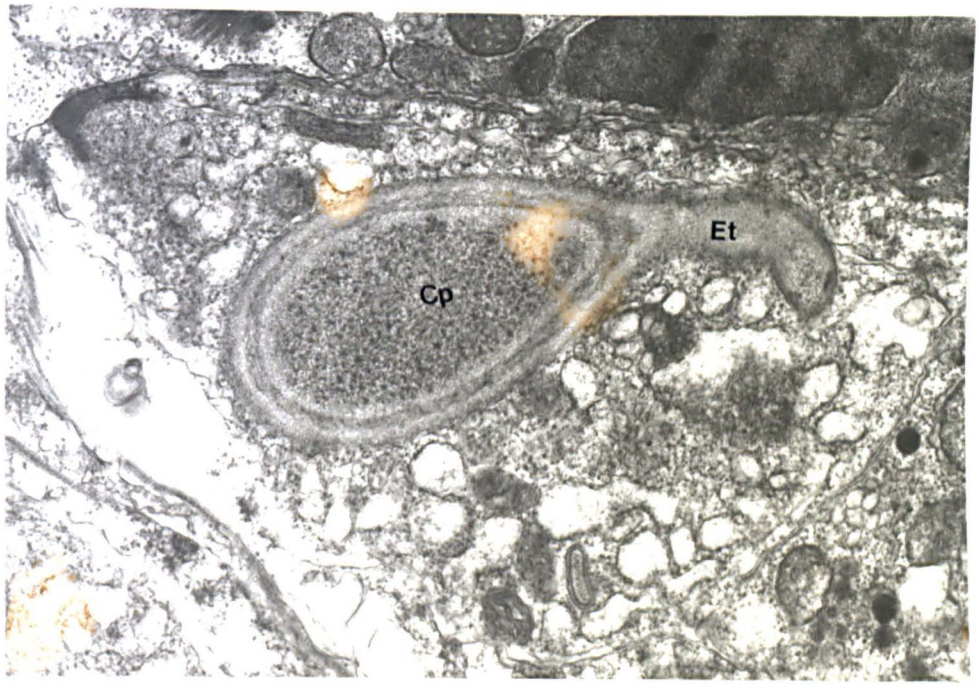

Fig 4.14 Longitudinal TEM section of developing spore showing bulb shaped capsular primordium (Cp) and long external tube (Et). Note capsular bulb surrounded by 4 layers (x29,400).

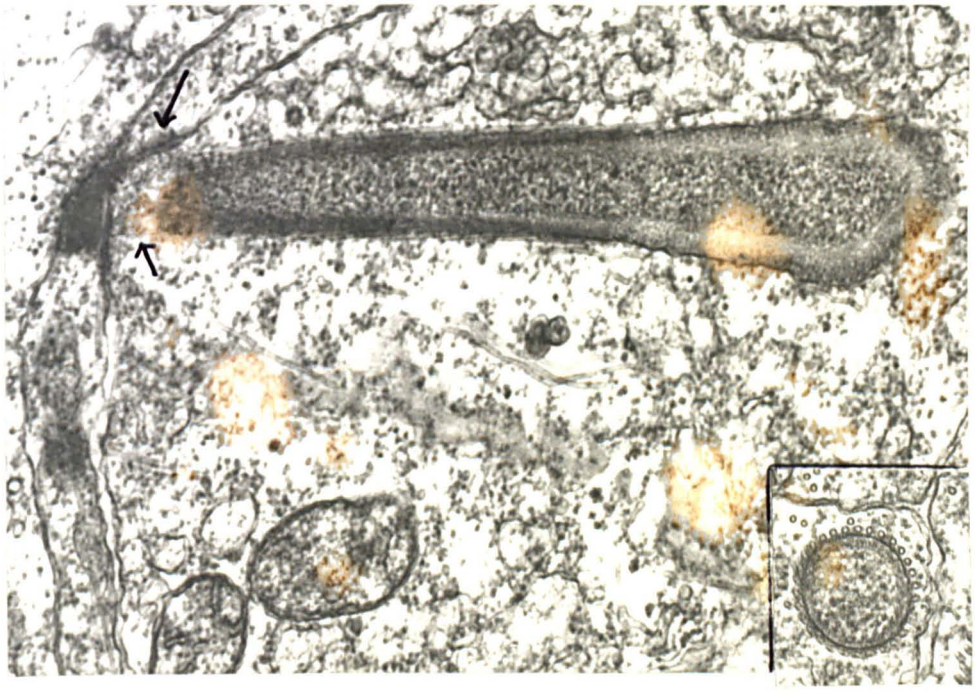

Fig 4.15 TEM section through the developing capsulogenic cell showing tip of the external tube fixed near future filament discharge canal (x39,200). Note microtubules (arrow) up to electron dense tip of the valvogenic cell. Inset: cross section of external tube surrounded by microtubules.

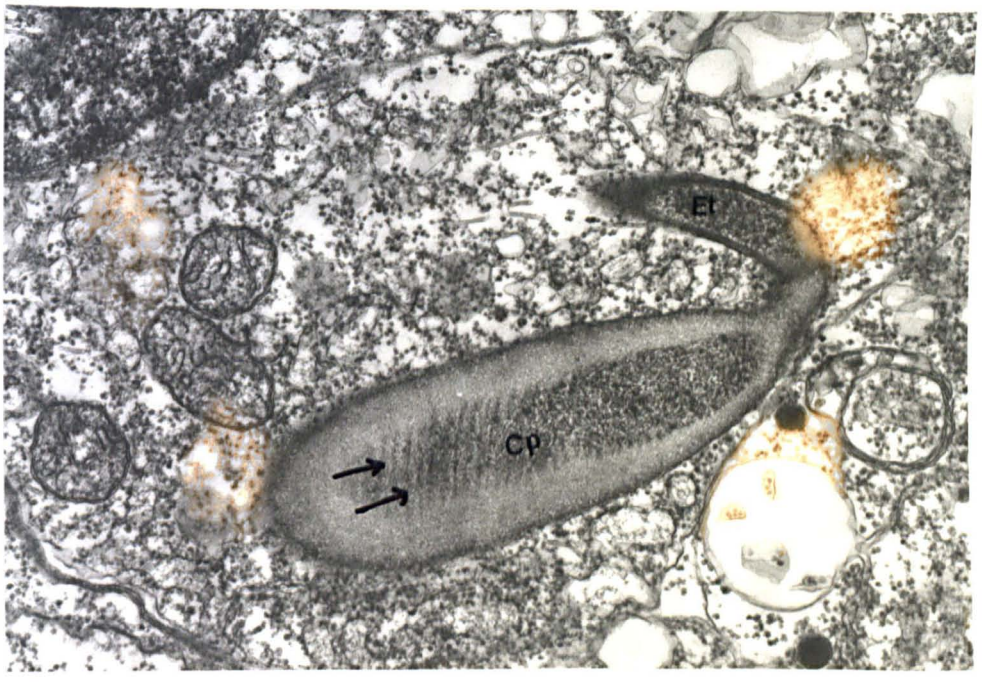

Fig 4.16 TEM section through developing capsule showing granular, oblique strands at the surface (arrows). Cp-bulb shaped capsular primordium; Et-external tube (x7,240).

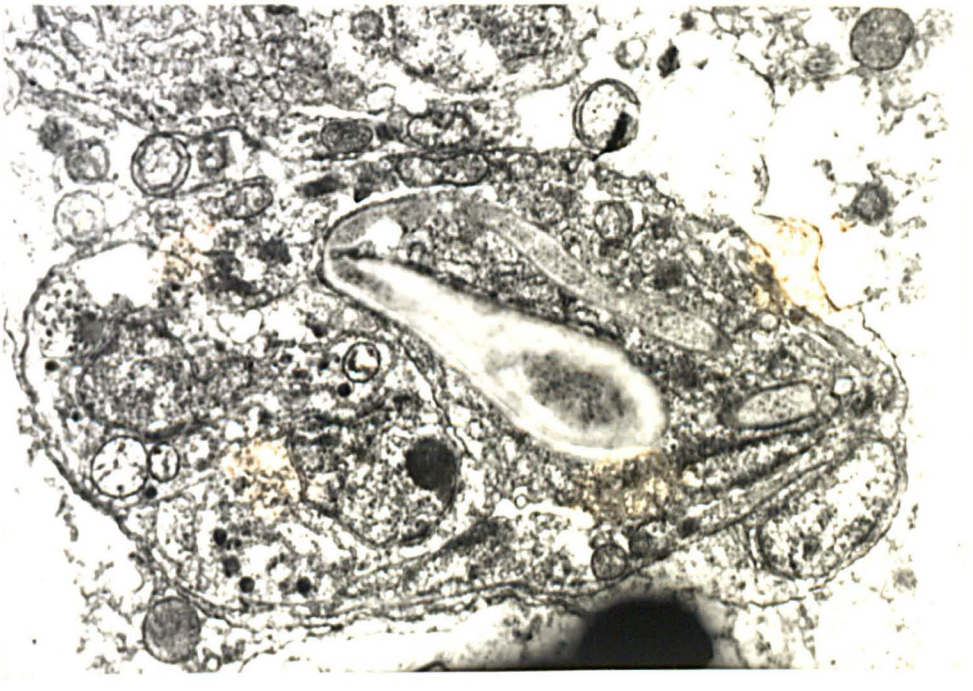

Fig 4.17 TEM section through developing capsule showing bulb shaped primordium positioned at the anterior part of the capsulogenic cell (x7,550).

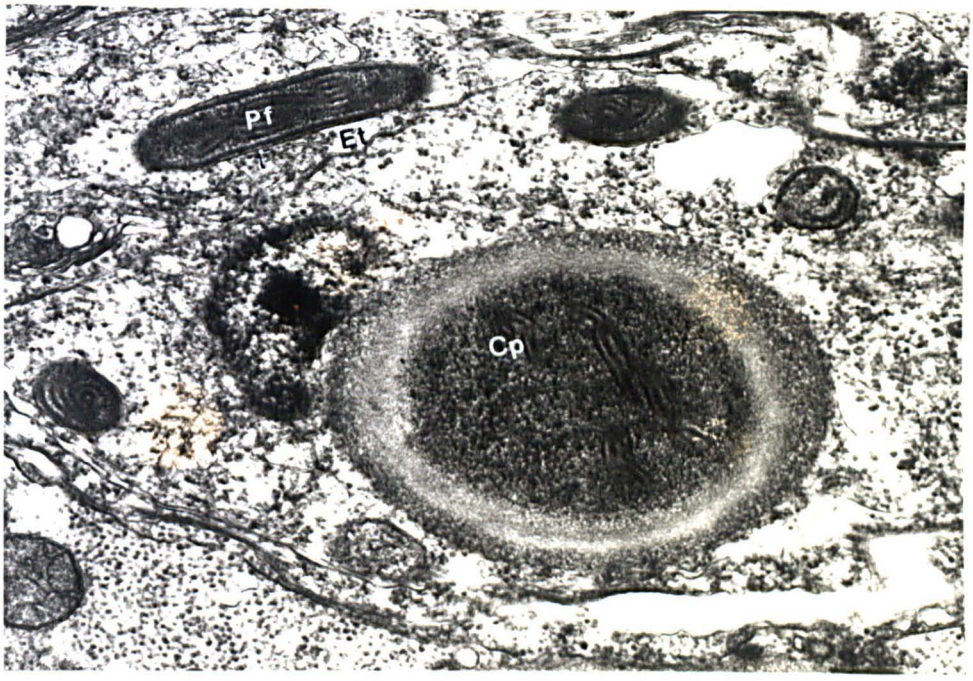

Fig 4.18 TEM section through developing capsulogenic cell. Note appearance of polar filament (Pf) at same time in external tube (Et) and bulb shaped primordium (Cp) (x26,000).

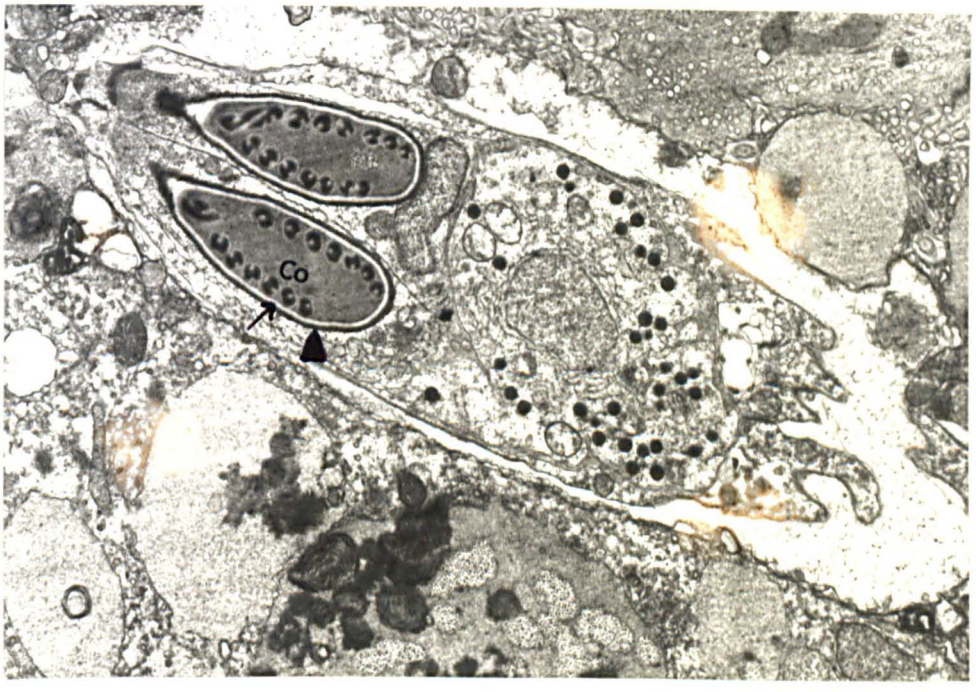

Fig 4.19 An electron lucent space (arrow) between polar capsule wall (arrow head) and central core (Co) giving the appearance of a two layered wall of the polar capsule (x11,210).

Development of polar capsules was mostly synchronous, although asynchronous development of the capsules was quite often observed (Fig.4.20).

The wall of the fully developed polar capsule consisted of an outer electron dense layer and inner electron lucent layer. The outer layer was continuous with the filament wall and the electron lucent layer formed a thin layer around the filament (Fig.4.21a).

An electron dense stopper formed at the anterior end of the polar capsule after the formation of filament coils (Fig.4.21b). An anterior cytoplasmic projection of the capsulogenic cell formed at the anterior of the stopper. The valvogenic cell became very electron dense over this cytoplasmic projection. The formation of this electron dense area commenced at the very beginning of valvogenic cell development. In longitudinal valvular sections of the spore the electron dense area at the anterior end of the valvogenic cell lay at the tip of the shell valves (Fig.4.21c). The membrane of the cytoplasmic projection was electron dense. An opening for polar filament discharge was not seen at the tip of the mature spore (Fig.4.21c).

A fully formed polar capsule occupied much of the area of a capsulogenic cell, with the nucleus, mitochondria and most of the capsular cytoplasm occupying the posterior part. Only a thin layer of cytoplasm was left around the polar capsule.

The fully developed polar capsule was elongated in shape and consisted of an outer electron dense layer, a middle electron lucent layer, and an inner polar filament layer surrounding a central heavily electron dense amorphous zone. Anteriorly it contained the filament discharge canal which was stoppered at the opening by an electron dense area. There was a cytoplasmic

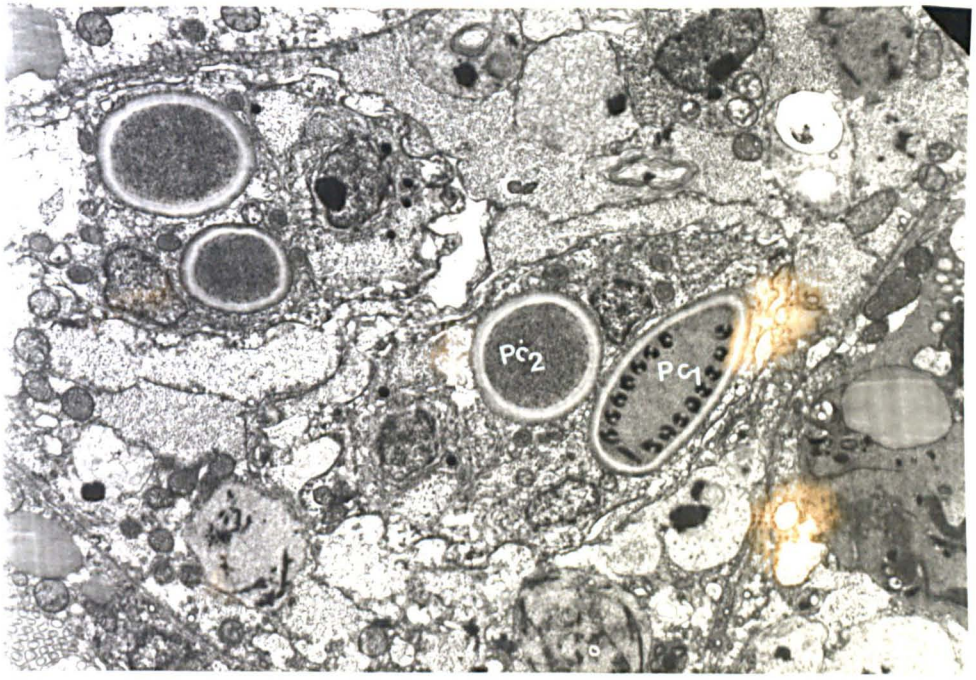

Fig 4.20 Asynchronous development of polar capsule. Note the two polar capsules one of which has a developed polar filament (Pc_1), while the other is still an early capsular primordium (Pc_2) (x8,850).

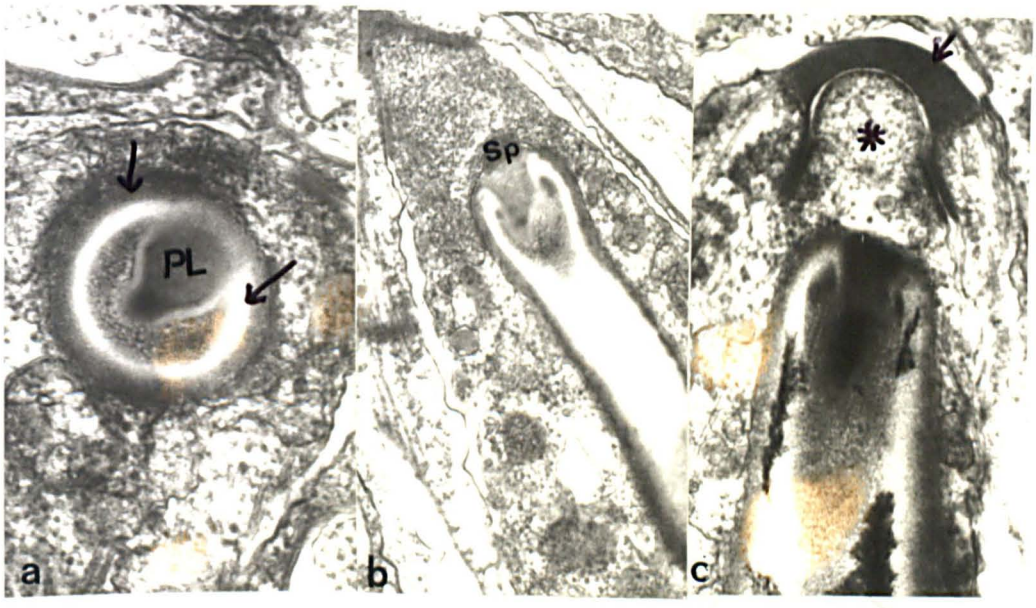

Fig 4.21 a, Mature polar capsule wall has an outer electron dense layer (arrow heads) and inner electron lucent layer (arrow). PL, polar filament (x5,600). b, An electron dense stopper (Sp) at the tip of the polar capsule (x32,500). c, Anterior end of a developing spore showing electron dense tip of valvogenic cell (arrow heads), plasmatic projection of capsulogenic cytoplasm (*) with electron dense surface (x54,000).

projection of the capsulogenic cell at the anterior end of the spore and at the tip, a very electron dense area of valvogenic cell (Fig.4.21c). The junctions between the capsulogenic and valvogenic cells were desmosomal junctions (Fig.4.21c).

Development of Sporoplasmic Cell.

At the very beginning of sporoblast development, sporoplasmic cells could be easily recognized by the presence of electron dense sporoplasmosomes within the cytoplasm (Fig.4.13). The single nucleus was large with an eccentric small nucleolus and evenly distributed heterochromatin. The cytoplasm of sporoplasmic cells contained many large round and a few elongated mitochondria with plate-like cristae. Rough endoplasmic reticulum was scattered in the cytoplasm which also contained many free ribosomes and two Golgi. As the sporoblast cells grow, the nucleus divided by endogenous division (Fig.4.13). Endoplasmic reticulum was accumulated around the newly formed nucleus, in which a nucleolus was not usually observed. With growth of the sporoplasm cell sporoplasmosomes increased in size and numbers and they became scattered throughout the cytoplasm of the cell. In the maturing spore the sporoplasmosomes continued to enlarge and show an electron dense central area surrounded by an electron lucent zone. With the complete maturation of the spore, the sporoplasmosomes became less electron dense (Fig.4.22).

Development of Valvogenic Cell.

The two valvogenic cells were assumed to be usually positioned at the anterior end of the spore where they were joined by a desmosomal junction, which ultimately formed the suture. With the development of capsulogenic and sporoplasmic cells the two valvogenic cells started to spread around the latter. During this process large, round mitochondria with tubular cristae

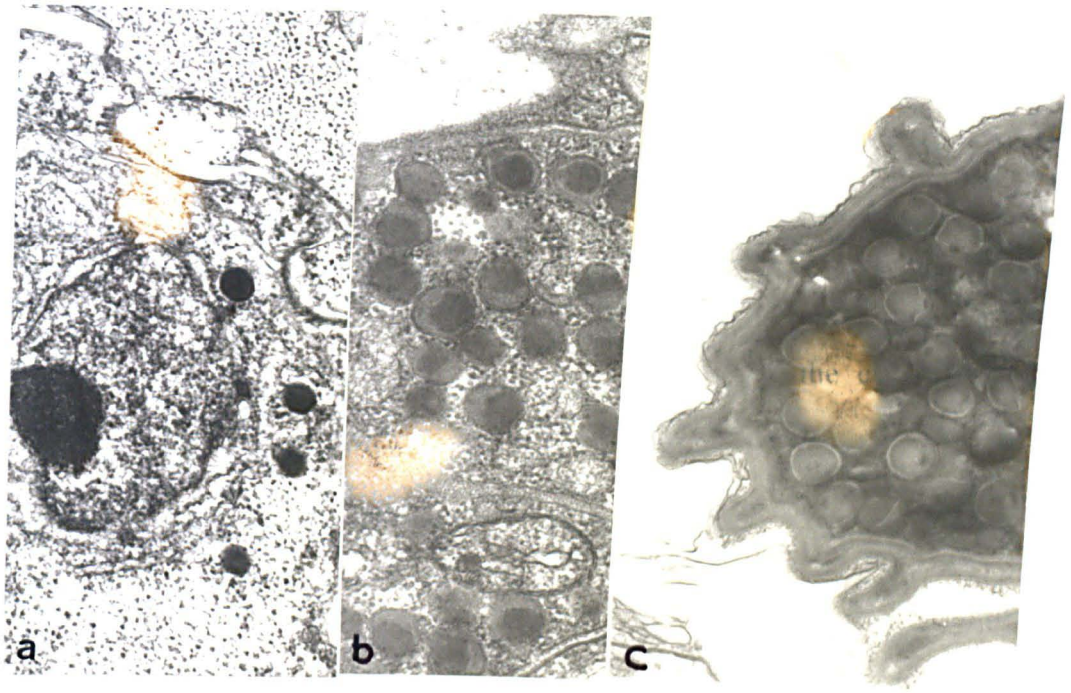

Fig 4.22 Maturation of sporoplasmosomes. (a). At the beginning of spore development sporoplasmosomes appeared as dark electron dense dots (x23,600); (b) later they form an electron lucent layer at the surface (x39,200), (c) in mature spores they became less electron dense (x5,2000).

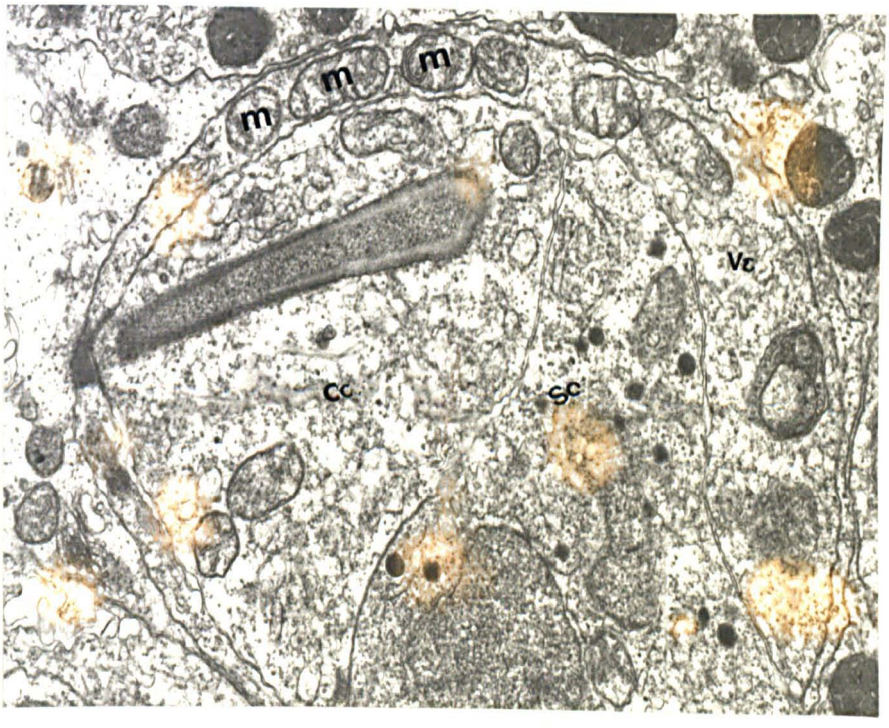

Fig 4.23 Development of valvogenic cell. Note mitochondrial (m) arrangement; Vc-valvogenic cell, Cc-capsulogenic cell, Sc-sporoplasmic cell; (arrow) dense anterior tip of valvogenic cell (x21,560).

become arranged serially at the periphery of the cell (Fig.4.23). The nucleus of the valvogenic cell moved posteriorly.

Beneath the outer cell membrane of the valvogenic cells there were numerous microtubules extended longitudinally. These were specially abundant near the sutural line (Fig.4.24a). The tips of the two valvogenic cells became very electron dense and they extended separately further above the suture (Fig.4.24b). At the

base of the sporoplasmic cell the two valvogenic cells meet to complete a continuous suture around the spore. In maturing spores the nuclei of valvogenic cells lie at the base of the sporoplasm cell. The cytoplasm of the valvogenic cells extends posteriorly below the suture to form the caudal appendages (Fig.4.25).

After joining at the suture on both sides, the outer surfaces of the valvogenic cells become wrinkled which may result in the formation of the surface ridges of the shell valves. There are 6 to 9 ridges on each valve. Usually the number of ridges is not equal in both valves but varies by one or two ridges. Posteriorly the ridges of the shell valves extended around the caudal appendages. The grooves between the surface ridges filled with cytoplasm of the enveloping cell. In cross sections of maturing spore sometimes it was observed that mitochondria of the enveloping cell lodged in the grooves of the shell valves (Fig.4.26). Internally the grooves were subtended by 18-24 longitudinally running microtubules. Additional microtubules were also scattered in the valvogenic cells. In longitudinal sections of the spore through the shell valves the ridges and the grooves appeared as interlocking with each other on the surface of the valves (Fig.4.27). In mature spores the shell valves appeared

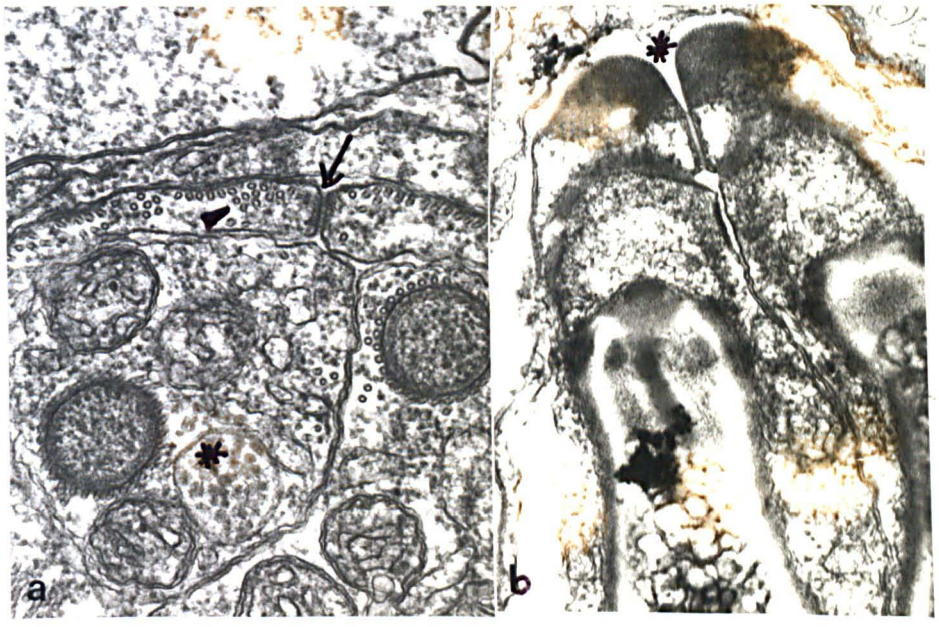

Fig 4.24 Developing capsulogenic and valvogenic cells. (a). Section of developing capsulogenic cell. Electron dense suture (arrow), large number of microtubules (arrow heads) scattered near suture. Note capsulogenic cell contains membrane bound vesicles (*) with electron dense bodies inside. Mitochondria (m) round, large with tubular cristae; (b) longitudinal section of anterior end of valvogenic cell showing desmosomal suture (arrow). Electron dense tips of valvogenic cells are separated (*) (x54,600).

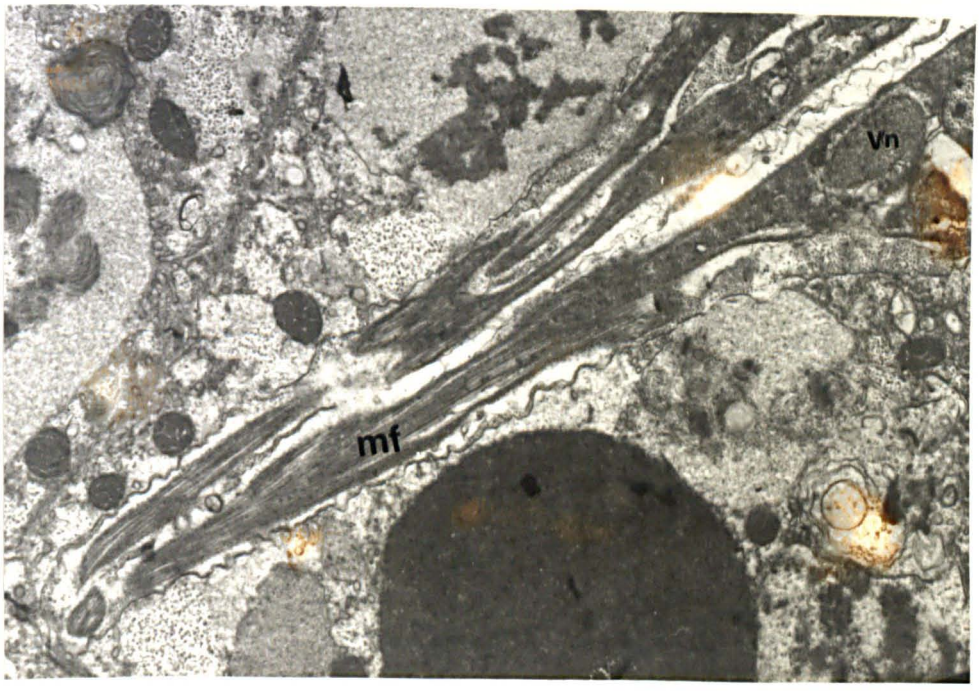

Fig 4.25 Section of caudal processes. Note microfilament (mf) of caudal processes. Nucleus of valvogenic cell (Vn) lies at the base of the caudal processes (x14,400).

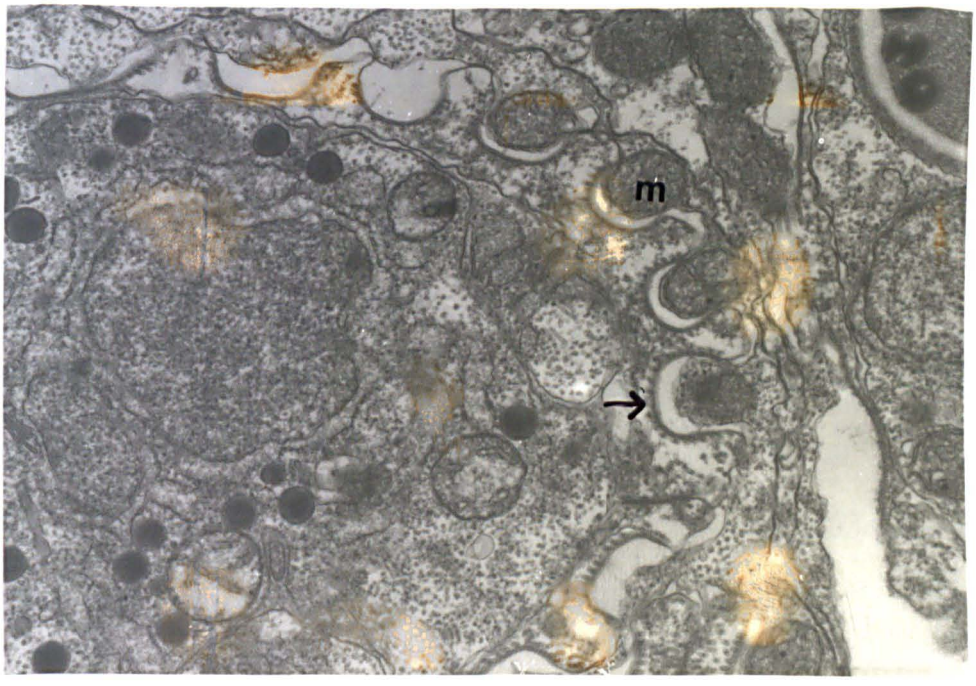

Fig 4.26 Surface ridges of the valvogenic cell (x27,440). Note mitochondria (m) of enveloping cell lodged within the grooves between ridges. Single layer of microtubules in the grooves (arrow).

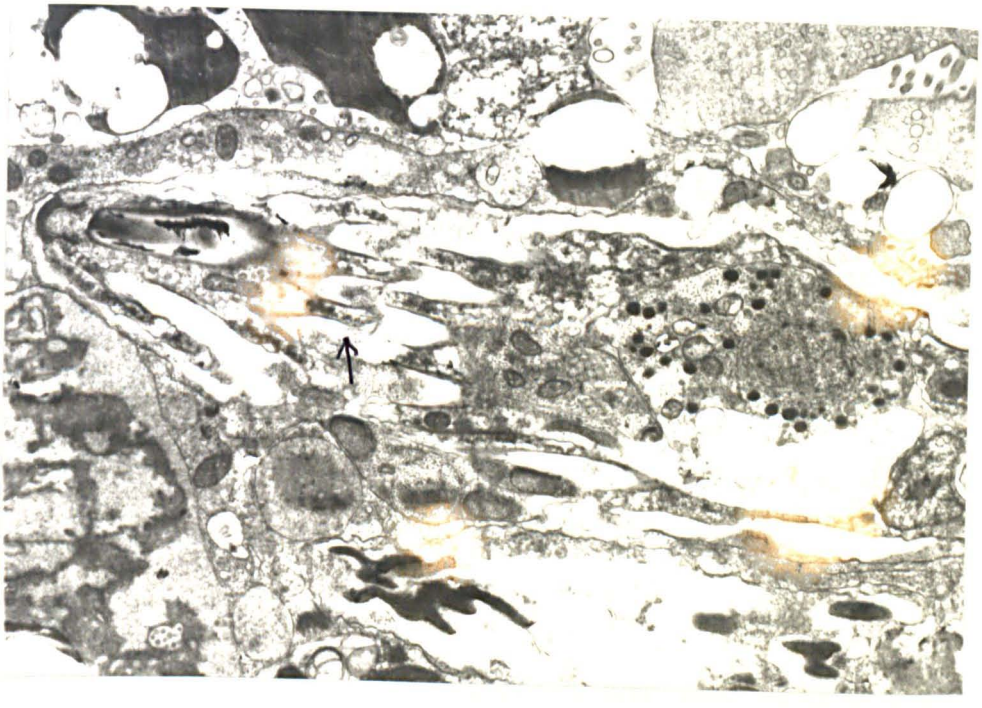

Fig 4.27 Section of the spore showing interlocking pattern of surface ridges and grooves (arrow) (x5,500).

as an electron dense thin double layer with a central narrow electron lucent zone (Fig.4.22).

