INTER-RELATIONSHIPS OF MYXOSPOREANS, INCLUDING PKX WITH CERTAIN FRESHWATER FISH.

by

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ABSTRACT

The prevalence and impact of proliferative kidney disease (PKD) and myxosporidiosis has been investigated in wild fish stocks in the UK, over 1,500 fish representing 17 species being examined. PKD was recorded in brown trout, grayling and pike, the causative agent, the PKX cell, being identified with the aid of light and electron microscopy. A further 27 myxosporean species were also noted, with *Myxobolus cotti* (syn. *M. jiroveci*), in the brain of bullheads *Cottus gobio* being recorded for the first time. Studies on the structure and development of *Myxidium lieberkuehni* in pike revealed several previously undescribed features.

Comparative morphological studies were undertaken to assess affinities of PKX with known myxosporean species. Results indicated similarities with early presporogonic stages of several myxosporean species, especially those belonging to the genus *Sphaerospora*. The apparent rarity of spore formation associated with PKX infections in the hosts examined focussed attention on species of *Sphaerospora* as possible sources of infection to salmonids. Studies concentrated on the possible involvement of the 3-spined stickleback, *Gasterosteus aculeatus* and its renal parasite, *Sphaerospora elegans*, in PKD transmission. A re-description of this parasite (recently elevated to "type species" for the genus), was prepared.

Laboratory experiments using rainbow trout PKX cells successfully transmitted the infection to rainbow trout, brown trout, brook trout and grayling; however sticklebacks challenged with PKX cells did not appear to become infected. Rainbow trout challenged with *S. elegans* spores and presporogonic stages showed no evidence of sphaerosporosis or PKD. Experiments designed to investigate the possible role of tubificid worms in PKD transmission provided inconclusive results. Field studies provided data on the pathogenesis of PKD in grayling and showed this species to be highly susceptible to the disease.

Parts of this work have been published or presented at national and international symposia:

1. Feist, S.W. & Bucke, D. (1987) Ultrastructural aspects of PKX, the causative agent of proliferative kidney disease in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases*, **10**, 323-327.

2. Feist, S.W. (1988) The stickleback (*Gasterosteus aculeatus* L.) and PKD in salmonids - culprit or innocent bystander? *Bulletin of the European Association of Fish Pathologists*, **8(5)**, 94-96.

3. Feist, S.W., Bucke, D. & Matthews, R.A. (1988) Proliferative kidney disease of salmonids-a problem with Myxozoan life cycles. 41st Annual Meeting of the Society of Protozoologists, Bristol, England.

4. Lom, J., Feist, S.W., Dyková, I. & Kepr, T. (1989) Brain myxoboliasis of bullhead, *Cottus gobio* L., due to *Myxobolus jiroveci* sp. nov.: light and electron microscope observations. *Journal of Fish Diseases*, **12**, 15-27.

5. Clifton-Hadley, R.S. & Feist, S.W. (1989) Proliferative kidney disease in brown trout *Salmo trutta*: further evidence of a myxosporean aetiology. *Diseases of Aquatic Organisms*, **6**, 99-103.

6. Lom, J., Dyková, I. & Feist, S.W. (1989) Myxosporea - induced xenoma formation in pike (*Esox lucius* L.) renal corpuscles associated with *Myxidium lieberkuehni* infection. *European Journal of Protistology*, **24**, 271-280.

7. Feist, S.W., Chilmonczyk, S. & Pike, A.W. (1991) Structure and development of *Sphaerospora elegans* Thélohan 1892 (Myxozoa: Myxosporea) in the sticklebacks *Gasterosteus aculeatus* L. and *Pungitius pungitius* L. (Gasterosteidae). *European Journal of Protistology*, **27**, 269-277.

8. Lom, J., Pike, A.W. & Feist, S.W. (1991) Myxosporean vegetative stages in the choroidal *rete mirabile* of *Gasterosteus aculeatus* infected with *Myxobilatus gasterostei* and *Sphaerospora elegans*. *Diseases of Aquatic Organisms*, **11**, 67-72.

9. Bucke, D., Feist, S.W. & Clifton-Hadley, R.S. (1991)   The occurrence of proliferative kidney disease (PKD) in cultured and wild fish: further investigations. *Journal of Fish Diseases*, **14**, 583-588.

10. Feist, S.W. & Bucke, D. (1993)   Proliferative kidney disease in wild salmonids. *Fisheries Research*, **17**, 51-58.

I would like to dedicate this thesis to my late father Alfred and to my mother Ingeborg for encouraging my early interest in things scientific.

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CHAPTER 1

GENERAL INTRODUCTION

Proliferative kidney disease (PKD) is a serious debilitating disease of farmed salmonids which has been recognised in several European countries as well as in Canada and the U.S.A. (Clifton-Hadley, Bucke & Richards, 1984; Hedrick, Kent, Rosemark, & Manzer, 1984; Olesen, 1985; Veselsky, 1986). Since its recognition as an emerging threat to salmonid aquaculture during the 1960s and 1970s, various aspects of the disease pathogenesis have been investigated (Ferguson & Needham, 1978; Ferguson, 1981; Hoffmann & Lommel, 1984; Ellis, McVicar & Munro, 1985; Clifton-Hadley, Bucke, & Richards, 1987; Clifton-Hadley, Richards & Bucke, 1987). Although much is known concerning the PKX cell within the fish host, very little is known of other stages in the life cycle of the parasite. The lack of knowledge of these stages has severely hampered the search for suitable control measures.

Originally, the PKX cell was considered to be an amoeba (Plehn, 1924) and later a member of the haplosporidia (phylum Ascetospora) (Seagrave, Bucke & Alderman, 1980a, b). Subsequent studies identified more advanced stages in the lumens of renal tubules which suggested a more correct placement to be within the phylum Myxozoa (Hedrick *et al.*, 1984; Kent & Hedrick, 1985a; Kent & Hedrick, 1986). Developmental stages of PKX exhibit features similar to the Sphaerosporidae, a myxosporean family typically infecting the kidneys and urinary bladders of marine and freshwater fish (Lom & Noble, 1984). Seagrave *et al.*, (1980a) postulated that salmonids, especially the rainbow trout, are aberrant hosts for the PKX parasite. The marked pathological response to the presence of the PKX cell and apparent failure of the parasite to complete sporogony were factors supporting this view. Subsequently, mature sphaerospores were found in the kidneys of rainbow trout that had recovered from PKD (Hedrick, Kent, Toth & Morrison, 1988; Odening, Walter & Brockhardt, 1988) and *Sphaerospora truttae* has been described from the kidneys of wild brown trout and grayling (Fischer-Scherl, El-Matbouli & Hoffmann, 1986; Walter, Odening & Brockhardt, 1991). The possible relationship between the sphaerospores and PKX was not confirmed and synonymy between PKX and *S. truttae* appears unlikely (Walter *et*

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al., 1991). Nevertheless the possibility remains that PKX will be synonymous with an already described species in which complete normal development occurs within another host.

The main objectives for this study were: (1) to assess the epidemiology of myxosporidiosis in wild fish in the UK, with emphasis on renal infections, and to determine the host range of PKX; (2) to conduct ultrastructural studies to determine structural affinities of PKX with already documented Myxozoans; (3) to assess the pathological impact of PKD in wild fish; and (4) to conduct field and laboratory experiments to investigate the involvement of documented Myxozoans and discover other stages in the life cycle including the possible role of invertebrates in the transmission of PKD to salmonids.

From these studies a better understanding of myxosporean infections, the impact of PKD on wild fish, and possible modes of transmission for these parasites will be gained. Such data is fundamental to the development of effective strategies for control of the disease in rainbow trout.

CHAPTER 2

LITERATURE REVIEW

2.1   BACKGROUND

The Myxosporea constitute a class within the phylum Myxozoa. All species are parasitic, chiefly in fish, although infections are recorded in reptiles, amphibians and Oligochaetes.   Over 1000 species have been recorded since the first myxosporean parasites were described in the middle of the 19th century.   In many of these early studies new species were created on the basis of spore morphology alone. More recent research suggests that the status of some species within certain genera is open to question (Mitchell, 1977; Lom & Arthur, 1989). Major descriptive works include those by Thélohan (1895), Kudo (1920), Shulman (1966), Bauer (1984), and Lom & Dyková (1992a). The most recent classification for the myxosporea has been proposed by Lom & Noble (1984) (Fig. 1).

Myxosporeans have been recorded from most tissue locations within the host. Coelozoic species inhabit body cavities: the gallbladder, urinary bladder and renal tubules being common sites for infection (Bauer, 1984). Histozoic species locate in intercellular spaces and usually form large multicellular trophozoites which may be visible to the naked eye as white round-oval cysts. Some species are intracellular and infect muscle tissue (Kabata, Whitaker & Bagshaw, 1986). Most infections are rather innocuous and cause little or no damage to the host. However, several species are significant pathogens and give rise to diseases causing major problems in aquaculture world-wide. Among the most important are *Myxobolus cerebralis* causative agent of whirling disease (Halliday, 1976; Wolf & Markiw 1985), *Ceratomyxa shasta*, a serious pathogen of salmonids of the Pacific north-west of the USA and British Columbia (Bartholomew, Rohovec & Fryer, 1989), *Sphaerospora renicola*, causing major problems to carp (*Cyprinus carpio*) culture in Europe (Dyková & Lom, 1982, 1988), and PKX, giving rise to proliferative kidney disease (PKD) in salmonids (Ferguson & Needham, 1978; Seagrave, Bucke & Alderman, 1980a,b; Kent & Hedrick, 1985a). Both farmed and wild stocks may be affected. Mortality rates can be high, especially in

Figure 1. Classification of the Phylum Myxozoa (after Lom & Noble, 1984) (number of genera are indicated in brackets).

Phlylum Myxozoa Grasse, 1960

Class	Actinosporea Stolc, 1899	Myxosporea Buetschli, 1881

Order	Bivalvulida Shulman, 1959	Multivalvulida Shulman, 1959

Suborder	Variisporina	Sphaeromyxina	Platysporina

Family	Ceratomyxidae (3)	Shaeromyxidae (1)	Myxobolidae (13)	Trilosporidae (2)
	Auerbachidae (2)			Kudoidae (2)
	Alatosporidae (2)			Pentacapsulidae (1)
	Parvicapsulidae (2)			Hexacapsulidae (1)
	Otholineidae (3)			Septemcapsulidae (1)
	Fabesporidae (1)			
	Chloromyxidae (3)			
	Myxidiidae (3)			
	Sphaerosporidae (5)			
	Sinuolineidae (8)			

Number of genera are indicated in brackets.

juvenile fish, although losses also occur as a result of poor growth rates or through increased susceptibility to other diseases.

2.2 TAXONOMY AND LIFE CYCLE BIOLOGY OF THE MYXOSPOREA

Most pre-spore stages in the myxosporean life cycle lack morphological features of use in taxonomy. This has resulted in classification systems primarily based on the structure of mature spores, (Shulman, 1966; Lom & Noble, 1984). Spores are multicellular units, comprising two or more shell valves enclosing an infective sporoplasm and one or more polar capsules each containing an extrudible coiled polar filament. For classification purposes the arrangement and numbers of shell valves and polar capsules are the most basic criteria used. In addition, overall dimensions, surface features and the presence or absence of a mucous envelope on the spore surface are also important in classification. Lom & Arthur (1989) provide useful criteria for the description of new species of myxosporea or re-descriptions of inadequately known species.

Myxosporean life cycles are still poorly understood (Fig. 2). It has long been thought that mature spores released from the host are capable of directly re-infecting susceptible hosts. This hypothesis prevailed, even though many authors, using fresh spores, were unable to induce infections from fish to fish under laboratory conditions (e.g. Thélohan, 1895; Hoffman, Dunbar & Bradford, 1962; Yunchis, 1984). Successful transmission has been reported when spores of *Myxobolus cerebralis* were used which had been "aged" for 4 months in sterile mud prior to challenge (see Halliday, 1976). The apparent need for a period of ageing was finally explained by Markiw & Wolf (1983), who reported the essential involvement of an intermediate host in the life cycle of *M. cerebralis*. In a series of experiments the authors subsequently demonstrated that ingested spores of *M. cerebralis* were infective to the oligochaete worm, *Tubifex tubifex*, and that the parasite transforms within the host to produce a new spore type previously associated with the Actinosporea (Wolf & Markiw, 1984; Markiw, 1986; Wolf, Markiw & Hiltunen, 1986). Spores released from the parasitised

Figure 2. Summary of current interpretation of myxosporean life cycles.
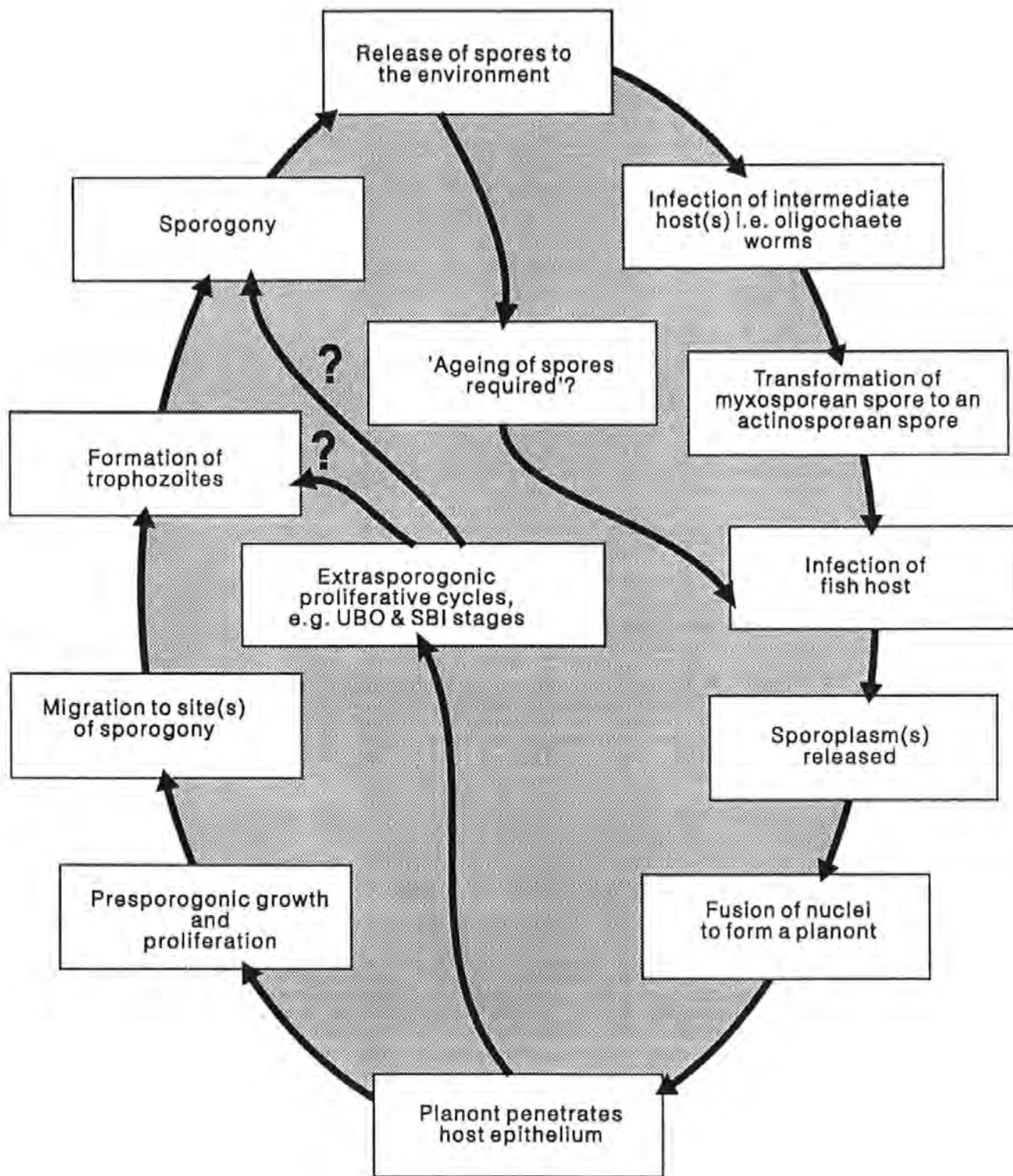
tubificids infect salmonids which eventually exhibit clinical signs of whirling disease. The formation of spores of *M. cerebralis* in the head cartilage completes the life-cycle.