4.3.2.2 Ultrastructural Development of Polysporous Plasmodia.

Early Stages

The earliest developmental stages observed in the tubular lumen, which appeared to develop into polysporous plasmodia, were irregular in shape with many finger like pseudopodial extensions projecting from the surface (Fig.4.28). The cytoplasm was not differentiated and it contained many large round and elongated mitochondria with tubular and plate like cristae, a few large vacuoles and some electron dense areas. The nucleus was not clearly detectable (Fig.4.28).

As the growth of these plasmodia proceeded they became more regular in shape, elongated or rounded with shortened surface extensions. Their polysporous nature was indicated by the presence of more than one generative cell and vegetative nuclei. Mitochondria increased in number and the cytoplasm became heavily vacuolated. The contents of the vacuoles sometimes had a frothy appearance (Fig.4.29).

Late Stages

The later stages of polysporous plasmodia were recognised by the presence of developing pansporoblasts (Fig.4.30). Their cytoplasm contained large round and oval mitochondria with plate like and tubular cristae respectively. Electron dense lipid inclusions of different sizes were common. The cytoplasm also contained vacuoles of different sizes with poorly defined electron dense and electron lucent substances. Some vacuoles appeared empty. Flat cisternae

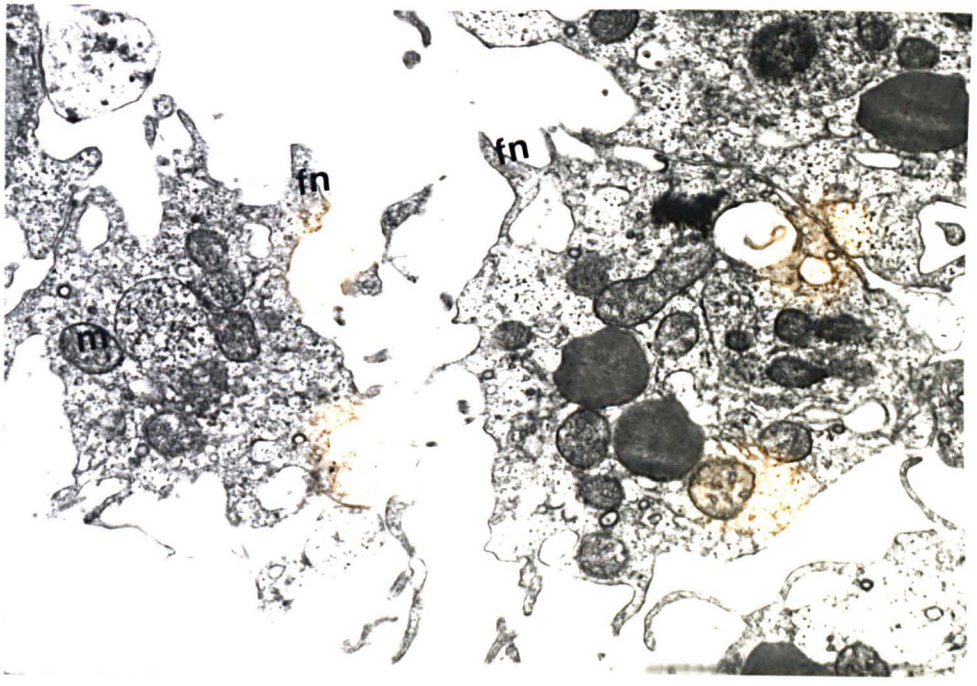

Fig 4.28 Early polysporous plasmodia within the lumen of the kidney tubules. Finger-like cytoplasmic processes (fn) extending from the surface. Large round mitochondria (m). Note nucleus is not detectable (x17,200).

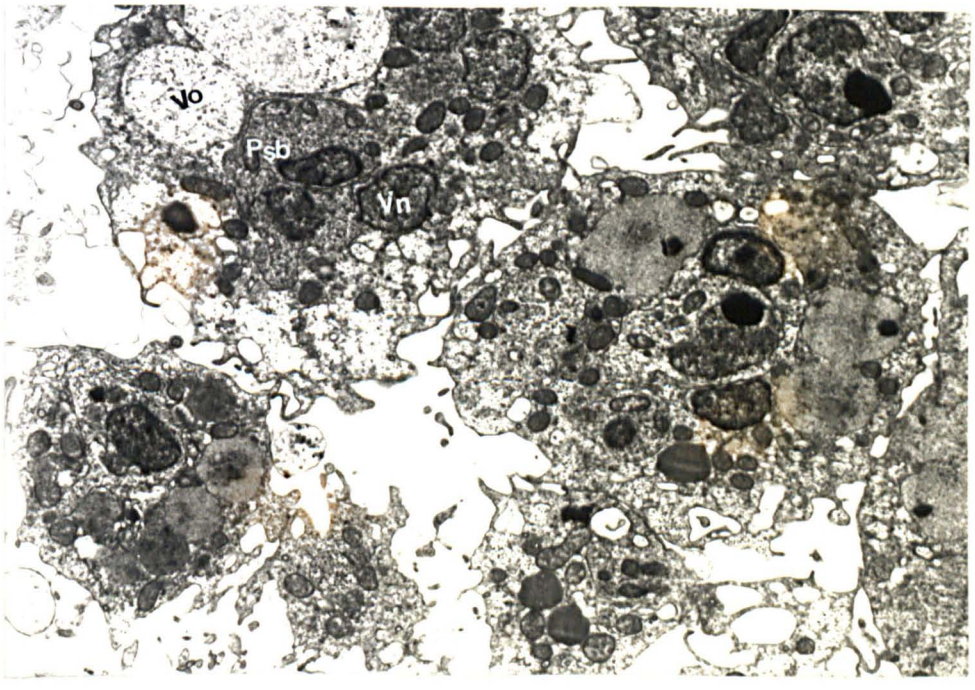

Fig 4.29 Polysporous plasmodia with a number of vegetative nuclei (Vn), pansporoblasts (Psb) and vacuoles of different types (Vo) (x9,460).

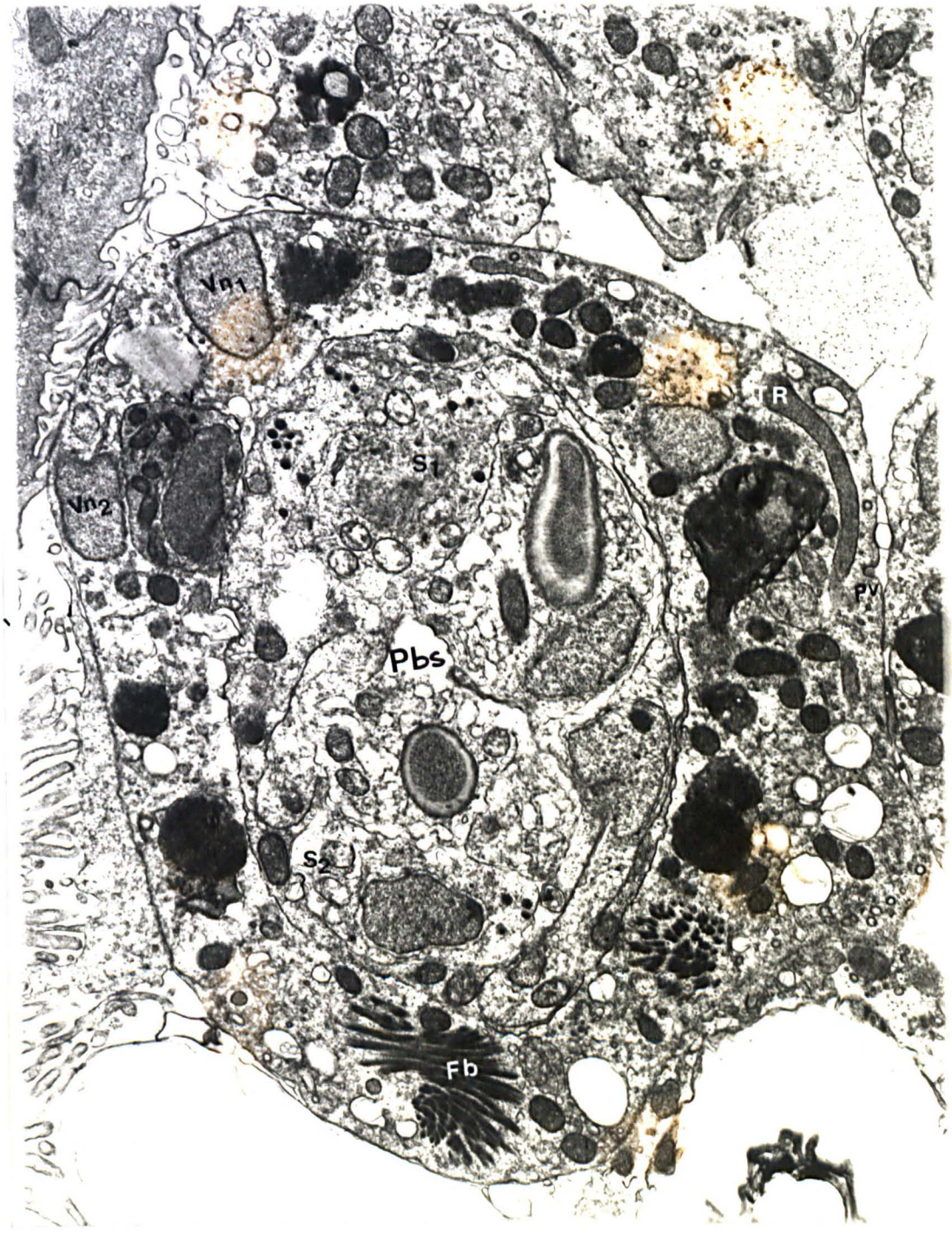

Fig 4.30 Polysporous plasmodia with pansporoblast (Pbs) containig two developing spores (S_1 & S_2), two vegetative nuclei (Vn_1 & Vn_2), generative cell (Gc). Note rod shaped tubular structure (TR), electron dense fibrillar bundles (Fb), pinocytotic vesicles (Pv) (x40,000).

of endoplasmic reticulum and numerous free ribosomes gave a darker appearance to the plasmodial cytoplasm. The Golgi apparatus was not discernable in the cytoplasm. The surface of the plasmodium was bounded by a single cell membrane. Invagination of the plasmodial wall to form pinocytotic vesicles was observed. Pinocytotic vesicles were also observed at the periphery and in the deeper cytoplasm of the plasmodium (Fig.4.30). A membrane bound elongated tubular structure with an electron dense inner coat was frequently observed in the cytoplasm. The appearance of cross and longitudinal areas of the tubular structure in the same plane of section showed that it may form several loops in the cytoplasm. Frequently some bundles of electron dense fibrils at the periphery of the cytoplasm near the cell membrane were observed.

4.3.2.3 Sporogenesis in Polysporous Plasmodia

Sporogenesis in polysporous plasmodia was initiated by the formation of the pansporoblast. Only the generative cells were involved in this process.

Generative Cell.

These were round to slightly elongated in shape with an average diameter of 20μm (n=10) (Fig.4.31). The nucleus was large and elongated in size with a dark staining, round, eccentric nucleolus. The nuclear membrane was not well developed and seemed loosely bound by endoplasmic reticulum. The cytoplasm contained a few mitochondria which were mostly round in shape,some free ribosomes and scattered endoplasmic reticulum and a Golgi apparatus close to the nucleus. The nucleolus was not always discernable. The generative cell was bounded by a single unit membrane. The cytoplasm of the generative cell appeared

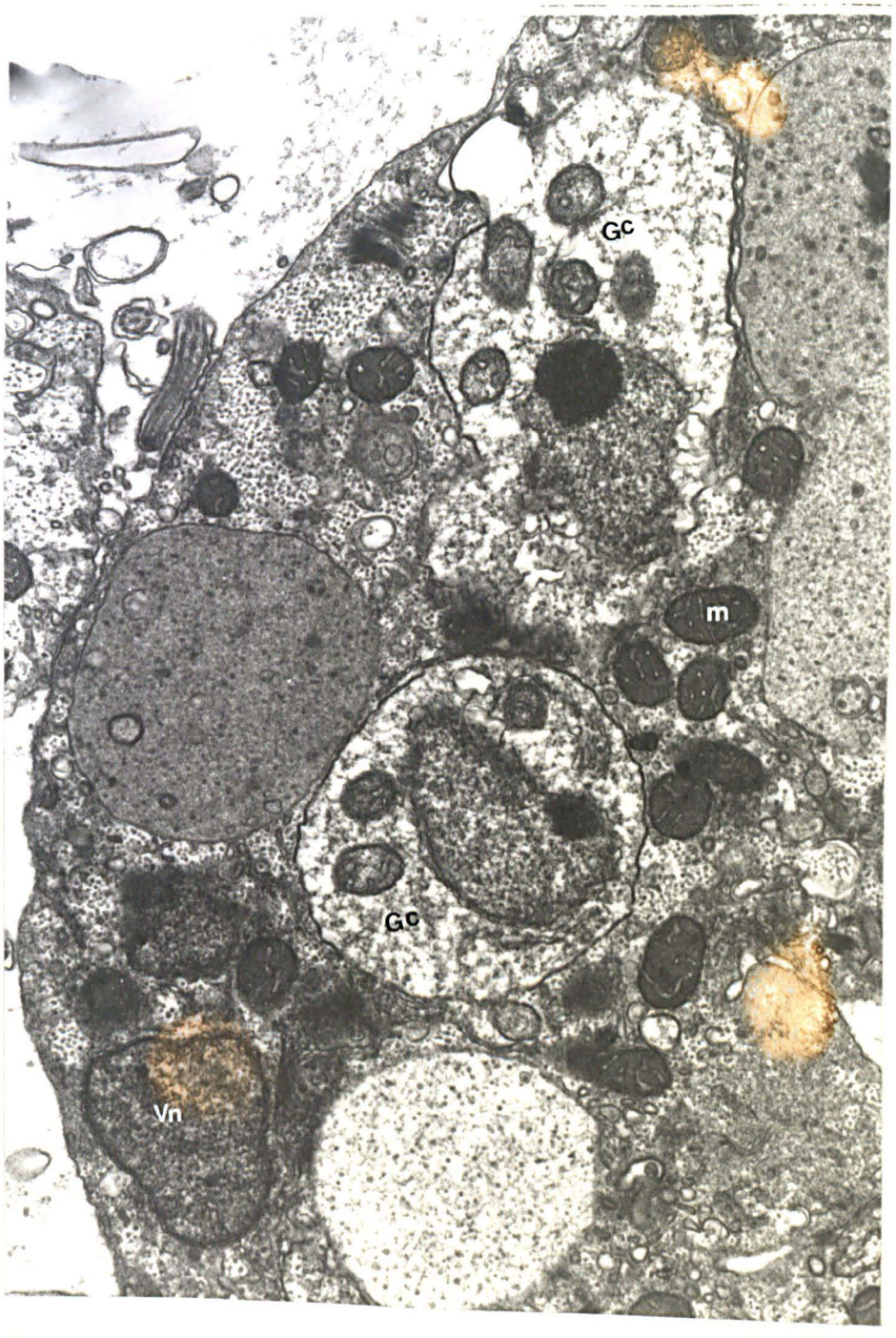

Fig 4.31 Section of polysporous plasmodia showing generative cells (Gc), vegetative nuclei (Vn) and mitochondria (m) (x14,700).

lighter than that of the plasmodium.

Formation of Pansporoblast.

Pansporoblast formation started with the envelopment of one generative cell by another (Fig.4.32). The enveloping cell acted as a pericyte and the enveloped cell as a sporont cell. The cytoplasm of the pericyte contained numerous scattered rough endoplasmic reticulum, free ribosomes and large round mitochondria and an elongate nucleus. In most cases a distinct nucleolus in the two pansporoblast cells was not detected during and after pansporoblast formation. After completion of encirclement of the sporont cell by the pericyte, another sporont cell started to form within it by endogenous division (Fig.4.33). The sporont cell cytoplasm contained large elongated mitochondria, scattered ribosomes and a small developing nucleus. At this time pericyte cytoplasm showed clusters of endoplasmic reticulum. Thus, each pansporoblast acquired two sporont cells which subsequently divided to create two complete spores. Each sporont cell divided to form 5 sporoblast cells. Thus 10 sporoblastic cells were formed from 2 sporont cells within the disporous pansporoblast (Fig.4.34). The sporoblastic cells differentiated to form two capsulogenic cells, two valvogenic cells and one binucleated sporoplasmic cell, which then further differentiated and organized to form 2 spores. The whole process of sporoblast differentiation and their arrangement seemed similar to that seen in mono and disporous plasmodia.

4.3.2.4 Ultrastructural Variations in Different Plasmodia.

The mono, di and polysporous plasmodia each showed some distinguishing characteristics. Mono and disporous plasmodia usually contained one vegetative nucleus and one or two

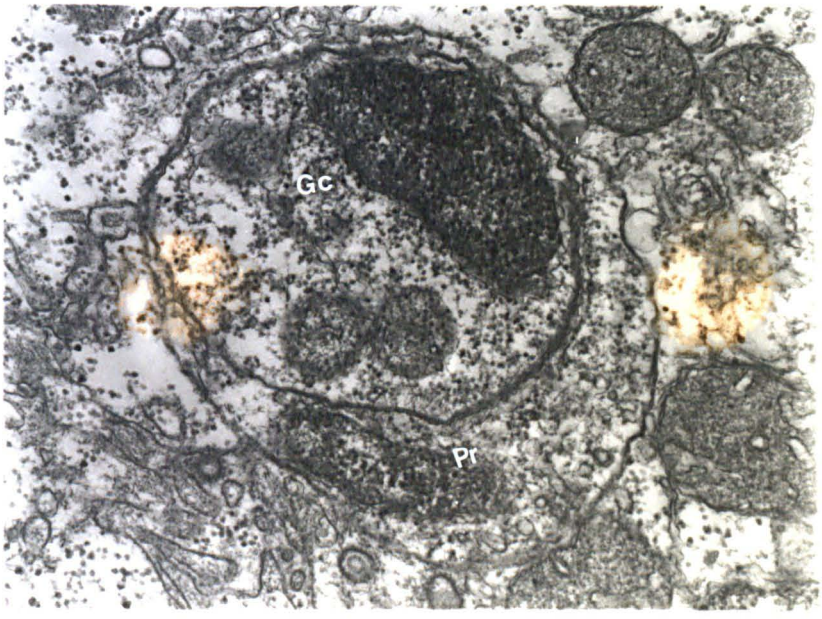

Fig 4.32 Pansporoblast formation with pericyte (Pr) enclosing one generative cell (Gc) (x36,400).

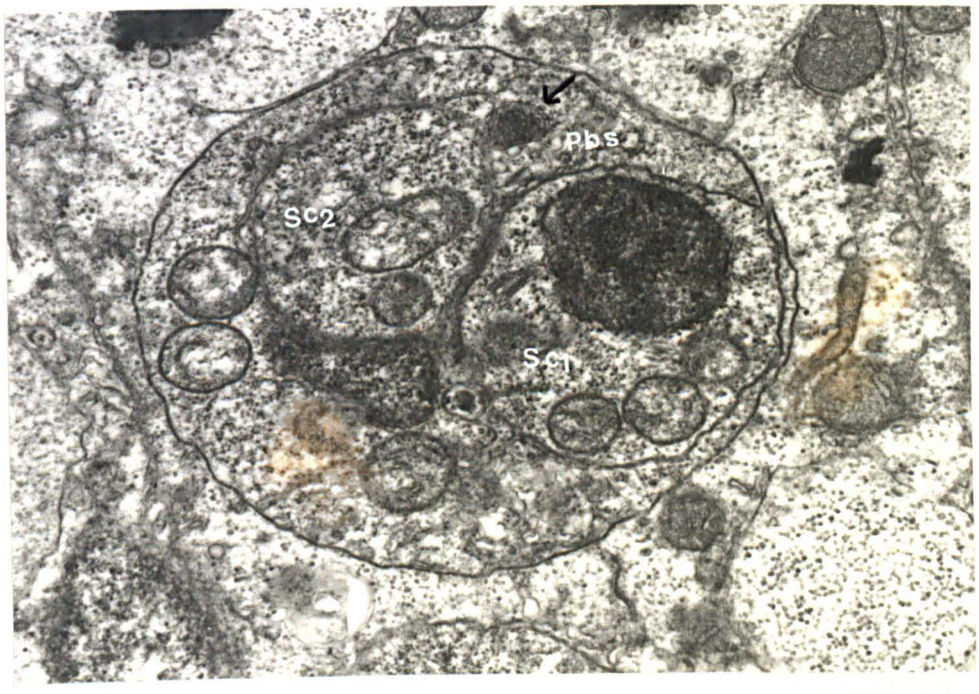

Fig 4.33 Division of sporogonic cell (Sc1) in pansporoblast (Pbs) to form another sporont cell (Sc2). Note cluster of endoplasmic reticulum (arrow) in the cytoplasm of pansporoblast (x38,000).

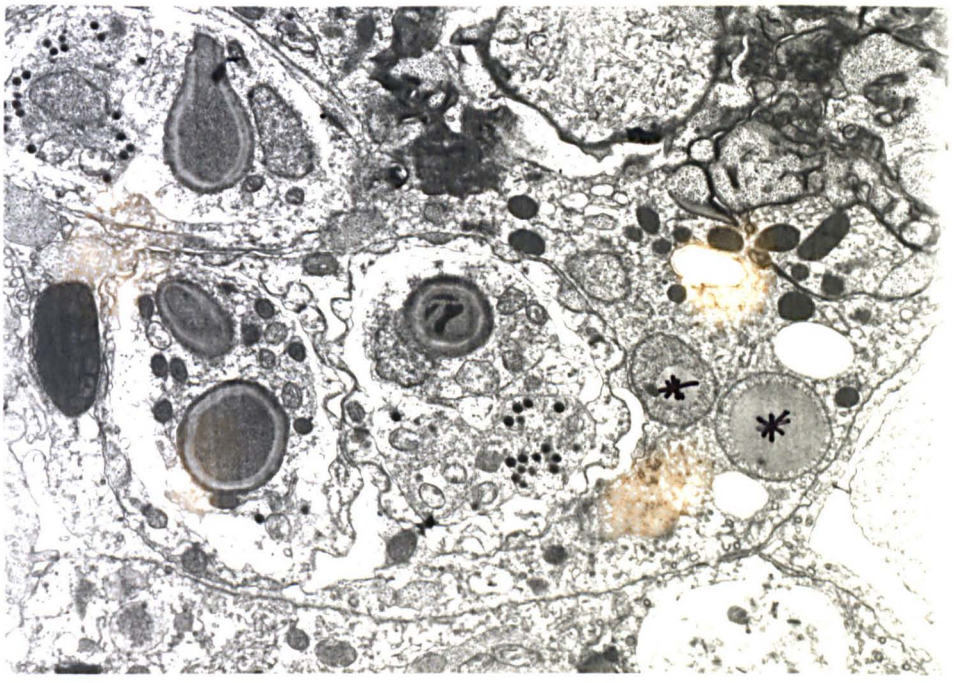

Fig 4.34 Polysporous plasmodia with ER bounded vesicles (*) some empty vacuoles (arrow head) and developing pansporoblasts (x11,800).

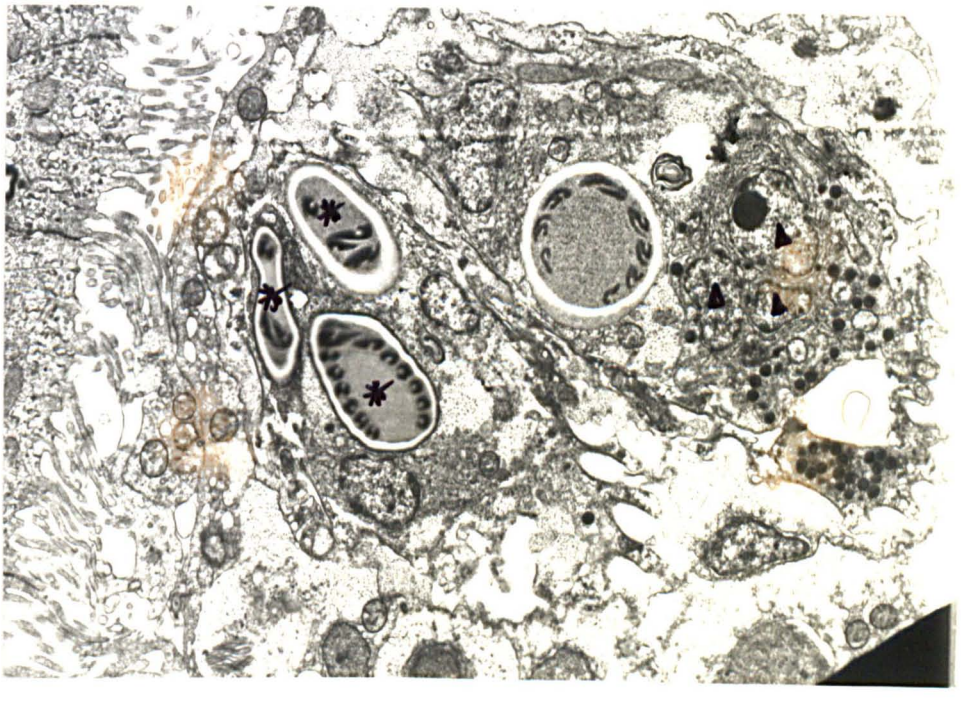

Fig 4.35 Spores with three polar (*) capsules and three sporoplasmic cells (arrow head) (x10,620).

sporogonic cells, but polysporous plasmodia contained more than one vegetative nucleus and many generative cells. In other respects the organelles shared almost the same characteristics as those of mono and disporous plasmodia. All the plasmodia had similar kinds of vacuoles filled with poorly defined electron dense or electron lucent substances. Ectoplasm and endoplasm were not differentiated in all three types. The surface wall of the plasmodium was made up of a thin single unit membrane (Fig.4.30). Pinocytotic vesicles were found in different stages of formation below the pellicle by invagination of the latter into the cytoplasm. Some pinocytotic vesicles were also found in the deeper cytoplasm. A rod shaped elongated structure was again seen in the cytoplasm forming several loops. An electron dense collection of fibrous strands was frequently observed in polysporous plasmodia.

Polysporous plasmodia were generally found in the lumen of the kidney tubules, whereas mono and disporous plasmodia were also found in Bowman's spaces and interstitial spaces. Disporous plasmodia which developed outside the tubular lumen were oval shaped, with irregular outlines. Their cytoplasm was less vacuolated than the plasmodia in the tubules. In some polysporous plasmodia inside the tubular lumen vesicles containing an electron lucent inner core encircled by an outer granular area and bounded by ER were found (Fig.4.34). In some disporous plasmodia single spores were found with three polar capsules and 3 sporoplasmic cells (Fig.4.35).

4.3.2.5 Abortive Development of Plasmodia.

Some plasmodia did not develop normally. In such plasmodia the sporoplasm nuclei and a deformed polar capsule were found in an incomplete membrane bound structure containing

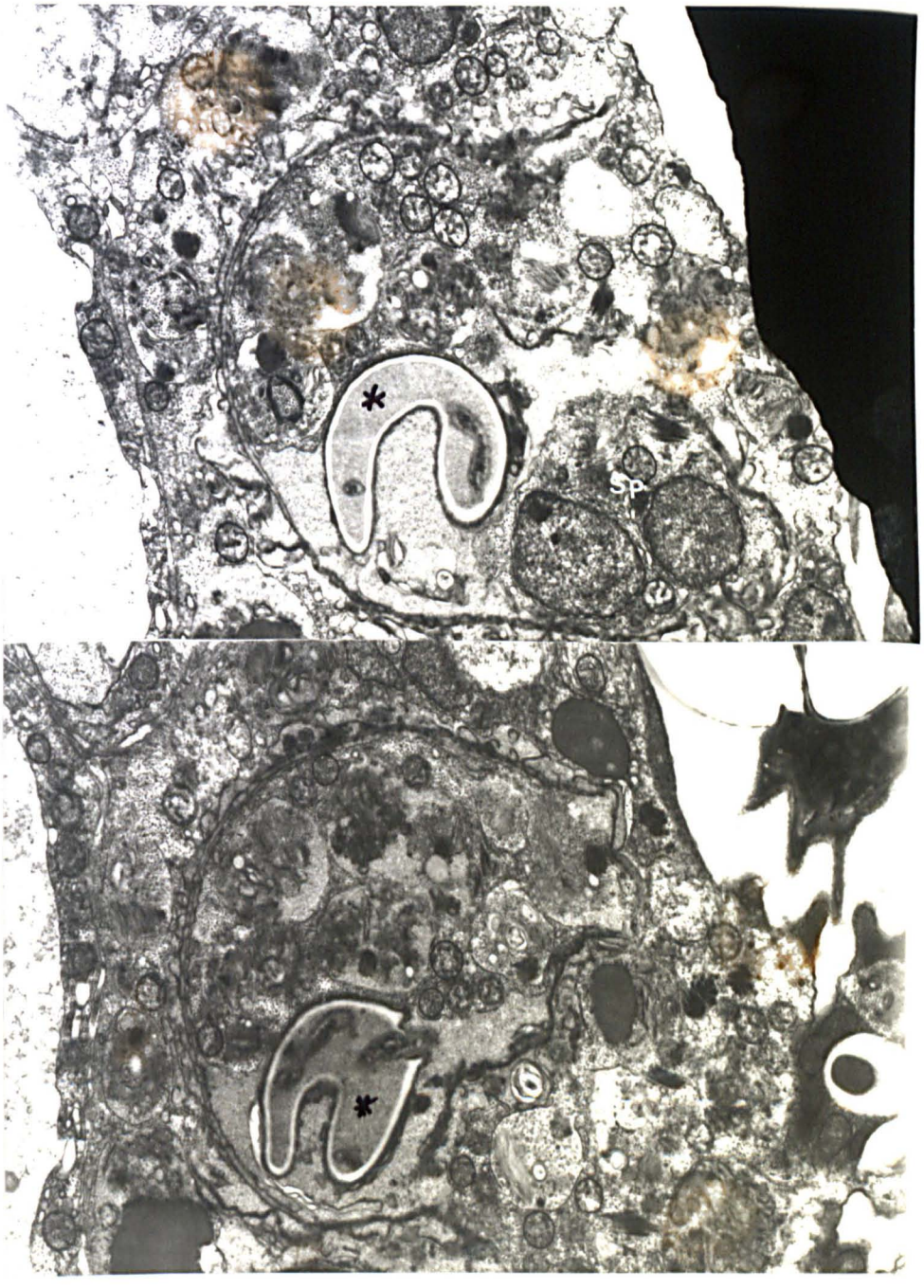

Fig 4.36 Abortive development of spores within the plasmodium, (*) deformed polar capsules, (Sp) binucleated sporoplasm (x12,000).

vesicles filled with electron dense amorphous substances (Fig.4.36a,b).

4.3.3 Scanning Microscopy of Spore and Plasmodia

Scanning electron micrography of the mature spore clearly revealed its surface topography. In sutural view the spore was spindle shaped with a sutural ridge running between the two polar capsules (Fig.4.37). In valvular view the spore was convex on one side and flattened at the other (Fig.4.38). The sutural ridge formed by the fused thick edges of the valves was straight and prominent. Both spore valves had 6 to 9 extrasutural ridges of more or less equal thickness running parallel to the sutural line. The two shell valves did not meet anteriorly and posteriorly they extended to form two caudal appendages. The thick sutural line ran posteriorly between the two caudal appendages. The extrasutural ridges converged at the anterior tapered end of the spore, and posteriorly they extended discontinuously into the caudal appendages. In one case an extruded coil of polar filament was visible and the filament appeared smooth.

Plasmodia of the spores were round to irregular in shape with various globular bodies on the surface, giving the plasmodia a rough appearance (Fig.4.39). The caudal appendages of the mature spores were frequently observed protruding outside the plasmodia.

4.3.4 Host Attachment and Pathogenicity

Histological study showed that large numbers of plasmodia caused dilation of the tubular lumen and flattening of the epithelial cells (Fig.4.40). Electron microscopy showed that

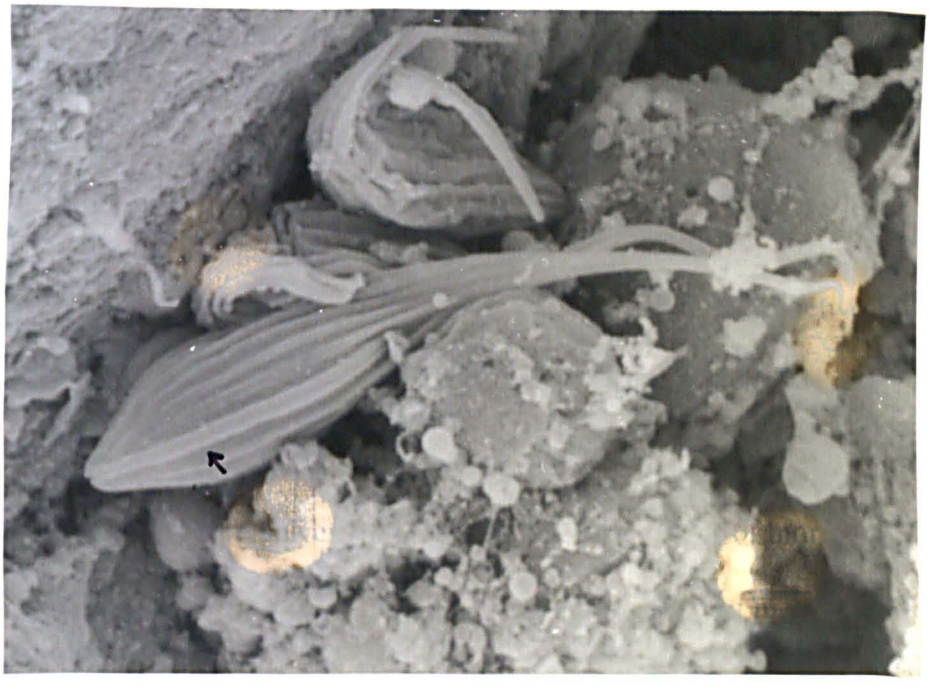

Fig 4.37 SEM of mature spore. Note sutural line (arrow) formed by the fused sutural ridges of each valve and running between the caudal processes (x8,750).

Fig 4.38 SEM of spore. Valvular view (dorsal view). Note surface ridges (arrows). One side of the spore is flattened (arrow head). Polar filament (Pf) is smooth. Some of the ridges extend into caudal processes(Cp) (x8,775).

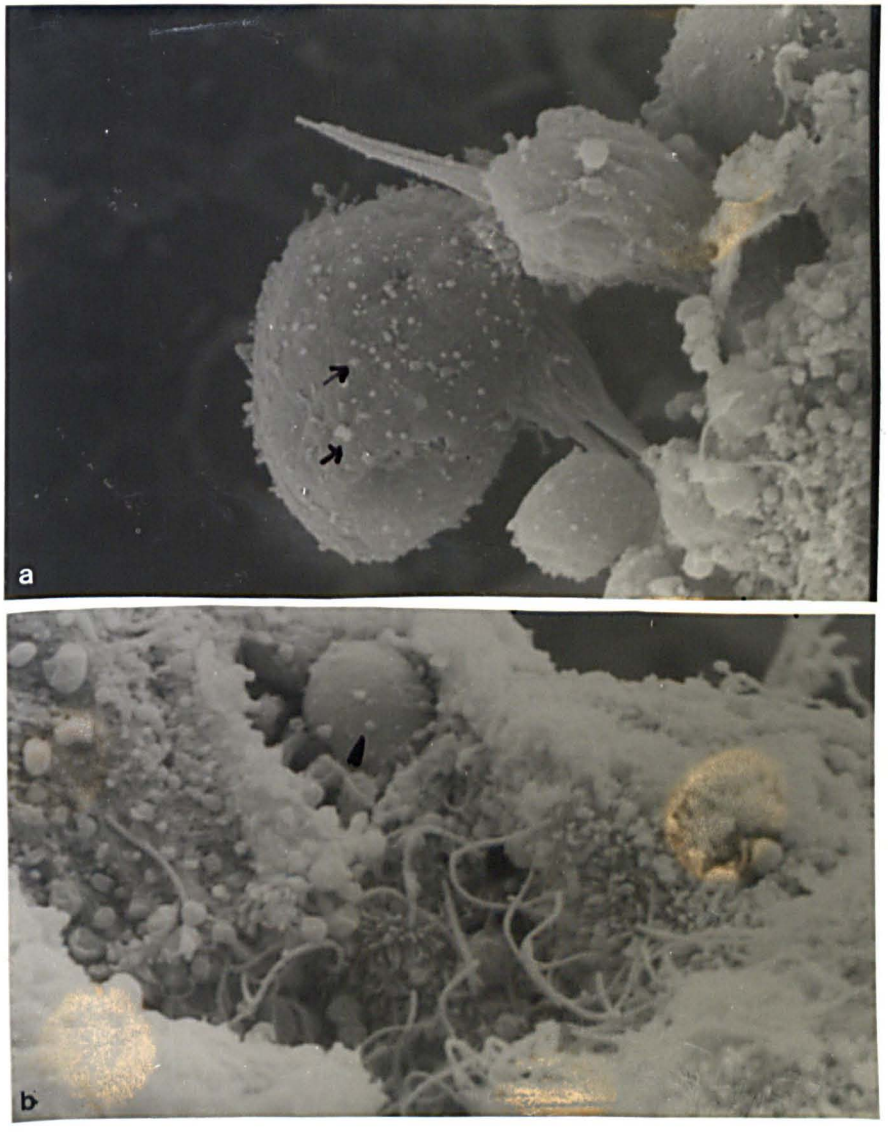

Fig 4.39 SEM of plasmodium surface showing globular bodies (arrows): (a). Caudal processes of mature spore protrude from the plasmodium (arrow heads) (x6,250). (b), Plasmodium inside the tubular lumen of kidney (arrow heads)(x5,000).

plasmodia were firmly attached to the epithelial brush border of the tubular wall. Long finger-like surface extensions of the plasmodial wall entered into the epithelial cells of the tubular wall (Fig.4.41). The point of attachment between the parasite and the epithelial cell was electron dense and the area of the epithelial cell near the site of parasite attachment became vacuolated (Fig.4.42).