Since the pioneering studies of Wolf & Markiw (1984), other workers claim to have confirmed their findings and have demonstrated similar cycles for other myxosporeans (El-Matbouli & Hoffmann, 1989, 1991; Hedrick, Wishkovsky, Groff & McDowell, 1989; Yokoyama, Ogawa & Wakabayashi, 1991). However, Hamilton & Canning (1987) presented results which did not support the findings of Markiw & Wolf (1983) and Wolf & Markiw (1984). If confirmed, the actinosporean involvement in myxosporean life-cycles would have dramatic implications for the taxonomy of Myxozoan parasites (Corliss, 1985) and Odening (1991) has recently proposed new nomenclature for Myxosporean developmental stages. The mature spore would be termed a 'gametophore', the pansporoblast a 'pangamoblast', the sporoplasm a 'gametoplasm' and the process of sporogony becomes 'gamogony'. For the purposes of this review the long established terminology for Myxosporean developmental stages is retained.

Conventionally it was thought that the myxosporean spore passes down the alimentary tract of the fish host and the action of physical and biochemical factors trigger the extrusion of polar filaments which attach the spore to the epithelial lining of the gut. The shell valves separate and the sporoplasm leaves the spore (by this stage the two nuclei of the sporoplasm have fused to complete the primitive sexual process of autogamy). This cell, now called a planont (Lom, 1989) is the only unicellular stage in the myxosporean life cycle. It is assumed that this cell penetrates the digestive epithelium and enters the blood or lymph systems and proceeds directly to the final site of infection where spore formation (sporogony) commences (Mitchell, 1977). The portals of entry by which actinosporean spores infect the fish host include the skin, buccal cavity and digestive tract (Markiw, 1989).

The sporogonic phase of myxosporean/actinosporean parasites in fish is preceded by a period of growth and vegetative proliferation during which the nucleus of the planont repeatedly divides to produce vegetative nuclei. Enveloped cells (generative cells) are produced by endogenous division. The entire structure becomes a

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trophozoite or plasmodium containing numerous vegetative nuclei and generative cells. The trophozoite of coelozoic species may itself proliferate by plasmotomy or budding. In species that form small uninucleate coelozoic pseudoplasmodia, in which vegetative nuclei are not produced, proliferation may occur by repeated growth and subsequent division. In addition to the well established method of vegetative proliferation shown by the large coelozoic trophozoites, two further types of presporogonic (extrasporogonic) development are now known. The first was described by Csaba (1976), who reported "an unidentifiable extracellular sporozoan parasite" in the blood of carp fingerlings (designated as an "unidentified blood organism" or UBO by Lom, Dyková & Pavlásková, 1983), which is now thought to be part of the life cycle of *Sphaerospora renicola*. Subsequently, similar blood stages were found in other fish species infected with species of *Sphaerospora*. (Lom, Pavlásková & Dyková, 1985; Baska & Molnár, 1988; Jurajda, 1989). The second type of extrasporogonic development also associated with *S. renicola* infections in carp, occurs in the swimbladder (so-called "SBI stages", referring to the swimbladder inflammation they initiate). Both UBO and SBI stages consist of primary cells containing enveloped secondary and tertiary cells, a characteristic feature of myxosporean development. It is possible that the PKX cell associated with proliferative kidney disease (PKD) of salmonids may one day be identified as an extrasporogonic stage of a *Sphaerospora* species. PKX is associated with a severe inflammatory response, especially in the spleen and renal interstitial tissues, although almost any tissue may be affected (Clifton-Hadley *et al.*, 1987). In the examples of extrasporogonic stages given above, including PKX, the parasites are able to proliferate rapidly by plasmotomy or binary fission. Sporogony only occurs when parasites enter renal tubules.

Within large coelozoic trophozoites sporogony is initiated by the union of two generative cells. One cell, the pericyte, envelopes the other which is then termed the sporogonic cell. The formation of this cell doublet or pansporoblast is characteristic of myxosporean developmental cycles, although it also occurs in other protistan groups such as the Haplosporea and Paramyxea (Lom & Dyková, 1986). The enveloped sporogonic cell divides to produce the requisite number of cells to form one or two

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sporoblasts depending on whether the pansporoblast is mono- or disporic. For species of *Sphaerospora* and some species of other genera, such as *Ceratomyxa shasta* the pseudoplasmodium is analogous to the pansporoblast and sporogony starts not by the envelopment of one generative cell by another but by direct division of generative cells to produce sufficient to construct one or two sporoblasts depending on the species of parasite. Following the formation of the sporoblast the constituent cells differentiate to form the structures of the mature spore. Typically, two cells, destined to become the sporoplasm, accumulate electron-dense bodies called sporoplasmosomes. Their function is unknown (Lom, Feist, Dyková & Kepr, 1989). Two more cells, the capsulogenic cells, are characterised by the large amounts of rough endoplasmic reticulum in their cytoplasm, and it is from this structure that the polar capsules originate. Lastly, a further two cells, the valvogenic cells, spread to enclose the sporoplasm and capsulogenic cells and form the shell valves of the mature spore.

Spores may be released from the host by a variety of routes, depending on the site of infection: by rupture of superficial cysts, passage through alimentary or urinary tracts or upon death and decomposition of the host.

2.3 PROLIFERATIVE KIDNEY DISEASE (PKD)

2.3.1 Background and the nature of the causative agent, the PKX cell.

The term "proliferative kidney disease" (PKD) was first used by Roberts & Shepherd (1974) to describe a debilitating condition of rainbow trout in which the major clinical and post-mortem signs were dark colour, abdominal swelling and gross kidney enlargement. The first detailed description of PKD is thought to have been by Plehn (1924). The condition she referred to as "Amobeninfektion der Niere" in "river trout" (thought to refer to brown trout, *S. trutta* L.) and rainbow trout, derives its name from the amoeboid nature of the characteristic cells associated with the disease, these perhaps being first described by Hofer (1906). Subsequent workers describing a condition similar to PKD have used various names, "Infektiose Nierenschwellung und Leberdegeneration" (Schaperclaus, 1954) and "hepatonephrite" by de Kinkelin & Gérard (1977) being examples.

The taxonomic status of the PKD organism has been controversial. In her description of PKD in Germany, Plehn (1924) refers to the causative agent as an amoeba, a view also supported by Ghittino, Andruetto & Vigliani (1977). Identification of pseudopodia in the parasite cells by Ferguson & Adair (1977) and Ferguson & Needham (1978) prompted the authors to agree that the parasite was "amoeba-like", but in addition they noted similarities to the amoeboid nature of myxosporean trophozoites. An alternative hypothesis was put forward by Seagrave, Bucke & Alderman (1980a), who referred to the parasitic cells as PKX cells. Principally, on the basis of ultrastructural evidence, they considered PKX to have close affinities with the haplosporea (phylum Haplosporidia, Perkins, 1989). The main features of PKX cited as showing kinship with the haplosporidia (especially *Marteilia* species) were the presence of multivesicular bodies, endogenous cell division, and the presence of characteristic electron-dense, membrane-bound, cytoplasmic bodies referred to as "haplosporosomes". These structures, measuring 140-200 nm in diameter were only found in the outermost or primary cell of PKX. On the basis of the report by Current & Janovy (1977) of similar structures in the cytoplasm of *Henneguya exilis*, affinities between *Marteilia* species and the myxosporea were discussed.

PKD was first recognised in North America in 1981 (Smith, Morrison, Ramsay & Ferguson, 1984). Subsequently, the disease was detected in salmonids in California (Hedrick *et al.*, 1984). Significantly, this report described the presence of myxosporean pseudoplasmodia and sporoblast stages in the renal tubules of hatchery stocks of steelhead trout recovering from PKD. The authors were later able to demonstrate that in parasite-free fish, inoculations with blood and spleen tissue from PKD-infected fish (which were known to contain PKX cells and not more advanced stages) resulted in infections with PKX and the development of renal intraluminal myxosporean stages, thus confirming the myxosporean nature of PKX (Kent & Hedrick, 1985b). The intraluminal sporoblasts were characterised by the presence of two spherical polar capsules, however, the spore valves were not fully developed. This immaturity precludes the precise identification of PKX, although there are strong similarities between PKX and certain developmental stages of members of the genus *Sphaerospora*.

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Feist & Bucke (1987) provided additional data concerning the Myxosporean nature of PKX.

The apparent inability of the PKX parasite to complete sporogenesis in the salmonid host has prompted several authors to examine wild fish from waters enzootic for PKD in the search for natural reservoirs of the parasite. The earliest investigations sought to determine the host range susceptible to the disease *per se* (Seagrave, Bucke, Hudson & McGregor, 1981). Attention focussed on hosts of *Sphaerospora* species present in water enzootic for PKD. Hedrick, Kent & Toth (1986) speculated that the *Sphaerospora* sp. in tui chub *Gila bicolor* might be related to PKX, but also reported a *Sphaerospora* sp. present in the renal tubules of sticklebacks *Gasterosteus aculeatus*. The possible role of the stickleback and its renal parasite, *Sphaerospora elegans*, in the transmission of PKD to salmonids was discussed by Feist (1988). Hedrick *et al.*, (1988) described stages of tui chub and stickleback sphaerospores that resembled PKX cells, and reported the presence of mature sphaerospores in a total of four out of several hundred rainbow trout from two hatcheries. The material was insufficient for species determination and considering the rarity of the find the authors regarded the advanced development of the parasite as highly abnormal. However, Odening *et al.*, (1988) also reported the coincidental infection of PKX and intraluminal renal sphaerospores in Germany. The authors were, however, unable to state with certainty that the luminal spores and the PKX cells were directly related. It is of interest to note that *Sphaerospora truttae* n.sp. was recently reported from brown trout (known to be susceptible to PKD), also in Germany (Fischer-Scherl *et al.*, 1986). However, the authors did not discuss possible affinities of *S. truttae* with PKX. At the present time, the myxosporean nature of PKX is accepted, although its precise identity has yet to be confirmed. Recent studies have shown that the infectious waterborne stage of PKX is less than 50 μm in size and that infectivity was also associated with material trapped on a 500 μm filter which contained the oligochaete *Stylaria lacustris* (Hedrick, Monge & de Kinkelin, 1992). These results, although unconfirmed, support the hypothesis that an invertebrate host is involved in PKX transmission (Hedrick, Monge, Kazanji, Pery, Marin & de Kinkelin, 1992).

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2.3.2 Geographic distribution

Since the original description of PKD in Germany, the disease has now been recognised in several other European countries, including Czechoslovakia, Denmark, France, Ireland, Italy, Norway, Spain, Sweden and the United Kingdom, as well as in Canada and the USA. Infected sites are almost invariably fed by river water, sites using spring or borehole water are rarely affected.

2.3.3 Economic significance

Mortalities directly attributable to PKD are usually between 5 and 10%, but much higher figures (up to 90%) have been recorded (Ferguson & Needham, 1978), although these are usually associated with stress with adverse environmental factors such as low dissolved oxygen levels and high water temperatures. Apart from the mortalities, decreased productivity results from poor growth rates, representing decreased food conversion efficiency, a reduction in the food quality of affected fish, and increased labour costs (Clifton-Hadley, Bucke & Richards, 1986). The authors calculated that the full cost of PKD in England and Wales was approximately £1 million per annum.

2.3.4 Host specificity and susceptibility

Most reports of PKD involve rainbow or steelhead trout, although outbreaks in brown trout and Atlantic salmon in Europe and in chinook salmon (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon in North America are common (Hedrick *et al.*, 1984; Ellis *et al.*, 1985). PKD has also been reported in feral cutthroat trout (*O. clarki*) from a reservoir in Montana in North America (MacConnell & Peterson, 1992). Experimental infections have confirmed the susceptibility of chinook and coho salmon and have shown that sockeye (*O. nerka*) salmon may be severely affected (Arkush & Hedrick, 1990). The Arctic charr (*Salvelinus alpinus*) and grayling (*Thymallus thymallus*) are also known to be hosts for the PKX parasite, and may exhibit clinical signs of the disease. The only non-salmonid fish known to be susceptible to infection is the pike (Seagrave *et al.*, 1981). In Europe, brown trout and grayling

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appear more resistant to the disease and clinical signs are usually less severe. In addition, the PKX parasite is able to penetrate the renal tubule epithelium and undergo sporogony, but not complete spore development, in these species (Clifton-Hadley & Feist, 1989; Feist & Bucke, 1993). Recent findings (Foott & Hedrick, 1987, 1990) have shown that resistance to the disease is induced by active infection with PKX, as well as previous exposure to the parasite. The nature of the resistance mechanism is at present unknown, although it is likely that cell mediated immunity to PKX is the major factor involved (Foott & Hedrick, 1987).

2.3.5 Pathogenicity and pathology

2.3.5.1 Clinical signs

Externally, the main presenting features of the disease are abdominal distension, bilateral exophthalmia and pale gills indicative of anaemia. Melanosis may occasionally be seen (Clifton-Hadley et al., 1987). Internally, the kidney may be grossly hypertrophied and straining against the capsule, giving a corrugated appearance. Characteristic greyish mottling may be present and occasional haemorrhagic areas will also be seen. The spleen may also be hypertrophied and have a "knobbly" appearance. In advanced cases ascites, contributing to the abdominal swelling, will be evident.

2.3.5.2 Pathogenesis

Outbreaks of PKD are seasonal. Most reports in the UK refer to the onset of clinical signs during the months July and August when water temperatures reach 15°C and above (Ferguson & Ball, 1979; Ferguson, 1981; Clifton-Hadley, Richards & Bucke, 1986). Investigations by Clifton-Hadley et al., (1986), Foott, Rosemark & Hedrick (1986), and Foott & Hedrick (1987) have shown the infectious agent to be present in the water supply at infected sites 8-12 weeks before clinical signs become apparent and to persist in sufficient numbers to produce a sub-clinical infection until October. Their results also indicated that susceptible fish became infected at temperatures as low as 10°C, but that the disease progressed more rapidly and was more severe as temperatures increased.

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Microscopically, the presence of numerous PKX cells is associated with marked histopathological changes. Although PKX cells may be found in most tissues and organs of the body, the kidney and spleen react most severely to the presence of the parasite (Ferguson & Needham,1978). The principle pathological response is one of chronic inflammation which, in renal tissue, results in the marked proliferation of the interstitial haematopoietic tissue and a reduction in the number of glomeruli and excretory tubules. These elements are dispersed by the hyperplastic interstitial tissue, but also exhibit degenerative changes during the course of the disease (Clifton-Hadley *et al.*, 1987). Vascular pathology, with possible haemoglobin crystallisation, has also been reported (Clifton-Hadley *et al.*, 1987). In advanced cases, granulomatous change, involving macrophages and neutrophils which surround the PKX parasites, are commonly reported (Clifton-Hadley *et al.*, 1987; Ellis *et al.*, 1985; Ferguson & Needham 1978; MacConnell, Smith, Hedrick & Speer, 1989). Other disease conditions may also be present and contribute to both the gross clinical appearance of the kidney and also to the histopathological changes observed. *Aeromonas salmonicida* is frequently present (Hoffman & Dangschat, 1981), and infection with the fungus *Exophiala* sp. has been reported in fish chronically infected with PKD (Alderman & Feist, 1985).

Eventually, the PKX cells are eliminated by the host response or manage to penetrate the tubule epithelium to initiate sporogony. The disappearance of PKX cells from the renal interstitial tissue is accompanied by the resolution of lesions and usually the complete recovery of affected fish.

2.3.6 Diagnosis

Presumptive diagnosis of PKD can be made from the appearance of clinical signs, the history of the stock, and post-mortem findings. The geographic location of the site needs to be taken into account, since generally only river-fed sites are likely to be affected.

A positive diagnosis depends on the recognition of the characteristic PKX cells (usually with host cells attached to the PKX cell membrane), as seen in stained

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histological sections. A more rapid diagnostic technique involves the use of Giemsa or May-Grunwald Giemsa techniques applied to fixed tissue imprints. The stained PKX cells appear characteristically mottled with nuclei and secondary cells clearly visible (Clifton-Hadley, Richards & Bucke, 1983) .

No histological dyes have been found which selectively stain PKX cells; however, the use of silver deposition methods, such as the Grocott & Gomori technique for fungal hyphae, have proved useful in PKX identification. Castagnaro, Marin, Ghittino & Hedrick (1991) have demonstrated the ability of various biotinylated lectins to bind with specific carbohydrate residues of PKX. The application of lectins histochemically allowed the detection of very low numbers of PKX parasites in *O. mykiss* kidneys affected with PKD, and may assist in the recognition of hitherto unknown infective stage. Similarly, the development of monoclonal antibodies to PKX has provided a powerful tool with which other life cycle stages of the parasite may be detected (Adams, Richards & Marin de Mateo, 1992).

2.3.7 Disease management
2.3.7.1 Control strategies

If contact with the infective stage cannot be avoided, delaying the transfer of fingerlings to infected sites until later in the season, when water temperatures fall, may help in reducing mortalities (Ferguson & Ball, 1979). Reduced water temperature does appear to suppress the effects of the disease (Clifton-Hadley *et al.*, 1986); however, the numbers of infectious units are also reduced later in the year and appear to be totally absent during the winter months (Foott & Hedrick, 1987). Mortalities can also be minimised by the avoidance of the stress involved in grading or transporting fish and by feeding only a maintenance diet. Ultraviolet treatment of water supplies has been found to be effective in removing the infectious agent (Hedrick, Kent & Smith, 1986).

2.3.7.2 Treatment regimes

Early attempts to treat PKD with antibacterial and antiprotozoal compounds were unsuccessful (Ferguson & Ball, 1979; Bucke, McGregor, Hudson & Scott, 1981).

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An apparently successful treatment for PKD was discovered by Clifton-Hadley & Alderman (1987). During attempts to treat infestations with the ciliate *Icthyophthirius multifilis* in laboratory-held PKD-infected fish, the use of malachite green (1.0 ppm) and formalin (20 ppm) flushes was found to delay the development of the disease. Further experiments indicated the malachite green to be the active agent involved in suppressing PKD. Good control of the disease using malachite green treatment in the field situation was subsequently reported by Alderman & Clifton-Hadley (1988). Unfortunately, malachite green is capricious in use, toxic in high doses, and possibly detrimental to the freshwater environment.

An alternative treatment was given by Hedrick, Groff, Foley & McDowell (1988), who showed the antibiotic Fumagillin DCH to be effective in controlling experimental infections with PKX. In a further study, Wishkovsky, Groff, Lauren, Toth & Hedrick (1990) confirmed the efficacy of Fumagillin DCH in controlling PKD in the field situation. Fish receiving 0.34 g Fumagillin/kg of diet (fed at 6% biomass per day) showed a highly significant reduction in both the numbers of PKX cells present and kidney inflammation; 7.9% in treated fish compared with 92.7% in the controls. However, the authors found Fumagillin to be toxic in higher doses and to have a cumulative toxic effect, making it a high risk treatment for the routine control of PKD.

CHAPTER 3

SURVEY OF MYXOSPOREAN INFECTIONS IN FRESHWATER FISH

3.1   INTRODUCTION

Myxosporean infections have been noted in several parasite surveys of British freshwater fish (Andrews, 1979; Campbell, 1974; Chubb, 1963,1965; Kane, 1966; Lee, 1977; Mishra, 1966; Mishra & Chubb, 1969; Rizvi, 1964). However, there have been very few published reports concerned specifically with the myxosporea of UK fish. Nicholas & Jones (1959) described *Henneguya tegidiensis* in the gwyniad *Coregonus clupeoides pennantii*, and Quadri (1962) reported the presence of three previously unrecorded species: *Myxidium scardini* in the rudd *Scardinius erythrocephalus; Zschokkella cyprini* in carp *Cyprinius carpio* and *Chloromyxum phoxini* from minnow *Phoxinus phoxinus*. Elson (1969) reported whirling disease caused by *M. cerebralis,* for the first time in the UK in cultivated rainbow trout from four Scottish trout farms. Subsequently, in 1981,the disease was identified in farmed rainbow trout in England (Bucke, Hudson & McGregor, 1982). A detailed account of myxosporean parasites from fish in the River Lugg (Herefordshire) was given by Davies, (1968). Walliker (1966) reported the presence of *Myxidium pfefferi* in roach *Rutilus rutilus* and of *Myxidium oviforme* in Atlantic salmon *Salmo salar* (Walliker, 1968). His thesis on protozoan parasites of fish included studies on the morphology and transmission of myxosporean parasites (Walliker, 1967). By 1974 a total of 26 myxosporean species had been reported in the UK (Kennedy, 1974). Copeland, (1982) reported the occurence of *Myxobolus dermatobia* in wild eels from the River Ouse in Yorkshire and the River Torridge in Somerset. Denham (1983) investigated a total of eleven myxosporean species in several freshwater fish species. Of particular importance for myxosporean taxonomy, he found that spore morphology was affected more by the tissue infected than by the host species involved. In only one instance has a myxosporean parasite been implicated in a mass mortality in the UK (Williams, 1964). However, skeletal deformities in juvenile chub *Leuciscus (Squalius) cephalus* associated with *Myxobolus ellipsoides* infection of the vertebral column have been reported (Bucke & Andrews, 1985). Similar infections have since been found in other

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fish species in the UK (Bucke, pers. comm., 1991; Lom, Pike & Dyková, 1991). Pathogenic changes associated with *Chloromyxum truttae* infections of the gallbladder of wild brown trout (*Salmo trutta*) were reported by Poynton & Bennett (1985). Cultured brown trout also examined as part of the study were uninfected. *Myxobolus* species were identified in cranial cartilage and brain of grayling (*Thymallus thymallus*) and brown trout examined for the presence of *M. cerebralis* spores (Bucke & Feist, unpublished). The first occurrence of *Myxobolus koi* in the UK was reported by Crawshaw & Sweeting (1986) who described numerous gill cysts on an imported koi carp (*C. carpio*). The authors discussed the the possible threat to native fish stocks from the accidental introduction of fish pathogens associated with the importation of exotic fish species.

The aim of the present study was to undertake a survey of fish myxosporidiosis in the UK. Primarily to assess which fish species were susceptible to PKD and renal myxosporidiosis, but in addition to supplement existing data on the myxosporean fauna of freshwater fish in the UK.

3.2 MATERIALS AND METHODS

3.2.1 Fish

Species and numbers examined are shown in Table 1.

3.2.1.1 Sources

Wild fish were examined from a total of 18 rivers and streams in England and Wales, although predominantly from the Wiltshire Avon, one of its tributaries (River Nadder), and at various sites on the Rivers Test and Itchen. In addition, small samples were obtained from Haweswater (Cumbria) and the Fleet lagoon in Dorset (Fig. 3).

Several methods for fish collection were employed, including electric-fishing and seine netting. Hand-netting was the preferred method for catching juvenilles and smaller fish. Fish were transported back live to the laboratory, either in water filled plastic bags placed in fibreglass or plastic tanks with aeration supplied by a battery operated pump or, where possible, by using the laboratory's fish transporter.

Table 1. Fish species and numbers examined.

Family and Species		No. examined
	Cyprinidae	
Carp	*Cyprinus carpio* L.	36
Gudgeon	*Gobio gobio* L.	1
Chub	*Leuciscus cephalus* L.	1
Dace	*L.leuciscus* L.	37
Roach	*Rutilus rutilus* L.	13
Minnow	*Phoxinus phoxinus* L.	117
	Gasterosteidae	
Three-spined stickleback	*Gasterosteus aculeatus* L.	470
Nine-spined stickleback	*Pungitius pungitius* L.	67
	Salmonidae	
Atlantic salmon	*Salmo salar* L.	83
Brown trout/Sea trout	*S. trutta* L.	62
Rainbow trout	*Oncorhynchus mykiss* Walbaum	38*
	Thymallidae	
Grayling	*Thymallus thymallus* L.	273
	Cobitidae	
Stone loach	*Barbatula barbatulus* L.	6
	Esocidae	
Pike	*Esox lucius* L.	42
	Cottidae	
Bullhead	*Cottus gobio* L.	156
	Coregonidae	
Gwyniad or Powan	*Coregonus lavaretus* L.	12
	Anguillidae	
Eel	*Anguilla anguilla* L.	101
	TOTAL	**1515**

* Rainbow trout routinely examined and used as a source of PKX-infected material are not included above. Most of these originated from a fish farm on the River Avon (Wilts.) with a known annual history of PKD outbreaks. The fish indicated here were fish farm escapees electrofished from the R. Avon.

Figure 3. Sampling locations for myxosporeans in England and Wales.

Fig. 3
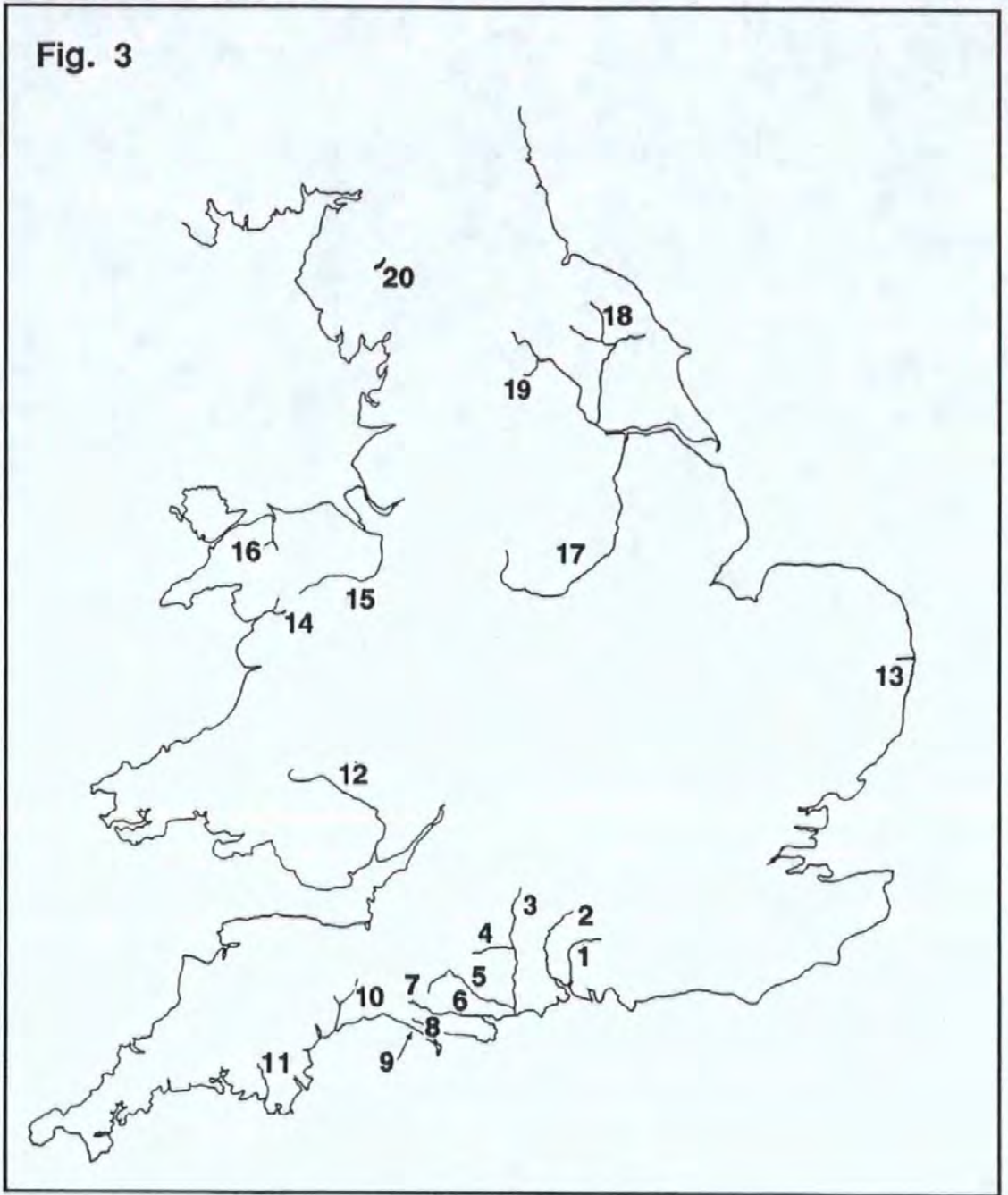
Key

1. River Itchen
2. River Test
3. River Avon
4. River Nadder
5. River Stour
6. River Frome
7. River Hooke
8. River Wey
9. The Fleet
10. River Weaver

11. River Erme
12. River Usk
13. The Kirkley Run
14. River Winion
15. River Dee
16. River Lledr
17. River Trent
18. River Dove
19. River Tutt
20. Haweswater

3.2.1.2 Maintenance.

Captured fish returned to the laboratory were held in 323 l fibreglass tanks or in 78 and 172 l troughs at ambient temperatures and supplied with constant aeration and a continuous flow of dechlorinated water. The majority of wild fish returned to the laboratory were sacrificed within one week. Fish maintained for longer periods were either weaned onto commercial dried diets (Ewos Baker trout pellets of various grades), fed frozen bloodworms or, when available, live foods such as maggots or bloodworm obtained from a local petshop.

Treatment for parasitic infections (predominantly Ichthyophthiriosis) in stock and test fish was by bath or flush methods using formalin at 20 ppm for 1 hour or salt at a 2% concentration for 1 hour. The use of malachite green was avoided, since it was known to be effective in the treatment of PKD and possibly other myxosporean infections (Clifton-Hadley & Alderman, 1987). Treatment for any bacterial infections arising in the samples of wild fish was made by using oxolinic acid-treated food at the rate of 10 mg/kg of fish per day for up to 14 days. Fish failing to respond to treatment were sacrificed for investigation before they became moribund.

3.2.2 Procedures for the detection of myxosporean parasites.

3.2.2.1 Dissection of the fish host.

Fish were first sacrificed by exposure to an overdose of the anaesthetic MS222 (0.1 g/l) (Sandoz, Basle, Switzerland), followed immediately by severance of the spinal cord posterior to the head. A small ventral incision was made transversely, just posterior to the pectoral fins and then making a bisecting incision longitudinally along the abdomen to the vent. On one side of the fish the first incision was extended to a point above the lateral line and then to the vent, thus allowing a flap of skin and muscle to be removed to expose the visceral organs for examination and/or further dissection. For fish less than 5 cm in length the alimentary tract, swimbladder and gonads were displaced, exposing the kidneys, and the whole fish placed in fixative for histological study. Alternatively, small portions of the kidneys other visceral organs and selected

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tissues were removed for fresh examination. In larger specimens organs were selectively removed for further study.

3.2.2.2 Fresh preparations

Certain visceral organs removed at necropsy were examined in the fresh state by squashing approximately 1 mm^3 of the tissue in a drop of phosphate buffered saline (PBS) between a slide and coverslip. Filter paper was used to protect the coverslip and moderate pressure exerted by the thumb or forefinger was applied to spread the tissue thinly and evenly. The contents of urinary and gall bladders were placed directly between agar-coated slides and coverslips without dilution.

Myxosporean cysts visible to the naked eye were examined as for tissue samples. However, it was found that by using this method the influence of Brownian motion on spores and developmental stages prevented high resolution micrographs being taken. The use of agar-coated slides (Lom, 1969), was found to be excellent in subduing Brownian motion. This technique also allowed mature spores to be presented in different attitudes, allowing a full description of overall morphology to be made.

Expulsion of polar filaments and subsequent measurement for taxonomic purposes was accomplished by treating spores with a saturated aqueous solution of urea (Lom, 1964). Observations on fresh preparations were made using phase contrast-optics on a Nikon 'Apophot' photomicroscope and Normarski interference contrast optics on a Reichert 'Polyvar' photomicroscope. In some instances ordinary transmitted light was found to give good contrast for photographic purposes.

Where sufficient numbers of parasites occured, the identification of myxosporean species was made by measurement of mature spores, and developmental stages if present, using an eyepiece graticule or photographic prints. These data were then compared with parasite descriptions in published keys (Bauer, 1984; Shulman, 1966). In several instances specific diagnosis was not possible and identifications were based on the general morphology of the spores and developmental stages and by taking account of the host and the tissues involved in the infection.