Developmental stages of the parasites were not usually found in the epithelial layer of the tubules. However, occasionally very early pre-sporogonic stages of the parasites were found in the epithelial cells, the nuclei of which were pyknotic. The parasites were found in vacuoles within the epithelial cell and were attached to the wall of the vacuole by their surface extensions (Fig.4.43).

In heavy infections it was observed that early pre-sporogonic stages accumulated between the basement membrane and the epithelial layer pushing the latter into the tubular lumen (Fig.4.44). The epithelial cells showed vacuolation around the host cell nucleus and the parasites. Some infected tubules showed a huge vacuolated area in the tubular wall with cellular necrosis (Fig.4.45). Intraluminal stages also caused destruction of the epithelial brush border. There was an increased number of rodlet cells within the tubular epithelium (Fig.4.46).

In Bowman's space of the glomeruli parasite stages caused vacuolation and degeneration of the basement membrane (Fig.4.47). In heavy infections parts of the kidney occasionally became a mass of parasites and cell debris with complete destruction of host cells (Fig.4.48). The pyknotic deformed nuclei of host cells were hardly recognisable. It is not

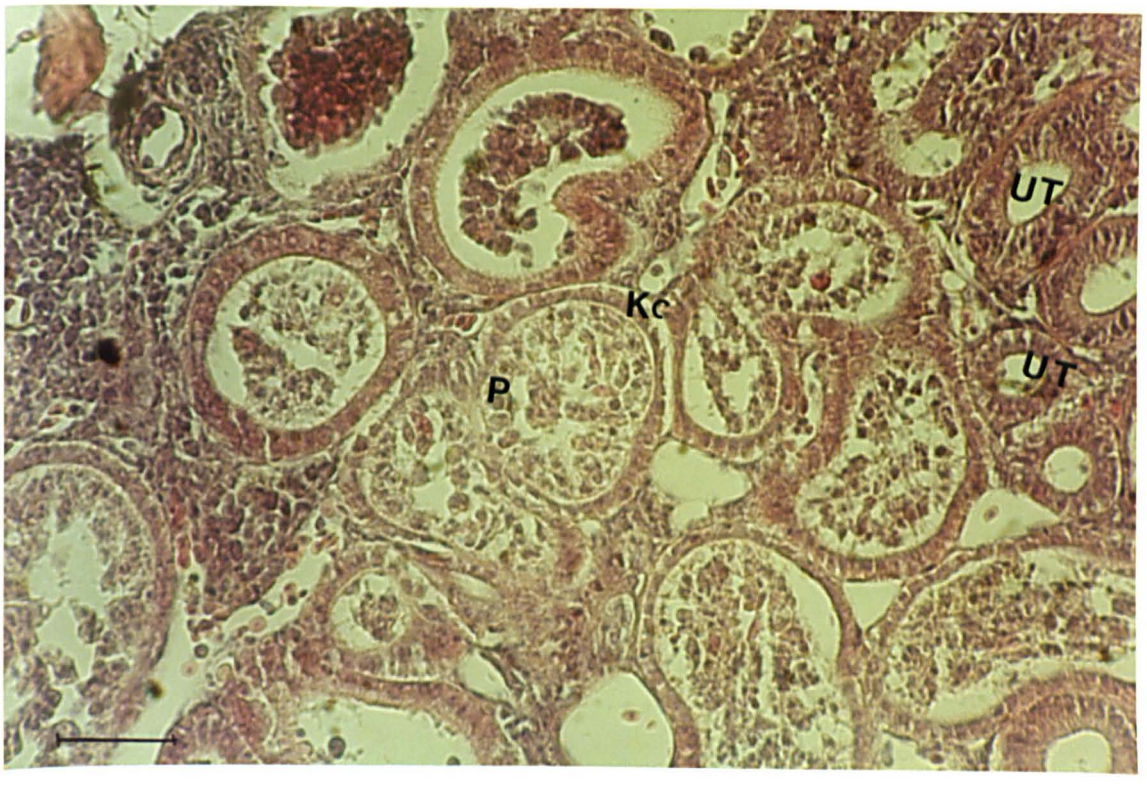

Fig 4.40 Histology of kidney showing parasites (P) in the tubular lumen. Lumen became dilated with flattening of epithelial cells (Kc). Some uninfected tubules (UT) can also be seen. (H&E bar=10μm).

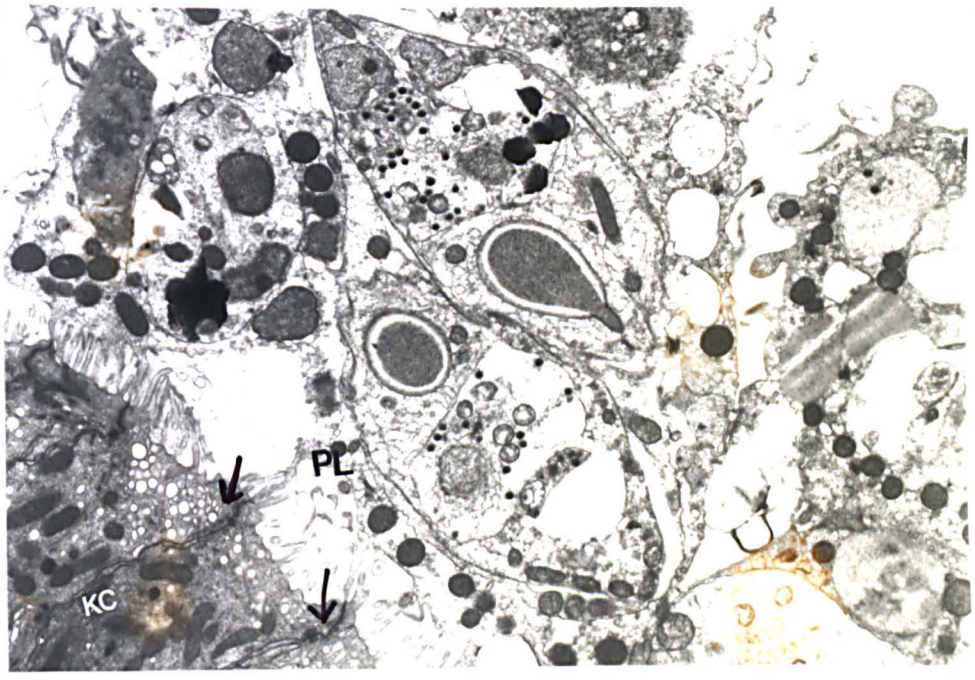

Fig 4.41 Attachment of plasmodium (PL) with the host cell (KC) by its cytoplasmic processes. Note attachment point has increased electron density (arrows) and host cell is vacuolated (arrow heads) (x7,740).

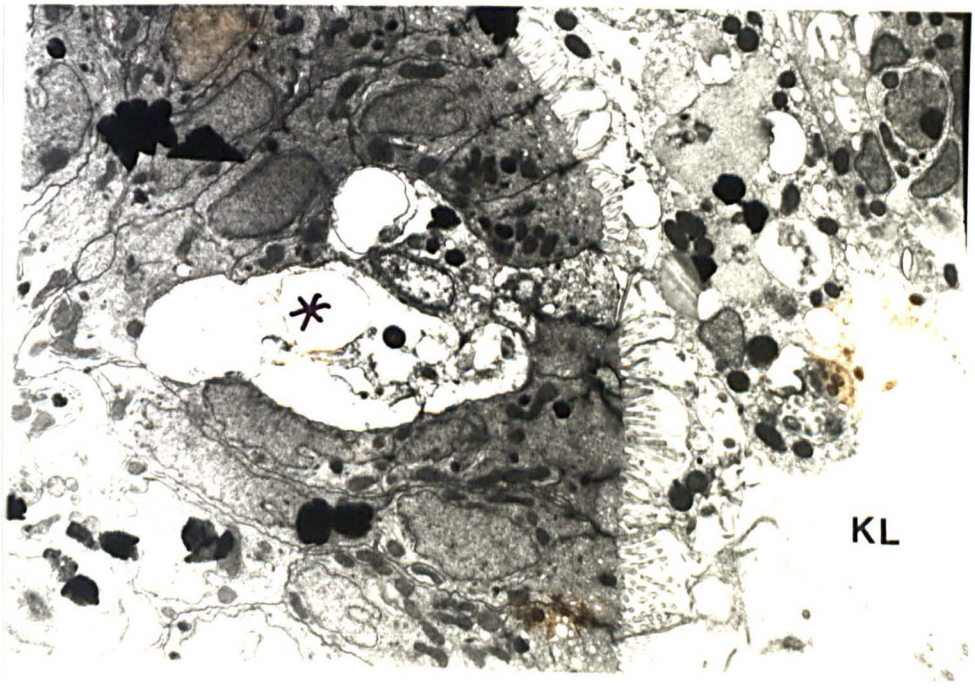

Fig 4.42 Intracellular early parasitic stages (Ps) in a kidney epithelial cell (KC). Nucleus of the affected cell has disappeared. Large vacuolations occur around the parasite (*). Adjacent host cell nucleus is pyknotic (arrow head). Cytoplasm of the parasite is about to drop into the tubular lumen (Kl) (x6,840).

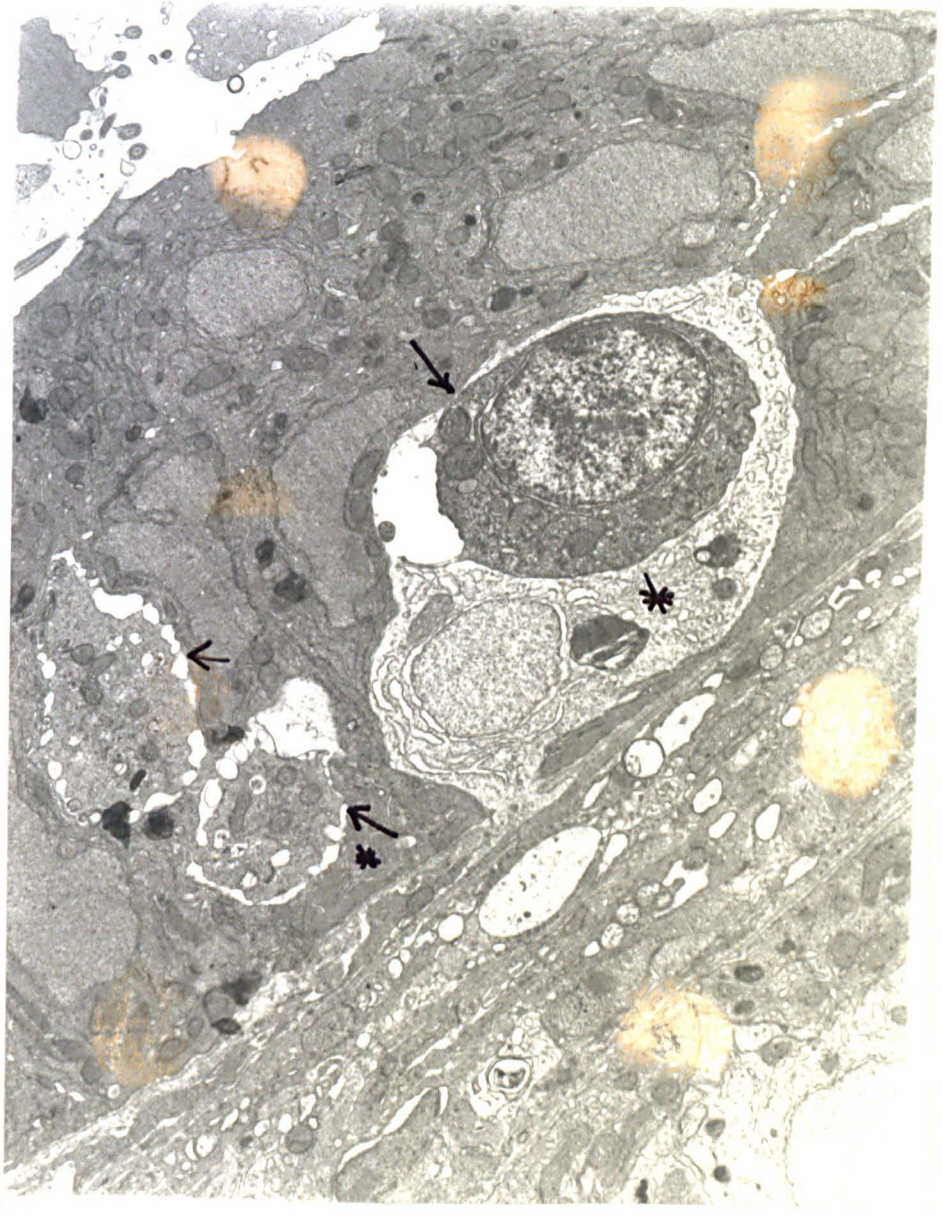

Fig 4.43 Intracellular developmental stages of *M. gasterostei* (arrow). Note host cell cytoplasm (*) is vacuolated and most cell organelles are lost (x9,460).

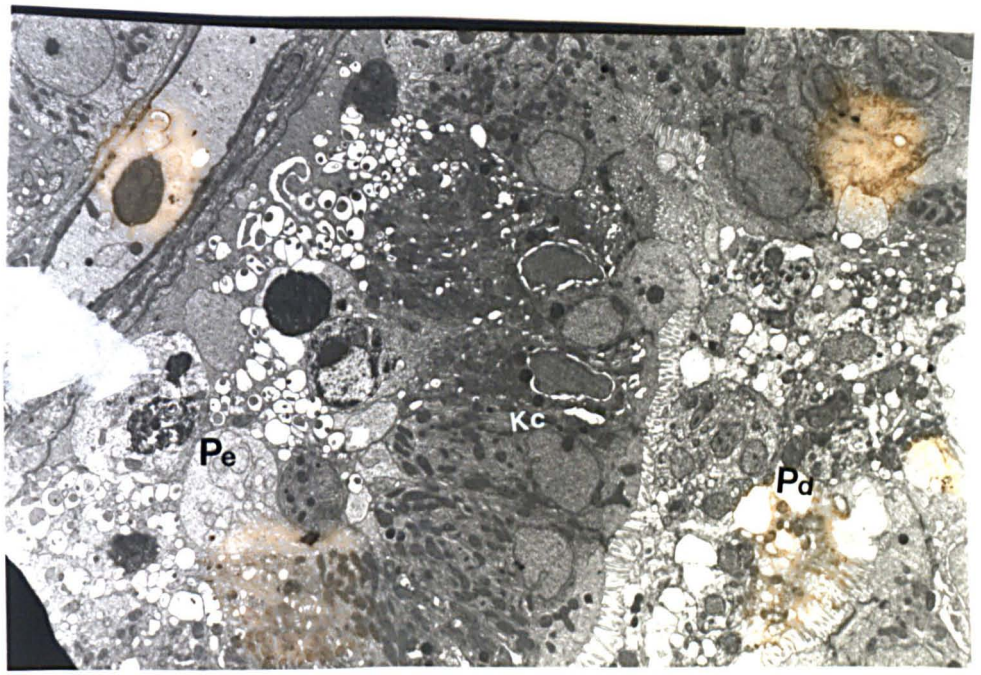

Fig 4.44 Early parasitic stages (Pe) under the basement membrane at the base of the epithelial cells of kidney tubule (Kc). Affected area vacuolated with dark inclusion bodies (arrow). Tubular lumen contains many parasites at different developmental stages (Pd) (x4,400).

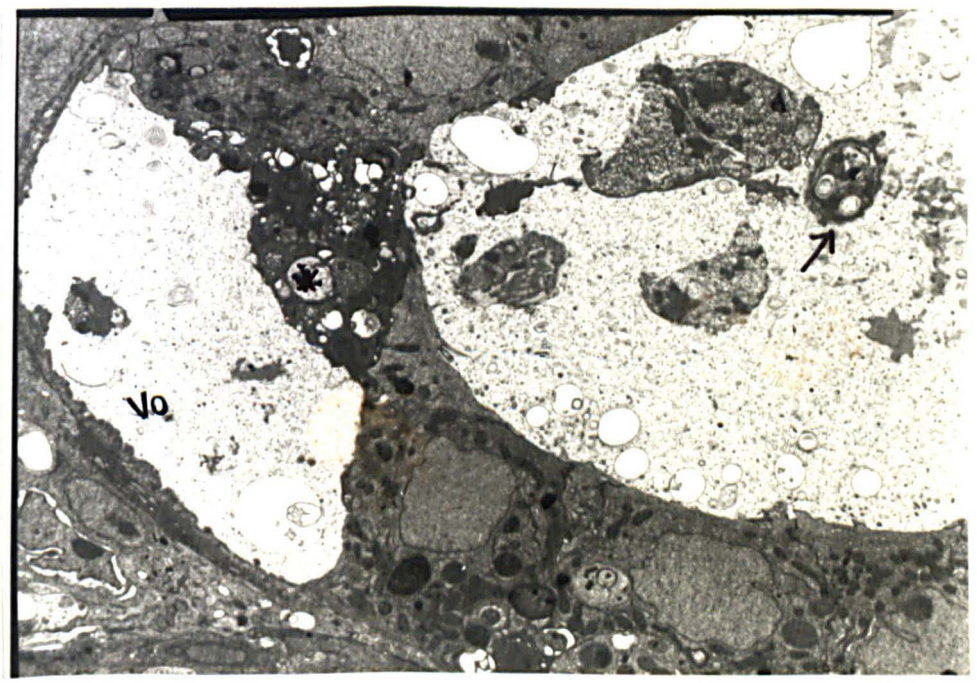

Fig 4.45 Vacuolation (Vo) and necrosis (*) of epithelial layer. Parasite in the tubular lumen (arrow) (x4,200).

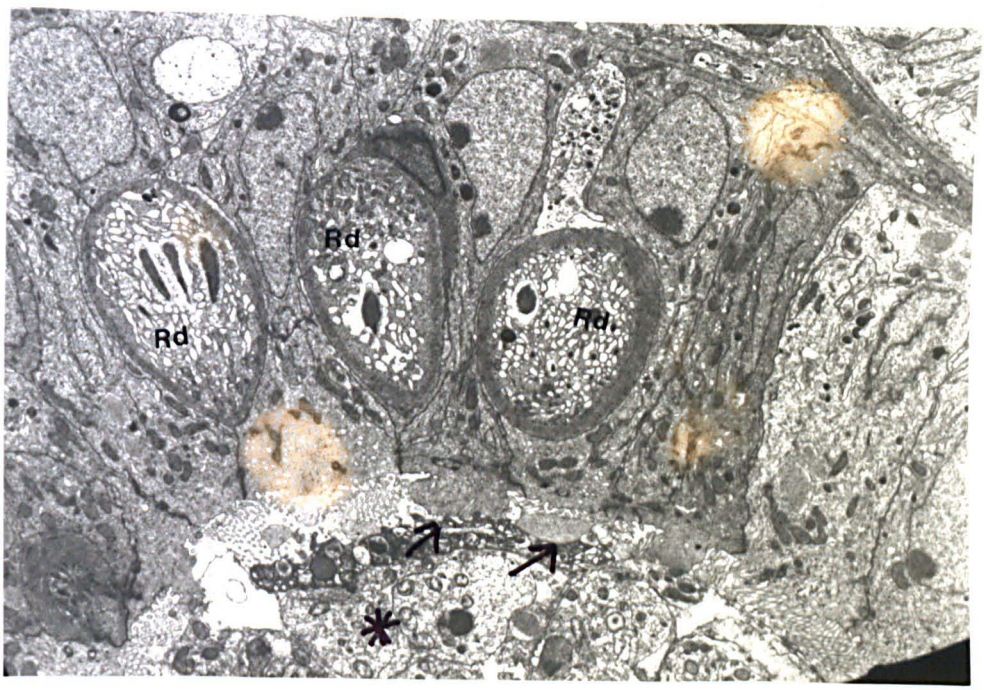

Fig 4.46 Number of rodlet cells (Rd) became increased in the epithelial layer of kidney tubule. Lumen of the tubule is full of parasites (*). Destruction of epithelial brush border (arrows) (x4,480).

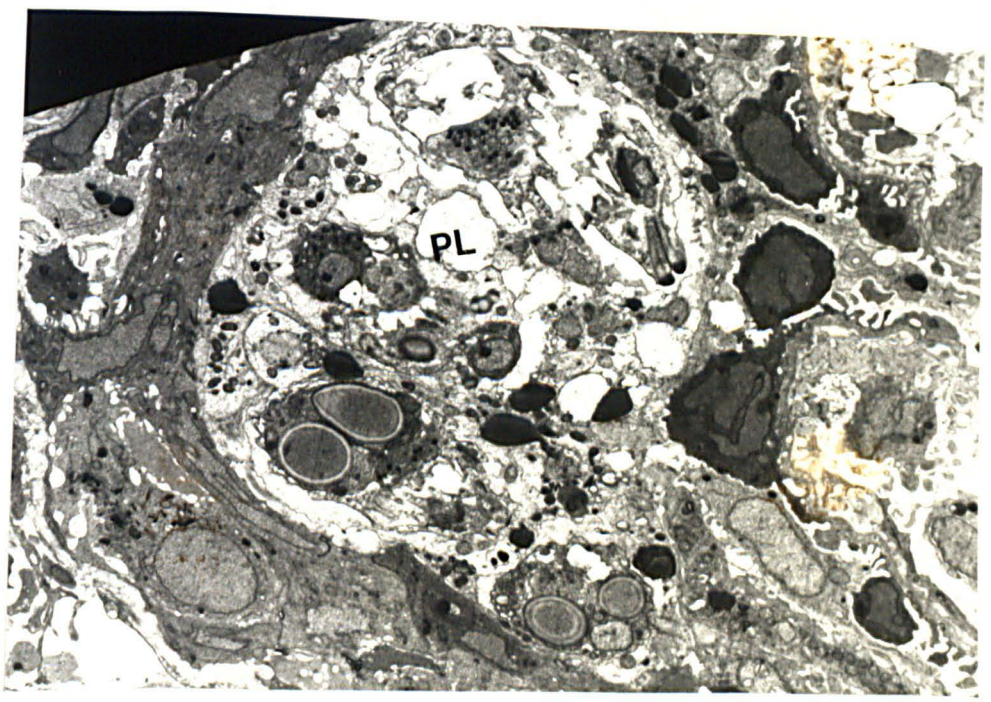

Fig 4.47 Polysporous plasmodium (PL) in the Bowman's space of the glomerulus (x5,140).

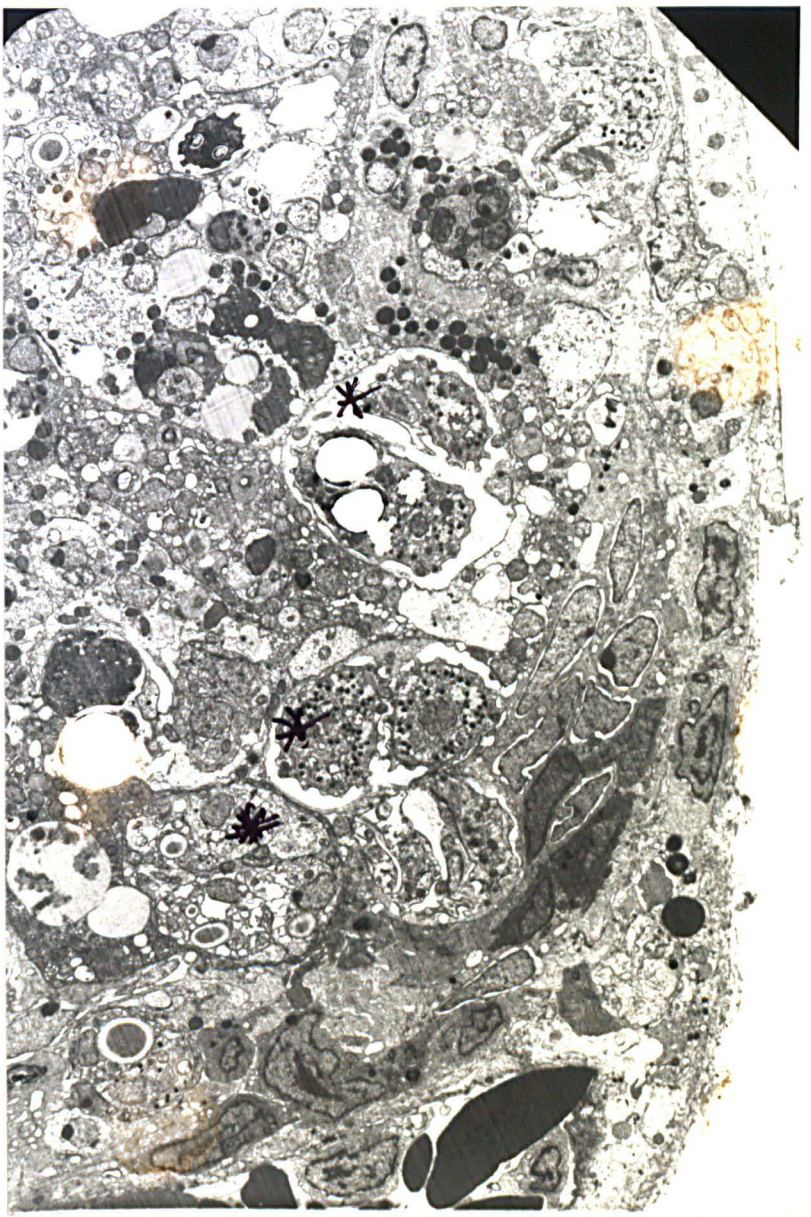

Fig 4.48 Parasites of different developmental stages (*) formed a huge mass in the kidney (x7,200).

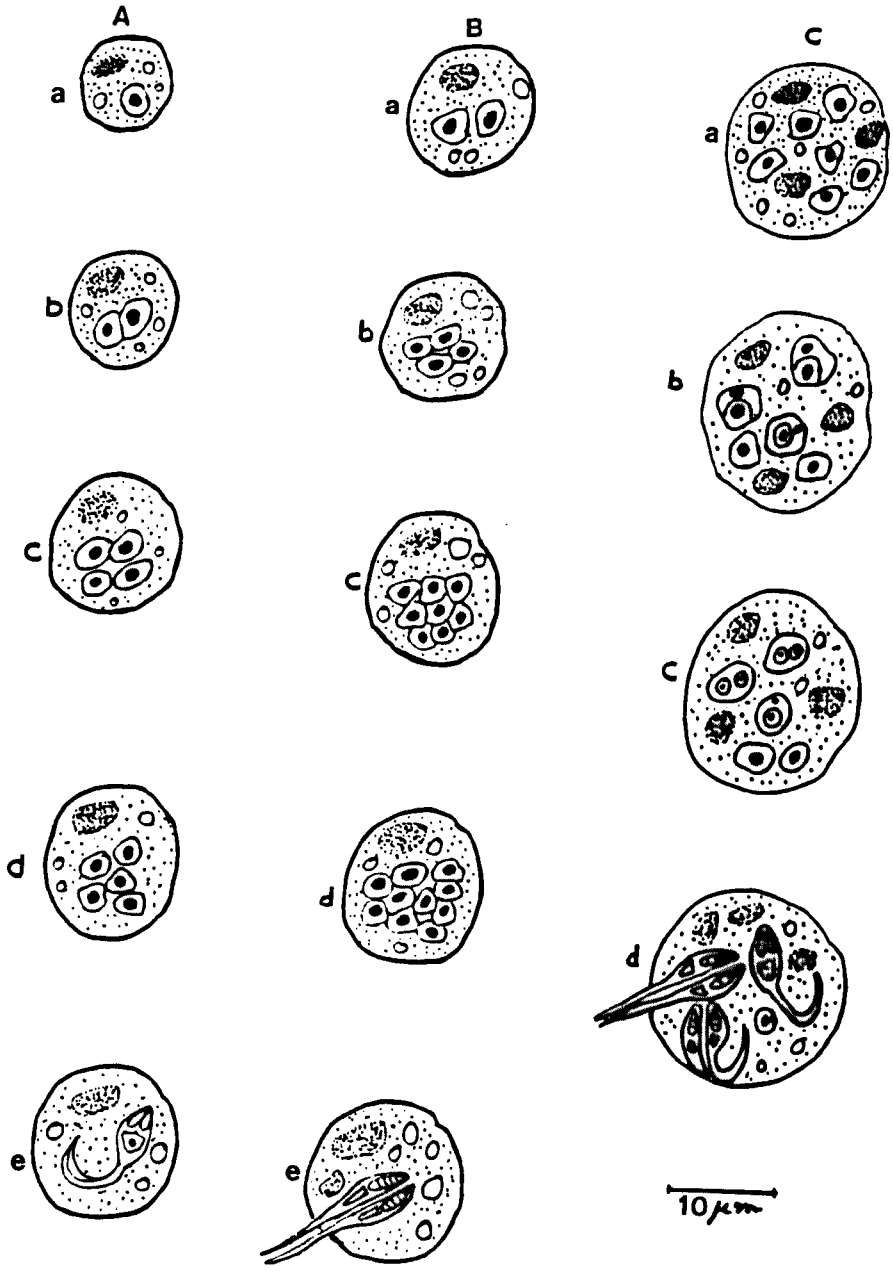

Fig 4.49 Drawings of the developmental cycle of *M. gasterostei* within the lumen of the kidney tubules as studied by light and electron microscopy.
A(a-e), development of monosporous plasmodia.
B(a-e), development of disporous plasmodia.
C(a-d), development of polysporous plasmodia. Note pansporoblast formation initiates sporogenesis (b).

clear whether these areas were derived from glomerular or tubular infections. Parasites were not found inside the capillary lumen of the renal corpuscles.

4.4 Discussion.

The developmental cycle of *M. gasterostei*, including the fine structure of the different developmental stages has been described in the present study. The parasite was mainly confined to the excretory part of the kidney. Although parasites were found in the Bowman's capsules and in the interstitial spaces of renal tubules, the lumen of the tubules seemed to be the most suitable site for parasitic development. Developmental stages were never found in the urinary bladder, but mature spores were occasionally observed. This is in contrast to the previous studies of Davis (1944) and Guilford (1965) who mentioned the common occurrence of spores and developing stages of *Myxobilatus* species in the urinary bladder of the host. Species of *Myxobilatus* have been subsequently reported from both kidney tubules and urinary bladder of the host (Arthur and Margolis, 1975; Molnár, 1988). The present observations are consistent with the original description of Parisi (1912, in Arthur and Margolis, 1975) who described *M.gasterostei* from kidney tubules (as *Henneguya*).

4.4.1 Light Microscopy.

The light microscopic structure of the parasite was similar to the description of Davis (1944) with monosporous, disporous and polysporous plasmodia (Fig.4.5). Davis (1944) described trophozoites (probably pre-sporogonic and sporogonic stages) of *Myxobilatus* as being very variable in shape, usually irregular or elongated with frequent bizarre forms. Davis (1944)

also described young stages which were rounded and slowly amoeboid and which later became attached to host cells by means of pseudopodia. This is very similar to the characteristics of the pre-sporogonic and sporogonic stages observed in this study. The irregular shape of sporogonic stages is due to the variable positioning of developing spores within the plasmodium. Younger stages are rounded in shape because little differentiation of the sporoblast cells has yet occured. The characteristics of pre-sporogonic and sporogonic stages are their surface globular refractile bodies (as seen in phase contrast microscopy) which Davis described as a coating of fine hair or bristles. Davis suggested such structures are characteristic of urinary bladder-dwelling myxosporea.

The morphological characteristics and development of *M. gasterostei* differs from descriptions of *Myxobilatus* spp. by other authors. Current and Booker (1981) demonstrated that *M. mictospora* within the urinary bladder of largemouth bass possessed three distinct forms of plasmodia which occurred seasonally. These were winter sheet like plasmodia and elongated finger like and spherical plasmodia that occurred in spring and summer. There was no information on the number of spores within these plasmodia. In *M. gasterostei* there were no such seasonal plasmodia. Molnár (1988c) described development of *Myxobilatus legeri* within the kidney tubule and urinary bladder from a range of cyprinid fish and observed development of the parasite in two stages, which were strictly seasonal. The author described intracellular vegetative proliferation in the epithelial cells of collecting tubules and plasmodial sporogony in the lumen of renal tubules, ureters and urinary bladder. *Myxobilatus gasterostei* did not have any seasonal vegetative proliferation within the epithelial cells. It would seem that *Myxobilatus* species may have a variety of developmental patterns.

Myxobilatus gasterostei forms three types of plasmodia. Lom and Dyková (1992) grouped those myxosporean plasmodia which form one, two or many spores as "mictosporic plasmodia". The polysporous plasmodia of *M. gasterostei* contained up to 8 spores. Molnár (1988c) observed up to 3 spores in *M. legeri* infecting the ureter and urinary bladder of white bream.

4.4.2 Mono and Di-sporous Plasmodia.

The earliest developmental stage found in mono and disporous plasmodia was a single sporogonic cell enclosed by another cell (Fig.4.8). Subsequent internal division of the sporogonic cell results in 5 sporoblast cells in monosporous (Fig.4.11) and 10 cells in disporous plasmodia (Fig.4.12). This is similar to the general sequence of spore development described for *Sphaerospora* species (Lom *et al.*, 1982, 1985a, 1985b; Desser *et al.*, 1983a; Hedrick *et al.*, 1990; Feist *et al.*, 1991; Sitja-Bobadilla and Alvarez-Pellitero, 1992, Supamattaya *et al.*, 1991, 1993) and for *S. elegans* as described in Chapter 3. This developmental sequence of mono and disporous plasmodia was further confirmed by electron microscopic observations. The general developmental pattern of myxosporean plasmodia producing one or two spores may be the same in all species.

4.4.3 Polysporous Plasmodia.

The polysporous plasmodia of *M. gasterostei* displayed characteristics of both histozoic and coelozoic myxosporeans. Lom (1969a) described a single unit membrane and villosities on the plasmodial surface of the coelozoic genus *Sphaeromyxa* infecting the gall bladder of

Litaris cyclopus and *Dasycottus* sp. which were very similar to those found in *M. gasterostei*. The formation of pinocytotic vesicles was also similar in both species. However, the plasmodial cytoplasm of *M. gasterostei* differed markedly from *Sphaeromyxa* in being undifferentiated. The cytoplasm of the plasmodia of *Sphaeromyxa* is differentiated into ecto- and endoplasm. The plasmodium of *Sphaeromyxa* has an amorphous sticky surface coating of the pellicular membrane (Lom, 1969a) which was not observed in the plasmodium of *M. gasterostei*. *M. gasterostei* commonly showed electron dense fibrillar bundles at the periphery of the cytoplasm (Fig.4.30), whereas *Sphaeromyxa* has a fine mesh work of simple fibrils concentrated at the ecto-endoplasmic boundary. The membrane bound tubular structure found in *M. gasterostei* can be compared with the ectoplasmic tubular elements seen in *Sphaeromyxa*. These tubular elements of *Sphaeromyxa* are variable in length and consist of a unit membrane and a more or less electron dense core and are often closely packed into irregular bundles. However, the tubular element of *M.gasterostei* appeared to be a single structure which formed several loops in the cytoplasm (Fig.4.30). The vacuoles of the plasmodium of *M. gasterostei* did not form a meshwork like that recorded in *Sphaeromyxa*.

The plasmodial structure of *M. gasterostei* was similar to that of other coelozoic species such as *Myxidium lieberkuhni* from urinary bladder of pike, *Esox lucius*, and *Henneguya psorospermica* from the gill capillaries of perch *Perca fluviatilis* (Lom and de-Puytorac, 1965) which are also bounded by a single, but highly folded unit membrane.

The major differences between the plasmodia of *M.gasterostei* and histozoic species lie in the structure of the plasmodial walls. The plasmodial wall of histozoic species may have single or double unit membranes, continuous with pinocytotic canals, that extend into the

cytoplasm of the parasite (Current and Janovy, 1977 1978; Desser and Paterson, 1978). These plasmodia also have different types of surface coat which was not the case in *M. gasterostei*. The type of pinocytosis seen in *M. gasterostei* was markedly different from that seen in histozoic species. In *M. gasterostei* pinocytotic vesicles formed by simple invagination of the outer membrane (Fig.4.30), whereas in histozoic species pinocytotic vesicles formed at the end of pinocytotic canals extending from the plasmodial membrane towards the endoplasm. Pinocytosis is presumably involved with the nutrition of the parasite, by taking host material inside the plasmodium (Current and Janovy, 1976). A common feature of both histozoic and coelozoic plasmodia are lipid inclusions and vacuoles of various types. The structural differences of coelozoic and histozoic plasmodia presumably depend on the specific habitat of the parasite within the host.

4.4.4 Generative Cell.

Grassé (1960, in Lom *et al.*, 1982) confirmed the existence of the generative cells in large polysporous myxosporea. Lom *et al.*, (1982) suggested that the generative cells of polysporous plasmodia are equivalent to the sporogonic cell of small mono and disporous pseudoplasmodia. These authors believed that vegetative nuclei acquire organelles and a limiting membrane to form a generative cell, however, there is no direct evidence for this assumption (Lom and de-Puytorac, 1965; Mitchell, 1977; Current and Janovy, 1976, 1979). Similarities between generative and vegetative nuclei have led some authors to support this view (Current *et al.*,1979). The nuclei of the generative cell of *M.gasterostei* showed a number of dissimilarities with the vegetative nuclei. The latter lacks a nucleolus whereas the generative cell nucleus usually has a distinct nucleolus (Fig.4.31). The generative cell nucleus

is bound by a loose membrane with a regular outline, but vegetative nuclei are closely bound by a membrane. The generative cell of *M.gasterostei* has some distinctive features which can be compared with other histozoic and coelozoic myxosporeans. The generative cells of *Sphaeromyxa* have long filiform pseudopodia which were not seen in *M.gasterostei*. Histozoic *Myxobolus* sp. have generative cells with an extremely irregular outline with a prominent central nucleolus and dense cytoplasm (Lom and de Puytorac, 1965), whereas *M.gasterostei* cells have a regular outline and a small eccentric nucleolus.

4.4.5 Pansporoblast.

The development of spores in pansporoblasts is commonly reported in histozoic species (Lom and de-Puytorac, 1965; Current and Janovy, 1977), but much less so in coelozoic myxosporeans. This process has been described in *Sphaeromyxa* (Lom, 1969a). In polysporous plasmodia of *M. gasterostei*, spore formation does takes place in pansporoblasts (Fig.4.32). However, in mono and disporous plasmodia pansporoblast formation was not observed and the earliest stage observed was a primary cell enclosing a single or double sporogonic cells. Current *et al.* (1979) suggested that the non-dividing enveloping cell plays an important role in compartmentalizing the dividing and differentiating sporont progeny. They also suggested that the envelope cell may provide nutrients for sporogenesis and molecular regulators to control sporogenesis.

After complete envelopment of the sporogonic cell, its division, differentiation and arrangement of the differentiated cells into a spore producing unit has been reported to occur very rapidly in many myxosporean species (Lom and de-Puytorac, 1965; Current and Janovy,

1976, 1977). It is thought that differentiation of sporoblast cells occurs after the formation of the necessary number (10) of sporoblast cells which are then arranged into 2 groups of 5 cells (Lom and de-Puytorac,1965; Current and Janovy, 1976, 1977, 1978). This may be also a rapid process in *M. gasterostei*. Only in a few cases were a complete set (10) of sporoblast cells observed before cell differentiation began (Fig.4.12), although this may be in part because few TEM sections would include all sporoblast cells.

However, in *M. gasterostei* differentiation of sporoblast cells did seem to occur before the complete division of sporoblast cells and the first indication of differentiation was formation of the capsular primordia in the capsulogenic cells (Fig.4.11a).

4.4.6 Capsulogenic Cell.

In *M. gasterostei* development of capsulogenic cells to form the polar capsule seemed more or less identical with that described in other myxosporean genera (*Henneguya, Myxobolus*, Lom and de-Puytorac, 1965; Current and Janovy, 1978; Current *et al.*, 1979). Abundant RER, large mitochondria, many microtubules and a capsular primordium in different degrees of development are the common features of a developing myxosporean capsulogenic cell as observed in the present study. Some authors (Schubert, 1968; Desser and Paterson, 1978) assumed that abundant RER are involved in the formation of capsular primordia. On the other hand, other authors suggested (Lom and de-Puytorac, 1965) that the microtubules of the capsulogenic cell might be involved in the formation of capsular primordia. In the present study the association of large numbers of microtubules around the capsular primordia and also their scattered occurrence throughout capsule cells supports the latter suggestion.

An unusual feature observed in the capsulogenic cell of *M. gasterostei* was the presence of one or two membrane bound vacuoles filled with a granular substance which might be glycogen (Fig.4.24). Glycogen particles in the capsular cell during the development of the polar capsule were also described in *Sphaerospora* sp. (Supamattaya *et al.*, 1991) and Desser and Paterson (1978) reported scattered β-glycogen materials in the cytoplasm of capsular cells of *Myxobolus* sp. from common shiner, *Notropis cornutus*.

Formation of Polar Capsule.

In *M. gasterostei,* as in other myxosporea, formation of the polar capsule began with the swelling of one end of the capsular primordia. The subsequent development of capsular primordia into mature polar capsules was also very similar to that in other myxosporeans. The proximal end of the capsular primordia were fixed at the time of early differentiation into the anterior part of the future polar capsule pointing towards the filament discharge canal (Fig.4.15). However, the distal swollen end appeared to change its orientation within the capsulogenic cell cytoplasm prior to becoming fixed in position. This swollen end formed the polar capsule of the mature spore, while the long external tube is involved in formation of the polar filament.

Desser and Paterson (1978) suggested that before formation of the polar capsule the capsular primordium undergoes its maximum elongation. Formation of the polar filament inside the capsular primordium is known to occur by the reorganization of membraneous material of the capsular primordium with its electron dense matrix material (Lom, 1964; Lom and de-Puytorac, 1965). This process was not very clear in *M. gasterostei*.

The appearance of the polar filament occurred simultaneously in the external tube and the bulb-shaped swollen end of the capsular primordia (Fig.4.18). This simultaneous appearance of the polar filament has also been reported in other myxosporeans (Current and Janovy, 1978, 1979). Lom (1969a) reported that initiation of the polar filament occurred in one part of the external tube. This process could not be followed in the present study, suggesting that it might be of very short duration.

Granular material in the wall of the capsular primordium was observed in *M. gasterostei* when sections were cut longitudinally through the capsular primordium (Fig.4.16). The granular material was arranged in spiral strands as reported from other groups of myxosporeans (Lom and de-Puytorac, 1965). Similar granular fibrils lining the inner surface of the external tube were reported by Desser *et al.* (1983a) in *S. angulata* and *S. carassii* and also by Voelker *et al.* (1978) from the polar filament of *Kudoa* sp in butterfly fish. In *M. gasterostei* these granular, spirally arranged strands were confined to the bulb like portion of the capsular primordium. The strands are probably involved in providing support to the developing capsular primordium.

Microtubular girdles around the external tube have also been observed in other myxosporeans, but the number of microtubules varies greatly between different species. There are 30 to 50 microtubules surrounding the external tube of *Sphaeromyxa* (Lom, 1969a) and *Henneguya adiposa* contains 12-28 microtubules around the external tube (Current, 1977). In cross sections of *M. gasterostei* 28 to 32 microtubules were observed around the external tube (Fig.4.15).

The filament discharge canal of *M. gasterostei* has a similar structure to that in other myxosporeans. However, the dense plug- like structure at the opening of the polar capsule has not been reported for all myxosporeans (Fig.4.21b). This plug was observed after complete development of the polar capsule. Thus the filament discharge canal consists of the electron dense stopper at the opening of the polar capsule and the cytoplasmic projection of the capsulogenic cell, which is covered anteriorly by the very electron dense area of the shell valves (Fig.4.21c). A similarly heavily electron dense cell membrane of the cytoplasmic projection has been reported in *Henneguya* and some other myxosporeans (Lom and de-Puytorac, 1965).

The presence of three polar capsules (Fig.4.35) occasionally observed in a single mature spore in *M. gasterostei* has also been reported for a few other myxosporeans. Current *et al.*, (1979)suggested that some spore-producing units contained 6 cells rather than the normal complement of 5 cells. The third capsulogenic cell probably resulted from an additional division of one of the sporont progeny before structural differentiation.

4.4.7 Sporoplasm Cell.

The characteristic features of the sporoplasm of *M. gasterostei* are the appearance of electron dense, membrane bound sporoplasmosomes before the differentiated cells become positioned for spore formation (Fig.4.13). Even in early stages of sporoblast formation they sometimes appeared as a few electron dense bodies. With the maturation of the sporoplasm they increased in number and size and were more densely distributed at the posterior of the spore. Sporoplasmosomes change their structure with the maturation of the spore (Fig.4.22). In

immature spores they consist of a central electron dense area surrounded by a less dense layer. In mature spores the central dense area becomes much lighter, and is surrounded by an electron lucent area. Sporoplasmosomes of *M. gasterostei* are generally similar to those found in other myxosporeans. There is no direct evidence of their origin. However, the dense "haplosporosomes", which are morphologically very similar to sporoplasmosomes, found in the primary cell cytoplasm of PKX of salmonids are thought to originate from Golgi complexes (Smith *et al.*, 1984). Some authors speculate that this could also be the source of myxosporean sporoplasmosomes (Azevedo *et al.*, 1989). The role of sporoplasmosomes has not yet been resolved.

The nuclei of the sporoplasm cell starts dividing after the differentiation of the sporoblastic cell (Fig.4.13). The dividing nuclei usually lack a nucleolus and are smaller in size than the original nucleus. Both the sporoplasmic nuclei are separately bounded by rough endoplasmic reticulum. At early stages the nucleolus has evenly distributed heterochromatin but later the heterochromatin is mostly distributed at the periphery. In contrast the two sporoplasmic nuclei of *Myxidium giardi* have peripherally located heterochromatin and both have a distinct nucleolus. The two nuclei later attach closely together and became very electron dense with homogeneously distributed material (Azevedo *et al.*, 1989). In *H. adiposa* the two sporoplasmic nuclei are nearly equal in size and both contain a distinctive nucleolus (Current, 1979).

Sporoplasm maturation of *M. gasterostei* complies with that of other myxosporean species (Current *et al.*, 1979; Current and Janovy, 1978) with an increase in the number of mitochondria and accumulation of β-glycogen like particles at later stages of sporoplasm

maturation. Current *et al.* (1979) suggested that maturation of the germ cell may involve storage of metabolic reserves and acquisition of aerobic metabolism which may provide the energy requirements for exsporulation and establishment of the parasite within a new host. In *M. gasterostei* two Golgi apparatuses were found in the binucleated sporoplasm (Fig.4.13). This has only been reported previously in *Sphaeromyxa* by Lom (1969a).

4.4.8 Valvogenic Cell.

As the two capsulogenic and sporoplasmic cells can easily be identified by their capsular primordia and electron dense inclusions, respectively, at an early stage of sporoblast formation, it is normal to consider the remaining sporoblast cells as the valvogenic cells. In *M. gasterostei* the two valvogenic cells became attached by electron dense junctions at the anterior side of the spore (Fig.4.23) and then spread posteriorly to cover the capsulogenic and sporoplasmic cells. Close to the suture the tip of the valvogenic cell also became very electron dense and the mitochondria became arranged at the periphery of the developing spore (Fig.4.23). These mitochondria may provide the metabolic energy necessary to allow the spread of the valvogenic cell around the capsulogenic and sporoplasmic cells.

The cell junctions between the valvogenic cells themselves and between the valvogenic and capsulogenic cells are regarded as one of the characters distinguishing myxosporeans from the majority of protists (Azevedo *et al.*, 1989). In myxosporea the junction is invariably characterised by deposition of electron dense material beneath and between the cell membranes. The junctions of *M. gasterostei* are very simple junctions with a thin deposit of dense material situated between the membranes and underlying them. An elaborate

desmosomal junction was observed in *Thelohanellus nikolskii* (Desser *et al.*, 1983b), where multilayered fibrillar structures were attached to the cytoplasmic face of the membrane. An intermediate form between the simple and elaborate junctions are the septate desmosomal junctions observed in *Myxidium giardi* (Azevedo *et al.*, 1989). The detailed structure of myxosporean cell junctions is not known satisfactorily.

Microtubules.

In most ultrastructural studies of myxosporean parasites, microtubules are predominantly observed in the developing valvogenic cells. As valvogenesis proceeded in *M. gasterostei* large numbers of microtubules appeared in the cytoplasm of the valvogenic cells and were especially abundant in the valve-suture-forming regions (Fig.4.24a). Some myxosporeans (*Myxidium* sp., Azevedo *et al.*, 1989; *Thelohanellus* sp., Desser *et al.*, 1983b) have a special arrangement of microtubules with different diameters in the suture forming regions. *M. gasterostei* showed two types of arrangement of microtubules in the developing shell valves, very similar to those seen in *Sphaeromyxa* (Lom, 1969a). One arrangement of microtubules was at the sutural ridge and the other was a single layer of 18-24 microtubules beneath the membrane of the shell grooves. *Sphaeromyxa* has a similar arrangement in the sutural ridge but the shell grooves contain 8-14 microtubules. Current (1979) suggested these microtubules are the supportive elements along the sutures and that they play an important role in suture formation. Microtubules were also present in the posterior region of valvogenic cells of *M.gasterostei* from which the posterior valve processes extended. The structure of the myxosporean valve processes has not previously been well described. Current (1979) described valve processes of *Henneguya* as surrounded by enveloping cell microfibrils. Valve processes of *M. gasterostei* also contained grooves and ridges. The valvogenic cell cytoplasm

extended within the valve processes up to a certain distance after which the processes seemed to be supported by fibrillar elements (Fig.4.25). A peculiar event observed in the nearly mature spore of *M. gasterostei* was the presence of large rounded mitochondria of the enveloping cell lodged within the grooves of the valvogenic cell (Fig.4.26). It has been observed that, as in other myxosporeans, microtubules gradually disappear in mature spores of *M. gasterostei*.

4.4.9 Intracellular and Abortive Development.

Myxosporeans are generally considered as parasites of body cavities and intercellular spaces (Lom and Dyková, 1986). *Myxobolus cyprini* develops within the myocytes of carp (Molnár and Kovács-Gayer, 1985a). *Mitraspora cyprini* and *S. renicola* also have intracellular stages in the renal tubule epithelium (Ahmed, 1973; Dyková and Lom, 1982). Occasionally intracellular pre-sporogonic stages of *M. gasterostei* containing a single or no sporogonic cell were observed in the tubular epithelium. Lom and Dyková (1986) mentioned that *Myxidium lieberkhuni* has intracellular stages in the renal tubules which contained sporogonic cells. However, in the present case no massive proliferation of the intracellular stages were seen as occurs in *Hoferellus cyprini*. The latter parasite showed massive proliferation in the renal tubules and destroyed the normal structure of epithelial cells, turning the tubular layer into an enormous cyst-like structure (Lom and Dyková, 1985; Molnár and Kovács-Gayer, 1986b).

Plasmodia with one or two abnormal spores and many irregular, poorly defined vesicles and cellular debris were occasionally observed within the tubular lumen (Fig.4.36). This abortive development of small plasmodia has also been reported from *Sphaerospora* species (Lom *et*

al., 1982; El-Matbouli per. com.), it has been suggested that abnormal development of plasmodia causes the formation of abnormal spores within them. Lom and Dyková (1986) considered that the abortive, aberrant development of myxosporeans may not only affect spores but may involve whole developmental sequences.

4.4.10 SEM Observation.

There is very little information on the SEM structure of myxosporeans. The general SEM structure of myxosporeans was described by Lom and Dyková (1992). The shell valves of mature myxosporean spores have a smooth or rigid surface, which may be drawn out into various projections, bear a secreted caudal appendage or be coated with a mucous envelope. This is the first ultrastructural description of the mature spore of the genus *Myxobilatus*. SEM observations of the mature spore of *M. gasterostei* showed the shell valves with 6-9 surface ridges which are drawn out into caudal projections at the posterior end of the spore (Fig.4.38). The surface ridges of *M. gasterostei* spores are comparatively fewer in number than those of other myxosporean spores in which they occur. Thus, Hine (1978, 1979) described that in *Myxidium zealandicum* infecting freshwater eels, surface ridges of mature spores varied from 11 to 20 and this was related to the site of infection. In *M. gasterostei* no such variations in numbers of ridges was observed, but usually one of the shell valves had 1 or 2 more ridges than the other, which might be due to the flattening of the spore body on one side. The patterns and number of spore ridges in other species of *Myxobilatus* is not yet known and it might a useful taxonomic characteristic in this genus.

Booker and Current (1981) described the SEM surface topography of *M. mictospora*

plasmodia and found it varied between different seasons, so that in winter sheet-like plasmodia had a surface topography of ridges or folds from which numerous branched microvilli extended, whereas in summer plasmodia were fingerlike and devoid of microvilli. In the present study seasonal variations in the SEM of *M. gasterostei* were not observed. The plasmodium showed some globular bodies of unknown function on the surface (Fig.4.39).

4.4.11 Pathogenicity.

The histopathology of kidney parasitised by *M. gasterostei* was supported by TEM observations. The lesions caused by *M. gasterostei* are generally moderate although marked lesions were occasionally observed in heavy infections. The histopathological changes seen are similar to those caused by other coelozoic myxosporea. Parasites at different stages of development were observed predominantly in the proximal part of the tubular lumen causing enlargement of the lumen with vacuolation and flattening of epithelial cells (Fig.4.40). In heavy infections the epithelial brush border was apparently destroyed. Light microscopy of infected renal corpuscles showed dilation of Bowman's space with shrinkage of the glomerular tufts. Electron microscopy showed occasional presence of intracellular stages which caused vacuolation of the epithelial cells near the site of plasmodial attachment. The surface of the plasmodium is thrown into cytoplasmic extensions between the epithelial cells with an increased electron density at the point of attachment (Fig.4.41). Lom *et al.* (1983b) observed an electron dense area at the point of attachment of *S. renicola* and its host tubular epithelium. Large masses of *M. gasterostei* in the kidney tubule caused vacuolation and necrosis of the epithelium, which involved accumulation of electron dense non-homogeneous substances in the epithelium. Dyková and Lom (1988b) described distension of the tubular

lumen with atrophy and necrosis of the epithelium in heavy infections of *S. renicola*. Lom (1969a) described hyperaemia and hypertrophy associated with coelozoic myxosporeans. Fischer-Scherl *et al.* (1986a) observed epithelial necrosis with electron dense non-homogeneous substances in the tubular epithelium of *Salmo trutta* due to the presence of *S. truttae*.

An increased number of rodlet cells was a regular feature of infected tubules in *M. gasterostei* infection (Fig.4.45). Alvarez-Pellitero and Sitja-Bobadilla (1993a) found an increase of rodlet cells in the epithelium of gall bladders parasitised by the early trophozoite of *Chloromyxum* spp. Supamattaya *et al.* (1992) described very similar histopathological changes in kidney of grouper due to the presence of *S. epinephali* which involved increased rodlet cells in the epithelium. The reason for this increase is not clear and the nature and function of the rodlet cells is not certain. Earlier workers postulated that they were protozoan parasites but Desser and Lester (1975) described the ultrastructure of rodlet cells and refuted the hypothesis that they are parasites. Desser and Lester suggested a possible glandular nature of these cells. Generally rodlet cells are widely distributed in fish including the epithelial lining of several organs. Rodlet cells do not appear to increase in other disease conditions of the kidney and may represent a specific host reaction to myxosporeans.

CHAPTER 5

EPIDEMIOLOGY OF *S. ELEGANS* AND *M. GASTEROSTEI* IN *G. ACULEATUS*.

5 EPIDEMIOLOGY OF *MYXOBILATUS GASTEROSTEI* AND *SPHAEROSPORA ELEGANS* IN *G. ACULEATUS*.

5.1 Introduction

A knowledge of the epidemiology of fish parasites is important in understanding the internal and external factors which influence their life cycles. A greater understanding of transmission biology, through epidemiological studies, may help to develop control measures and effective management practices to combat parasitic disease. Although there are numerous studies on helminth parasites of fish (Lester and Adams, 1974a,b; Kennedy, 1977; Chubb, 1977) the epidemiology of fish parasitic protozoans is largely unknown.

Most of the epidemiological data available on myxosporeans is limited to records of prevalence over a very limited period, in many cases only a single sample. Mitchell (1977) reviewed the available literature on the seasonal cycle and epizootiology of myxosporean parasites of fish. Subsequently epidemiological studies of fish myxosporeans have been more numerous and involved mainly economically important cultured fish species (Ferguson, 1981; Ching and Munday, 1984; Wolf and Markiw, 1984; Gonzalez-Lanza and Alvarez-Pellitero, 1984; Amandi *et al.*, 1985; Grupcheva *et al.*, 1985; Foott and Hedrick, 1987; Sitja-Bobadila and Alvarez-Pellitero, 1993; McGeorge, 1994). Although cultured fish represent ideal populations for epidemiological study, since they provide a cohort which can be followed from hatching and regularly sampled over a prolonged period (McGeorge, 1994), epidemiological studies on wild fish populations are also important because they provide data on natural parasite infections unmodified by the constraints of aquaculture.

5.1.1 Seasonality in Myxosporean Infection.

Studies on myxosporean seasonality provides examples of different situations. Some species show all year round infectivity to fish, with no clear seasonal influence, for example *Myxobolus pavlovskii* infecting silver carp and bighead (Molnár, 1979b), while others show a definite seasonal peak in their life cycle (Kovács-Gayer and Molnár, 1983). Brummer-Karvenkontio *et al.* (1991) observed no clear seasonal patterns in a number of myxosporean species in roach.

Many myxosporeans show a single or double peak of infectivity in their life cycle. According to Kovács-Gayer and Molnár (1983) myxosporeans with a one year life cycle show a single peak of infectivity. In this case host fish become infected by the parasite at a particular time of the year followed by the production of mature spores. *Thelohanellus nikolskii* and *T. hovorkai* in common carp (Molnár, 1982; Molnár and Kovács-Gayer, 1986a) show this type of life cycle.

Some species of myxosporeans show two peaks of infectivity each year including *Myxobolus basilamellaris* in carp (Kovács-Gayer and Molnár, 1983) and *Myxobolus farionis* and *M. ibericus* in trout (Gonzalez-Lanza and Alvarez-Pellitero, 1984).

5.1.2 Factors Affecting Seasonality.

Several authors have suggested that the pattern of myxosporean infection in a particular host and a given habitat is due to the interaction of environment and host related factors (Bucher

et al., 1992; Lom and Dyková, 1992). Among environmental factors the most important is believed to be temperature. In temperate climates temperature has an important influence on the rate of development and transmissibility of a number of myxosporeans (Mitchell, 1977). Schafer (1968) reported infection of *Ceratomyxa shasta* proceeded at a slower rate than normal at temperatures below 10^0c. In North Central California the infection with this parasite occurred from May to November, but from January to March when the temperature was below 10^0c infection did not occur. The effect of temperature in ceratomyxosis was established more precisely by Udey *et al.* (1975), who indicated that ceratomyxosis in coho salmon, *Oncorhynchus kisutch* can be suppressed by water temperatures of 6.7^0c or below.

Ferguson (1981) found that low temperature suppressed the development of PKD in rainbow trout *Oncorhynchus mykiss*. An effect of temperature on the host-parasite relationship has also been recorded for *M. cerebralis* (Wolf and Markiw, 1984). Gonzalez-Lanza and Alvarez-Pellitero (1984) described the influence of temperature on the seasonal patterns of *Myxobolus* species in *Salmo trutta* and suggested other limnological factors may also be involved. Grupcheva *et al.* (1985) demonstrated the strict seasonality of occurrence of extrasporogonic and sporogonic stages of *Sphaerospora* species in carp fingerlings.

A number of myxosporean species are known in which infection increases with the lowering of the temperature. Lom (1970) observed that infections of *Henneguya psorospermica* in perch, *Perca fluviatilis*, occurred only during winter, with peak infections from January to March in central Europe. A similar pattern of seasonal infection has been reported for *Myxobolus dujardini* in juvenile squafish in the western United States (Mitchell, 1977). A greater level of prevalence and intensity of infection in the colder months was also reported

for *Hoferellus carassii* infecting goldfish (Molnár *et al.*, 1989; Fischer-Scherl *et al.*, 1992; Yokoyama *et al.*,1990), *Myxobolus areus* in carp (Ogawa *et al.*, 1992) and *Ceratomyxa labaricus* and *C. diploidae* in cultured sea bass (Alvarez-Pellitero and Sitja-Bobadilla, 1993a).

On the other hand, Foott and Hedrick (1987) showed experimentally that not only temperature but availability of infective stages in the environment at a particular time of year also favoured PKD infection.

Other Factors.

Mitchell (1977) suggested that in spite of direct or indirect effects of temperature on seasonal cycles, other limnological factors, such as crowding of hosts, photoperiod and seasonal differences in host feeding habits may influence seasonal patterns of myxosporean infection. El-Matbouli *et al.* (1992) considered that eutrophication is one of several environmental factors that probably favour the appearance and distribution of these parasites in a biotope. Environmental stress acts as a predisposing factor increasing the susceptibility of fish to infection by myxosporeans.

Bucher *et al.* (1992) reported that the prevalence of *Zschokkella nova* in bullhead, *Cottus gobio*, increased in a polluted river. They assumed that pollution related eutrophication might lead to a multiplication of oligochaete alternate hosts. However, Brummer-Korvenkontio *et al.* (1991) found no increase in infection of a number of myxosporeans in a polluted lake despite the presence of high numbers of tubificid worms.

Host Factors.

The prevalence of myxosporean infection is known to be extremely variable. In many cases 100% of hosts can be parasitized (Mitchell, 1977) and parasites can thrive in the host irrespective of the season. Much of the literature on myxosporean epidemiology suggests that host size and age appear to be an important factor in prevalence of infection. Mitchell (1977) noted that in most cases very young hosts are usually most susceptible to diseases caused by myxosporeans. Some reports show that prevalence and intensity are higher in older fish (Molnár and Kovács-Gayer, 1985a; Sitja-Bobadila and Alvarez-Pellitero, 1993). These authors suggested that older fish accumulated parasites over time. On the other hand, a decreasing rate of infection with increasing age of fish is reported by other authors (Brummer-Korvenkontio *et al.*, 1991; Gonzalez-Lanza and Alvarez-Pellitero, 1984), which they suggested is due to age related physiological changes in the fish which makes them less suitable for infective stages of the parasite, or due to the death of infected hosts within the population. Information on the influence of host sex on myxosporean infection is very scarce. Janovy and Hardin (1987) found that females of *Fundulus zebrinus* were more susceptible to infection with *Myxosoma funduli* than males.

Lom (1969c) suggested that the seasonal occurrence of myxosporeans is influenced by the antibody response of the host. Massive infections may develop in cold weather because antibody titres of host fish are low at that time. However, studies on immunity against myxosporean infections show that in some cases fish infected in their first year became resistant to infection in subsequent years (Ferguson, 1981; Foott and Hedrick, 1987; Kent, 1992; Hoffmann *et al.*, 1992; Odening *et al.*, 1988). In many cases the infectivity of myxosporeans is clearly correlated with the life cycle of the fish host. Mitchell (1977)

recorded a high prevalence of *Henneguya* sp. in channel catfish in spring which coincided with the hatching of juvenile fish. Development of *S. testicularis* is linked with gonad maturation in sea bass (Sitja-Bobadilla and Alvarez-Pellitero, 1993).

Availability of Infective Stages.

A number of authors have agreed that the seasonality of some myxosporeans primarily depends on the presence of the infectious stage in the environment (Foott and Hedrick, 1987; Alvarez-Pellitero and Sitja-Bobadilla, 1993a). If an alternate host cycle is generally applicable for myxosporeans, i.e. myxosporean spores need to be ingested and to develop in oligochaete worms to form infective actinosporean stages (Wolf and Markiw, 1984; Markiw, 1986; El-Matbouli and Hoffmann, 1989; Yokoyama *et al.*, 1991; Benajiba and Marques 1993), then the influence of the alternate host (oligochaete) life cycle must have some effect on myxosporean seasonality.

5.1.3 Seasonality of *Sphaerospora* spp.

Members of the genus *Sphaerospora* are increasingly being considered as an important pathogens of freshwater and marine fish (Dyková and Lom, 1988; Molnár, 1988a; Sitja-Bobadilla and Alvarez-Pellitero, 1992). Although there is now considerable information on development and pathogenicity, there remains a lack of epidemiological studies which are necessary to understand several aspects of the host-parasite relationship and to establish adequate prophylactic measures. Grupcheva *et al.* (1985) reported from Bulgaria that there is a marked seasonal fluctuation in the prevalence and intensity of *S. renicola* and myxosporean blood stream stages in a study over three carp fingerling generations. Farmed

carp from waters with *S. renicola* were either heavily infected or uninfected, because of multiplication of extrasporogonic stages in infected hosts. *Sphaerospora* species from sea bass had a higher prevalence in wild than in cultured fish and the infection level increased with host age (Sitja-Bobadilla and Alvarez-Pellitero, 1993). Feist *et al.* (1991) observed a 0 to 82% prevalence of *S. elegans* in three-spined stickleback in waters from England, Scotland and France. Juvenile *G. aculeatus* from the River Avon in the UK were mildly infected during July and August but the data was not adequate to identify seasonal influences on the development of the infection. These authors also reported co-infections of *Myxobilatus gasterostei* but provided no information on prevalence and developmental cycles.

5.1.4 Seasonality of *Myxobilatus* spp.

Seasonal studies on the genus *Myxobilatus* are very limited. Booker and Current (1981) found a strict seasonal influence in the development of the plasmodium of *Myxobilatus mitraspora* in largemouth bass and described the influence of temperature on the formation of three distinct forms of plasmodia. Molnár (1988c) found that development of *M. legeri* in cyprinid fish from natural waters of Hungary is strictly seasonal. Vegetative proliferation of the parasite occurrs intracellularly in autumn whereas sporogenesis occurs in the tubular lumen of the kidney in spring. The exact seasonal influence on development was not the same in all cyprinid hosts.

5.1.5 Associations between Myxosporeans.

Mixed infections of myxosporeans have been reported from some host species and in some

cases there appears to be a positive association (Moser, 1976; Gonzalez-Lanza and Alvarez-Pellitero, 1984; Feist *et al.*, 1991; Alvarez-Pellitero and Sitja-Bobadilla, 1993). Interspecific influences between these parasites may affect the seasonality of individual species (Moser, 1976).

5.1.6 Intensity of Infection.

Exact data on the intensity of myxosporean infections is very difficult to obtain because of problems in accurately counting the number of parasites from the infected host. Several methods have recently been devised to measure the level of intensity of infection semi-quantitatively in epidemiological studies (Alvarez-Pellitero *et al.*, 1983; Gonzalez-Lanza *et al.*, 1984; Grupcheva *et al.*, 1985; Sitja-Bobadilla and Alvarez-Pellitero, 1993; Alvarez-Pellitero and Sitja-Bobadilla, 1993a). Knight *et al.* (1977) described the summer epizootiology of *Myxosoma funduli* on the gills of *Fundulus kansae*. By counting the total plasmodia it was found that the parasite population was overdispersed, and fitted the negative binomial distribution.

5.1.7 Objectives.

The objective of the present study was to describe the occurrence of *S. elegans* and *M. gasterostei* in a population of three-spined stickleback in its natural habitat and to provide information on the seasonal fluctuations in parasite abundance and development, particularly in relation to host biology, to determine the influence of host age and sex on infection and to investigate interactions between the two parasite species.

5.2 Materials and Methods.

Three-spined sticklebacks were collected from Airthrey Loch, every two weeks from January 1993 to February 1994. A total of 26 samples and 797 fish were examined in the 14 month sampling period. Each sample contained 15 to 60 fish. After collection fish were maintained and examined as described in Chapter 2.

Infection was determined by the presence of developing stages and mature spores of the parasites as seen in fresh mounts of the kidney and, for extrasporogonic stages, occasionally the circulating blood and choroidal rete-mirabile of the eye. Prevalence was calculated as the percentage of the total number of fish infected with parasitic stages by examination of the kidneys of all fish in a sample. The two samples taken each month were combined to determine the monthly prevalence rate. Prevalence was determined separately for extrasporogonic, developing and spore stages. The prevalence of extrasporogonic stages in the blood and choroidal rete of the eye and in the kidney was determined by examining sub-samples (5-10 fish) from each monthly sample.

Intensity of infection was assessed from the presence of mature spores in histological sections of the kidney. The intensity of the infection was calculated semi-quantitatively and graded according to the number of infected kidney tubules per microscopic field at x 25 magnification. For this purpose histological sections were standardized by examining the sections from the same part of the kidneys. Three fields from the central part of 10 longitudinal sections (i.e 30 fields) of the kidney of each fish were examined. The intensity of infection was categorized as follows:

No. of infected tubules/field	Intensity
1 - 5	+
6 - 11	1+
12 - 20	2+
21 - 35	3+
> 35	4+

Statistical Analysis

A chi- square test ($P < 0.05$) was used to analyze the influence of both length and sex on the infection rate (Sokal and Rohlf, 1981) and also to determine mutual influences of the two species of myxosporea.

5.3 Results.

The combined prevalence of *S. elegans* and *M. gasterostei* in three-spined stickleback was 84.19%. Overall data on prevalence and intensity of myxosporean infection in the three-spined stickleback are presented in Table 5.1. Of the 797 fish examined, 494 fish were infected with *S. elegans* giving an overall prevalence rate of 61.98%. Among the *S. elegans* infected fish, 176 were male and 289 female with prevalence rates of 64.94% and 65.38% respectively. The slight difference in the prevalence of infection of male and female fish was not statistically significant. The sex of 29 infected fish could not be determined. The mean number of infected kidney tubules with *S.elegans* was 9 for both male and female fish, i.e. 1+ (Table 5.1).

Table 5.1: Overall Infection of *S. elegans* and *M. gasterostei* in three-spined sticklebacks.

No. Fish Examined	No. of Fish with *S. elegans*	No. of Fish with *M. gasterostei*	Prevalence% *S. elegans*	Prevalence% *M. gasterostei*	Mean Intensity *S. elegans*	Mean Intensity *M. gasterostei*	Chi-square analysis	Mean no. of Infected Tubules (range) *S. elegans*	Mean no. of Infected Tubules (range) *M. gasterostei*
Male 271	176	182	64.94	67.15	1+	1+	*S.elegans*(♂♀)x^2= 0.014(NS)	9(3-14)	10(3-9)
Female 442	289	355	65.38	80.31	1+	1+	*M.gasterostei*(♂♀) x^2=15.645(S)	9(3-14)	16(3-20)
Sex not detected 84	29	78	34.52	92.85					

S= Significant (P<0.05)
NS= Not significant (P<0.05)

Six hundred and fifteen fish were infected with *M. gasterostei* with an overall prevalence rate of 77.16 %. There were 182 male and 355 female fish infected with prevalence rates of 67.15% and 80.31% respectively. The sex of 78 infected fish was not determined. The higher prevalence rate observed in females was statistically significant (Table 5.1). In *M.gasterostei* the mean number of infected tubules in male fish was 10, i.e. 1+ and in female fish it was 16, i.e. 2+ (Table 5.1).

5.3.1 Prevalence and Intensity of Extrasporogonic Stages.

Extrasporogonic stages of *S. elegans* were found in the choroidal rete mirabile of the eye, the circulating blood and in the blood sinuses of the interstitial spaces of the kidney. The occurrence of extrasporogonic stages during the whole sampling period is shown in Fig.5.1. Extrasporogonic stages in the eye were found in only two months; in January 1993 with a prevalence of 42.85% and in June 1993 with a prevalence of 33.33%. Extrasporogonic stages in the circulating blood were also found in only two months, in June 1993 and January 1994, with prevalence rates of 20% and 25% respectively. In the kidney, extrasporogonic stages were observed only in February with a prevalence rate of 40%. The intensity of infection of extrasporogonic stages was apparently very low, with only one to two stages found in up to 17 fields per slide. Extrasporogonic stages of *M. gasterostei* were not found.