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3.2.2.3 Histology and electron microscopy.

Material for histological examination was taken at necropsy, as soon as possible after death of the fish. For routine light microscope studies, tissues were fixed in either 10% neutral buffered formalin (NBF) or Bouin's fluid. Mild decalcification of transverse 'steaks' of heads or whole fish was accomplished using either the proprietary rapid decalcifying solution 'RDC' (R.A.Lamb Ltd), or 'Decal Rapid' (National Diagnostics (UK) Ltd) for 1-2 h. Alternatively, an aqueous solution of 10% formic-acid was used. Subsequent treatment of tissues through to paraffin wax was undertaken with the aid of carousel-type tissue processors or with a more advanced vacuum infiltration processor (VIP 2000) (Miles Laboratories equipment). Specimen dehydration was through ascending grades of alcohols, and 'clearing' was either with chloroform using the carousel-type processor, or xylene or a proprietary clearing agent 'Histoclear' in the VIP processor, prior to embedding in paraffin wax.

Sections of 4-5 μm thickness were cut with the aid of a Leitz rotary microtome. The cut sections were routinely stained with haematoxylin (Shandon Activity No.3) and eosin (H&E) for general morphological interpretation of parasitic infections and pathology. A variety of specialised stains were employed (Table 2) to enhance specific details of parasitic infections and host reaction.

Fresh kidney impressions and blood smears spread on cleaned microscope slides were air-dried and then fixed in a solution of 5% acetic-methanol for a minimum of 5 minutes prior to staining with May-Grünwald Giemsa. This method was routinely used to identify Myxosporea and to describe developmental stages of the parasites.

Histological preparations and stained smears were observed with the aid of standard optics on the above mentioned microscopes. Black and white photographs were taken at various magnifications using Ilford PanF or Kodak Tech Pan film through a green filter. Colour prints or slides were taken using Kodak Ektachrome film through a blue filter. All photographs were taken on a Reichert 'Polyvar' photomicroscope.

Tissues for ultrastructural investigation were removed for fixation immediately on death. Tissue blocks, 1mm3, were placed in a solution of chilled 4% glutaraldehyde in 0.1 M cacodylate buffer (ph 7.2) for a minimum of 1 hour. Followed by several

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washes in 0.1 M cacodylate buffer. Secondary fixation was in 1% osmium-tetroxide in 0.1 M cacodylate buffer for 1 hour. The fixed tissues were subsequently washed with several changes of 0.1 M cacodylate buffer. Dehydration was in graded alcohols starting at 50% absolute alcohol through to two changes of 100% absolute alcohol. Tissues, cleared in two changes of propylene-oxide, were embedded in an epoxy resin ('Emix', Agar Scientific Ltd.) and cured at 60°C for a minimum of 24 hours.

One micron, semi-thin sections were cut with the aid of glass knives prepared using an LKB knifemaker and stained with a 1% solution of toluidine blue in a 1% solution of aqueous borax. Following microscopic evaluation of these preparations, the selected embedded tissue blocks were further trimmed and ultra-thin sections were cut with a diamond knife. These sections were collected on uncoated copper grids and allowed to dry. Subsequently, they were double-stained using uranyl acetate (either as a saturated solution in 70% alcohol, or as a saturated aqueous solution) followed by treating with Fahmy's lead citrate (Lewis & Knight, 1977). The stained sections were examined using a JEOL 100CX electron microscope operated at 100 kV.

3.3   RESULTS

A total of 1515 fish representing 17 species and 9 families were examined for the presence of clinical PKD, PKX cells and other myxosporean infections. The emphasis of this investigation was on the renal infections (Tables 2, 3 & 4). Representatives of eight myxosporean genera were found and nine species were positively identified. A further 23 species were recorded and were identified to genus, see Table 4. Myxosporidiosis was diagnosed in every species except the eel *Anguilla anguilla*. In most instances parasite identifications were based on the examination of fresh material and histological sections of paraffin-embedded tissues.

Results are conveniently presented below under fish species, in alphabetical order as shown in Table 3. Data on the source, age of fish and number examined, month of sampling, prevalence of myxosporidiosis and location in the host are also given in Table 3. Additional information on selected infections is presented below and in Chapter 5.

Table 2. Histological staining techniques for the study of Myxosporeans

Technique	Application	Reference
Periodic Acid Schiff (PAS) with diastase control	Carbohydrates	McManus, 1946*
Feulgen	DNA Myxosporean nuclei	Feulgen & Rossenbek, 1924*
Giemsa	Parasites Myxosporean polar capsules and shell valves	in Bancroft & Stevens, 1977
May-Grünwald Giemsa	Cellular morphology and parasite structure in tissue and blood smears	in Disbrey & Rack, 1970
Gomori 1-step trichrome	Connective tissue	in Drury & Wallington, 1973
Masson trichrome	Connective tissue	in Disbrey & Rack, 1970
Haematoxylin and eosin	Routine staining technique	in Bancroft & Stevens, 1977
Ziehl-Neelson	Acid fast bacilli and myxosporean shell valves	Ziehl, 1882* Neelsen, 1883*
Gordon & Sweets	Reticulin fibres	Gordon & Sweets, 1936
Grocott & Gomori	Fungal hyphae, Reticulin fibres and myxosporean trophozoite argyrophilia	Grocott, 1955*; Gomori, 1946*

* The original reference is provided. Technical procedures were carried out according to those given in Bancroft & Stevens, 1977 and Disbrey & Rack, 1970.

Table 3.　　　Results of survey for fish myxosporidiosis in the UK

Table 3. Results of survey for fish myxosporidiosis in the UK

Specimens examined	Ref. no. (Year/ Accession No.)	Age if known and number examined ( )	Source	Month of examination	Finding and prevalence	Location in host
Anguilla anguilla	HE 87/44	(1)	River Avon (Wilts)	June	NEM (various tissues)	-
	HE 87/97	(25)	"	August	NEM (kidney)	-
	HE 88/52	Elvers (50)	Bristol Channel (Avon)	April	NEM (various tissues)	-
	HE 87/88	(10)	River Frome (Dorset)	August	NEM (kidney)	-
	HE 87/94 (PM 87/144)	(15)	River Trent (Notts)	"	NEM (kidney)	-
Barbatula barbatulus	HE 87/64	0+ (2)	River Avon (Wilts)	July	-	-
	HE 89/18	Adult (4)	" " (")	April	Sphaerospora sp. PS	Kidney tubules
Coregonus lavaretus	HE 87/49	(2)	Haweswater (Cumbria)	June	Sphaerospora sp. (2/2)	Kidney
	HE 87/62B	(10)	"	July	NEM (kidney)	"
Cottus gobio	HE 86/58	0+ (5)	River Avon (Wilts)	August	Myxobolus cotti (2/5)	CNS
	HE 86/78	Adult (39)	"	October	Zschokkella nova (3/39)	Liver and bile ducts
	HE 86/91	Adult (6)	"	"	Myxobolus cotti (6/6)	CNS
	HE 86/79	Adult (100)	River Wey (Dorset)	"	Myxobolus cotti (23/100)	CNS
	HE 89/69	Juvenile (6)	River Avon (Wilts)	July	NEM (various tissues)	-
Cyprinus carpio	HE 87/85	(5)	River Avon (Wilts)	August	NEM (kidney)	-
	HE 87/196	(10)	R.Weaver (Devon)	November	Hoferellus cyprini (9/10)	Kidney (tubule epithelium
	HE 88/7	(5)	R.Weaver (")	February	-	- and lumens)
	HE 88/32	(6)	" (")	"	-	-
	HE 89/13	1+ (10)	" (")	"	Sphaerospora sp. PS (5/10)	Kidney tubules
Esox lucius	HE 86/20	(5)	River Avon (Wilts)	April	Myxidium Lieberkuehni (ML) (3/5)	Kidney and urinary bladder (UB)
					Unidentified Myxozoan (1/5)	Liver
					Henneguya psorospermica (1/5)	Gill
	HE 87/36	(4)	"	April	(ML) (4/4)	Kidney
					Henneguya psorospermica (1/4)	Gill
	HE 88/55	(1)	"	April	-	-
	HE 88/86	(3)	"	May	-	-
	HE 87/42	Various (3)	"	"	ML (3/3)	Kidney and UB
	HE 86/68	(4)	"	September	ML + PKD with PKX cells (2/4)	" " "
	HE 87/107	(7)	"	"	ML (7/7)	" " "
	HE 87/189	0+ (2)	"	October	ML (2/2)	" " "
					PKX (1/2)	Spleen
	HE 87/92	(3)	River Frome (Dorset)	August	Slight ML (2/3)	Kidney and UB
	HE 87/108	(2)	" " (")	September	ML (2/2)	" " "
	HE 87/12	(1)	River Itchen (Hants)	January	ML (1/2)	" " "
	HE 86/87	(5)	" " (")	October	ML + PKD with PKX cells (1/5)	" " "
	HE 87/61	1+ (2)	River Stour (Dorset)	July	ML (2/2)	" " "
Gasterosteus aculeatus	HE 88/56	(10)	River Avon (Wilts)	April	Sphaerospora elegans (SE)(7/10) Myxobilatus gasterostei (MG)(3/10)	Kidney tubules " "
	HE 88/61	(6)	" " (")	May	SE (3/6) MG (3/6)	" "
	HE 87/45	(4)	" " (")	June	MG (4/4)	" "
	HE 88/68	(10)	" " (")	June	SE (9/10) MG (6/10)	" "
	HE 88/70	(3)	" " (")	June	SE (3/3)	" "
	HE 88/73	(5)	" " (")	July	SE (2/5) MG (1/5)	" "

Table 3 (cont'd.)

Specimens examined	Ref. no. (Year/ Accession No.)	Age if known and number examined ( )		Source	Month of examination	Finding and prevalence	Location in host
Gasterosteus aculeatus	HE 88/74		(6)	River Avon (Wilts)	July	SE (3/6) MG (1/6)	Kidney tubules
	HE 86/54	Fry	(3)	" " (")	August	NEM (kidney)	-
	HE 88/88	0+	(22)	" " (")	August	SE (6/19) MG (11/19)	" "
	HE 88/92		(36)	" " (")	August	SE (17/33) MG (17/33)	Kidney tubules & choroidal rete (3/36)
	HE 88/96	Adult(7)(17) 0+ (10)		" " (")	August	SE (11/17) MG (12/17)	Kidney tubules
	HE 88/103	0+	(5)	" " (")	August	MG (4/5)	" "
	HE 87/101A	0+	(10)	" " (")	September	SE + MG (10/10) plus PKX-like cell (1/10)	Kidney interstitium
	-	0+	(19)	" " (")	September	SE + MG (15/19)	Kidney tubules
	HE 88/29		(10)	River Erme (Ludbrook) (Devon)	February	NEM	-
	HE 88/77		(10)	" " (")	July	NEM	-
	HE 88/93		(14)	" " (")	August	NEM	-
	HE 87/105	0+	(10)	The Fleet (Abbotsbury) (Dorset)	September	SE (8/10)	Kidney tubules
		0+	(5)	" " (")	September	SE (5/5)	" "
	HE 87/106	0+	(7)	" " (")	September	SE (6/7)	" "
	HE 88/50		(10)	The Kirkley Run, Lowestoft (Suffolk)	April	MG (2/10)	" "
	HE 88/57		(10)	" "	April	SE (1/10) MG (7/10)	" "
	HE 88/63		(10)	" "	May	SE (1/10) MG (5/10)	" "
	HE 89/56		(6)		June	NEM	-
	HE 89/12	Adult	(25)	River Weaver (Devon)	February	SE (10/25)	Kidney tubules
	HE 88/30	Adult	(10)	" " (")	"	SE (4/10)	" "
	HE 88/31		(11)	" " (")	"	NEM	-
	HE 88/33		(10)	" " (")	"	SE (2/10)	-
	HE 88/36		(5)	" " (")	March	NEM	-
	HE 88/85		(10)	" " (")	May	SE (1/10)	Kidney tubules
	HE 88/94	Adult(20)(40) 0+ (20)		" " (")	August	NEM	-
	HE 88/95	Adult	(4)	" " (")	August	SE (2/4)	Kidney tubules
	-	0+	(27)	" " (")	September	SE (1/27)	" "
	-	0+	(20)	" " (")	October	SE (3/20) v.mild infection	" "
	-	0+	(15)	" " (")	November	(1/5) Possible infection - no spores found	" "
	HE 89/12	Adult	(25)	" " (")	February	SE (10/25)	" "
	HE 89/49	Adult	(10)	River Hooke (Dorset)	May	SE (3/10)	" "
	HE 90/57	Adult	(10)	" " (")	June	SE (3/10) MG (1/10)	" "
Gobio gobio	HE 86/63	2+	(1)	River Avon (Wilts)	September	Myxobolus sp.	Various tissues
Leuciscus cephalus	HE 86/75		(1)	River Avon (Wilts)	September	Myxobolus muelleri	Various tissues
Leuciscus leuciscus	HE 88/7		(1)	River Avon (Wilts)	February	-	-
	HE 87/37	1+	(7)	" "	June	Chloromyxum sp. (1/7) Myxobolus muelleri (5/7) Myxobolus sp. (5/7) Hoferellus sp. (3/7)	Bile Kidney interstitium " " Kidney tubule epithel.

Table 3 (cont'd.)

Specimens examined	Ref. no. (Year+ Accession No.)	Age if known and number	examined ( )	Source	Month of examination	Finding and prevalence	Location in host
Leuciscus leuciscus	HE 87/37					PS + IS (4/7)	Kidney tubules
						Myxobilatus sp. (1/7)	Urinary bladder
						Myxobolus sp. (2/7)	Gill
						" (1/7)	Somatic musculature
		1+	(7)	River Avon (Wilts)	June	Zschokkella sp. (1/7)	Bile
	HE 86/52	Fry	(5)	" " (")	August	Sphaerospora sp. (1/5)	Kidney
	HE 86/62	Fry	(5)	" " (")	September	Myxobolus sp. (2/5)	Connective tissue
	HE 86/64	1+	(2)	" " (")	"	Myxobolus muelleri (1/2)	Kidney/musculature
	HE 86/76	2+	(10)	" " (")	"	Myxobolus sp. (10/10)	Various tissues
Oncorhynchus mykiss	HE 89/11		(10)	River Avon (Wilts)	February	PKX (1/10)	Kidney and spleen
	HE 86/78B		(28)	" " (")	October	NEM	-
Phoxinus phoxinus	HE 87/38	Adult	(5)	River Avon (Wilts)	May	Sphaerospora sp. PS (2/5)	Kidney tubule + collecting ducts
				" " (")		Myxobolus sp. (2/5)	Kidney
	HE 88/60	Adult	(6)	" " (")		unidentified forms	Gall + urinary bladders
	HE 88/76	Fry	(50)	" " (")	July	-	-
	HE 87/65	Adult	(5)	" " (")	"	Myxobolus sp. (1/5)	Kidney
	HE 86/56	1+	(5)	" " (")	August	Myxobolus sp. (5/5)	Various tissues
						PS (1/5)	Kidney
						Myxobolus sp. (2/5)	Gill cysts
	HE 86/53	Fry	(24)	" " (")	August	PS (3/24)	Kidney
	HE 86/80	Adult	(17)	" " (")	October	Myxobolus sp. (2/17)	Somatic musculature
	HE 86/82	Adult	(5)	" " (")	"	Myxobolus sp. (5/5)	Various tissues
						Myxobolus sp. (2/5)	Gill cysts
Pungitius pungitius	-	0+	(1)	River Avon (Wilts)	September	NEM	Kidney
	HE 87/101B	0+	(10)	" " (")	September	(6/8) sections of kidney with mild SE	"
				" " (")			
	-	0+	(1)	" " (")	September	NEM	"
	-	0+	(1)	" " (")	September	SE (1/1)	"
	-	0+	(1)	" " (")	"	NEM	"
	-	0+	(2)	" " (")	"	NEM	"
	-	0+	(10)	" " (")	November	SE (8/10)	"
	HE 88/51		(10)	The Kirkley Run, Lowestoft (Suffolk)	April	SE (4/10)	-
	HE 88/53		(10)	" "	April	SE (3/10)	-
	HE 88/62		(11)	" "	May	SE (4/11) MG (1/11)	-
	HE 88/66		(10)	" "	June	SE (4/10)	-
Rutilus rutilus	HE 86/45,47,50	0+	(10)	River Avon (Wilts)	July/August	NEM	-
	HE 86/66	1+	(3)	" " (")	September	Myxobolus sp. (3/3)	Various tissues
						Myxidium rhodei (1/3)	Kidney
Salmo salar	HE 86/102		(10)	River Lledr (Gwynedd)	November	Sphaerospora sp. (2/10)	Kidney tubules
	HE 87/223		(8)	" " (")	December	Sphaerospora sp. (3/8)	" "
	HE 87/3		(3)	River Itchen (Hants)	January	NEM	-
	HE 87/2		(6)	River Test (Hants)	January	Sphaerospora sp. (2/6)	Kidney tubules
	HE 87/4		(8)	" " (")	January	Sphaerospora sp. (2/8)	" "
	HE 87/5		(5)	" " (")	January	NEM	-

Table 3 (cont'd.)