5.3.2 Prevalence of Pre-sporogonic and Sporogonic Stages.

S. elegans.

For convenience of data analysis pre-sporogonic and sporogonic stages are collectively

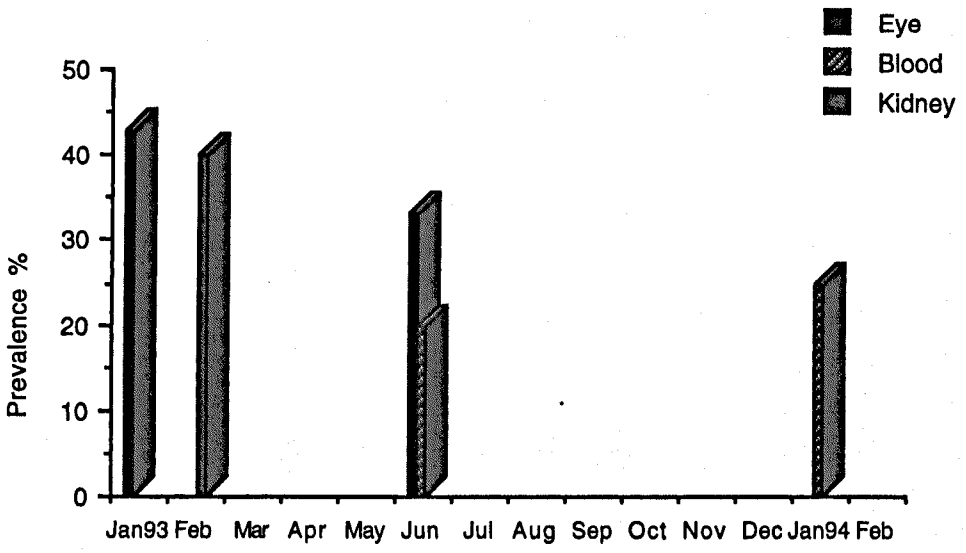

Fig: 5.1 Prevalence of extrasporogonic stages of *S.elegans* in different months.

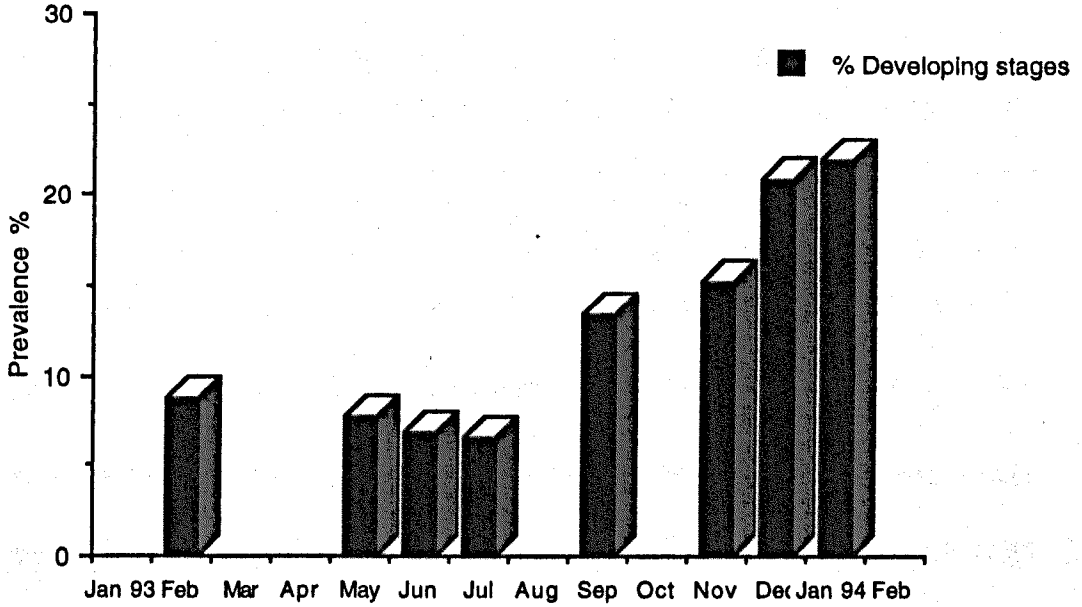

Fig: 5.2. Prevalence of developing stages of *S.elegans* in different months.

referred to here as developing stages and when mature spores are found with developing stages, these are together denoted as spore stages. Figures 5.2-5.3 show the prevalence of developing and spore stages of *S.elegans* throughout the sampling period. The prevalence of developing stages in different months of the sampling period is shown in Fig.5.2 whilst Fig.5.3 shows the prevalence of developing and spore stages. The prevalence of developing stages was much lower than that of spore stages and in some months they were absent altogether. The prevalence of developing stages was highest between September 1993 and January 1994 reaching 21.8% in the latter month.

The prevalence of spore stages was comparatively much higher than that of the developing stages. The highest rate was (>80%) in January 1993 and February 1994. On the other hand, in February 1993 prevalence was lowest (23.21%).

Figure 5.4 shows the prevalence of all stages of *S. elegans* in fish of different lengths. Fish in the largest group were infected only with sporogonic stages. Extrasporogonic stages were only found in L2 (2.1-3 cm.) fish with a prevalence of 33%. Developing stages were present in L2 and L4 fish with a prevalence of 19.35% and 25.19% respectively. Spore stages were present in over 75% of all fish in the L2 and L4 classes and all fish in the L3 and L5 classes.

M. gasterostei.

Figures 5.5-5.6 show the prevalence of developing and spore stages of *M. gasterostei* over the sampling period. Developing stages (Fig.5.5) were observed in every month except January and March 1993 although generally at a low prevalence. Higher prevalence was observed in May, June, July and December 1993 and in January 1994. Figure 5.6 shows the

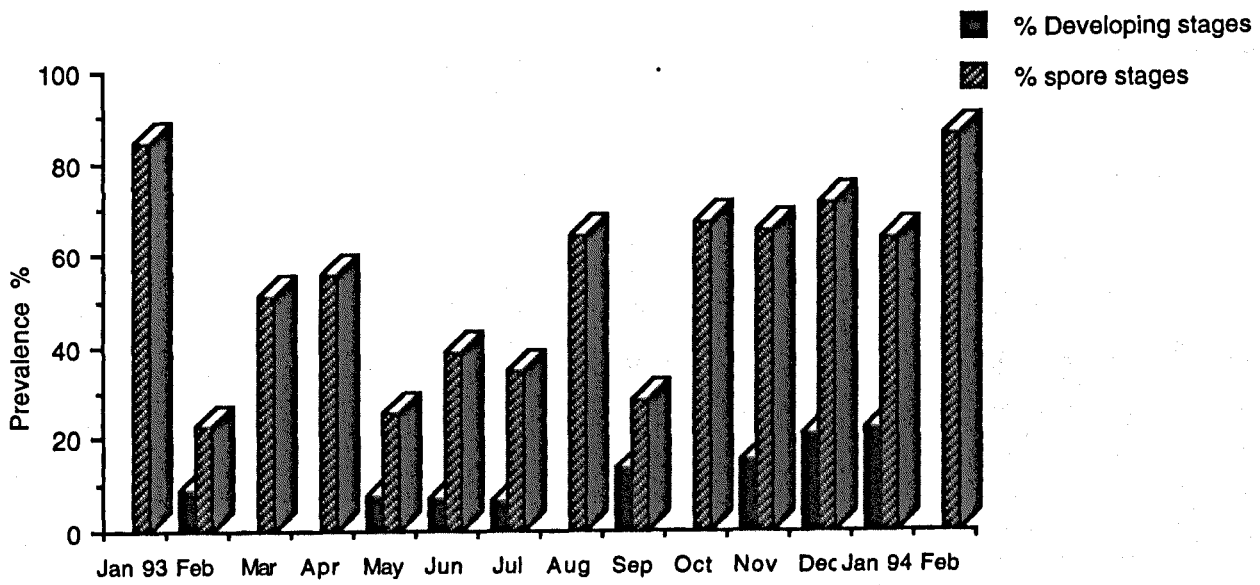

Fig: 5.3. Prevalence of developing and spore stages of *S.elegans* in different months.

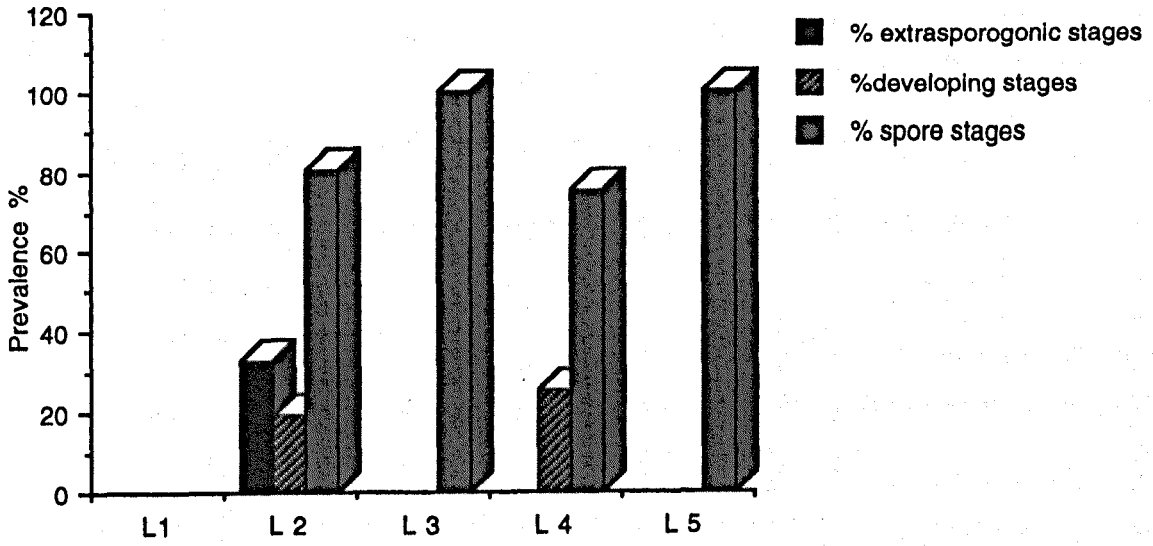

Fig: 5.4. Prevalence of extrasporogonic, developing and spore stages of *S.elegans* in different length classes of sticklebacks.

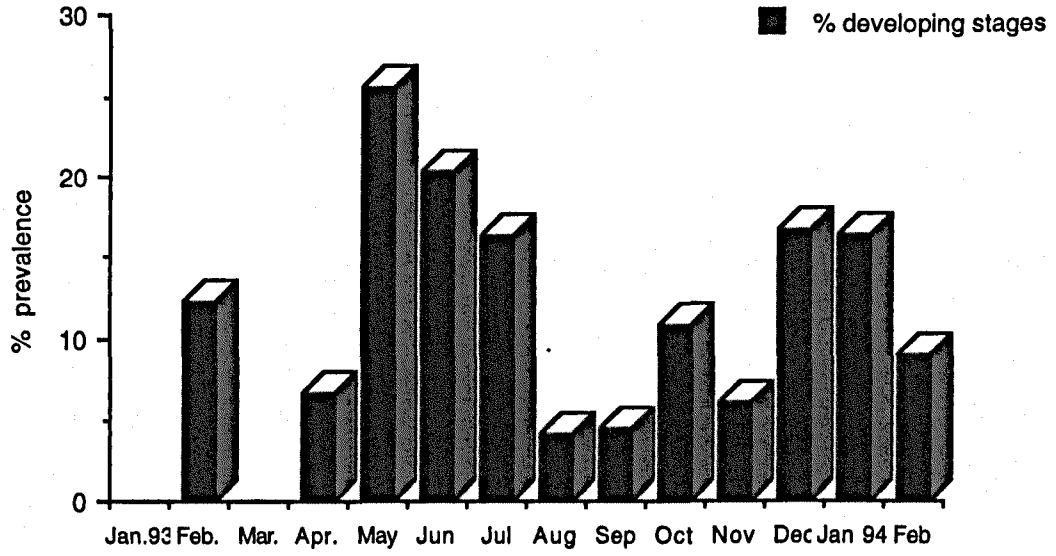

Fig:5.5. Prevalence of developing stages of *M.gasterostei* in different months.

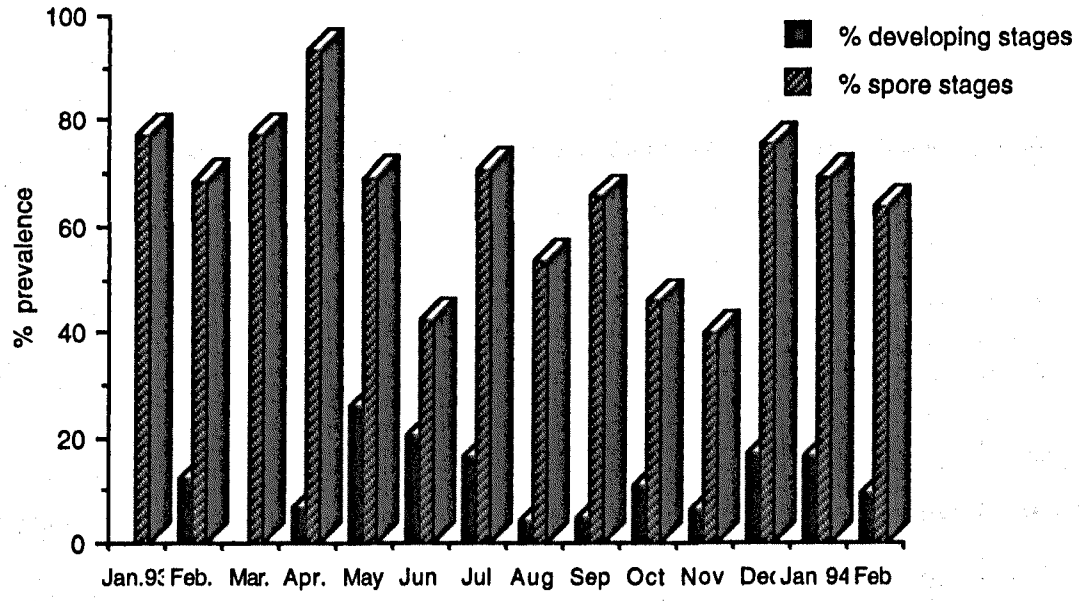

Fig: 5.6. Prevalence of developing and spore stages of *M.gasterostei* in different months.

prevalence of developing and spore stages of *M.gasterostei*. Prevalence of spore stages was generally high (>50%) throughout the sampling period but a little lower in June, October and November 1993.

Figure 5.7 shows the prevalence of developing and spore stages in fish of different length groups. The highest prevalence (53.57%) of developing stages was recorded in the L1 class of fish and was much lower in other groups. Spore stages were not found in the L1 class but prevalence was highest in the L2 and L3 classes (>70%).

The intensity of infection of pre-sporogonic stages of *S. elegans* and *M. gasterostei* could not be determined due to the difficulty of distinguishing them in histological slides.

5.3.3 Overall Prevalence and Intensity of Infection in Different Months.

S. elegans.

S. elegans was present in at least 30% of the fish examined in each month. There were wide variations in the prevalence between months (Fig.5.8). Highest prevalence rates were observed in January 1993 and from November 1993 to February 1994. Prevalence rates were generally lower, although fluctuating, between February and October 1993. *S. elegans* showed the highest intensity of infection in January and February 1994, which coincided with a period of high prevalence. Intensity was at a lower level throughout the rest of the sampling period (Fig.5.9).

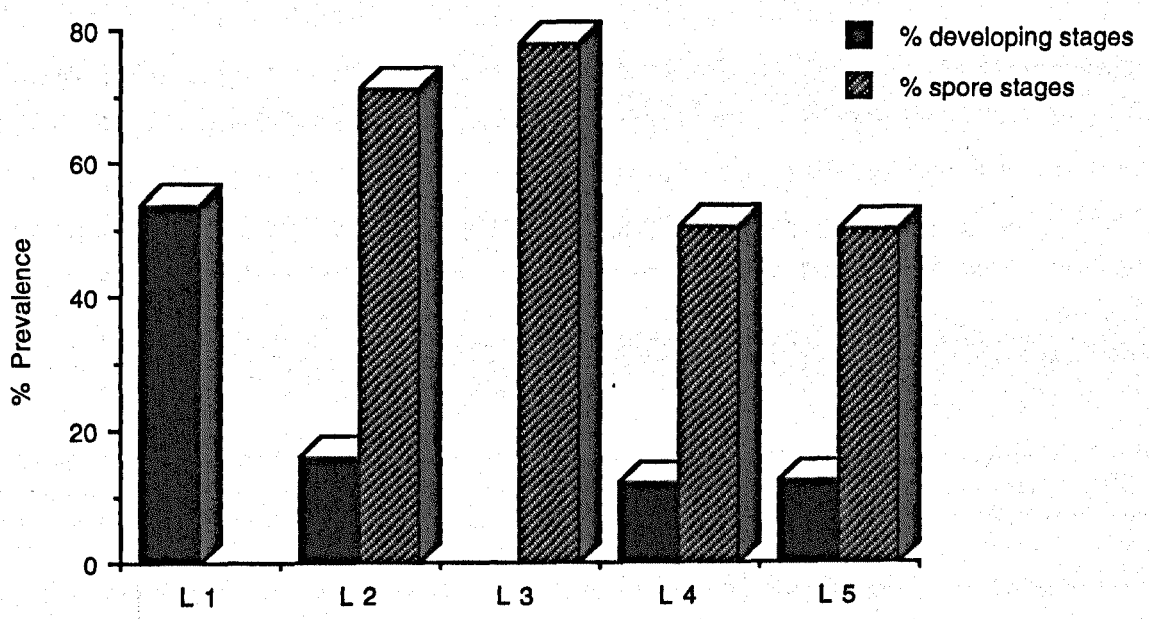

Fig: 5.7 Prevalence of developing and spore stages of *M.gasterostei* in different length classes of sticklebacks.

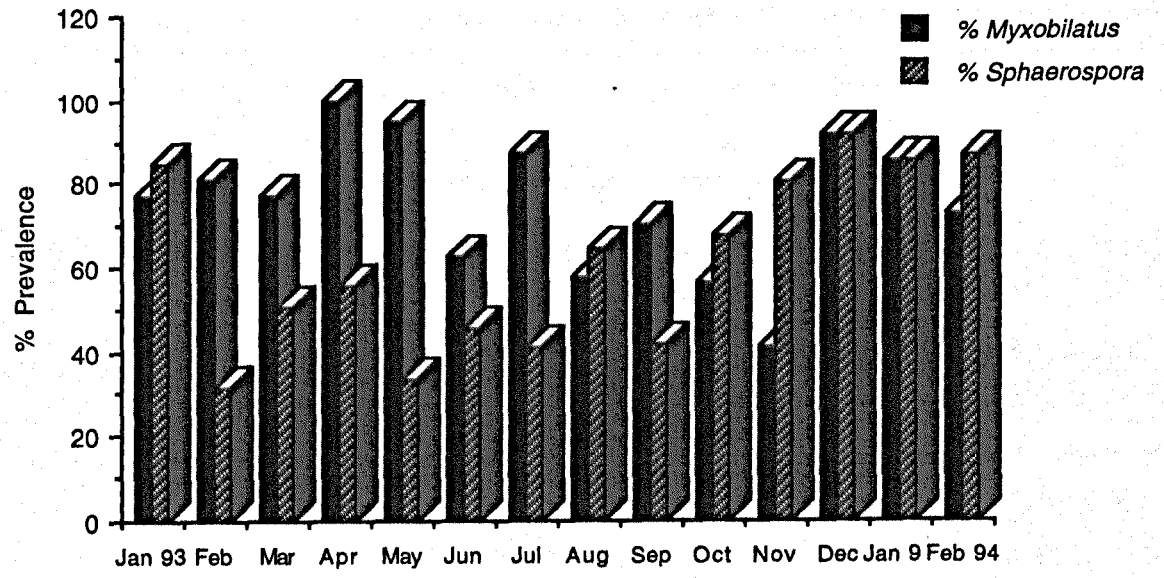

Fig:5.8 Prevalence of *S.elegans* and *M.gasterostel* in different months.

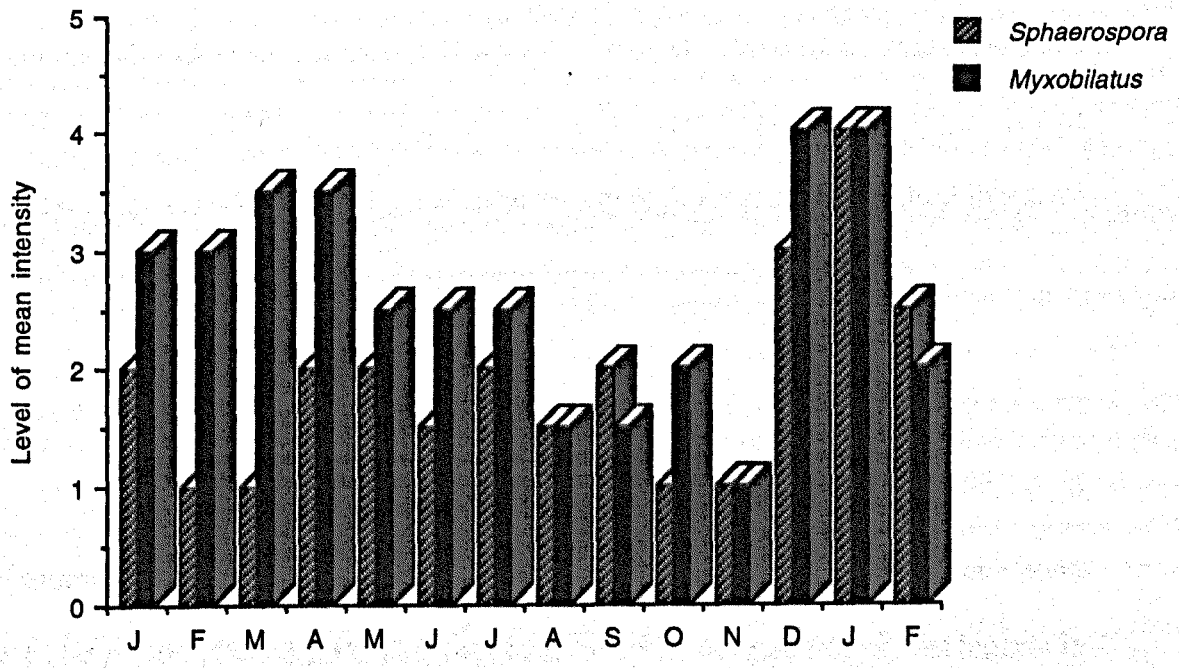

Fig: 5.9. Level of mean intensity of *S.elegans* and *M.gasterostel* in different months.

M. gasterostei.

Prevalence of *M. gasterostei* was generally highest from January to May 1993 and December 1993 to February 1994. Between June and November 1993 the prevalence was lower except in July 1993 (Fig.5.8).

The highest level of intensity of infection with *M.gasterostei* occurred in January-August 1993 and November 1993 to February 1994, but levels were generally much lower in the intervening period, and generally followed prevalence rates (Fig.5.9).

5.3.4 Overall Prevalence and Intensity of Infection in Different Seasons.

S. elegans.

The prevalence of *S. elegans* was lowest (48.12%) in spring and summer (51.29%) but was higher in autumn (66.29%) and winter (87.75%)(Fig.5.10). The difference in the prevalence of infection in different seasons was statistically significant ($x^2_{obs}=63.952 < x^2_{.05}=9.488$,d.f.4). The level of intensity of *S. elegans* was highest in winter 1993, while in other seasons the level of intensity was similar (Fig.5.11).

M. gasterostei.

The highest prevalence (>80%) of *M. gasterostei* occurred in winter and spring with lower levels in summer and autumn. The difference in the prevalence was significant ($x^2_{obs}=129.132 < x^2_{.05}=9.488$, d.f.4). The level of intensity of *M. gasterostei* was highest in spring and winter 1993 which parallels the prevalence of infection.

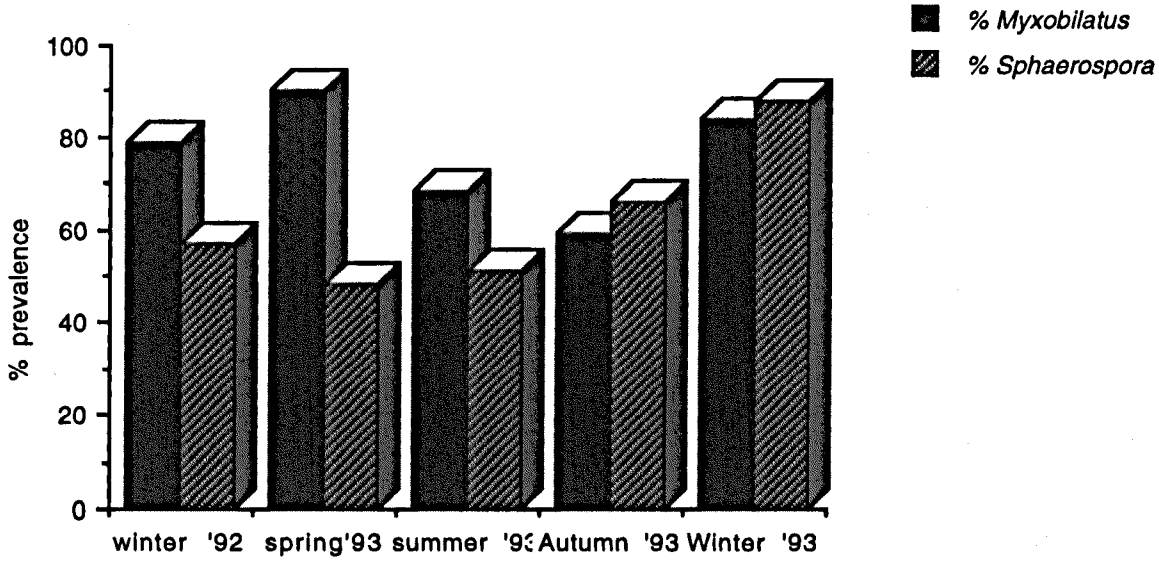

Fig: 5.10. Prevalence of *S.elegans* and *M.gasterostei* in different seasons.

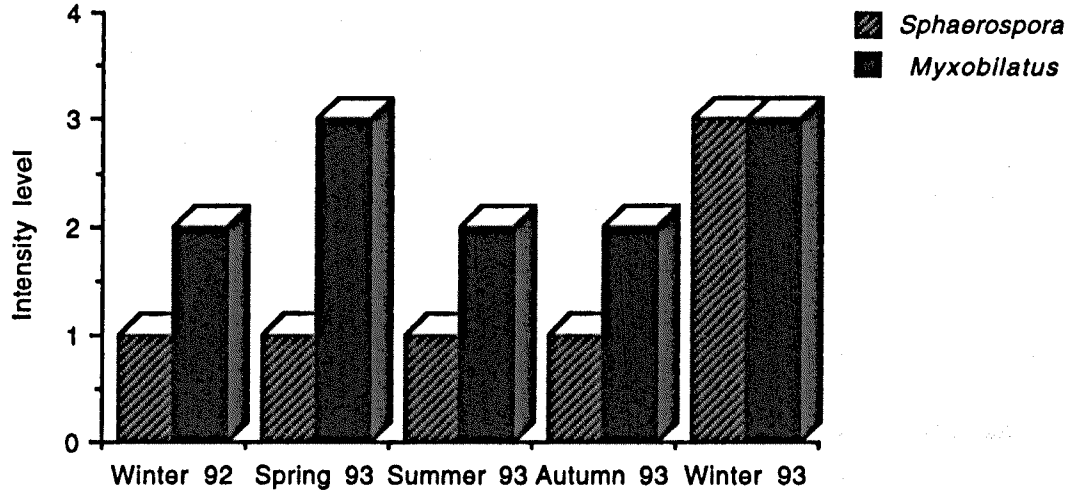

Fig 5.11. Level of mean intensity of *S.elegans* and *M.gasterostei* in different seasons.

5.3.5 Prevalence and Intensity of Infection in Male and Female Sticklebacks.

S. elegans.

The prevalence of *S. elegans* in both sexes of fish is shown in Fig.5.12. The prevalence of *S. elegans* in male sticklebacks decreased slightly between winter 1992 and summer 1993 and then increased in autumn and winter 1993 to a maximum level. In females prevalence of *S. elegans* was very similar throughout the sampling period except for winter 1993 when it also reached a maximum level. These variations in prevalence of male and female fish in different seasons were not statistically significant ($x^2_{obs}=10.625>x^2_{.05}=9.488$, d.f.4). The intensity of infection of female and male fish with *S. elegans* was identical in most months. There were no very obvious seasonal variations (Fig.5.13).

M. gasterostei.

Figure 5.14 shows the prevalence of *M. gasterostei* in both male and female sticklebacks. The prevalence of *M. gasterostei* in male sticklebacks was quite uniform except in autumn 1993, when it was at its lowest level. Prevalence in female fish was generally a little higher (>60%) and was maximum in winter 1993. In autumn and winter 1993 the prevalence of infection of female fish was significantly higher than that of males ($x^2_{obs}=6.340<x^2_{.05}=9.488$, d.f.4).

M. gasterostei was present at an equal or usually higher intensity of infection in female compared with male sticklebacks in all months (Fig.5.15). The intensity of infection in males was highest from January 1993-April 1993 but lower during the rest of the sampling period, with the exception of December 1993. In females intensity was lowest in August, September and November 1993.

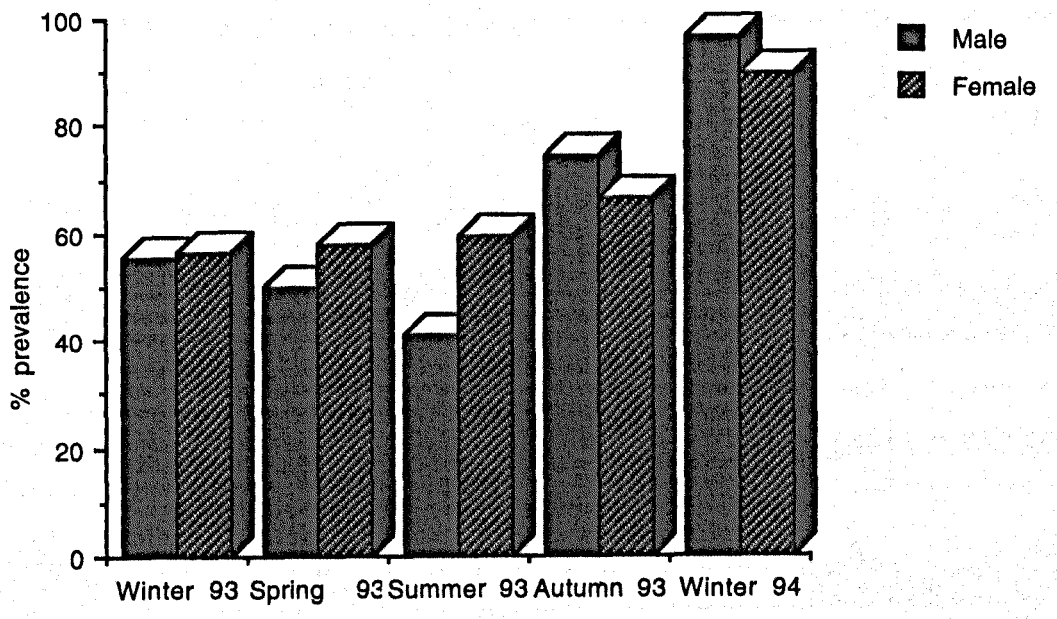

Fig: 5.12. Prevalence of *S.elegans* in male and female sticklebacks in different seasons.

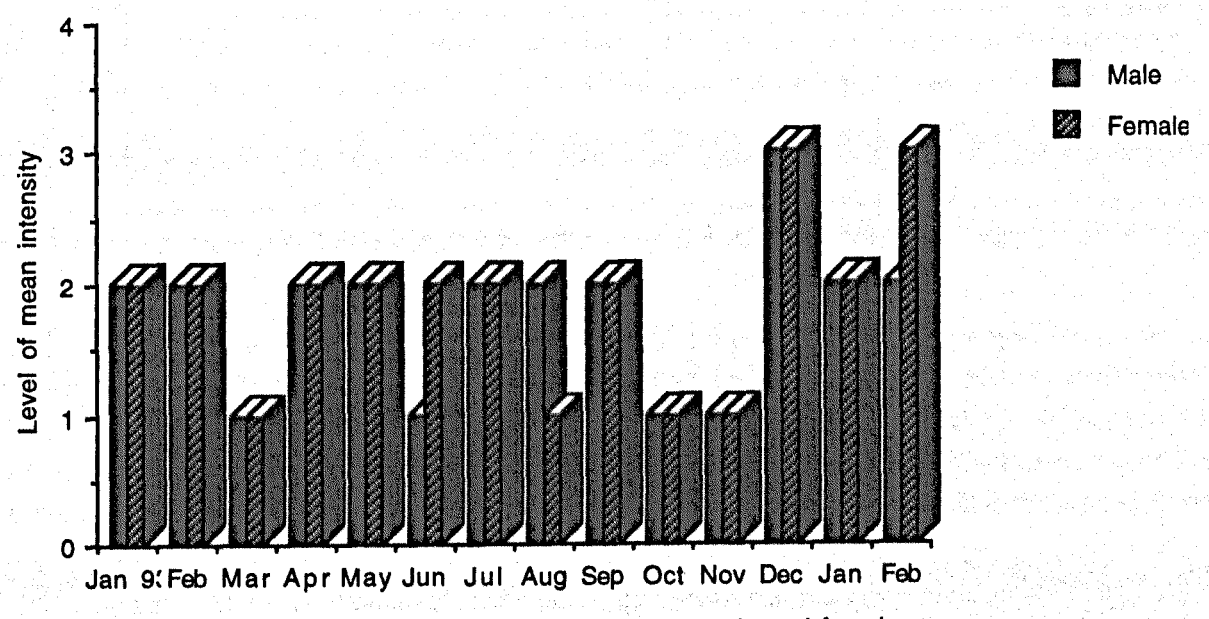

Fig:5.13. Level of mean intensity of *S.elegans* n male and female sticklebacks in different months.

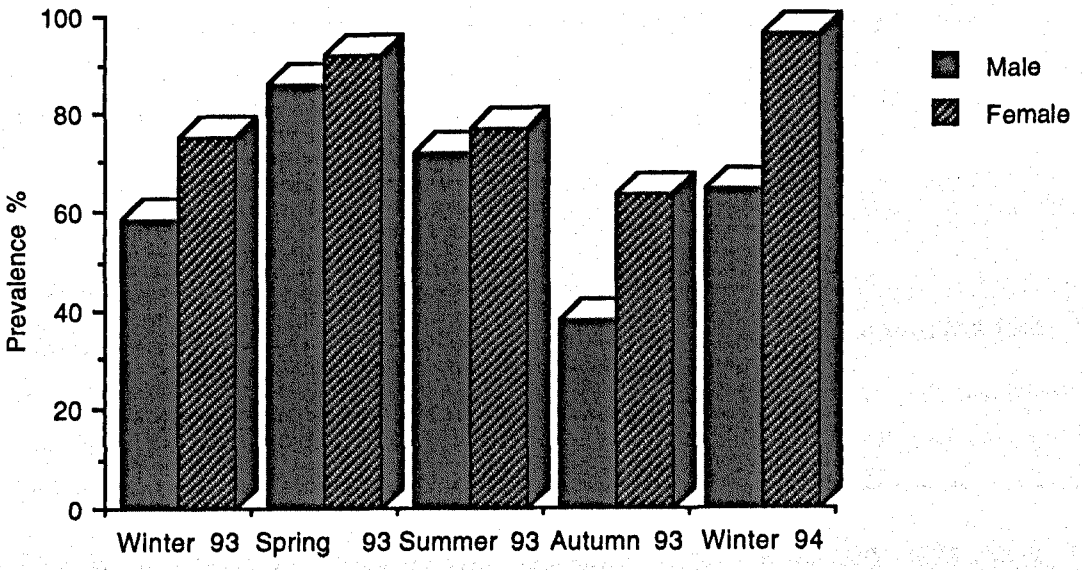

Fig: 5.14. Prevalence of *M.gasterostei* in male and female sticklebacks in different seasons.

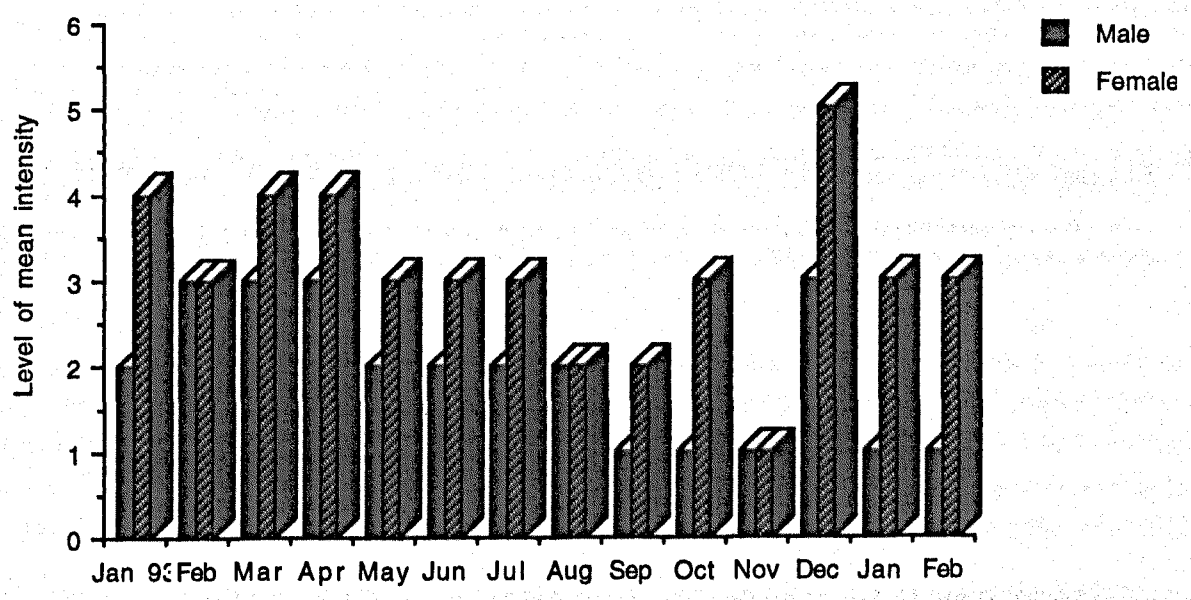

Fig: 5.15 Level of mean intensity of *M.gasterostei* in male and female sticklebacks in different months.

5.3.6 Overall Infection in Fish of Different Length Classes.

Figure 5.16 shows the overall prevalence of infection in stickleback of different length classes from all samples combined.

S. elegans was not found in the smallest length class of fish, but the prevalence rate was similar in the larger length classes and was between 50% and 66.77%. The difference in the prevalence of infection in different length classes was statistically significant ($x^2_{obs}=66.095<x^2_{.05}=9.488$, 4 d.f). In contrast, *M. gasterostei* was found in the smallest fish of 1-2 cm. length with a prevalence rate of 53.57%. The prevalence reached its highest level 86.97% in L2 fish and then gradually decreased with increasing length of fish. The variations in the prevalence of infection in different length classes were statistically significant ($x^2_{obs}=45.992>x^2_{.05}=9.488$, 4 d.f).

Figure 5.17 shows the intensity level of both parasites in different length classes of fish. *S. elegans* had a highest intensity of infection in L3 fish which subsequently declined to a lowest level in L5 fish. L2 and L3 fish had a high intensity of infection with *M. gasterostei* which decreased sharply in larger fish.

5.3.7 Seasonal Prevalence and Intensity in Different Length Classes of Fish.

Table 5.2 shows the prevalence and intensity of infection of *S. elegans* and *M. gasterostei* in different length classes of fish.

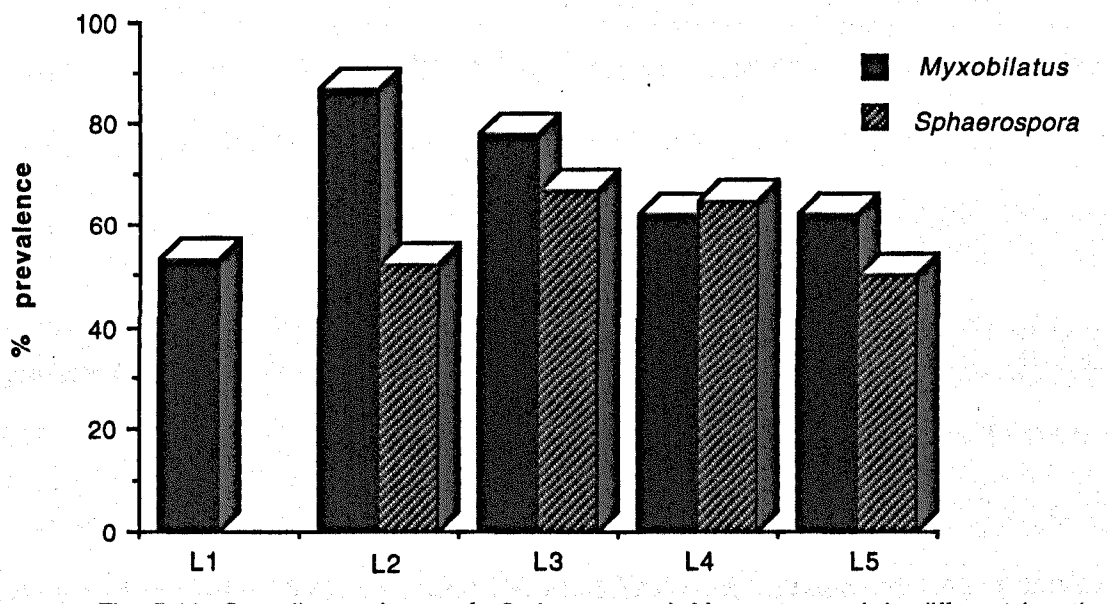

Fig: 5.16. Overall prevalence of *S.elegans* and *M. gasterostei* in different length classes of sticklebacks.

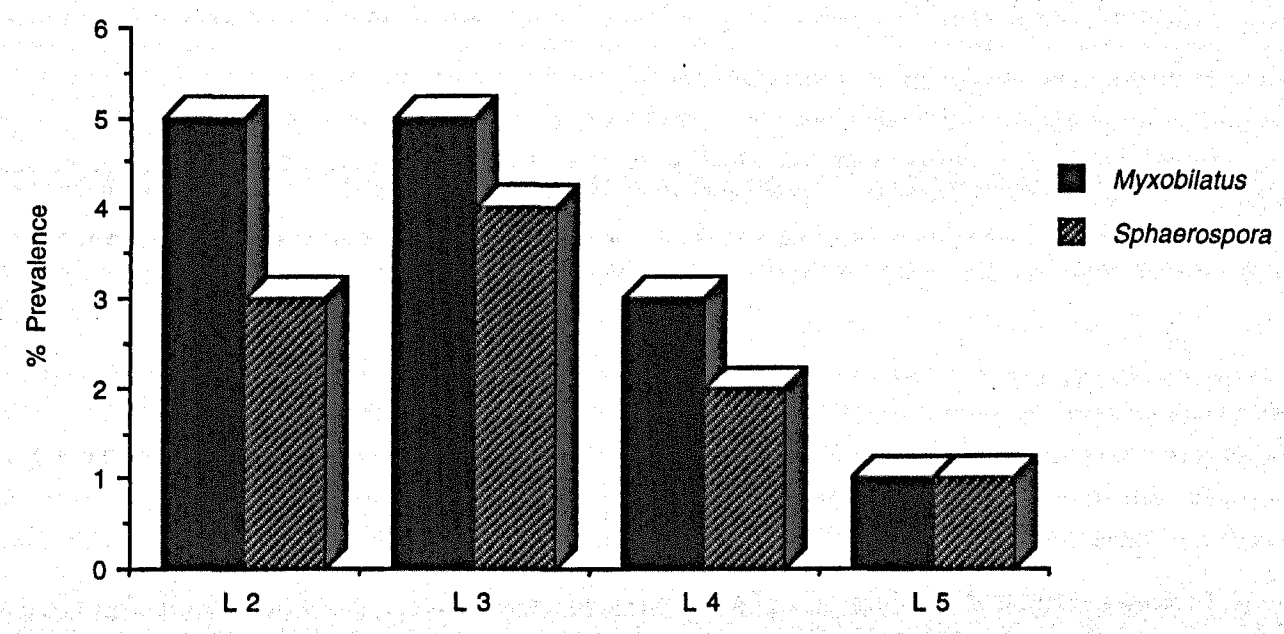

Fig: 5.17. Level of mean intensity of *S.elegans* and *M.gasterostei* in different length classes of sticklebacks.

S. elegans.

S. elegans was not found in L1 fish. In L2 fish the prevalence was similar from winter 1992 to summer 1993 and then increased to a maximum of 75% in winter 1993. Similarly in the L3 class prevalence was similar in winter 1992 and spring 1993 and then increased to reach a maximum of 90% in winter 1993. In the L4 class there was an increasing prevalence throughout the period of study reaching a maximum of 90.47% in winter 1993. Changes in the level of intensity generally paralleled changes in prevalence in all length classes.

M. gasterostei.

In the L1 class prevalence was low in summer 1993 but increased to 100% in autumn. In other seasons these fish were not present in the samples. In the L2 class a 100% prevalence was observed in winter 1992 and spring 1993 followed by a decrease in summer and autumn and a rise to 91% in winter 1993. The level of intensity paralleled the prevalence of infection. Prevalence in the L3 and L4 classes was highest in winter and spring but somewhat lower in summer and autumn. The level of intensity was higher in winter and lower in summer. In summer the prevalence rate was 50% in the L5 class and reached 75% in autumn while the intensity level was similar in both seasons; although these results are based on only a few fish.

5.3.8 Mixed Infections of the Parasites.

Mixed infections with both parasites were observed in 438 fish giving a total prevalence of 54.95%. A total of 56 fish were infected with only *S. elegans* (7.02%) and 177 fish were infected with only *M. gasterostei* (22.20%). A total of 126 fish were uninfected. Although

Table 5.2: Seasonal Prevalence and Level of Mean Intensity in Different Length Classes of Sticklebacks.

Length Group	Seasons	No. of Fish Examined	*S. elegans*		Mean Intensity	*M. gasterostei*		Mean Intensity Level
			No. of Fish Infected	%		No. of Fish Infected	%	
	Winter 92	0						
L1	Spring 93	0						
	Summer 93	16	0			3	18.75	+
	Autumn 93	12	0			12	100	+
	Winter 93	0						
	Winter 92	21	9	42.85	2+	21	100	2+
L2	Spring 93	50	20	40	2+	50	100	4+
	Summer 93	56	22	39.28	1+	44	78.57	1+
	Autumn 93	55	31	56.36	2+	41	74.54	2+
	Winter 93	56	42	75	2+	51	91.07	4+
	Winter 92	52	22	42.3	1+	44	84.61	4+
L3	Spring 93	54	25	46.29	1+	53	98.14	2+
	Summer 93	69	47	68.11	2+	53	76.81	1+
	Autumn 93	71	56	78.87	1+	40	56.33	1+
	Winter 93	49	47	95.91	2+	40	81.63	5+

Table 5.2 contd.

	Winter 92	36	6	16.66	2+	21	58.33	1+
L4	Spring 93	44	26	59.09	2+	35	79.54	2+
	Summer 93	40	30	75	2+	26	65	1+
	Autumn 93	40	31	77.5	3+	12	30	1+
	Winter 93	42	38	90.47	3+	32	76.19	3+
	Winter 92	0						
L5	Spring 93	12	6	50	+	6	50	2+
	Summer 93	12	0	0		9	75	2+
	Autumn 93	0						
	Winter 93	0						

a high number of single infections occurred with *M. gasterostei* the differences were not statistically significant ($x^2_{obs}=43.390>x^2_{.05}=3.85$, 1d.f).

5.4 Discussion

The present study has described the epidemiology of the two myxosporeans *S. elegans* and *M. gasterostei* from the three-spined stickleback collected from a natural habitat. The total prevalence of both parasites was quite high (>80%) and both were present in the host population all year round.

5.4.1 Extrasporogonic Stages.

The prevalence of extrasporogonic stages of *S. elegans* was between 20% and 42.85%, which is lower than that of the sporogonic stages. Extrasporogonic stages are usually reported as having a lower prevalence (Lom *et al.*, 1985b; Supamattaya *et al.*, 1993). Extrasporogonic stages are part of the developmental cycle of *Sphaerospora* and thus all fish harbouring *S. elegans* must have at some time carried these stages. However, their overall prevalence may appear lower than the sporogonic stages because they are not available throughout the year, as are the latter, and also because they are relatively difficult to detect. The extrasporogonic stages of *S. elegans* were found in only 2 months. Feist *et al.* (1991) found that the rete mirabile stages of *S. elegans* had a prevalence of 25%, but did not mention their seasonality of occurrence or the intensity of infection. The prevalence of these stages in this study was a little higher than that observed by Feist *et al.* (1991), but their intensity was very low, probably due to the difficulty of isolating them from the firmly attached rete mirabile

capillaries. A number of reports describe a high prevalence of extrasporogonic stages of *Sphaerospora* spp. Foott and Hedrick (1987) found extrasporogonic stages of PKX at a higher prevalence than that of sporogonic stages, but this is presumably because spore formation does not properly occur, so the extrasporogonic stages take part only in an extensive proliferation. Hedrick *et al.* (1990) found extrasporogonic stages of *S. ictaluri* with a prevalence of 40% to 80%.

Extrasporogonic stages of *S. elegans* may have a very short duration in the host population since samples taken within one week of their detection were negative. The extrasporogonic stages of some *Sphaerospora* species follow a regular seasonal pattern. Grupcheva *et al.* (1985) described marked seasonal fluctuations in the prevalence and intensity of *Sphaerospora* blood stages in three generations of carp fingerlings. They found maximum infection in autumn (August and October) in carp fry of the year, which sharply decreased to zero in the winter and increased again to a second peak in the following spring (February and April). The blood stages coincided with kidney infection only in spring. In the present case blood stages simultaneously occurred with the kidney infection of *S. elegans* which indicates that *Sphaerospora* blood stages do not always precede the appearance of sporogonic stages as also described in PKX (Foott and Hedrick, 1987) and salmon *Sphaerospora* (McGeorge *et al.*, 1994). The extrasporogonic stages observed in the present study undoubtedly belong to *S. elegans*, although the hosts were co-infected with *M. gasterostei*, since only *Sphaerospora* species are known to have extrasporogonic stages in the blood (Lom *et al.*, 1985b). Why the extrasporogonic stages of *S. elegans* are so restricted in their time of appearance while *Sphaerospora* sporogonic infection occurs throughout the year is not resolved. The effect of temperature is not very clear since they appeared both in warm and

cold seasons. Reports on extrasporogonic stages show that they are more common in younger fish of a given species (Lom *et al.*, 1985b; Baska and Molnár, 1988). In the present study extrasporogonic stages were confined to the L2 class of fish so those infected in June must have been hatched that year. McGeorge *et al.* (1994) suggested a development of immunity in fish against salmon *Sphaerospora* which meant that only first year fish became infected, a similar situation occurs in PKX (Ferguson and Ball, 1979)

5.4.2 Seasonal Prevalence.

Developing and spore stages of *S. elegans* were present almost all the year round, although the prevalence varied irregularly. Feist *et al.* (1991) did not identify a seasonal pattern of *S. elegans* infection, but they reported a prevalence rate between 0 and 82%.

In Airthrey Loch monthly prevalence rates were between 45% and 100%. Grupcheva *et al.* (1985) described large fluctuations in kidney infection by *S. renicola* in carp fingerlings. In autumn and winter the kidney infection disappeared while in spring and summer the infection rate was highest. The pattern of seasonal fluctuation of *S. elegans* is markedly different from *S. renicola*. In *S. elegans* a higher prevalence of infection was observed in winter whereas a lower prevalence was observed in spring and summer. This is undoubtedly because most of the previous years' cohort of fish died after breeding in the spring and the young of the year sticklebacks were not yet heavily infected when they first appear in the samples in summer.

Myxobilatus gasterostei was also present in the sticklebacks throughout the year. A high

prevalence was observed in spring and winter and a lower rate in summer and autumn. Booker and Current (1981) found that *Myxobilatus mictospora* in largemouth bass had finger like plasmodia in spring and summer which produced large numbers of spores whereas in winter plasmodia were sheet-like and produced fewer spores. Molnár (1988c) found that in *M. legeri* infecting a range of cyprinid fish, the vegetative proliferation (i.e. probably the presporogonic stages) occurred intracellularly in autumn and spore formation in the kidney tubule mostly occurred in spring. Seasonal development of *M. legeri* was not the same in all infected hosts. In rudd, *Scardinius erythrophthalmus*, the developmental cycle differed in that sporogonesis was demonstrable in winter. *M. gasterostei* differed from these parasites by showing year round sporogenesis. *M. gasterostei* also showed a peak of prevalence in winter and early spring. This probably reflects the aging of the host population so that most fish hatched during the spring and early summer have become infected by winter. The drop in infection in summer is caused by the death of the previous years' cohort and the arrival in the population of young uninfected fish.

A number of authors have suggested that seasonal variations in myxosporean infections resulted from the developmental cycle of the parasite, the availability of susceptible hosts or the effect of environmental factors (Ferguson, 1981; Foott and Hedrick, 1987; Gonzalez-Lanza and Alvarez-Pellitero, 1984; Ching and Munday, 1984; Wolf and Markiw, 1984). In general temperature has been considered as the major factor influencing both the development of the parasite and the immunological state of the host. Both of the species in sticklebacks showed a high prevalence of infection at low temperature in winter. Increase in infection with falls in temperature has also been observed in some other myxosporeans. Mitchell (1977) reported the maximum infection of *Myxobolus dujardini* in northern squafish during cold

months. Alvarez-Pellitero and Sitja-Bobadilla (1993a) reported that the seasonal pattern of infections of *Ceratomyxa* species correlated negatively with the temperature in Meditterrean sea bass, as maximum infection was observed in winter and minimum in summer.

Many myxosporeans show a positive correlation between infectivity and temperature. *Ceratomyxa shasta* shows a generally higher frequency of infection in summer (Udey *et al.*, 1975; Ratliff, 1983; Ching and Munday, 1984). Ferguson (1981) suggested that PKD is temperature dependent and that low temperature inhibits the development of the disease. *Sphaerospora renicola* showed a high prevalence of kidney infection in summer when the fry had hatched but disappeared in autumn and in winter months, and peaked again in spring (Grupcheva *et al.*, 1985). *Sphaerospora dicentrarchi* has a tendency to increase in prevalence with increase in temperature in cultured sea bass, whereas in wild fish the prevalence was 100% all year round (Sitja-Bobadilla and Alvarez-Pellitero, 1992). According to Schafer (1968) the availability of the infective stage (as trophozoites) more than temperature seems to condition the seasonal distribution. Foott and Hedrick (1987) suggested that water temperature may influence the emergence or presence of an infectious stage of PKD in the environment and the susceptibility and subsequent development of the disease in the salmonid host. These authors also provide evidence that seasonal occurrence of PKD corresponds to the time when infectious stages are present in the water supply.

5.4.3 Influence of Host Sex

In most cases there is no correlation between myxosporean infections and host sex (Alvarez-Pellitero and Sitja-Bobadilla, 1993a), but *Sphaerospora testicularis* parasitizes only male sea

bass while Janovy and Hardin (1987) reported higher prevalence of *Myxosoma funduli* in female *Fundulus zebranicus*. Wootton (1976) stated that the prevalence and intensity of parasitic infections is not significantly different in male and female sticklebacks. In this study both sexes of sticklebacks were equally susceptible to *S. elegans*. However, *M. gasterostei* had a significantly higher prevalence in female sticklebacks but the reasons for this are unknown.

5.4.4 Size and Age.

A relationship between host age and prevalence rate has frequently been reported for fish parasites. According to Holloway (1987) a statistically positive relationship between host age and the prevalence of infection of several parasites is a consequence of a higher probability of infection in older fish. On the other hand, Mitchell (1977) reported that very young fish are usually most susceptible to infection by myxosporeans. *Sphaerospora renicola* affects most commonly in carp fingerlings (Lom *et al.*, 1982). In *S. dicentrarchi* a positive statistical interdependence was observed between age and prevalence in sea bass where prevalence increased in young fish but then decreased in older fish (Sitja-Bobadilla and Alvarez-Pellitero, 1993).

In sticklebacks prevalence of *S. elegans* was highest in L3 (3.1-4 cm.) fish and *M. gasterostei* in L2 (2.1-3 cm.) fish. In both cases prevalence decreased with increase in length (i.e. increase in age). The lower prevalence in larger fish may indicate the acquisition of immunity, as reported for other parasites (*Ceratomyxa shasta* in chinook salmon, Ratliff, 1983; *Myxobolus dentium* in *Esox masquinongy*, George *et al.*, 1977; *Myxobolus* spp in

brown trout, Mitchell, 1988). Alternatively larger infected fish may die or infected fish may not grow so rapidly. Feist *et al.* (1991) mentioned that juvenile stickleback in the U.K were mildly infected with *S. elegans* in July and August with very few mature spores being present in the kidney. This is in agreement with the present study.

5.4.5 Source and Time of Infection.

Infection with *M. gasterostei* was first detected in very young sticklebacks between 1 and 2cm in size. The stickleback attains this size 18 to 30 days after fertilization (Wootton, 1976) and starts to feed on microscopic organisms. The exact timing of infection is uncertain. Spore formation in *M. gasterostei* was observed in fish less than 2cm in length which implies a very rapid sporogenesis. Infection with the presumed actinosporean stage must first occur very soon after hatching. It is not known how *M. gasterostei* infects the host, whether this is associated with feeding or by direct invasion of the parasite. It cannot be ruled out that infection can occur at the egg stage. Markiw (1991) reported laboratory infection of eyed eggs and newly hatched sac fry of rainbow trout with *Triactinomyxon*, the infective stage of *Myxosoma cerebralis*. Eyed eggs and one day old sac fry became infected but the infection did not successfully establish. Only in two day old sac fry of rainbow trout did the parasite become properly established. Myxosporean infection can occur orally (Markiw and Wolf, 1983; El-Matbouli and Hoffmann, 1989) or externally through the skin or gill (Yokoyama *et al.*, 1994). In the present case any successful infection at the egg stage must be very low as the prevalence of infection in the small sticklebacks was also very low. In the L2 and larger length classes of fish there was a rapid increase in prevalence and the route of infection might be orally or by direct invasion of the body surface.

Sphaerospora elegans was not found in fish below 2cm in length which suggests a slightly later invasion of the host population. On the other hand, it may be that early stages in younger fish were not detected. It is not certain whether sticklebacks become infected with either parasite only once in their life cycle or whether infection is a continuous process. The presence of early plasmodia of *M. gasterostei* throughout the year and the occurrence of extrasporogonic blood stages of *S. elegans* in June and January suggests the latter may be the case. Infection throughout the year was also reported for *Myxidium rhodei* (Shulman,1966) and *Myxobolus pavlovskii* (Molnár, 1979b)

In stickleback spores of *S. elegans* and *M. gasterostei* are present throughout the year in Airthrey Loch and are also shed throughout the year (see Chapter 7) and are thus available to infect potential oligochaete alternate hosts. If fish become infected by feeding on alternate hosts the seasonal variation in feeding activity will affect the number of spores consumed. Bucher *et al.* (1992) reported that the diet of bullhead, *Cottus gobio*, in a polluted river included large quantities of oligochaetes which transmitted *Zschokkella nova* infection.

A number of authors have studied the diet of stickleback (Hyne, 1950; Chappell, 1969) and reported that a wide variety of organisms may be eaten by these fish, although specific dietary components varied according to season. Seasonal variations in the diet of stickleback from two sites in England indicated that in one site (Birket River) the oligochaete consumption rate was highest in December, January and February, and very low in autumn (Wootton, 1976). On the other hand quantitative analysis of the data indicated that there is a slight decrease in the amount of food eaten during winter. In spring the food consumption increases due to breeding which imposes a high demand for energy by both males and

females. If oligochaete are the alternate hosts of *S. elegans* and *M. gasterostei* and the intake of oligochaete by sticklebacks increases in winter, there may be a possible relationship between this and the observed peak of infection with both parasites in winter.

5.4.7 Mutual Interdependence

A number of myxosporean parasites from the same host species show positive or negative relationships between them. Alvarez-Pellitero *et al.*, (1983) described a positive relationship between *Myxidium carinae* and *Chloromyxum complicatum* infection in the barbel as the number of mixed infections was significantly higher than single species infections. Gonzalez-Lanza and Alvarez-Pellitero (1984) found no inter-relationship between *Myxobolus farionis* and *M. ibericus* in *Salmo trutta*. Sitja-Bobadilla and Alvarez-Pellitero (1993) and Alvarez-Pellitero and Sitja-Bobadilla (1993a) reported no interdependence between the *Sphaerospora* sp. and *Ceratomyxa* spp. infecting sea bass. In the present study a high population of mixed infections occurred, but there was no significant interdependence between the prevalence of two parasite species ($P > 0.05$). Moser (1976) reported apparent positive and negative associations between different myxosporeans of macrourid fish, which he considered to be probably due to seasonal or ecological preferences of the parasites.

Myxobilatus gasterostei was usually present at higher intensity than *S. elegans* in the kidney tubules (Fig.5.16) and both species commonly occurred in the same tubules. Whether this represents a greater competitive success of *M. gasterostei* or a greater proliferative capacity of this species in the kidney is not clear.

CHAPTER 6

MYXOSPOREAN SPORE SHEDDING FROM *G. ACULEATUS*.

6 MYXOSPOREAN SPORE SHEDDING FROM *G. ACULEATUS*.

6.1 Introduction.

The dynamics of spore release by myxosporeans is an important factor in the host parasite relationship and the epidemiology of infection. It may also be important in determining possible control measures. There are no published experimental studies on spore release of myxosporeans. A number of authors have suggested possible exit routes from the host for myxosporean spores (Mitchell,1977; Lom,1987; Hoffman,1990).

Hoffman (1990) and Lom and Dyková (1992) suggested that in histozoic myxosporean species with plasmodia at the body surface mature spores may shed directly into the water by the rupture of the plasmodial wall. In the species sited in the gills or intestine, where there is a natural outlet, spores may be released from the encapsulated plasmodia and the resulting gill tissue lesion repaired. At sites in deeper tissues, once the spores are mature the plasmodia is attacked by host tissue reaction and is destroyed through granuloma formation. Usually spores are ingested by macrophages and transported to the melanomacrophage centres in the kidney, spleen and liver where they are completely digested (Dyková, 1984). Lom and Dyková (1992) suggested that transmission of some histozoic species depends on the death of the host and its decomposition or perhaps ingestion by other animals to release the spores. Coelozoic species may release spores from plasmodia or pseudoplasmodia in to the gut,urinary system or reproductive fluids of the fish and thus spores pass to the environment (Lester,1974).

The objective of the present study was to determine the pattern and rate of spore shedding

by *S.elegans* and *M.gasterostei* from the three-spined stickleback in relation to the environmental temperature and host factors.

6.2 Materials and Methods.

After collection from Airthrey Loch fish were acclimatized for one week at ambient and laboratory temperatures in spore free dechlorinated water.

During experiments fish were maintained in 600 ml beakers containing 200 ml spore free water which was aerated. The sides of beakers were covered with black paper to avoid excess light. In each experiment three beakers were kept at ambient temperature In the open air (labelled a, b, c) and three were kept at laboratory temperature (labelled a1, b1, c1). One to three fish were kept in each beaker and the water filtered using a plankton centrifuge after every 12 or 24 hours for 5 days and every 48 hours for 10 days. Fish were not fed during the experimental period. After the experimental period fish were killed and the kidney was examined individually by a squash preparation to determine whether they were infected by *S.elegans* and / or *M.gasterostei*.

Experiment 1. A total of 14 fish of L3 and L4 length classes were maintained for 5 days at ambient (0-7^{0}c) and laboratory temperatures (18^{0}c). Water was filtered after every 24 hours.

Experiment 2. A total of 12 fish of L2 and L3 length classes were maintained for 5 days at ambient (16^0-20^{0}C) and laboratory temperatures (20^{0}C). Water was filtered after every 12 hours.

Experiment 3. A total of 12 fish of L3 and L4 length classes were maintained for 5 days at ambient (16^0-18^0C) and laboratory temperatures (20^0C). Water was filtered after every 24 hours

Experiment 4. A total of 12 fish of length class L3 were maintained for 10 days at ambient (16^0-20^0C) and laboratory temperatures (20^0C). Water was filtered after every 48 hours.

Experiment 1 was carried out in November and December 1993 while the rest of the experiments were carried out in June and July 1994.

Filtration of Water.

Water from the beakers was filtered using a plankton centrifuge according to the method described by O'Grodnick (1975). Water from each beaker was poured into a separating funnel emptying into the centrifuge. A low flow of water was passed from the separating funnel into the centrifuge which was set at high speed Water from the first few runs was collected and recentrifuged but spores were not deteced in the recentrifuged filtrate and thus this step was later discarded.

After centrifugation of 200ml water, 5 to 6ml of filtrate was obtained from the centrifuge. This filtrate was collected in a bottle and samples were taken directly from this to count the number of spores released by fish.

Spore Counting.

Before sampling the filtrate, a bottle was shaken for about 5 minutes to obtain a

homogeneous suspension. A drop of filtrate was poured into the counting chamber of a Neubauer ruled bright line haemocytometer. Spores were counted in 80, 1 mm square areas at a magnificaion of x20 to x40. A total of 40 samples were taken from each filtrate and counted in the haemocytometer.

The ruled surface of the haemocytometer is 0.1mm below the coverglass. The volume over a 1mm square area is $0.1mm^3$ or $1x10^4cc$. The average number of spores counted over a $1mm^2$ area multiplied by 10^4 results in an estimation of the number of spores in a $1mm^2$ volume. The following formula was used to calculate the number of spores in 1 ml of filtrate:

$$\frac{\text{No. of spores}}{1\text{ml}} = \frac{\text{Total no. of spores counted x } 10^4}{\text{Number of } 1mm^2 \text{ areas counted.}}$$

Since all released spores were concentrated in 5 to 6ml of water so the number of spores concentrated in that filtrate was obtained by multiplying the number of spores per ml by the volume of filtrate.

As 1 to 3 fish were kept in each beaker the number of spores obtained in the total filtrate was divided by the number of fish in the beaker to gave the mean number of spores released by each fish.

To determine the total number of spores of *S.elegans* and *M.gasterostei* in the kidney of

individual sticklebacks 10 fish were examined immediately after capture from the Loch and a further 10 after acclimatization for 5 days. Fish were killed and their kidneys were removed and macerated individually in a mortar and pestle. Three ml of water were added to this macerated kidney and the suspension was poured into a bottle. Ten samples from each fish were taken directly from the kidney suspension and spores were counted in a haemocytometer as described above.

6.3 Results.

Tables 6.1-6.6 show results of the experimental studies on the shedding of spores of *S.elegans* and *M.gasterostei* by the three-spined stickleback.

6.3.1 Experiment 1.

Ambient temperature: Six fish were observed at ambient (0-7^{0}c) temperature (Table 6.1). Both *S.elegans* and *M.gasterostei* spores were released on days 1-4 but spores were not shed in each beaker on each sampling day. Spore release was fairly consistent over the first four days of the experiment. Spores were not found on the 5th day. One fish died from beaker a on the 3rd day and one from beaker c on the 4th day. Both of the fish in beaker c died on the 5th day.

Laboratory temperature: A total of 8 fish were maintained at a laboratory temperature of 18^{0}c (Table 6.2). Again, spores were released from fish in each beaker over days 1-4. Spores of *S.elegans* released were fewer in number than those of *M. gasterostei* and on some days no

spores of this species were found in the filtered water. No spores of either species were found in day 5. Both fish in beaker c1 died on the 5th day.

Examination of kidney squashes showed that all fish from ambient and laboratory temperatures were infected with mature spores and developing stages of both parasites.

6.3.2 Experiment 2.

No spores of any parasites were found in the filtered water at ambient and laboratory temperatures. However, examination of the the kidney squashes showed that all fish were infected with the pre-sporogonic, sporogonic and mature spore stages of *S.elegans* and *M. gasterostei* at both temperatures.

6.3.3 Experiment 3.

Ambient temperature: Six fish of L3 and L4 size were maintained in the ambient temperature of 16 to 20^{0}c (Table 6.3). Spores of *M.gasterostei* were shed in beaker a on days 1-4 but in beakers b and c only on days 1 and 2. Spores of *Sphaerospora elegans* were released in beaker a on days 1 and 2 and in b and c only on day 2. Numbers of spores released by both species were generally similar. One fish in beaker b and both fish in beaker c died on the 3rd and 4th day. Spores were not found in any of the remaining beakers on day 5.

Laboratory temperature: Six fish of L3 size were observed at a constant laboratory temperature of 20^{0}c (Table 6.4). Spores of *M. gasterostei* were shed in beaker a1 on days

1, 3 and 4, in beaker b1 on days 1 and 2 and in beaker c1 on days 2, 3 and 5. Spores of *Sphaerospora elegans* were shed on days 1-4 in beaker a1, on days 1 and 2 in beaker b1 and in beaker c1 on days 2 and 5. Again numbers of spores shed were similar in both myxosporean species. One fish died from beaker b1 on the 3rd day and all fish in beakers a1 and b1 died on the 5th day.

Examinations of kidney squashes showed that all fish at ambient and laboratory temperatures were infected with both parasites.

6.3.4 Experiment 4.

Ambient temperature: Six fish were maintained at an ambient temperature of 16-20^{0}c (Table 6.5). Spores of *M. gasterostei* were not shed in beaker a on day 4 and in c on days 2 and 5. Spores of *S. elegans* were shed in beakers a and b on days 1, 2, 3 and 5, but in beaker c only on days 3 and 4. Two fish each from beaker b and c died on the 4th and 5th day.

Laboratory temperature: Six fish were observed at a constant laboratory temperature of 20^{0}c (Table 6.6). Spores of *M. gasterostei* were shed in all the beakers on days 1 and 2, but in beakers b1 and a1 no spores were shed on days 3, 4 and 5. Spores of *S. elegans* were shed in all beakers on day 1, but on days 2, 3 and 4 spores were found in two of the three beakers, whereas on day 5 spores were found only in beaker c1. One fish from beaker a1 died on the 3rd day.

Examinations of kidney squashes showed both of the parasites were present in the kidney of all fish at ambient and laboratory temperatures.

6.3.5 No. of Spores of *S. elegans* and *M. gasterostei* in kidneys of Individual Sticklebacks.

Kidneys of stickleback were prepared individually to estimate the number of spores present in each fish. Table 6.7 shows the numbers of spores isolated from fish immmediately after collection from Airthrey Loch. Kidneys of ten fish of L2, L3 and L4 length classes were used and all fish were infected with *M.gasterostei* and *S.elegans*. In 8 of the 10 fish spores of *M.gasterostei* were more abundant, usually considerably so than those of *S.elegans*. Spores of *M.gasterostei* were more abundant in L3 and L4 fish than in L2 fish, but this was not the case with *S.elegans*.

Table 6.8 shows spores isolated from the kidneys of fish maintained experimentally for 5 days. In total 10 kidneys from L3 and L4 size fish were prepared. Again in most cases spores of *M.gasterostei* were higher in number than those of *S.elegans*. One fish of the L3 length class was not infected with *S.elegans*. There was a little difference between the number of spores of either myxosporean species in the two length classes of fish.

6.4 Discussion.

The accuracy of the plankton centrifuge technique, especially on water samples where the number of spores are relatively low, is uncertain and the figures presented here should

Table 6.1 Experiment 1: spores released at ambient temperature.

Days	No. of Fish	No. of Spores Counted		No. of Spores Counted per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
Ist day	(Size)	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a	2 (L3)	4	1	500	125	2000	500	1000	250
b	2 (L4)	2	3	250	375	1000	1500	500	750
c	2 (L4)	0	0	0	0	0	0	0	0
2nd day									
a	2	3	2	375	250	1500	1000	750	500
b	2	0	0	0	0	0	0	0	0
c	2	0	2	0	250	0	1000	0	500
3rd day									
a	1	2	0	250	0	1000	0	500	0
b	2	1	0	125	0	500	0	250	0
c	2	0	0	0	0	0	0	0	0
4th day									
a	1	0	0	0	0	0	0	0	0
b	2	2	1	250	125	1000	500	500	250
c	1	3	3	375	375	1500	1500	750	750
5th day									
a	1	0	0	0	0	0	0	0	0
b	1	0	0	0	0	0	0	0	0
c	0	0	0	0	0	0	0	0	0

Table 6.2 Experiment 1: spores released at laboratory temperature.

Days	No. of Fish	No. of Spores Counted		No. of Spores Counted per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
1st day	(Size)	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a1	2(L3)	0	0	0	0	0	0	0	0
b1	3(L4)	2	0	250	0	1250	0	416.66	0
c1	3(L4)	2	2	250	250	1250	1250	416.66	416.66
2nd day									
a1	2	2	2	250	250	1250	1250	625	625
b1	3	2	0	250	0	1250	0	625	0
c1	3	0	0	0	0	0	0	0	0
3rd day									
a1	2	1	0	125	0	625	0	312.5	0
b1	1	1	0	125	0	625	0	312.5	0
c1	3	0	0	375	0	0	0	0	0
4th day									
a1	2	2	1	250	125	1250	625	625	625
b1	1	3	0	375	0	1875	0	1875	0
c1	2	3	2	375	250	1875	1250	937.5	625
5th day									
a1	1	0	0	0	0	0	0	0	0
b1	1	0	0	0	0	0	0	0	0
c1	2	0	0	0	0	0	0	0	0

Table 6.3 Experiment 3: spores released at ambient temperature.