Specimens examined	Ref. no. (Year/ Accession No.)	Age if known and number examined ( )	Source	Month of examination	Finding and prevalence	Location in host
Salmo salar	HE 88/1	(1)	River Test (Hants)	January	NEM	-
	HE 88/2	(1)	" " (")	January	NEM	-
	HE 88/3	(1)	" " (")	January	NEM	-
	HE 86/100	(19)	River Usk (Gwent)	November	Sphaerospora sp. (1/19)	Kidney tubules
	HE 86/101	(13)	" " (")	November	Sphaerospora sp. (4/13)	Kidney tubules
	HE 87/222	(8)	River Winion (Gwynedd)	December	Sphaerospora sp. (3/8)	" "
Salmo trutta (brown trout)	HE 86/78A	(17)	River Avon (Wilts)	October	Sphaerospora sp. (1/17)	Kidney tubules
	HE 89/59	0+ (1)	" " (")	June	NEM	" "
	HE 89/70	0+ (3)	" " (")	July	PKX/Sphaerospora sp. (2/3)	" "
	HE 86/101	(2)	River Dee (Clwyd)	November	Sphaerospora sp. (2/2)	" "
	HE 90/146	(7)	River Wey (Dorset)	October	NEM (kidneys only)	-
	HE 87/62A	(5)	Haweswater (Cumbria)	July	Chloromyxum sp. (3/5)	Kidney tubules
Salmo trutta (sea trout)(anadromous)	HE 87/223	(9)	River Lledr (Gwynedd)	December	-	-
	HE 87/192	1+ (1)	River Tutt (Yorkshire)	September	Marked PKD inc.PS	Kidney
	HE 87/221	(6)	River Usk (Gwent)	December	Sphaerospora sp. (1/6)	-
	HE 87/222	(11)	River Winion (Gwynedd)	December	Sphaerospora sp. (1/11)	-
Thymallus thymallus	HE 88/5	(5)	River Avon (Wilts)	January	NEM (various tissues)	-
	HE 88/7	(1)	" " (")	February	NEM (" ")	-
	HE 88/34	(15)	" " (")	February	PS (1/15)	Kidney
	HE 88/35	(10)	" " (")	March	NEM (kidneys only)	-
	HE 87/35	1+ (40)	" " (")	April/May	PS (24/40) IS (2/40)	Kidney
	HE 87/79	(1)	" " (")	July	Rare PS	"
	HE 88/117	Adult (5)	" " (")	September	PS (1/5)	"
	HE 87/88	Various (11)	" " (")	October	PS (3/11) Poss. PKD (3/11)	"
	HE 87/190	Various (10)	" " (")	October	PS (4/10) Poss. PKD (8/10)	Kidney
	HE 88/127	Adult (25)	" " (")	October	PS (23/25)	Kidney tubules
	HE 87/199	(10)	" " (")	November	IS (1/10)	" "
	HE 89/58	(2)	" " (")	June	NEM (various tissues)	-
	HE 86/98	(1)	River Dove (Yorkshire)	November	PKD with PKX cells	Kidney & spleen
	HE 86/85	(17)	River Itchen (Hants)	October	PKD (15/17), PKX (5/17) PS (12/17) IS (5/17)	Kidney Kidney tubules
	HE 86/18	(5)	River Nadder (Wilts)	April	PS (2/5)	" "
	HE 86/35	(5)	" " (")	June	PS (3/5) IS (2/5)	" "
	HE 86/37	(13)	" " (")	June	PS (8/13) IS (5/13)	" "
	HE 86/39	(5)	" " (")	June	PS (4/5) IS (1/5)	" "
	HE 87/1	(5)	River Test (Hants)	January	NEM (kidneys only)	-
	HE 87/6	(5)	" " (")	January	NEM (" ")	-
	HE 87/11	(41)	" " (")	January	NEM (" ")	-
	HE 87/27	(1)	" " (")	March	Rare PS	Kidney
	HE 87/34	1+ (8)	" " (")	April	PS (4/8)	Kidney
	HE 86/87	(4)	" " (")	October	NEM	-
	HE 86/88	(10)	" " (")	October	PKD (2/10) PKX (2/10) PS (1/10)	Kidney
	HE 90/143	(18)	" " (")	October	NEM (kidneys only)	-

PKD - Proliferative kidney disease;
MG - Myxobilatus gasterostei Parisi, 1912 (Davis, 1944);
CNS - Central nervous system;

PKX - Proliferative kidney disease organism 'X';
PS - Pseudoplasmodia;
IS - Immature spores;

SE - Sphaerospora elegans Thelohan, 1892;
ML - Myxidium lieberkuehni Buetschli, 1882;
NEM - No evidence of myxosporidiosis;

Table 4.   Summary of myxosporeans identified

Host	Parasite species	Site of infection
Barbatula barbatulus	*Sphaerospora* sp.	Renal tubules
Coregonus lavaretus	*Sphaerospora* sp.	Renal tubules
Cottus gobio	*Myxobolus cotti* *Zschokkella nova*	CNS bile ducts
Cyprinus carpio	*Hoferellus cyprini* *Sphaerospora* sp.	Kidney (tubule epithelium) Renal tubules
Esox lucius	*Myxidium lieberkuehni* " " *Henneguya psorospermica* PKX	Renal tubules Glomeruli Gills Kidney and spleen
Gasterosteus aculeatus	*Sphaerospora elegans* *Myxobilatus gasterostei*	Kidney and choroidal *rete* Kidney
Gobio gobio	*Myxobolus* sp.	Various tissues
Leuciscus cephalus	*Myxobolus muelleri*	Various tissues
Leuciscus leuciscus	*Chloromyxum* sp. *Myxobolus muelleri* *Myxobolus* sp. *Hoferellus* sp. *Myxobilatus* sp. *Myxobolus* sp. *Myxobolus* sp. *Sphaerospora* sp. *Zschokkella* sp.	Gall bladder Renal interstitium " " Renal tubule epithelium Urinary bladder Somatic musculature Gills Renal tubules Gall bladder
Oncorhynchus mykiss	PKX	Various tissues
Phoxinus phoxinus	*Myxobolus* sp. *Myxobolus* sp. *Sphaerospora* sp.	Renal interstitium Somatic musculature Gills Renal tubules
Pungitius pungitius	*Sphaerospora elegans*	Kidney
Rutilus rutilus	*Sphaerospora* sp. *Myxidium rhodei* *Myxobolus* sp.	Renal tubules Renal interstitium Various tissues

Table 4. (cont'd)

Host	Parasite species	Site of infection
Salmo salar	*Sphaerospora* sp.	Renal tubules
Salmo trutta	*Sphaerospora* sp.	Renal tubules
	Chloromyxum sp.	" "
	PKX	Renal interstitium and spleen
Salmo trutta (anadromous)	*Sphaerospora* sp.	Renal tubules
Thymallus thymallus	*Sphaerospora* sp.	Renal tubules
	PKX	Various tissues

(a)   *Cottus gobio* L, Bullhead.

Myxobolus cotti and *Zschokkella nova* were the only two species of Myxosporean recorded in *Cottus gobio*. The former was selected for a detailed investigation and was described by Lom *et al.*, (1989). Identification of *Z. nova* was based upon comparative morphology of the spore with the original description by Klokacéwa (1914). Although the parasite has previously been reported from the gall-bladder and bile ducts (Fig. 4) invasion of the hepatic parenchyma is also reported here for the first time. These latter infections were associated with cellular infiltration and granuloma formation (Fig.5).

(b)   *Cyprinus carpio* L, Carp.

Hoferellus cyprini Doflein, 1898 was diagnosed in 9/10 *C. carpio* examined in November 1987. Typical lesions were observed in kidney tubule epithelium (Fig. 6). Numerous parasitic stages were seen to occupy the grossly enlarged epithelial cells of the renal tubules. In addition to this parasite, *Sphaerospora* sp. pseudoplasmodia were observed in *C. carpio* sampled in February 1989 (Fig. 7).

(c)   *Esox lucius* L, Pike.

Myxidium lieberkuehni Buetschli, 1882 was identified in most (79%) pike examined (Table 3.). Although the urinary bladder was the primary site of infection for this parasite, the renal tubules were also consistently infected. Extrasporogonic stages of *M. lieberkuehni* were observed in the renal glomeruli of 52% of fish examined. Investigations of these parasites are reported in Chapter 5. Several pike examined, harboured cysts of *Henneguya psorospermica* in the gill tissues (Fig. 8). PKX cells were recorded in only four pike examined. In three of these cases, the PKX cells were observed in the kidneys and were associated with histological changes typical for PKD in rainbow trout.

(d)   *Leuciscus cephalus* L, Chub.

Only one fish became available for examination during this study. Numerous myxosporean cysts were observed on the swimbladder. Spores from these cysts were of a *Myxobolus* sp. which measured 14.1 (13.7-15.3) μm in length and 11.3 (10.6-12.5) μm in width, (n=15) (Fig. 9). The parasite was identified as *M. muelleri* Buetschli,

Figure 4.   Micrograph of spores (arrowed) and trophozoites (T) of *Zschokkella nova* in distended hepatic bile ducts of *Cottus gobio*. Giemsa, x410.

Figure 5.   Granulomatous focus in the hepatic parenchyma of *Cottus gobio* associated with the presence of *Zschokkella nova* spores (arrowed). Giemsa, x410.
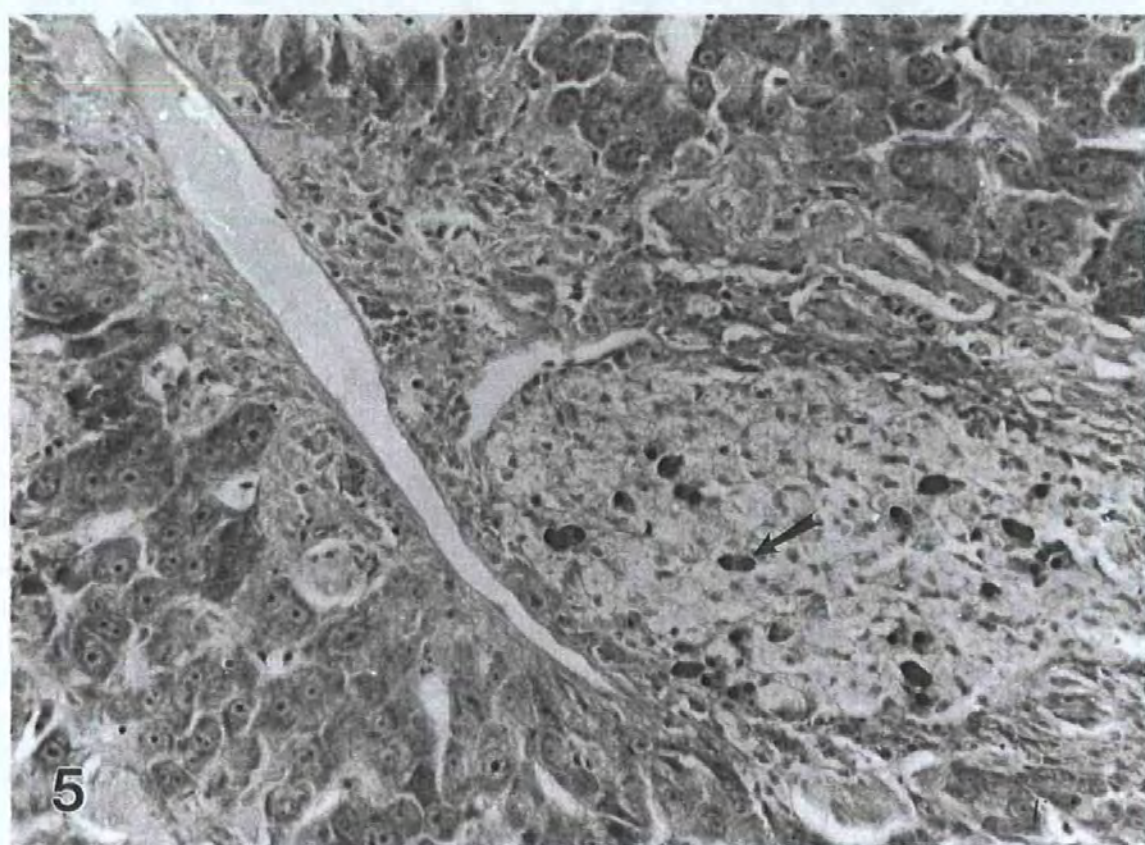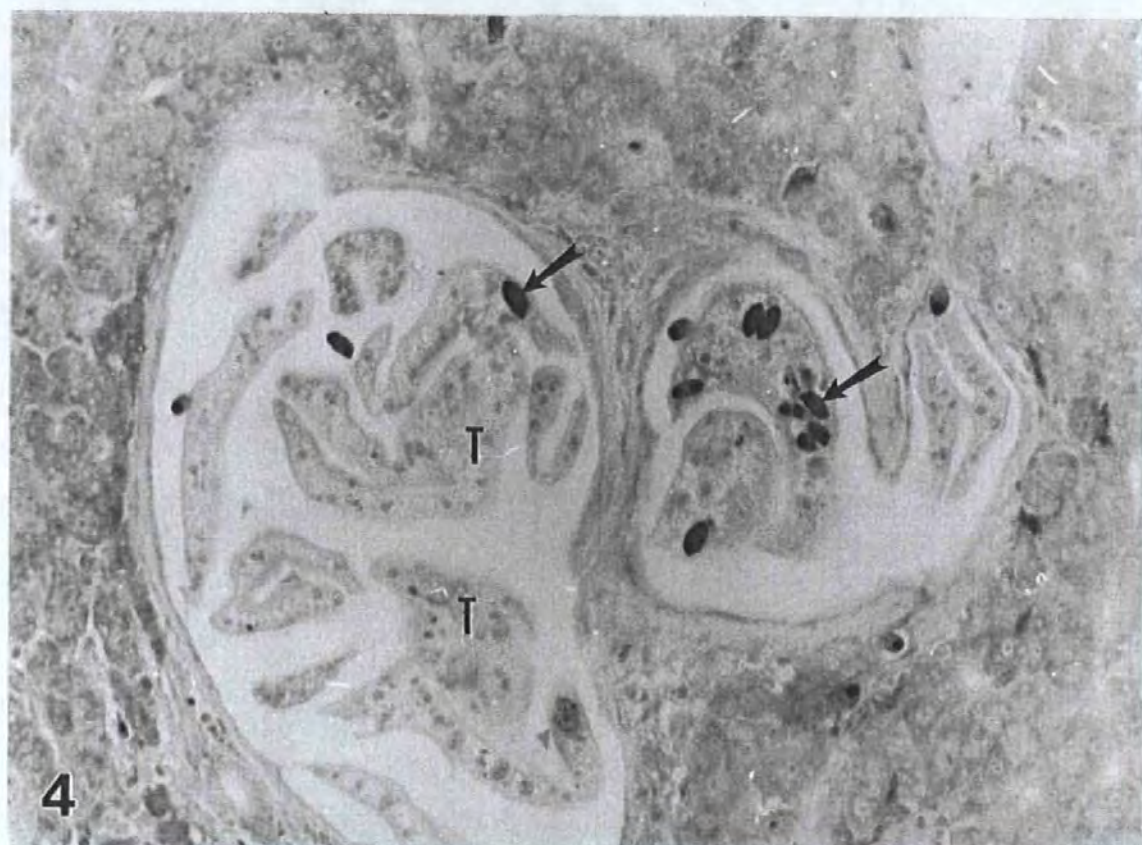
Figure 6.   Developmental stages of *Hoferellus cyprini* in the renal tubule epithelium of *Cyprinus carpio.* Lumen of the affected tubule is arrowed. H & E, x395.