Days	No. of Fish	No. of Spores Counted		No. of Spores Counted per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
Ist day	(Size)	*M. Gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a	2(L3)	3	3	375	375	1875	1875	937.5	937.5
b	2(L3)	2	0	250	0	1375	0	687.5	0
c	2(L4)	2	0	250	0	1250	0	687.5	0
2nd day									
a	2	2	1	250	125	1250	687.5	625	343.7
b	2	2	2	250	250	1250	1250	625	625
c	2	2	3	250	375	1250	1875	625	937.5
3rd day									
a	2	4	1	500	125	2500	625	1250	312
b	1	0	0	0	0	0	0	0	0
c	2	0	0	0	0	0	0	0	0
4th day									
a	2	2	2	250	250	1375	1375	687.5	687.5
b	1	0	0	0	0	0	0	0	0
c	0	0	0	0	0	0	0	0	0
5th day									
a	2	0	0	0	0	0	0	0	0
b	1	0	0	0	0	0	0	0	0
c	0	0	0	0	0	0	0	0	0

Table 6.4 Experiment 3: spores released at laboratory temperature.

Days	No.of Fish	No. of Spores Counted		No. of Spores Counted per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
1st day	(Size)	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a1	2	2	2	250	250	1250	1250	625	625
b1	2	2	2	250	250	1375	1375	687.5	687.5
c1	2	0	0	0	0	0	0	0	0
2nd day									
a1	2	0	2	0	250	0	1250	0	625
b1	2	3	2	375	250	1875	1250	937.5	625
c1	2	2	2	250	250	1250	1250	625	625
3rd day									
a1	2	3	1	375	125	1875	625	937.5	312.5
b1	1	0	0	0	0	0	0	0	0
c1	2	3	0	375	0	1875	0	937.5	0
4th day									
a1	2	2	2	250	250	1375	1375	687.5	687.5
b1	1	0	0	0	0	0	0	0	0
c1	2	0	0	0	0	0	0	0	0
5th day									
a1	0	0	0	0	0	0	0	0	0
b1	0	0	0	0	0	0	0	0	0
c1	2	2	3	250	375	1250	1875	625	937.5

Table 6.5 Experiment 4: spores released at ambient temperature.

Days	No. of Fish	No. of Spores Counted		No. of Spores per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
1st day		*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a	2	2	2	250	250	1750	1750	875	875
b	2	4	2	500	250	3500	1750	1750	875
c	2	2	0	250	0	1750	0	875	0
2nd day									
a	2	4	3	500	375	3500	2625	1750	1312.5
b	2	4	4	500	500	3500	3500	1750	1750
c	2	0	0	0	0	0	0	0	0
3rd day									
a	2	7	2	875	250	6125	1750	3062.5	875
b	2	5	3	625	375	4375	2625	2187.5	1312.5
c	2	8	3	1000	375	7000	2625	3500	1312.5
4th day									
a	2	0	0	0	0	0	0	0	0
b	1	3	3	375	375	2812.5	2812.5	1406.25	1606.25
c	2	3	3	375	375	3000	3000	1500	1500
5th day									
a	2	3	4	375	500	2625	35000	1312.5	1750
b	1	2	2	250	250	1750	1750	875	875
c	1	0	0	0	0	0	0	0	0

Table 6.6 Experiment 4: spores released at laboratory temperature.

Days	No.of Fish	No. of Spores Counted		No. of Spores Counted per ml.		Total Spores		Mean no. of Spores Released by Each Fish	
1st day		*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
a1	2	3	3	375	375	2625	2625	1312.5	1312.5
b1	2	2	3	250	375	1750	2625	875	1312.5
c1	2	4	2	500	250	3000	1750	1500	875
2nd day									
a1	2	2	2	250	250	1750	1750	875	875
b1	2	5	3	725	375	4375	2625	2187.5	1312.5
c1	2	5	0	625	0	4375	0	2187.5	0
3rd day									
a1	1	2	2	250	250	1750	1750	1750	1750
b1	2	0	0	0	0	0	0	0	0
c1	2	3	2	375	250	2625	1750	1312.5	875
4th day									
a1	1	0	0	0	0	0	0	0	0
b1	2	6	4	750	500	5250	3500	2625	1750
c1	2	7	3	875	375	6125	2625	3026.5	1312.5
5th day									
a1	1	0	0	0	0	0	0	0	0
b1	2	9	0	1125	0	7875	0	3937.5	0
c1	2	5	2	625	250	4375	1750	2187.5	875

Table 6.7: Spores isolated from individual kidneys of stickleback after collection from the natural habitat.

Size of Fish	No. of Spores Counted		No. of Spores per ml.		Total Spores per kidney	
	M. gasterostei	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
L2	5	3	5000	3000	15000	12000
L4	7	2	7000	2000	21000	6000
L4	6	5	6000	5000	18000	15000
L3	6	2	6000	2000	18000	6000
L3	5	6	5000	6000	15000	18000
L2	4	6	4000	6000	12000	18000
L3	6	3	6000	3000	18000	9000
L4	5	3	5000	3000	15000	9000
L3	7	2	7000	2000	21000	6000
L2	5	1	5000	1000	15000	3000

Table 6.8: Spores isolated from individual kidneys of experimental fish.

Size of Fish	No. of Spores Counted		No. of Spores per ml.		Total Spores per kidney	
	M. gasterostei	*S. elegans*	*M. gasterostei*	*S. elegans*	*M. gasterostei*	*S. elegans*
L3	4	3	4000	3000	12000	9000
L3	4	0	4000	0	12000	0
L3	5	4	5000	4000	15000	12000
L4	5	3	5000	3000	15000	9000
L4	4	2	4000	2000	12000	6000
L4	4	4	4000	4000	12000	12000
L3	6	3	6000	3000	18000	9000
L3	5	6	5000	6000	15000	18000
L4	3	2	3000	2000	9000	6000
L4	6	4	6000	4000	18000	12000

perhaps be taken as an indication of relative spore abundance rather than an absolute measure of abundance. However, the results obtained were consistent between experiments which gives some confidence that they accurately reflect the pattern of spore release. These experiments have shown that spores of both *S.elegans* and *M.gasterostei* are shed throughout the year and that temperature apparently has little effect on the rate of spore release. The rate of spore release of the two myxosporean species appeared to be similar.

The rather artificial experimental conditions might well have affected the physiological state of the fish and thus spore release. Rate of urine flow would obviously be important in influencing the quantity of spores shed. Hoar and Randall (1969) described how the urine flow of fish depends on the permeability of the body surface to water. Thus, fish living in freshwater normally have large weight specific urine flows, which is associated with their large permeable skin surface. Skin surface permeability to water can be influenced and increased by a number of factors including handling, stress, injury to the integument and rising temperature. The death of at least some fish in many of the beakers during the experiments suggests that they were under considerable stress. Spores were not found in the filtrates from fish examined every 12 hours, probably due to the frequent handling which was very stressful to the fish. It is not clear how long spores of *S.elegans* or *M.gasterostei* are retained in the kidney before release. It might be expected that once spores are released from the plasmodia they would be flushed out of the kidney tubules and urinary bladder quite rapidly. Spores were not found in every filtrate, even though they were known to be present on the kidney, which might suggest that shedding is not a continuous process, although again disturbances in the physiology of the experimental fish may have influenced this. At the end of the experimental period spore counts decreased in many cases, although kidney squashes

showed that mature spores were still present in the kidney. This may also reflect increasing stress over time.

Lom and Dyková (1992) noted two types of spore release in coelozoic myxosporeans. Some species have a limited release period during which all spores are released. These species usually have an annual cycle like *S.renicola*. Other coelozoic myxosporeans such as *Myxidium lieberkhuni* have an indefinite period of spore release. *S.elegans* and *M.gasterostei* seem to belong in the latter category, despite their annual occurrence in the stickleback population in Airthrey Loch. This pattern of continued spore production suggests that infection of any alternate host would occur all year round.

The total number of spores isolated from individual kidneys did not differ greatly between experimental fish and natural fish. In fish taken from the Loch, total spores of *M. gasterostei* per fish were 15,000 to 21,000 and *S. elegans* 3,000 to 18,000. In experimental fish total spores of *M. gasterostei* were 9,000 to 18,000 and of *S. elegans* were 6,000 to 18,000 per fish. Prasher *et al.*(1971) also using the plankton centrifuge, estimated an average of 32,430 spores of *Myxosoma cerebralis* from the head of a 4-7cm rainbow trout. The number of spores was even greater in larger rainbow trout. Hoffman (1968, in O'Grodnick,1975) recovered $4.2x10^5$ to $4.3x10^6$ spores of *M. cerebralis* per fish, whereas O'Grodnick recovered $3.4x10^4$ to $9x10^6$ spores. Estimation of numbers of myxosporean spores from other organs of fish has not been carried out.

The numbers of spores of both *S.elegans* and *M. gasterostei* obtained from sticklebacks are fairly consistent with those given by Prasher *et al.*(1971). *M. cerebralis* are not released

except on the death of the host and thus will accumulate in the fish over time, whereas spores of *S. elegans* and *M. gasterostei* are continuously released and thus do not build up in the host. Spore production over the life of a stickleback would be much greater than the numbers obtained here.

In the present study the rate of spore release per fish was very high relative to the total number of spores counted per kidney for both *M. gasterostei* and *S. elegans*. Even allowing for inaccuracies in the plankton centrifuge technique this variation suggests a continuous and rapid production and release of spores of both myxosporeans throughout the year.

CHAPTER 7

EPIDEMIOLOGY OF *GYRODUCTYLUS GASTEROSTEI* GLASER, 1974 ON *G. ACULEATUS*.

7 EPIDEMIOLOGY OF *GYRODACTYLUS GASTEROSTEI* GLASER, 1974 ON *G. ACULEATUS*.

7.1 Introduction

Monogenean parasites of the genus *Gyrodactylus* are widespread in both marine and freshwater fish and presently over 400 species of this genus are known (Harris,1993). In recent years there have been detailed biological studies on some species, notably *G. bullatarudis* and *G. turnbulli* on guppies (Scott, 1982; Scott and Anderson, 1984; Harris, 1988, 1989; Harris and Lyles 1992) and *G. salaris* on salmon (Bakke *et al.*, 1990, 1992a,b, Harris *et al.*, 1993). The three-spined stickleback is a host of a number of *Gyrodactylus* species including *G. gasterostei* (Table 1.1).

7.1.1 Taxonomy.

Gyrodactylus gasterostei has apparently been misidentified by many authors. For example, Chappell (1969) confused the species with *G. rarus* whereas Powell (1966) described it as *G. pungitii* (Harris, 1982). Glaser (1974) formally synonymised these species as *G. gasterostei*.

Harris (1982) described *G. gasterostei* as a large species with a cylindrical body and a distinct posterior haptor, armed with a single pair of ventral hamuli. The hamuli possess straight shafts and parallel roots. There is a long ventral bar with small processes and a slender triangular membrane which joins the hamuli. Sixteen articulated marginal hooks have

straight shafts and claw shaped sickles at their tip. The anterior of the body has two small cephalic lobes which bear uniciliate sense organs. There is a sub-terminal pharynx with a long pharyngeal process. Just behind the pharynx the gut divides into two and extends the length of the body.

7.1.2 Reproduction and Population Growth.

As *Gyrodactylus* is viviparous and directly reproduces on the surface of the host it may show explosive exponential population growth on individual fish (Scott and Anderson, 1984).

Harris (1993) reviewed the diverse reproductive strategies of *Gyrodactylus*. The parasite contains an embryo, which also contains a further developing embryo, a phenomenon termed hyperviviparity by Cohen (1977). The cytological details of this unusual development are still not clear, but there is evidence that the first and second embryos can develop without cross or self fertilization (Braun, 1966; Lester and Adams, 1974b; Scott, 1982; Harris, 1985; Jansen and Bakke, 1991) and only the third and subsequent embryos can develop sexually (Malmberg, 1957; Braun, 1966; Harris, 1989).

To fully understand the nature of the parasite-host interaction, it is necessary to know the population growth of the parasite within the host. This can be obtained from a knowledge of the reproductive rate and mortality of the parasite. A number of authors (Turnbull, 1956; Bychowsky, 1957; Lester and Adams, 1974a; Harris, 1980b; Scott, 1982) determined the maximum potential rate of reproduction for *Gyrodactylus* using direct observations.

It is believed that the relative importance of sexual reproduction depends on the population age structure and mortality of the parasite. Harris (1989) described in *G.turnbulli* from guppies alternate periods of predominantly asexual multiplication, when the parasite population is small, and phases of cross-insemination when the population is large. *G.turnbulli* is short lived with exponentially increasing age specific mortality, which results in less than 1% surviving to give birth a third time. On the other hand *G.salaris* exhibits a different pattern on salmon, with low mortality and 10-15% of the individuals surviving to reproduce sexually (Harris,1993). Inseminated individuals of this parasite were found in light infections and sexual reproduction forms a normal part of the biology of this species. Harris (1982) observed experimentally that *G.gasterostei* on *Gasterosteus aculeatus* showed increased mortality at 15^0c, but at 10^0C mortality increased less rapidly with the age of the parasite.Harris also mentioned that sexual reproduction was very rare in natural populations of this species.

The measurement of actual population growth on the host and parasite mortality has been attempted for *G. alexanderi* by Lester (1972) and Lester and Adams (1974a,b) and for *G. bullatarudis* by Scott (1982). It has been observed that populations of *G. alexanderi* on *G. aculeatus* reached a peak and then subsequently declined. Lester and Adams (1974b) suggested this decline was due to a host response. Studies on population dynamics of *Gyrodactylus* (Lester and Adams, 1974a,b; Harris, 1980b; Scott, 1982) suggest that rapid reproduction is the major source of population increase upon the host and the role of immigration and transmission between hosts is less important. On the other hand, according to Harris (1982), transmission of *Gyrodactylus* species take place throughout the life cycle of the parasite and this has an effect on overall dynamics of the parasite suprapopulation.

7.1.3 Transmission.

Unlike most monogeneans transmission of *Gyrodactylus* can take place throughout the life cycle and it is necessary to understand the mechanism of transmission in order to describe the overall dynamics of the parasite population. Bakke *et al.* (1992b) reviewed four routes of gyrodactylid transmission: -

1. Direct contact between hosts.
2. Contact between hosts and detached parasites on the substrate.
3. Contact between hosts and detached parasites drifting in the water current.
4. Contact between living hosts and infected dead hosts.

The most important method of transmission of gyrodactylids is host to host contact. Harris (1982) observed that parasites can pass through a mesh barrier presumably by crawling through it and reinfect fish. Transmission may also occur via detached parasites. Gyrodactylids may become detached from their host through accident, active migration or due to host response (Lester, 1972; Scott and Anderson, 1984). Bakke *et al.* (1992a) suggested this method of transmission is important for gyrodactylids of benthic hosts. Malmberg (1970) reported that gyrodactylids may abandon the host after its death, while Harris (1980a) suggested that the increased activity of gyrodactylids upon dead fish may increase their chance of transmission to living fish. Bakke *et al.* (1992a) suggested that this transmission route in gyrodactylid epidemiology is limited by parasite survival after host death and by the speed at which the parasites leave the dead fish and find a living host. Harris (1980b) observed in *G. gasterostei* up to 50% of the parasites left dead fish within 18 hours at 15^{0}c. Bakke *et al.*, (1992b) mentioned that transmission by dead fish can also be

limited by the number of infected hosts dying, because in many gyrodactylid host interactions host mortality is very small. However, Scott and Anderson (1984) showed parasite induced host mortality to be important for dispersion of *G. bullatarudis*.

7.1.4 Host Specificity.

Generally monogeneans are considered as narrowly host specific, due to their long period of co-evolution with the fish host (Bychowsky, 1957). Bychowsky (1957) considered gyrodactylids as the least specific group of monogeneans, but others have supported the idea of a high degree of specificity in gyrodactylids (Hoffman and Putz, 1964; Malmberg, 1970; Glaser, 1974). Harris (1982) interpreted two extreme possibilities of gyrodactylid specificity, that *Gyrodactylus* species are narrowly host specific, each host supporting an assemblage of distinct parasite species, or that only a few non-specific species exist which show small morphological variations between different hosts, which are only a reflection of their genetic adaptation to the host concerned. Bakke *et al.* (1992b) analyzed previously published host records of 319 gyrodactylid species and suggested that these in fact represented only 76 species. According to these authors gyrodactylids are less host specific than commonly thought and narrow host specificity is an artifact, based on numerous species descriptions of material collected from only a single host species. They concluded for example, that *G. salaris* has a particularly broad range of specificity.

Wood and Mizelle (1957 in Bakke *et al.*, 1992b) found that 22% of a total of 28 *Gyrodactylus* species may be found on more than one host species. Margolis and Arthur (1979) listed 33 *Gyrodactylus* species of which 58% could be characterized as being host

specific, 39% as narrowly host specific and 3% as having a broad host specificity. Bakke *et al.* (1992b) concluded that at least three mechanisms maintain the specificity of *Gyrodactylus* species.

Firstly, behavioral mechanisms may maintain the parasite on the normal host. Harris (1982) observed in laboratory experiments that up to 10% of *G. gasterostei* transmitted to uninfected *G. aculeatus*, whereas under the same conditions transmission to other hosts (*Pungitius pungitius* and *Phoxinus phoxinus*) was only 1%. *G. salaris* also showed a similar behaviour, with a reduced probability of attachment to other hosts in comparison to salmon (Bakke and Sharp, 1990; Bakke *et al.*, 1990). A second mechanism is physiological, where parasites infect a host, persist for some days, but cannot feed or reproduce and eventually die. An example of this is *G. salaris* infection in eels (Bakke and Sharp, 1990; Bakke *et al.*, 1990). The third mechanism maintaining gyrodactylid specificity involves responses of the hosts to the parasite and is seen in *G. salaris* infecting salmonids (Bakke *et al.*, 1992a). It is presumed that the differences in innate resistance and timing and efficacy of the host reaction between fish species are genetic.

7.1.5 Distribution of Parasite Within the Host Population.

The distribution of parasites within the host population has been the subject of considerable attention (Crofton,1971a,b ; Pennycuick,1971b; Boxshall,1974; Anderson and May,1978; May and Anderson, 1978). Generally parasite populations are normally overdispersed within the host population (Crofton, 1971b). Crofton (1971b) developed a model of parasite population regulation, where death of heavily infected hosts removed parasites from the

population, thus regulating their reproductive output.

According to Anderson and Gordon (1982), distribution patterns of parasites within their host populations can be broadly divided into three categories: under-dispersed, random and over-dispersed. These distribution patterns are commonly described by three probability distributions:

a. Positive binomial for under-dispersed patterns of parasite distribution

b. Poisson for random patterns of parasite distribution.

c. Negative binomial for over-dispersed patterns of parasite distribution.

A simple measure of the degree of dispersion that has been used widely is the variance to mean ratio of parasite number per host.

In natural habitats the identification of the processes responsible for generating the observed distribution patterns of parasites is fraught with difficulties and has attracted relatively little attention (Anderson & Gordon, 1982). However, distribution patterns in natural habitats may provide important clues concerning the influence of the parasite on the dynamics of the host population.

Harris (1980b) described how the regulation of *Gyrodactylus* populations by host death and host reaction was complicated by the survival of the detached parasites, especially during periods of high host density when the possibility of reattachment to host fish is increased. He suggested the regulation of gyrodactylid populations occurs at two levels :

1. On the individual host where stochastic fluctuations in birth and death rates and density dependent processes may limit population size.
2. In the population as a whole where interaction of the distribution of the parasite within the host population, parasite transmission and host death are important in determining the size of the parasite supra-population.

Environmental factors may influence the patterns of distribution of parasites in single hosts or within the host population. Among the environmental factors temperature and host density are the most important factors. Temperature has an influence on the reproductive rate of an organism in a complex way (Wieser, 1973). Increased temperature may reduce the carrying capacity of an environment due to an increased metabolic rate of the organism (Hutchinson, 1978).

7.1.6 Seasonal Occurrence.

Generally gyrodactylids can reproduce continuously throughout the year. However, studies on the seasonal occurrence of *Gyrodactylus* species shows that there is a strict influence of temperature on the prevalence and intensity of infection which generates a definite seasonal cycle of the parasite. Harris (1982) described two groups of gyrodactylids on the basis of their seasonal abundance. One group shows maximum abundance in summer, and the other a maximum in winter. Most of *Gyrodactylus* species, including *G. salaris,* belong to the former group. Relatively few species show maximum abundance in colder seasons but these include *G. meidus* infecting *Onos mustela* (Srivastava and James, 1967), *G.stephanus* infecting *Fundulus heteroclitus* (Dickenson and Threlfall, 1975) and *G. atratuli* in *Notropis*

spilopterus (Kirby, 1981). On the other hand, Rawson and Rogers (1973) described *G. macarochiri* as being most abundant in spring and autumn at an intermediate temperature. Apart from temperature many other factors such as host parasite interaction and interactions of parasite populations with each other may influence the seasonal dynamics of *Gyrodactylus*.

7.1.7 Objectives.

The epidemiology of gyrodactylids from natural environments has been studied for only relatively few species (Chappell, 1969; Rawson and Rogers, 1973; Kirby, 1981; Harris, 1982; Chubb, 1977; Jansen and Bakke, 1993). A number of laboratory based epidemiological studies have also been made (Bakke *et al.*, 1990; Jansen and Bakke, 1991; Bakke and Mackenzie, 1993; Harris, 1980b, 1988; Lester and Adam, 1974a,b). The aim of the present study was to investigate the epidemiology of *G. gasterostei* on three-spined sticklebacks from a natural habitat. Special emphasis has been given to the importance of processes that may influence the interaction of the parasite with the different life cycle stages of host.

7.2 Materials and Methods

7.2.1 Preparation of Fish for Examination.

Fish were collected and maintained in the laboratory until examination as described in Chapter 2. Fish were killed, measured and allocated to length class according to the method described in Chapter 2.

Immediately after a fish was killed, it was placed in a plastic Bijoux pot with 1/4000

formalin (250 ppm) for a minimum of 15 minutes, with occasional shaking (Davis, 1961; Lester and Adams, 1974a). This method caused the *Gyrodactylus* to drop off the fish. This procedure was repeated twice to ensure that all parasites were recovered from the fish. The contents of the Bijoux pot were poured into a Petri dish and observed under the dissecting microscope at X1 and X4 magnification. The parasites from each fish were counted three times and the mean number taken as the total number of parasites in that fish.

7.2.2 Identification of *Gyrodactylus*.

Ten specimens of *Gyrodactylus* from each fish were prepared for microscopic examination to determine specific identity.

Parasites were placed on glass microscope slides with a drop of 1/4000 formalin and the cover slip placed over the parasite. Excess solution was absorbed using a filter paper. The whole mount was examined using a phase contrast microscope under oil immersion at x100 magnificaion.

Some parasite specimens were fixed under the coverslip by adding a drop of ammonium picrate-glycerin to the edge of the cover slip (Malmberg, 1957). The fixing medium quickly and totally embedded the parasite allowing detailed examination.

Identification of the parasite was made according to the characteristics described by Malmberg (1970) and Harris (1982). Prevalence of infection was calculated by the number of infected host species divided by the number of hosts examined x100 (Margolis *et al.*,

1982). The mean intensity is the mean number of parasites per infected host in a sample.

7.2.3 Statistical Methods.

Observed parasite distributions were fitted to the theoretical negative binomial (Elliot, 1977), and variance/mean ratio was calculated (Gordon and Anderson, 1978). Significance levels were determined by the Chi-square test (Elliot, 1977). A probability of $p<0.05$ was considered significant. The prevalence rate between the two sexes was compared by using a 2x2 contingency table. The relationship of the intensity of infection to the size of the fish and differences in intensity between sexes of sticklebacks were tested by using the non-parametric Kruskal Wallis test (Sokal and Rohlf, 1981). All statistical methods were performed by using computer packages (Minitab and Quattro pro, version 1.00).

7.3 Results.

Gyrodactylus gasterostei was found on the skin and fins of the three-spined stickleback from Airthrey loch. A total of 369 fish were infected, giving a prevalence rate of 90.83%. Of the infected fish 134 were male, 212 were female and 23 were of undetermined sex. The prevalence of infection in males was 91.89% and in females 94.22%. The difference in prevalence rate between male and female sticklebacks was not statistically significant.

The overall mean intensity of infection was 18.92. The mean intensity of infected male fish was 22.72 and in females it was 19.85. This difference was not significant. Most of the fish of undetermined sex were very small with a comparatively low mean intensity of 10.95.

7.3.1 Seasonal Prevalence and Intensity of Infection.

Figure 7.1 presents prevalence and mean intensity of infection in each month of the study period. The prevalence of infection was above 90% in most months. A prevalence rate of 100% was recorded in April and December. The prevalence was relatively constant between January and May before falling to a minimum level of 45.5% in June. Subsequently it recovered again to a high level in August and remained so until the end of the sampling period.

Figure 7.2 shows that the mean intensity of infection in general paralleled the prevalence rate. The intensity of infection fluctuated between January and November 1993 but was generally lowest over the summer period. From November 1993 to February 1994 the mean intensity reached high levels of 28-35 *G. gasterostei* per fish. This level of infection was much higher than in the previous winter.

7.3.2. Prevalence and Intensity of Infection in Different Length Classes of Fish.

Figure 7.3 shows the prevalence of *G. gasterostei* in each length class of fish. The parasite was found in all length classes but the prevalence rate and mean intensity of infection were lowest in the smallest fish. The maximum number of parasite in this length class was only 2. In fish of length class L2 and above *G. gasterostei* was very abundant with prevalence rates of over 90% and a mean intensity of infection of 15.73 to 41.30. Although the prevalence rate remained fairly constant in classes L2-L4 the mean intensity rose markedly with increasing length of the fish (Fig.7.4). To test the relationship between intensity of

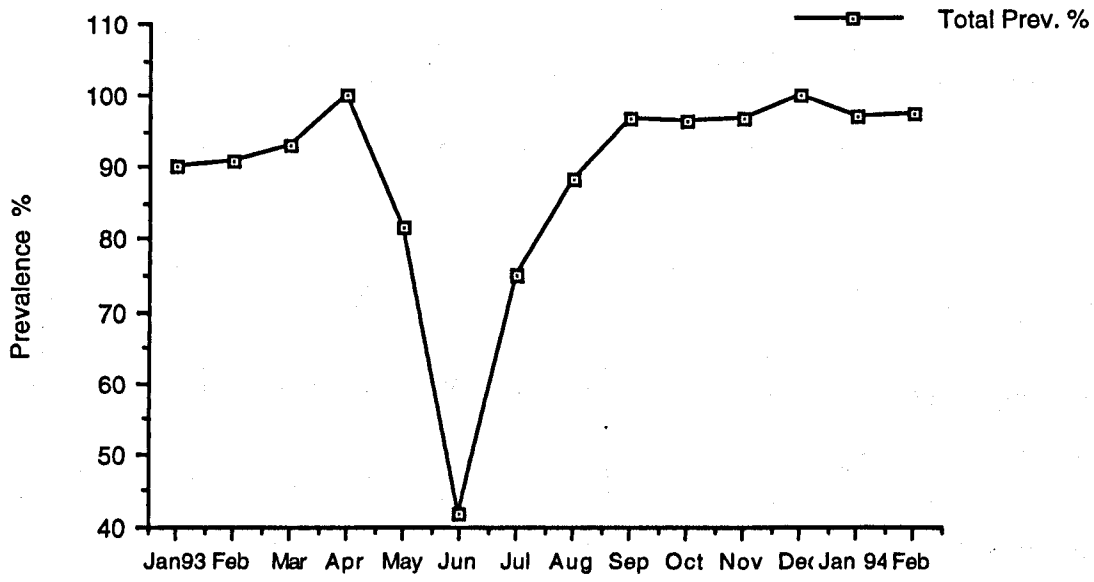

Fig: 7.1 Overall prevalence of *G. gasterostei* in different months.

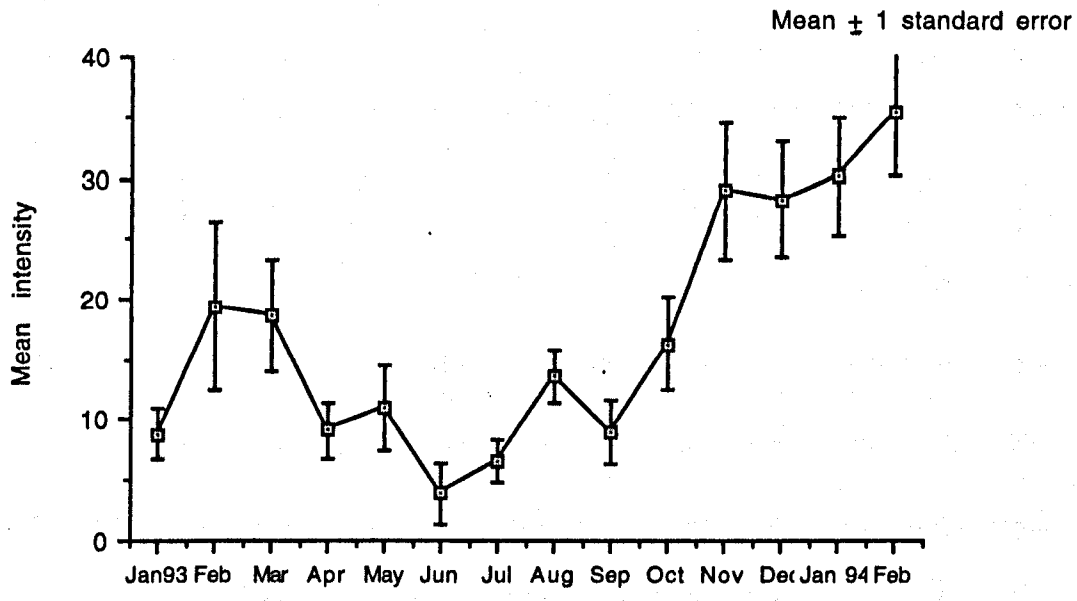

Fig: 7.2 Mean intensity of *G. gasterostei* in different months.

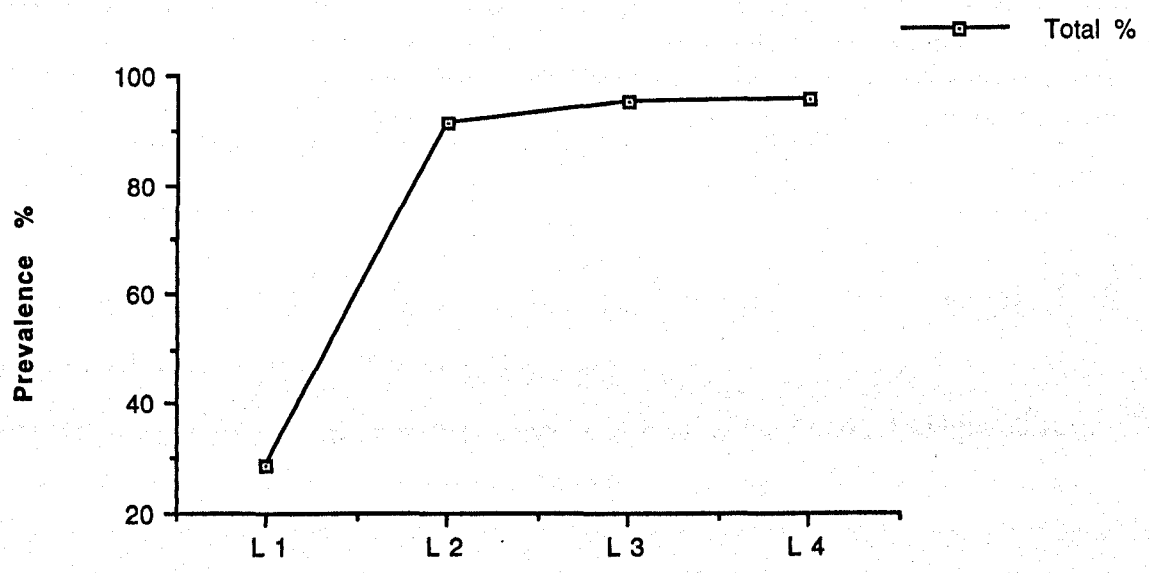

Fig:7.3 Total prevalence of *G.gasterostei* in different length classes of sticklebacks.

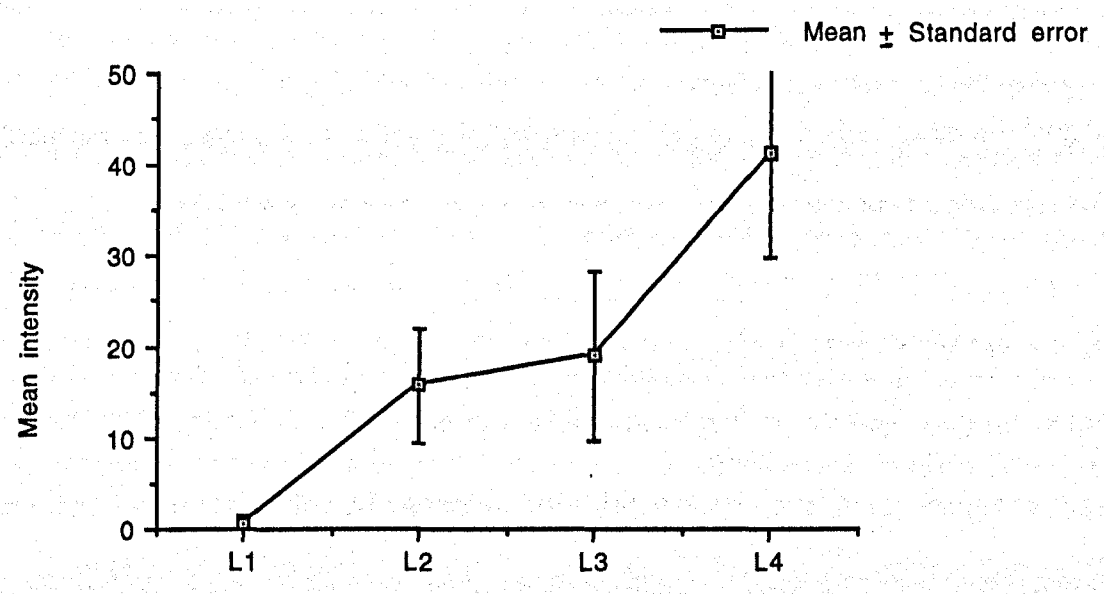

Fig:7.4 Mean intensity of *G. gasterostei* in different length classes of sticklebacks.

infection and the size of fish the Kruskal-Wallis test (Sokal and Rohlf, 1981) was applied and this confirmed that there were significant differences between the mean intensity in the different length groups ($p < 0.05$).

7.3.3 Prevalence and Intensity of Infection in Different Length Classes of Stickleback in Different Seasons.

In winter 1992 L1 fish were not present in the samples (Table.7.1). Only a very small number of L2 fish were caught. Prevalence rates were high in both L3 and L4 length classes. Highest intensity was observed in L3 fish.

In spring 1993 prevalence was more than 90% in the L2, L3 and L4 length classes of fish, while the L1 class was not present in the samples. Intensity was much higher in the L3 and L4 classes (Table 7.2).

Table 7.3 shows the prevalence and intensity of infection in summer 1993. The L1 length class appeared in samples for the first time but with a very low prevalence and intensity of infection. In the L2 and L3 length classes prevalence and intensity were lower than in other seasons. Table 7.4 shows the prevalence and intensity in autumn where prevalence and intensity of infection were increased in all length classes of fish. In winter 1993 infection levels were even higher in all length classes of fish (Table 7.5).

Table 7.1: Occurrence of *G. gasterostei* in different length classes of stickleback in winter 1992.

Length Class (cm)	No. of Fish Examined	No. of Fish Infected	Mean number of Parasites (±SD)	Prevalence %	Variance\Mean
L1	0				0
L2	5	5	32(29.80)	100	27.76
L3	14	13	17.66(4.74)	92.85	3.22
L4	9	8	10.4(9.07)	88.88	12.6

Table 7.2: Occurrence of *G. gasterostei* in different length classes of stickleback in spring 1993.

Length Class (cm)	No. of Fish Examined	No. of Fish Infected	Mean number of Parasites (±SD)	Prevalence %	Variance\Mean
L1	0				
L2	21	19	5.71(6.72)	90.47	7.91
L3	37	36	13.08(16.08)	97.29	19.77
L4	10	10	45.61(35.71)	100	27.95

Table 7.3: Occurrence of *G. gasterostei* in different length classes of stickleback in summer 1993.

Length Class (cm)	No. of Fish Examined	No. of Fish Infected	Mean number of Parasites (±SD)	Prevalence %	Variance\Mean
L1	15	6	0.6(0.91)	40	1.38
L2	32	22	8.21(15.9)	68.75	30.79
L3	54	46	9.16(9.31)	85.18	9.46
L4	3	3	28(36.76)	100	48.28

Table 7.4: Occurrence of *G. gasterostei* in different length classes of stickleback in autumn 1993.

Length Class (cm)	No. of Fish Examined	No. of Fish Infected	Mean number of Parasites (±SD)	Prevalence %	Variance\Mean
L1	2	1	6(6)	50	6
L2	41	39	10.78(9.95)	95.12	9.19
L3	35	34	24.13(29.81)	97.14	36.83
L4	10	10	38.4(39.54)	100	40.72

Table 7.5: Occurrence of *G. gasterostei* in different length classes of stickleback in winter 1993.

Length Class (cm)	No. of Fish Examined	No. of Fish Infected	Mean number of Parasites (±SD)	Prevalence %	Variance\Mean
L1	0				
L2	48	47	21.97(26.21)	97.91	31.28
L3	45	45	30.82(29.24)	100	27.74
L4	25	24	77.11(35.05)	96.00	15.93

7.3.4. Distribution of Parasites within Host Population.

When all fish sampled were grouped together, the frequency distribution of *G. gasterostei* was found to be overdispersed, with the variance greater than the mean number of parasites. The truncated negative binomial distribution of the parasite (Crofton, 1971a,b) is shown in Fig.7.5. The truncation were chosen to avoid lengthy calculations and extravagant use of computer time.

Frequency Distribution of *G. gasterostei.*

The over-dispersion of *G. gasterostei* was also confirmed by fitting the observed data for each season to the truncated negative binomial distribution. There was no significant difference of the observed data from the theoretical negative binomial distribution ($P < 0.05$). Samples were pooled by season in order to avoid the distorting effect of small samples (Fig.7.6).

Figure 7.7 shows the distribution of the parasite in different length classes of fish. The parasite population was over-dispersed in all length classes and the distribution was fitted by the negative binomial. Data for the L1 length class could not be pooled for analysis due to the small sample size of that group.

Variance/Mean Ratio.

The variance/mean ratio and the mean number of parasites in each fish sample is shown in Fig.7.8. The variance to mean ratio was always greater than 1 which indicates an over-dispersed parasite population and in most months was more than 20, which indicates a high

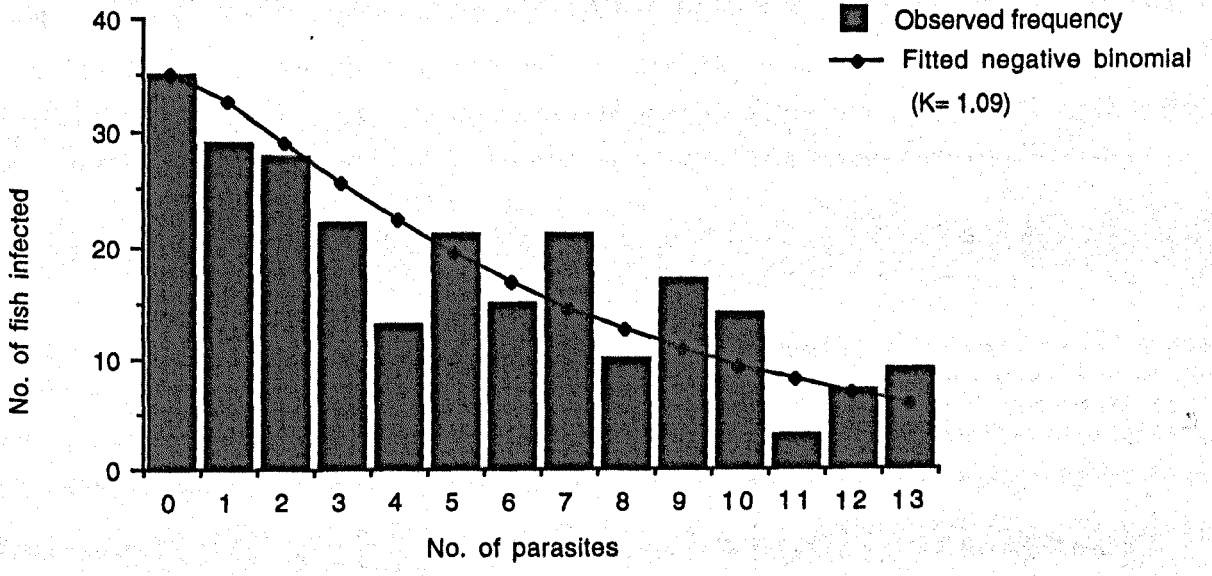

Fig : 7.5 Truncated negative binomial distribution of *G. gasterostei* in overall samples .

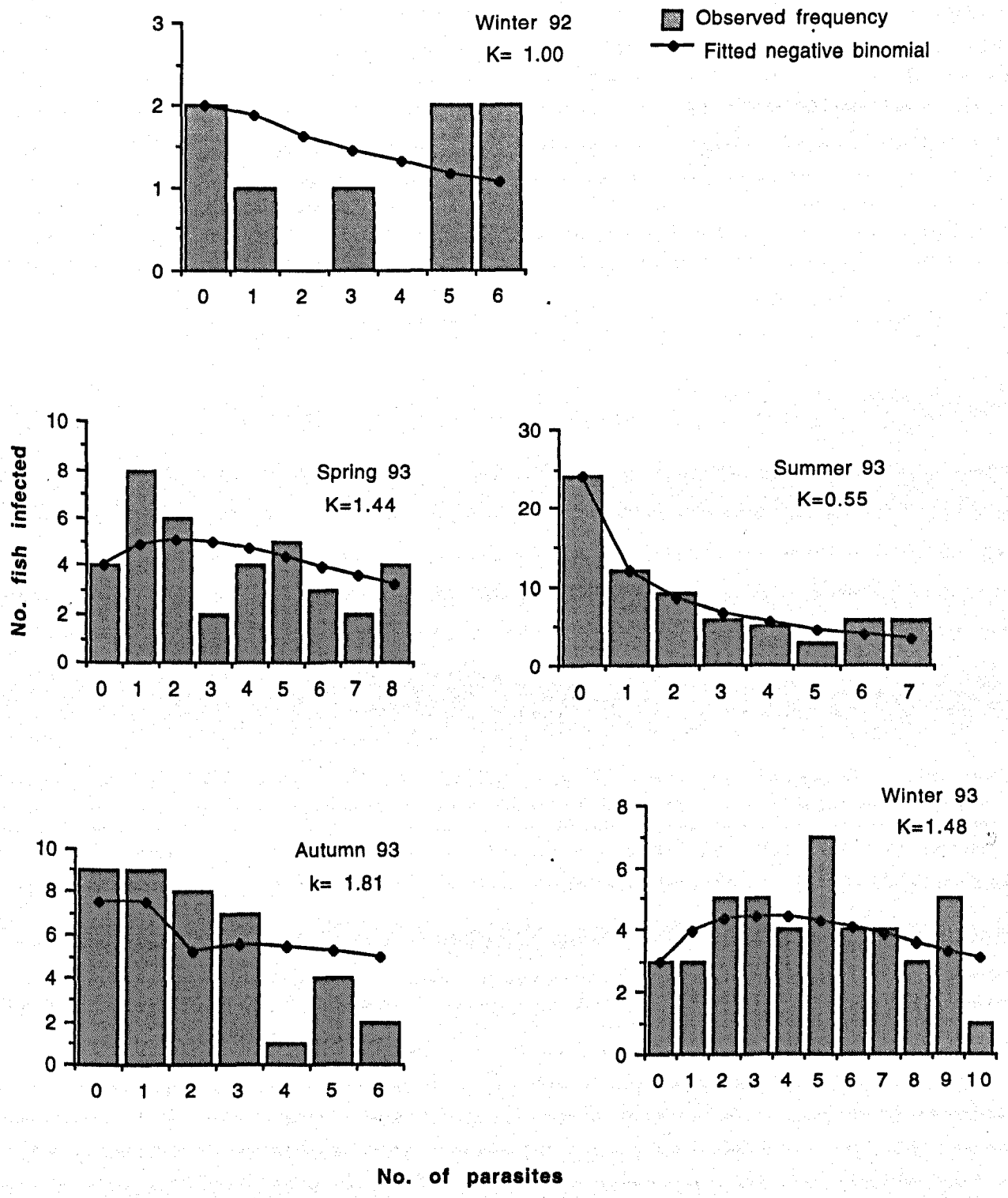

Fig: 7.6 Truncated negative binomial distribution of *G.gasterostei* in different seasons.

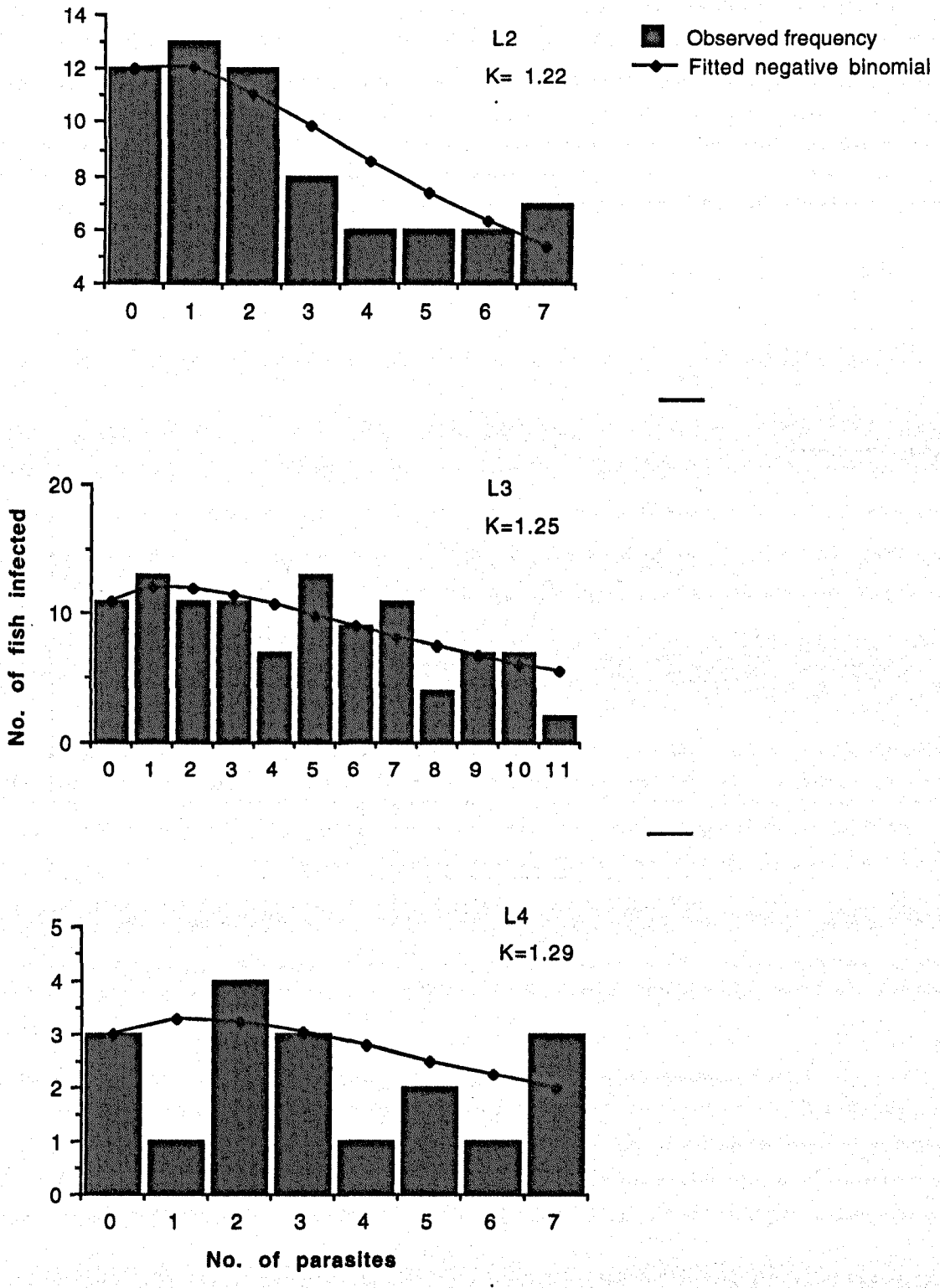

Fig :7.7. Truncated negative binomial distribution of *G. gasterostei* in different length classes of sticklebacks.

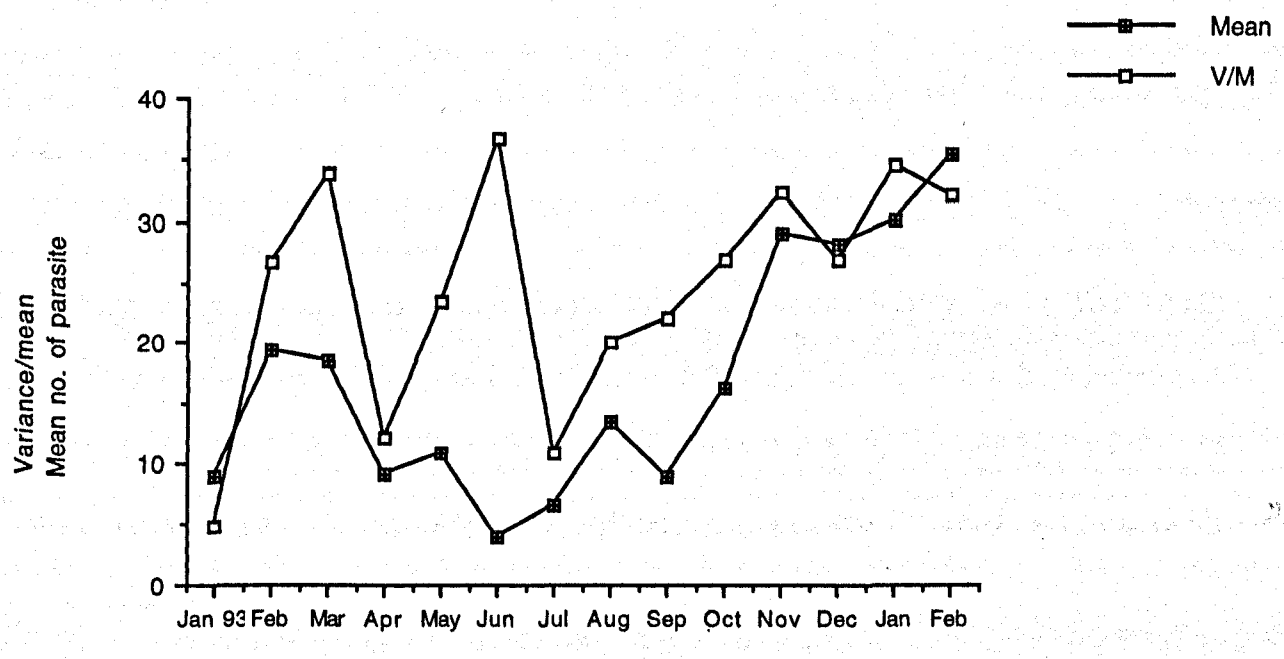

Fig: 7.8.Variance to mean ratio and mean no. of *G. gasterostei* in different months.

degree of overdispersion.

The variance/mean ratio was higher in summer 1993 than in any other season (Table 7.6), whilst the lowest ratio was observed in winter 1992, although data for this period is limited. In spring and winter 1993 the ratio was very similar, while in autumn it was lower. Table 7.7 shows the variance/mean ratio in each length class of fish. Variance/mean is always greater than unity which indicates an over-dispersion in all length classes. The ratio is highest in the L3 fish.

7.4. Discussion.

Gyrodactylus gasterostei was abundant in sticklebacks from Airthrey Loch. The overall prevalence rate was 90% with mean intensity around 20 parasites per fish. A high prevalence of *Gyrodactylus* species studied from natural populations has been reported by a number of authors (Rawson and Rogers, 1973; Kirby, 1981; Hanzelova and Zitnan, 1982; Mo, 1992; Jansen and Bakke, 1993).

Generally the parasite population showed a distinct pattern of seasonal variation of abundance with a higher prevalence and intensity in winter and spring and lower in summer, although the prevalence rate was between 90 to 100 % in most months. This pattern is very similar to that described by Chappell (1969) for *G. rarus* (*G. gasterostei*) from *G. aculeatus* from a pond in Yorkshire. Harris (1982) observed a similar seasonal cycle of *G. gasterostei* from a natural population of three-spined stickleback in England with 100% prevalence and an mean intensity of 10-20 parasites per fish in winter. However, the results of the present study

Table 7.6: Variance/Mean Ratio of *G. gasterostei* in Different Months.

Seasons	No. of Fish Examined	No. of Fish Infected	Mean	Variance	Variance/Mean	Chi-squre
Win 92	28	26	9	35.1579	3.9064	74.22
Spr 93	68	65	8.16	58.13	7.12	434.53
Sum 93	104	77	6.27	60.38	9.63	914.86
Aut 93	88	84	8.81	44.89	5.09	317.96
Win 93	118	116	11.26	80.91	7.18	517.39

Table 7.7: Variance/Mean Ratio in each Length Class of Fish.

Lengths	No. of Fish Examined	No. of Fish Infected	Mean	Variance	Variance/Mean	Chi-squre
L1	17	7				
L2	147	132	5.74	25.69	4.47	447.41
L3	185	174	9.16	61.21	6.67	1035.01
L4	57	55	7.55	28.76	3.80	113.26

differ slightly from those of Harris as he observed the peak of abundance and intensity in early summer and lowest levels in late summer and autumn. Harris assumed this decline was due to disappearance or death of adult fish at that period. This variation between the present study and that of Harris (1982) is clearly due to differences in the seasonal cycles of the sticklebacks, which spawned earlier in Airthrey Loch. A number of authors have suggested that the prevalence and intensity of gyrodactylid monogeneans are greatly influenced by the life cycle stages of the host fish (Malmberg, 1970; Chubb, 1977; Chappell, 1969; Harris, 1982, 1985).

Malmberg (1970) found that infection by *G.anguillae* was directly related to the seasonal occurrence of the host fish, the elvers of *Anguilla anguilla*. Chappell (1969) suggested fluctuations in numbers of *G. rarus* resulted mainly from the seasonal cycles of the stickleback and Harris (1982) also agreed that the annual life cycle of the stickleback can impose a marked seasonal pattern of abundance upon gyrodactylids.

The results of the present study support the observations of previous authors on *G. gasterostei*. Sticklebacks from Airthrey Loch showed a marked seasonal cycle (Fig.7.1) with fish hatching between summer and autumn in one year spawning and then probably dying the following year. The young fish of the year hatch in summer depending on water temperature (Wootton, 1976). This coincides with a comparatively lower prevalence of *G. gasterostei* on older sticklebacks. Stickleback fry have a high initial population density and quickly acquire *G. gasterostei*. Prevalence increases very rapidly in this new generation of sticklebacks in subsequent months and is maintained until the next breeding season. Jansen and Bakke (1993) described the behaviour of *Gyrodactylus salaris* as "opportunistic behaviour" where the

abundance of the parasite is influenced by the coincidence of newly hatched fish and heavily infected pre-smolts which facilitate a rapid spread of the parasite within the young of the year population. However, this opportunistic behaviour does not truly apply to *G. gasterostei* because recruitment of young sticklebacks and the most rapid build up of infection coincides with the presence of less infected adult fish. However, transmission to the new generation does occur quite rapidly and parasite numbers increase rapidly indicating the strong potential of *G. gasterostei* for rapid spread within host populations.

It is generally accepted that members of the viviparous genus *Gyrodactylus* spread between fish by means of physical contact between hosts (Glaser, 1974; Harris, 1980a; Scott and Anderson, 1984). Infection of newly hatched fish might occur by direct contact with an infected adult fish as the fry and parent stickleback initially live in intimate association in a nest.

Chappell (1969) suggested that newly hatched fish acquired *G. gasterostei* after the parasite transferred from the breeding fish to the deposited eggs.

Alternatively infection might occur by direct contact of fry with detached parasites. *G. gasterostei* may detach from older sticklebacks after their death following breeding or as a result of a host reaction. However, Harris (1982) found that in laboratory experiments the survivability of reattached *Gyrodactylus* was very poor. In addition, after fry have left the nest there is a spatial segregation between adults and young fish since the latter shoal in shallow water which also would reduce transmission of detached parasites to fry.

However, increases in parasite abundance in the late summer and autumn may be in part due to the shoaling behaviour of young fish which would facilitate host to host transmission of the parasite, one of the main routes for gyrodactylid transmission (Bakke *et al.*, 1993).

Many studies suggest that temperature is an important factor determining seasonal variation of ectoparasite populations (Srivastava and James, 1967; Rawson and Rogers, 1973). Several *Gyrodactylus* species have population numbers dependent upon temperature. Kirby (1981) reported the density of *G. atratuli* on spotfin shiners was highest when fish were schooled and temperature was below 15^{0}C, and lowest in summer, when fish were dispersed and temperature was above 15^{0}C. This seasonal pattern is similar to that seen in *G. gasterostei*. Sticklebacks school before the breeding season in winter and spring, when the temperature is also low. Other gyrodactylids show an increase in population with increasing water temperature. For example *G. macrochiri* in largemouth bass showed the highest population density in spring and autumn when water temperature increased to 20-25^{0}C (Rawson and Rogers, 1973). The abundance of *G. salaris* on 1+ and older salmon parr is positively related to water temperature (Jansen and Bakke, 1993).

The results of the present study suggest that the annual cycle of the host fish is the most important factor in regulating the size of the *G. gasterostei* population. The influence of water temperature upon the abundance of *G. gasterostei* is considered less important, although it may play a part. Adult fish taken in summer samples harboured relatively few parasites which may indicate the temperature indeed had some negative influence on the parasite. The reproductive rate of *Gyrodactylus* is strongly influenced by temperature (Lester and Adams, 1974a; Harris, 1982). An experimental laboratory study of *G. gasterostei*

(Harris, 1982) showed that at high temperatures (10-15^{0}C), the parasite initially increased in number and than underwent a period of decline and subsequently disappeared from the fish. At lower temperatures (5^{0}C), the parasite population continued to grow throughout the experimental period. Harris considered the decline of the parasite population at high temperatures, was due to a specific host response against the parasite. At lower temperatures the parasite population continuously grew, possibly because of the supression of the host response. In the present study the lower overall prevalence of *G. gasterostei* in summer was mainly due to the small size of individuals in the samples. Lower prevalence and intensity in adult fish in summer might be due to a specific host response as suggested by Harris (1982).

Differences in parasitic infection in fish of different sexes are probably mainly of physiological origin, including factors such as mucus, colour and hormonal states (Pickering and Christie, 1980). These authors examined skin parasites of the brown trout, *Salmo trutta,* during their spawning period and observed that prevalence and intensity of a number of ectoparasites, including *Gyrodactylus* species were higher in sexually mature males than the immature fish of either sex or in females. No other studies appear to have considered differences in infection of *Gyrodactylus* between host sexes.

In general the severity of most ecto and endoparasitic infections increases with the age of the host fish (Hanek and Fernando, 1978). The size of the host stickleback was an important factor favouring the number of *G. gasterostei.* This agrees with the results of other authors for gyrodactylid populations (Chappell, 1969; Harris, 1982). In the present study a low level of prevalence and intensity was observed in summer, mainly in June, when most of the fish

sampled were newly hatched individuals of 1-2cm in size. Subsequently the parasite population increased as the host also grew in size. Jansen and Bakke (1993) observed negative correlation between *G. salaris* intensity and fish length which they explained as due to heterogeneity in susceptibility or resistance to *G. salaris* among host individuals. As the three-spined stickleback has a yearly life cycle, heterogeneity in susceptibility to *G. gasterostei* infection could not be determined.

The distribution of *G. gasterostei* within the host population in Airthrey Loch was over-dispersed throughout the study period. The distribution corresponded to the negative binomial. The tendency to over-dispersion of parasite populations in their natural host populations has been well described by several authors (Crofton, 1971a,b; Pennycuick, 1971b; Anderson, 1974; Gordon and Rau, 1982; Janovy and Hardin, 1987). Harris (1982) suggested that the negative binomial distribution describes the distribution of *Gyrodactylus* more closely than any other.

According to Crofton (1971b), the negative binomial distribution may be generated by the exposure of the host to many waves of infection, by the spatial clumping of the infective stages of the parasite or by changes in the probability of infection caused by previous infections. Anderson and Gordon (1982) suggested that over-dispersed patterns of parasite numbers/host in natural habitat act to enhance the density-dependent regulation of both host and parasite abundance. According to Anderson and Gordon (1982), the mechanism of "environmental stochasticity" implies the rate processes which govern the population growth of parasite species. These rate processes are birth, death, immigration and emigration rates which are not constant for a given species and depend on environmental factors such as

climate, host susceptibility and host behaviour. They suggested that for parasites in which a host is the main environment for population growth, differences in host behaviour or host susceptibility are the major factors generating over dispersion in the distribution of the parasite within the host population.

Over-dispersion of *Gyrodactylus* species has been reported by other authors (Srivastava and James, 1967; Scott, 1982). Baily (1964) and Scott (1982) considered that over-dispersion may arise due to stochastic variations in population growth rate in individual fish, which can generate a negative binomial distribution. Harris (1982) considered heterogeneity in the susceptibility of the fish to *Gyrodactylus* infection,which might be genetic or due to a host reaction against the parasite, to be important in the generation of over-dispersion. Harris found considerable variation in gyrodactylid population growth rates between individual fish, which might also be true for *G. gasterostei* in natural conditions.

Possible mechanisms regulating the population of *G. gasterostei* within the host population may be summarised as follows:

i) By the rate of parasite immigration from other hosts or reattachment of free living individuals.

ii) By host death after breeding or due to predation or other factors.

iii) By host susceptibility to the parasite.

It is not possible to determine which of these is of the greatest importance and they in turn might be affected by factors such as temperature and host density.

CHAPTER 8

SUMMARY AND CONCLUSIONS.

8 SUMMARY AND CONCLUSIONS

The present study has investigated aspects of the developmental cycle and epidemiology of two myxosporeans and one monogenean parasite of the three-spined stickleback, *G.aculeatus*.

Light microscopy, TEM and SEM studies on the development of *S. elegans* have indicated a number of interesting features. One of the most important of these is the possible proliferation of extra-sporogonic blood stages by budding and plasmotomy. The earliest stage of the extra-sporogonic cycle was a primary cell without secondary cells. These stages had highly vacuolated cytoplasm and thin surface projections. In the present study extrasporogonic stages could not be recovered in TEM sections. Future ultrastructural studies will be needed to define the extrasporogonic stages of *S. elegans* more clearly and in particular their relationship to the pre-sporogonic stages of *S. elegans* within the capillary lumen of renal corpuscles. It is suggested that extrasporogonic stages are the precursors of sporogonic stages and transmitted to the kidney via the circulation.

A number of authors have suggested that extrasporogonic triple forms (secondary cells with two tertiary cells within the primary cell) represent pseudoplasmodia with two sporogonic cells (Baska and Molnár, 1988; McGeorge *et al.*, 1994) as found in the kidney. In *S. elegans* such triple forms may be the normal stage which exists in the kidney tubules, but primary cells with no secondary cells or only one secondary cell may invade the kidney and correspond to early pseudoplasmodial stages as listed below.

Extrasporogonic stages in blood.	Corresponding stages in kidney.
Early primary cell with no secondary cells.	Early pseudoplasmodia with no sporogonic cell.
Primary cells with single scondary cell (rarely found).	Occasionally monosporous pseudoplasmodia were found.
Primary cell with two secondary cells or two tertiary cells within the secondary cell of a primary cell (very common).	Pseudoplasmodia mostly with two sporogonic cells (disporous).

Ultrastructural studies on the development of *S. elegans* showed the earliest undifferentiated stages of pseudoplasmodia had very few cell organelles and lacked any sporogonic cells. The formation of the sporogonic cell within the pseudoplasmodium was not completely clear. However, occasionally early pseudoplasmodia were found with one or two less dense areas in the cytoplasm and these gradually acquired cell organelles and a definite cell membrane. These new formations thus became sporogonic cells within the pseudoplasmodium. Further ultrastructural studies on the early pseudoplasmodium might better demonstrate the origin of sporogonic cells. There was no evidence of the endogenous division of a vegetative nucleus to form sporogonic cells (Current and Janovy, 1977, 1978).

Light, electron and scanning microscopy of *M. gasterostei* showed that this species was a typical example of a mictosporic myxosporean producing mono, di and polysporous plasmodia (Lom and Dyková, 1992). Polysporous plasmodia of *M. gasterostei* displayed

characteristics of both histozoic and coelozoic myxosporeans. In common with coelozoic species *M. gasterostei* plasmodia had a surface unit membrane with simple cytoplasmic extensions, whereas the pinocytosis, more than one vegetative nucleus and many generative cells and initiation of sporogenesis by pansporoblast formation were characteristics of histozoic species.

TEM study of plasmodia of *M. gasterostei* showed some unusual characteristics including a membrane bound tubular structure and some electron dense bundles of microfibrils of unknown function in the plasmodial cytoplasm. Their role and significance is unknown and future TEM study may suggest their function.

Differentiation of myxosporean sporoblast cells usually occurs after completion of the necessary number of sporoblast cells. In *M. gasterostei* capsular primordia were frequently observed in very early sporoblast cells suggesting capsulogenic cell differentiation may occur just after sporoblast formation. Developing capsulogenic cells often contained two membrane bound vacuoles filled with electron dense material. As capsulogenic cells have often been reported to contain glycogen particles, it may be assumed that the electron dense material within these vacuoles is glycogen. Thiery's (1967, in Desser and Paterson, 1978) reaction should be used to test the identity of this material.

Epidemiological studies on the myxosporeans *S. elegans* and *M. gasterostei* demonstrated that both parasites are present throughout the year and both apparently produce spores continuously. It is not clear whether the infective stages are available to fish throughout the year or whether infection occurs over a limited period. This problem could be resolved either

by identifying with certainty the earliest infective stage in the fish, or by placing known uninfected fish in infected water at different times of the year. As the extrasporogonic stages of *Sphaerospora* species are considered the earliest stages of infection in fish (Grupcheva *et al.*, 1985; Kent and Hedrick, 1988; Supamattaya *et al.*, 1993; McGeorge *et al.*, 1994) the appearance of extrasporogonic stages, in the present study, in summer and winter might indicate year round new infections. These stages have a short duration, probably due to an immunological reaction of the host or to environmental effects or to a genetically fixed period of development. The main site of extrasporogonic development was the circulating blood from which parasites reached to the rete mirabile of the eye and the kidney.

Host sex did not influence abundance of *S. elegans* but the prevalence of infection of *M. gasterostei* was significantly lower in male fish. It was not possible to determine the mechanisms for this distinction.

The annual life cycle stages of the stickleback can be considered an an important factor controlling the epidemiology of *S. elegans* and *M. gasterostei*. The appearance of newly hatched uninfected stickleback fry in summer caused a marked drop of prevalence and intensity of both parasites.

One of the most important factors in the epidemiology of myxosporeans is the involvement of alternate hosts in the life cycle. A number of actinosporeans developing in oligochaete worms have already been identified as a life-cycle stage of certain myxosporeans (Wolf and Markiw, 1984; Wolf *et al.*, 1986; El-Matbouli and Hoffmann, 1990, 1992). It is very probable that such stages are involved in the life cycle of *S. elegans* and *M. gasterostei* and

controlled experimental studies are required to identify them. These were not possible within the time span of the present study.

Both *S. elegans* and *M. gasterostei* shed spores with no obvious influence of temperature or season. Without a knowledge of the possible alternate stages it is not certain what effect this may have on the seasonal abundance of the parasites. Experimental studies on spore release suggests that there is a rapid production of spores of both species in sticklebacks.

Pathological effects of *S.elegans* and *M.gasterostei* were quite marked although no clinical signs were evident even in heavy infections. Larger size groups of sticklebacks were less heavily infected with *M. gasterostei* which may indicate an effect on host mortality or growth rate. The pathological damage was different according to the species of parasite. Thus the intralumenal stages of *S. elegans* caused vacuolation and accumulation of electron dense material in the epithelial layer, whereas intralumenal stages of *M. gasterostei* were associated with large vacuolated areas in the tubular epithelium with destruction of the epithelial brush border. *S. elegans* did not cause destruction of the epithelial brush border. Intralumenal stages of *M. gasterostei* also caused flattening of epithelial cells and dilation of the lumen. The glomerular damage, including enlarged Bowman's spaces, compressed glomeruli and destruction of the glomerular cells was similar with both parasites and an increased number of rodlet cells in tubule epithelium occurred in both infections. A comparison of the epidemiology, development and pathogenicity of *S. elegans* and *M. gasterostei* is presented in Table 8.1.

Table 8.1: A comparison of the epidemiology, development and pathogenicity of *S. elegans* and *M. gasterostei* in the three-spined stickleback.

S. elegans.	*M. gasterostei.*
1. Single infections were very low (7%).	1. Single infections were significantly higher (22.20%).
2. Sex of host has no influence on the prevalence of infection.	2. Female hosts were more susceptible than male.
3. Infection not found in fish below 2cm. in length and L3 size fish showed the highest prevalence.	3. Infection found in fish below 2cm. and highest prevalence was observed in L2 size fish.
4. Extrasporogonic stages found in the circulating blood, choroidal rete and kidney.	4. Extrasporogonic stages were not found.
5. Formed mostly disporous and occasionally monosporous pseudoplasmodia.	5. Formed monosporous, disporous and polysporous plasmodia.
6. Under light microscopy developmental stages appeared as oval or round with smooth surface and regular outline. Sometimes inner sporogonic cells were visible and countable.	6. Under light microscopy developmental stages were round, elongated to irregular, with rough outer surface. Several refractile bodies observed on the surface. Sporogonic cells hardly visible.
7. In TEM sections pseudoplasmodia with different degrees of development were found as round to oval with smooth regular outline. No cytoplasmic extensions present.	7. In TEM sections plasmodia were oval, elongated to irregular with finger-like cytoplasmic extensions. Mature plasmodia were always very irregular in shape.
8. Six sporoblastic cells in monosporous and twelve in disporous pseudoplasmodia.	8. Five sporoblastic cells in monosporous plasmodia and 10 in disporous plasmodia and pansporoblasts.
9. Capsulogenic cells with lipid inclusions and glycogen granules scattered in the cytoplasm.	9. Capsulogenic cells with large round mitochondria with loosely arranged tubular cristae and membrane bound vacuoles containing electron dense granular materials.
10. Two uninucleated sporoplasm cells without sporoplasmosomes.	10. Binucleated single sporoplasm cell with electron dense sporoplasmosomes.
11. Valvogenic cell forms two protuberances at the posterolateral sides of the immature spore.	11. Valvogenic cells were more regular in shape while immature and developed surface ridges with the maturation of spore.
12. The attachment point of pseudoplasmodia and the host cells was not electron dense and often hair like surface extensions extended from the pseudoplasmodia to the epithelial brush border.	12. The attachment points with the host host cells were electron dense and plasmodial surface extensions extended among the tubular epithelium.
13. Pseudoplasmodia contained few internal organelles which disappeared with the maturation of the spores. In mature spores pseudoplasmodial cytoplasm became thinly spread around the mature spore.	13. Plasmodia with many complicated internal organelles became vacuolated and lost most of its structure after maturation of the spore.
14. Accumulation of electron dense material, does not cause flattening of epithelial cells and dilation of lumen.	14. Caused large vacuolations area in the tubular epithelial layer. Epithelial cells became flattened and lumen dilated.

G. gasterostei was present in sticklebacks throughout the year but was least abundant in summer (Chapter 7), this may simply reflect the recruitment of young sticklebacks into the population at that time and the death of older and larger fish. *G. gasterostei* was more abundant on larger fish which may reflect both their age and greater surface area. Within this wild population there was no evidence of a host response affecting the parasite, although Harris (1982) suggested this occurred in experimental infections. Sex of the stickleback has no obvious influence on the overall infestation with *G. gasterostei* and the parasite population was heavily overdispersed at all times. It would be of interesting to determine how sticklebacks initially acquire infestation of *G. gasterostei*. Experimental studies are necessary to determine if this occurred by direct contact with an infected fish, e.g in the nest, or whether parasites detached from older fish can invade juveniles.

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Appendix 1. Showing sampling months, mean temperature, numbers of fish examined and infected with two myxosporeans and one monogenian parasite during January 1993 to February 1994.

Months	No. of Fish Examined for Myxosporean	No. of Fish Examined for *G.gasterostei*	Subsamples of Extrasporogonic stages of *S.elegans*	No. Fish Infected with *S. elegans*			No. of Fish Infected with *M.gasterostei*		No of Fish with *G.gasterostei*	Mean temperature of sampling site
				Presporogonic & sporogonic stages	Presporogonic, sporogonic and spore stages	Extrasporogonic stages	Presporogonic & sporogonic stages	Presporogonic, sporogonic & spore stages		°c
Jan 93	52	10	7	0	44	3	0	40	9	5
Feb 93	57	11	5	5	13	3	7	39	10	3.5
Mar 93	61	30	7	0	31	0	0	47	28	5
Apr 93	60	20	5	0	33	0	4	56	19	9
May 93	39	20	10	3	10	0	10	27	19	13
Jun 93	59	22	12	4	23	6	12	25	10	16
Jul 93	61	24	10	4	21	0	10	43	18	19
Aug 93	73	60	5	0	47	0	3	39	54	17.5
Sep 93	67	30	7	9	19	0	3	44	29	13.5
Oct 93	55	29	7	0	37	0	6	25	28	5
Nov 93	66	30	10	10	43	0	4	23	33	1
Dec 93	48	33	10	10	34	0	8	36	33	0
Jan 94	55	46	12	12	35	3	9	38	45	0
Feb 94	44	40	10	0	38	0	4	28	38	0.5