Figure 7.   Pseudoplasmodia (arrowed) and developing spores of *Sphaerospora renicola ?* in a renal tubule of *Cyprinus carpio.* Inset-higher magnification of a pseudoplasmodium (*), note the presence of refractile granules (arrow) and polar capsules of the developing spore (double arrow). Normarski interference contrast, x410 and x825 respectively.
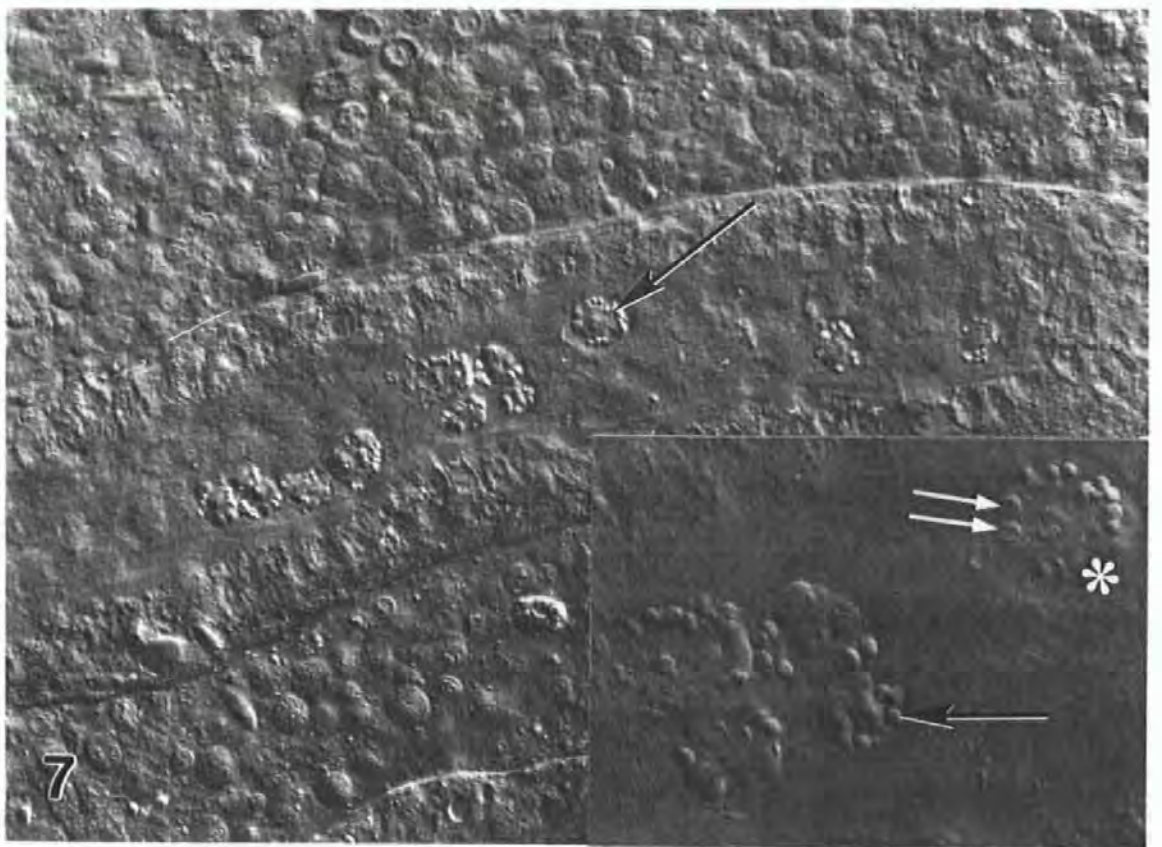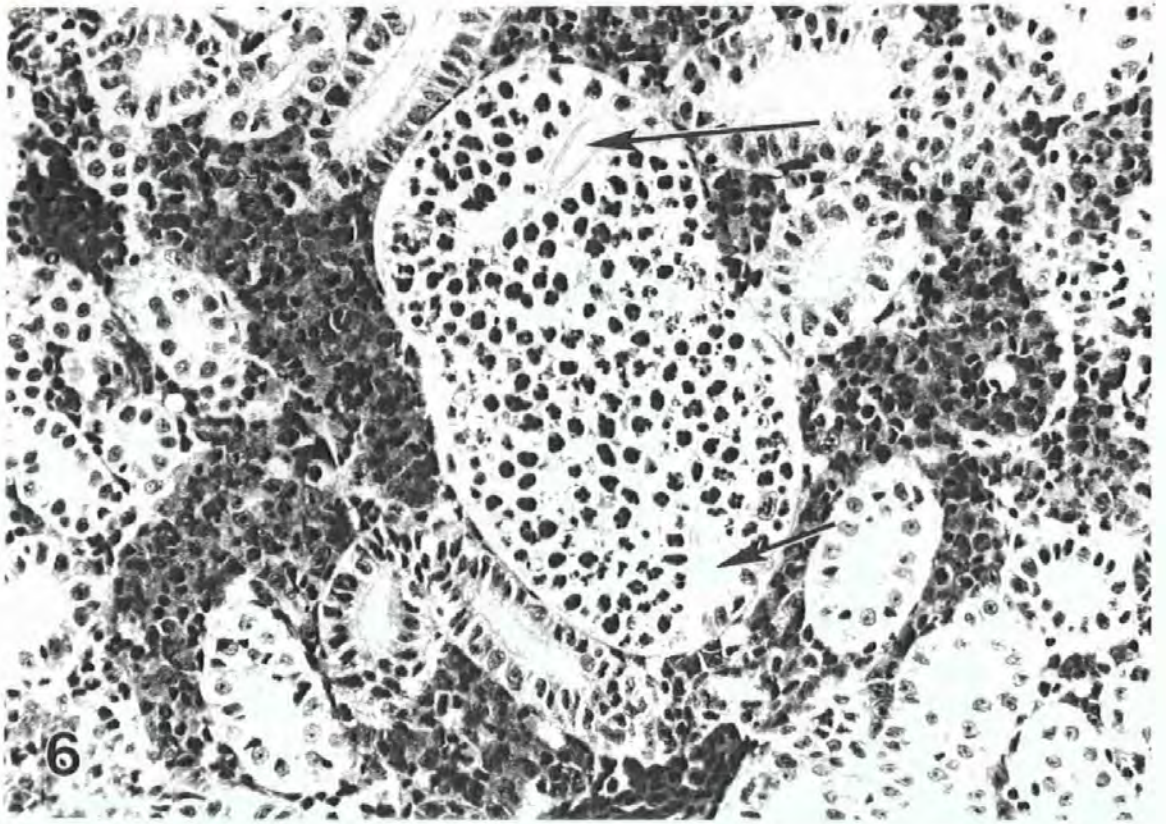
Figure 8.   Mature spores and sporoblasts of *Henneguya psorospermica* from *Esox lucius* gill. Normarski interference contrast, x1,275.
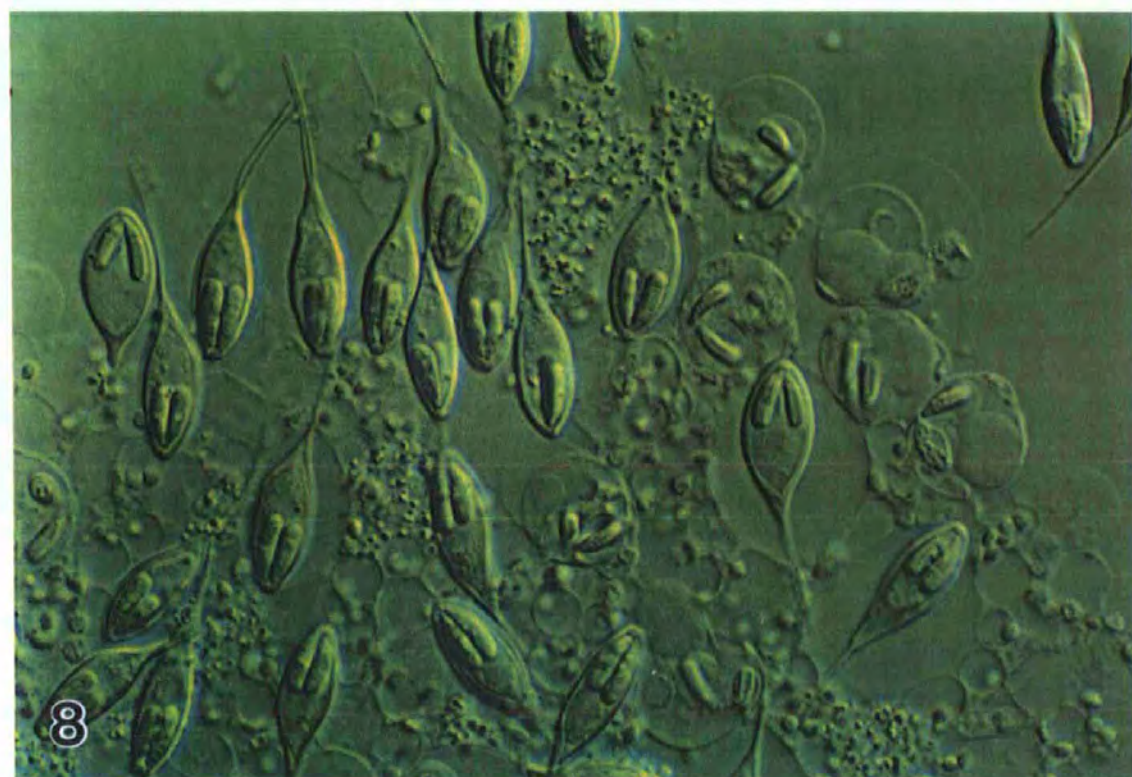
1882 on the basis of spore size and form. Spores of another *Myxobolus* sp. were observed histologically in several other tissues, predominately skeletal muscle and gill.

(e)   *Leuciscus leuciscus* L, Dace.

A total of 30 dace, all from the River Avon, were examined. A *Chloromyxum* sp. was found in the gallbladder of one fish and a *Zschokkella* sp. in one other (Fig. 10). Cysts of several *Myxobolus* species were located histologically in the somatic musculature and gills, in neither tissue was it possible to identify the parasite involved from histological sections. A *Myxobilatus* sp. was observed in the urinary bladder. Spores measured 23.3 (18-24) μm in length (including the caudal appendage) and 6.7 (5.4-7.6) μm in width, (n=7) (Fig. 11). 3 out of 7 fish sampled in June 1987 harboured intracellular myxosporean parasites within the renal tubule epithelium (Fig. 12). Since mature spores of this parasite were not located, their specific identity could not be determined. Pseudoplasmodia of a *Sphaerospora* sp. were observed in renal tubules of one fish (Fig. 13). Several *Myxobolus* species were observed in the renal interstitium of 5 fish. However, spores of only one species were present in sufficient numbers for morphometric analysis. These measured 10.3 (8.6-12.2) μm in length and 7.95 (7.1-8.6) μm in width (n=10) (Fig. 14). Of approximately 18 species of *Myxobolus* known to infect *L. leuciscus* the species recorded here most resembles *M. muelleri*.

(f)   *Phoxinus phoxinus* L, Minnow.

Sphaerospora sp. pseudoplasmodia were identified in 6/43 1 yr+ fish examined, the majority being adults (Fig. 15). Mature spores were not observed in these infections.

Several *Myxobolus* species were observed in various tissues of *P. phoxinus*, see Tables 3 & 4. Histologically, cysts of *Myxobolus* sp. were observed in the somatic musculature and gills. It was not possible to identify the species involved. Spores of a further *Myxobolus* sp. were found associated with renal tissue (Figs. 16). Developmental stages were absent. Spores measured 10.55 (10.1-11.4) μm in length and 8.1 (7.8-8.4) μm in width (n=16).

(g)   *Rutilus rutilus* L, Roach.

Small white cysts were noted on the surface of the kidney of a single fish

Figure 9. Spores of *Myxobolus muelleri* from *Leuciscus cephalus* swimbladder cyst. Normarski interference contrast, x1,275.

Figure 10. A *Zschokkella* sp. spore from *Leuciscus leuciscus* gallbladder. Normarski interference contrast, x912.

Figure 11. *Myxobilatus* sp. spore from *Leuciscus leuciscus* urinary bladder. Normarski interference contrast, x919.
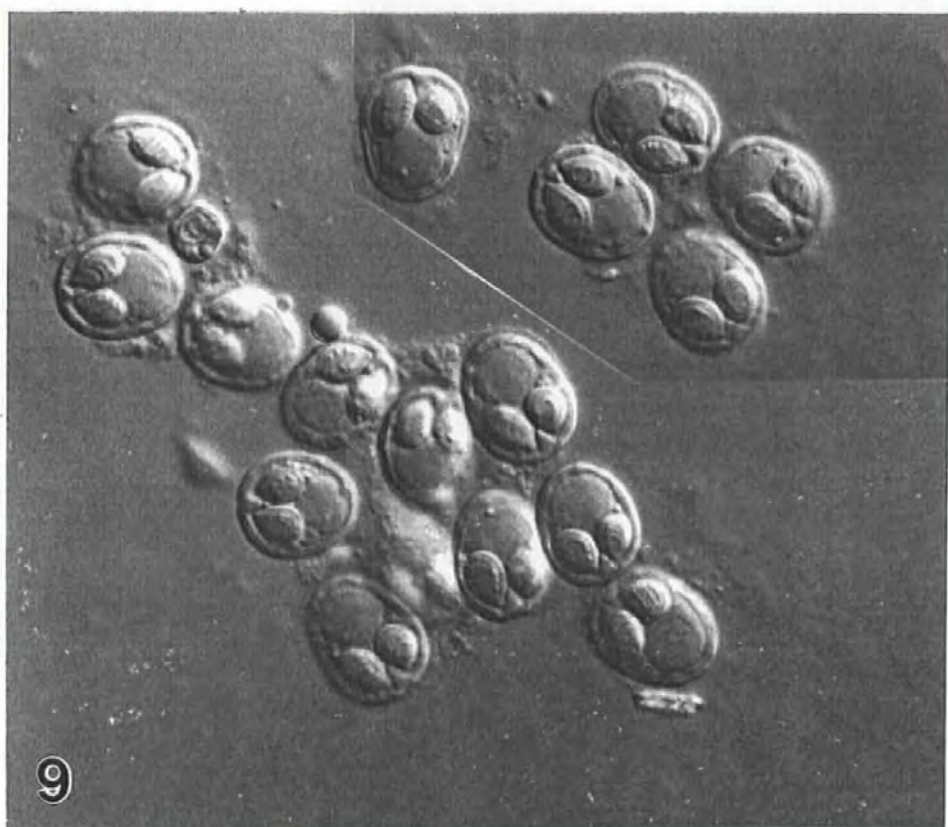
Figure 12. *Hoferellus* sp. cf. *cyprini* affecting the renal tubule epithelium of

Leuciscus leuciscus. H & E, x176.

Figure 13. *Sphaerospora* sp. pseudoplasmodia in a renal tubule lumen from

Leuciscus leuciscus. Normarski interference contrast, x367.
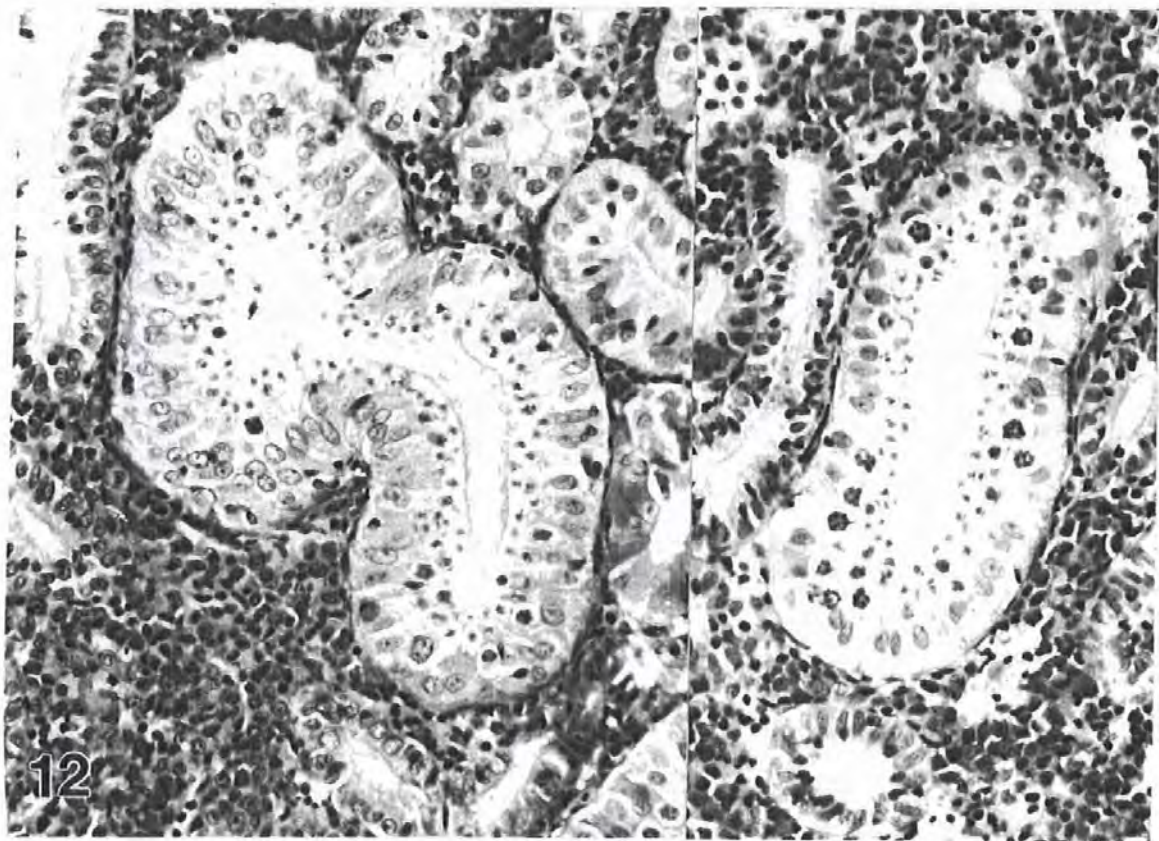
Figure 14.   *Myxobolus* sp. spores from the renal interstitium of *Leuciscus*
leuciscus. Normarski interference contrast, x1,275.

Figure 15.   *Sphaerospora* sp. pseudoplasmodia in a renal tubule lumen from
Phoxinus phoxinus. Normarski interference contrast, x832.
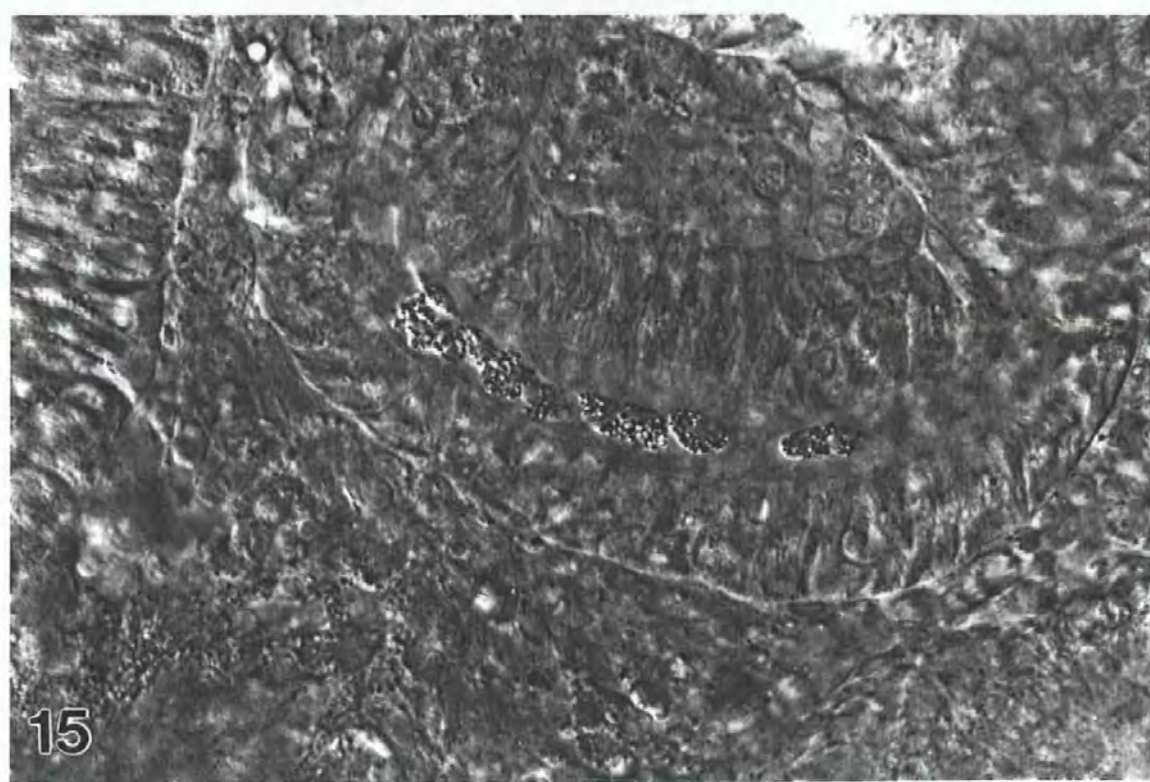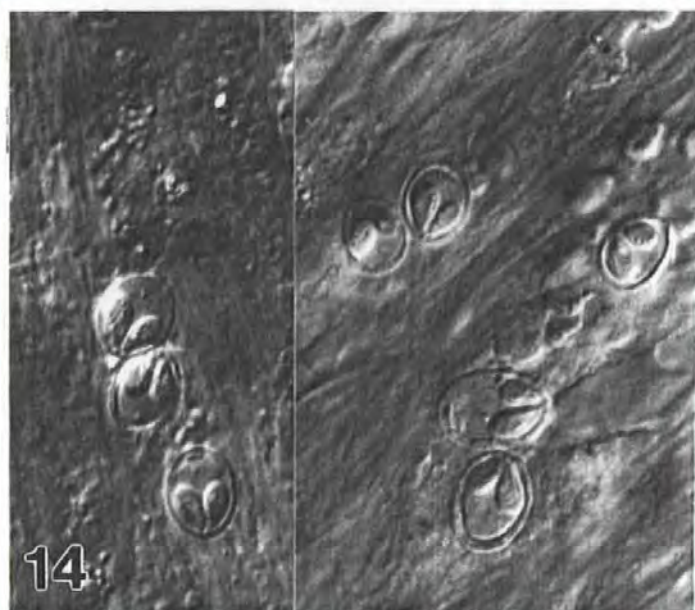
sampled in September 1986. Examination of cyst contents revealed the presence of numerous spores of *Myxidium rhodei* (Fig. 17). Spores measured 11.9 (11.0-12.5) µm in length and 4.9 (4.7-5.1) µm in width (n=10).

Pseudoplasmodia of a *Sphaerospora* sp. were observed in histological sections of renal tubules. Mature spores were absent. Cysts of *Myxobolus* sp. were frequently observed in various other tissues (Fig. 18).

(h)   *Salmo trutta* L, Brown trout.

Relatively few fish became available for examination during this study, see Table 3. PKX cells, associated with clinical PKD were observed in the kidneys of two fish sampled from the R. Avon in July 1989 (Fig. 19 & 20). These fish also harboured pseudoplasmodia and developing spores of a *Sphaerospora* sp. in the renal tubules (Figs. 21 to 24). From kidney impression smears the basic structure of the tubule lumen forms could be discerned (Figs. 25 & 26). Pseudoplasmodia appeared to be monosporous and characterised by the presence of numerous cytoplasmic vacuoles. Capsulogenic, valvogenic and sporoplasm cells could be seen. The capsulogenic cells frequently contained small densely staining capsular primordia (Figs. 25 & 26). The ultrastructure of the sporoplasm cells revealed the presence of numerous electron-dense sporoplasmosomes (Fig. 27) which lacked the characteristic 'bar' of PKX haplosporosomes. The ultrastructure of capsulogenic cells confirmed the presence of the external tube associated with the capsular primordium seen at the light microscope level in kidney impression smears (Fig. 28).

Spores of a *Chloromyxum* sp. were observed in the renal tubules of 3 fish sampled from Haweswater (Cumbria) (Fig. 29).

(i)   *Thymallus thymallus* L, Grayling.

PKD was diagnosed in grayling from the rivers Avon, Dove, Itchen and Test (see Table 5). Typical PKD lesions were seen in kidney sections of these fish, with numerous PKX cells present in the renal interstitium in most cases (Figs. 30-32). Ultrastructural examination of grayling PKX cells revealed them to be morphologically identical with PKX cells from rainbow trout, with numerous characteristic 'haplosporosomes' present in the primary cell (Figs. 33-34). A description of the

Figure 16. *Myxobolus* sp. spores from the renal interstitium of *Phoxinus phoxinus.* Normarski interference contrast, x837.

Figure 17. Spores of *Myxidium rhodei* from the kidney of *Rutilus rutilus.* Normarski interference contrast, x1,275.
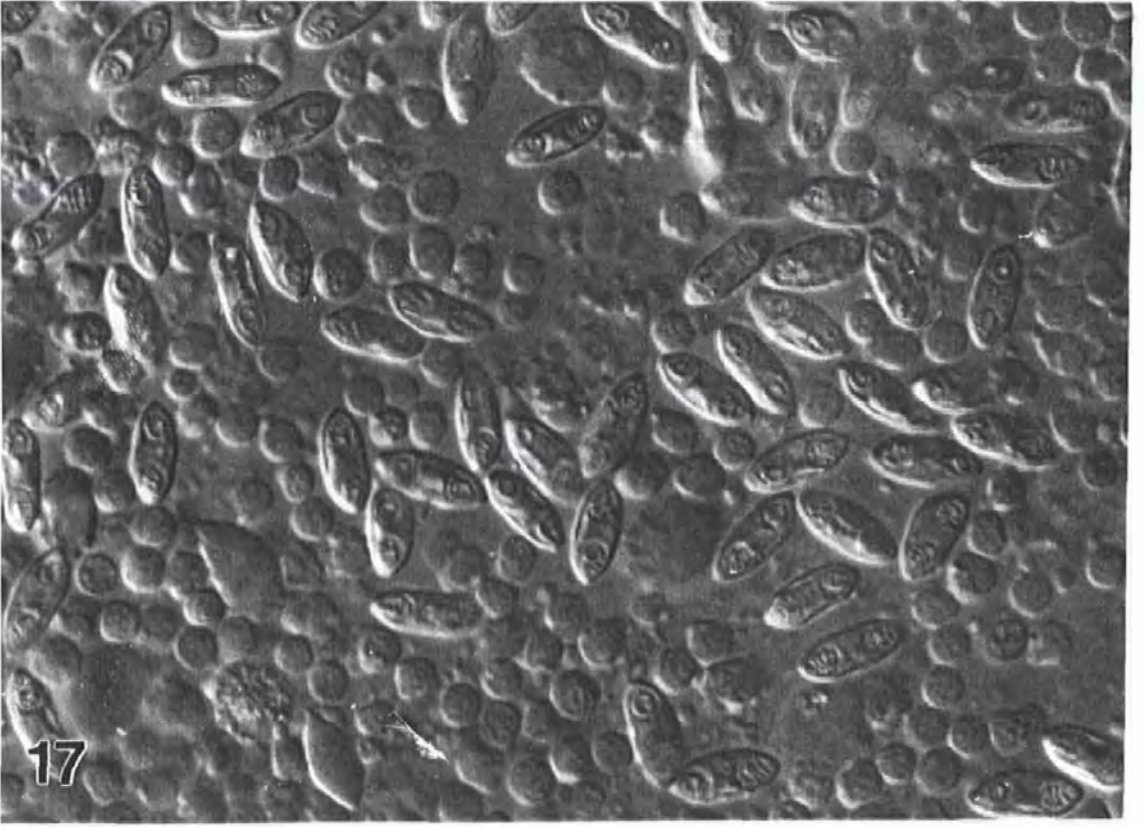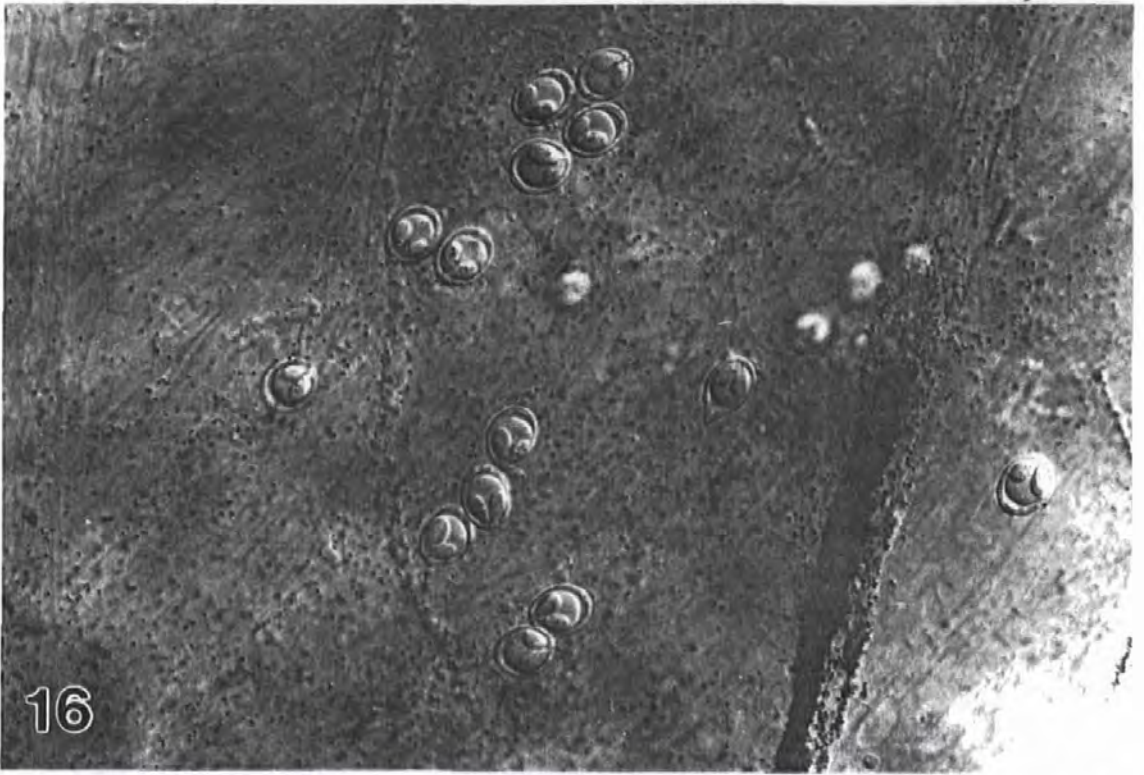
Figure 18. Accumulation of *Myxobolus* sp. spores located at the base of the secondary gill lamellae in *Rutilus rutilus* (arrowed). H & E, x83.

Figure 19. Histological section of kidney from *Salmo trutta* clinically infected with PKD. Several PKX cells are present (arrowed) associated with the proliferation of interstitial haemopoietic tissue. H & E, x330.
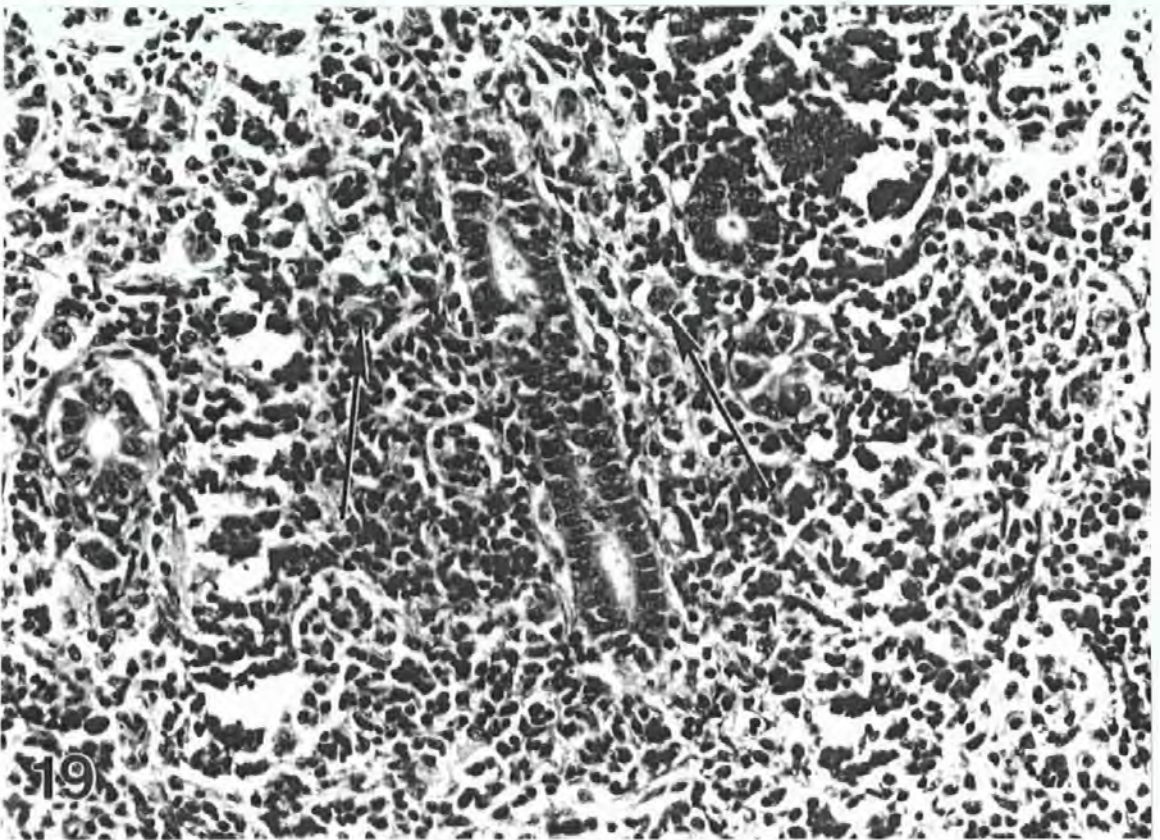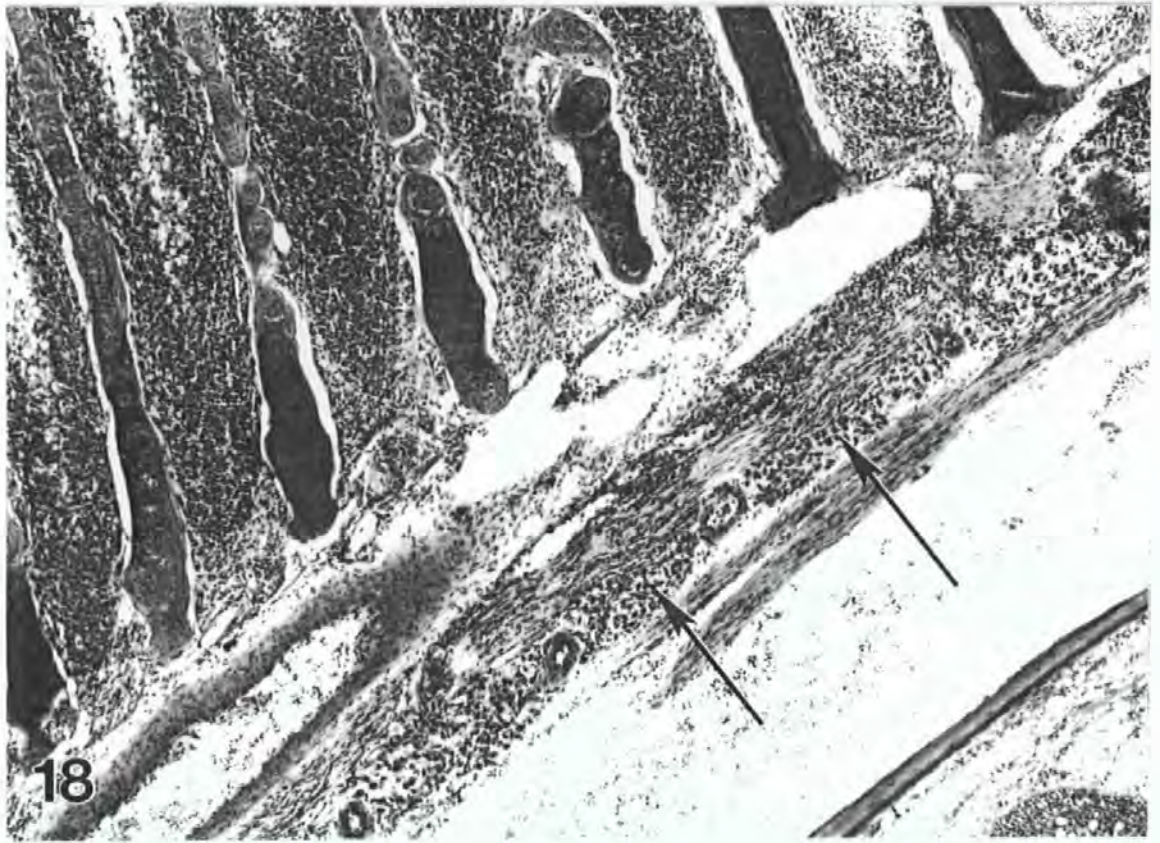
Figure 20.   A PKX cell from fresh unfixed kidney from *Salmo trutta* clinically affected by PKD. Note the presence of refractile granules in the primary cell cytoplasm (arrowed) and larger structures possibly corresponding to secondary cells. Normarski interference contrast, x950.

Figure 21.   A PKX cell (arrowed) in *Salmo trutta* migrating through the renal tubule epithelium (E) to the lumen (L). Normarski interference contrast, x950.

Figure 22.   *Sphaerospora* sp. pseudoplasmodia in the renal tubule lumen from *Salmo trutta*. Normarski interference contrast, x950.
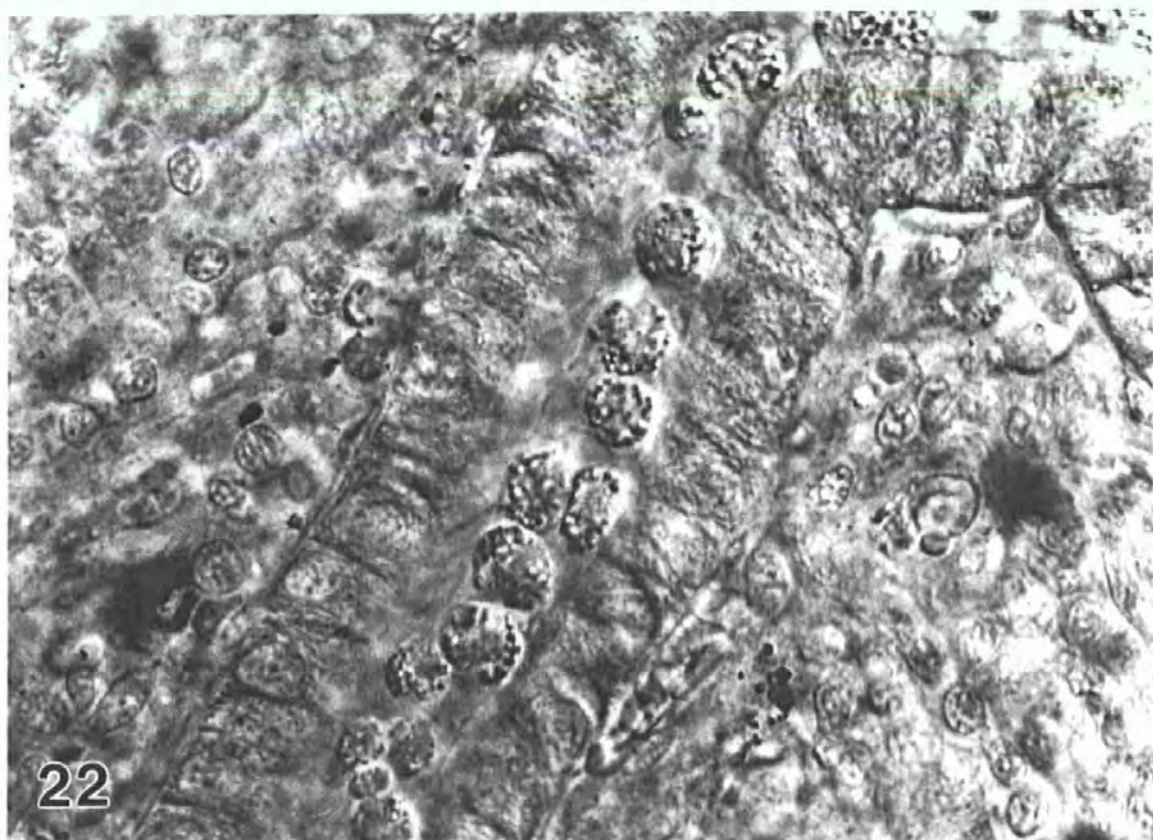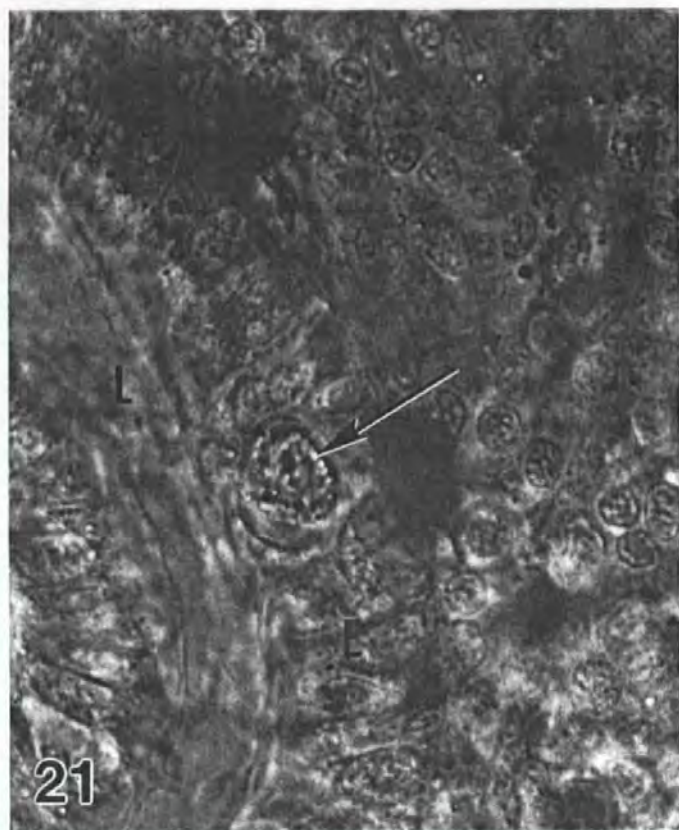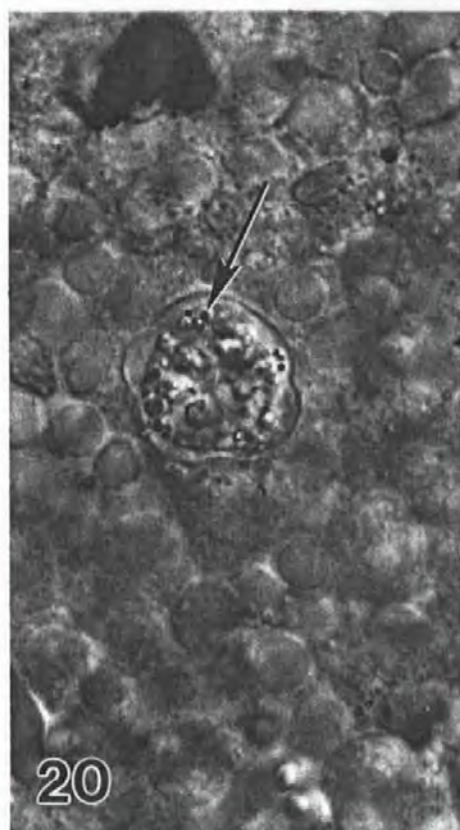
Figure 23.   Luminal stages of a *Sphaerospora* sp. in *Salmo trutta* kidney (see Fig. 22).   In this case sporogenesis has begun and developing polar capsules can be seen (arrow).   Normarski interference contrast, x980.

Figure 24.   Histological section showing developing spores of a *Sphaerospora* sp. in a renal tubule from *Salmo trutta* (arrowed). Giemsa, x620.

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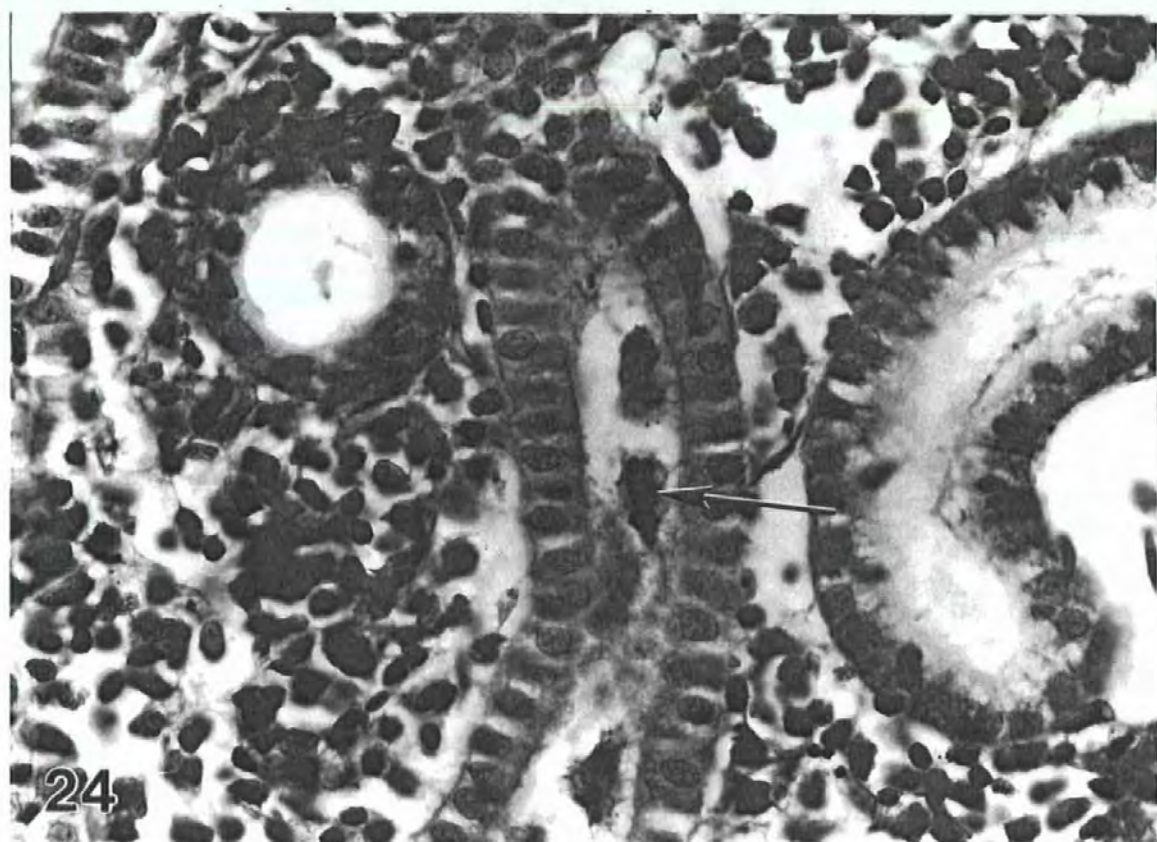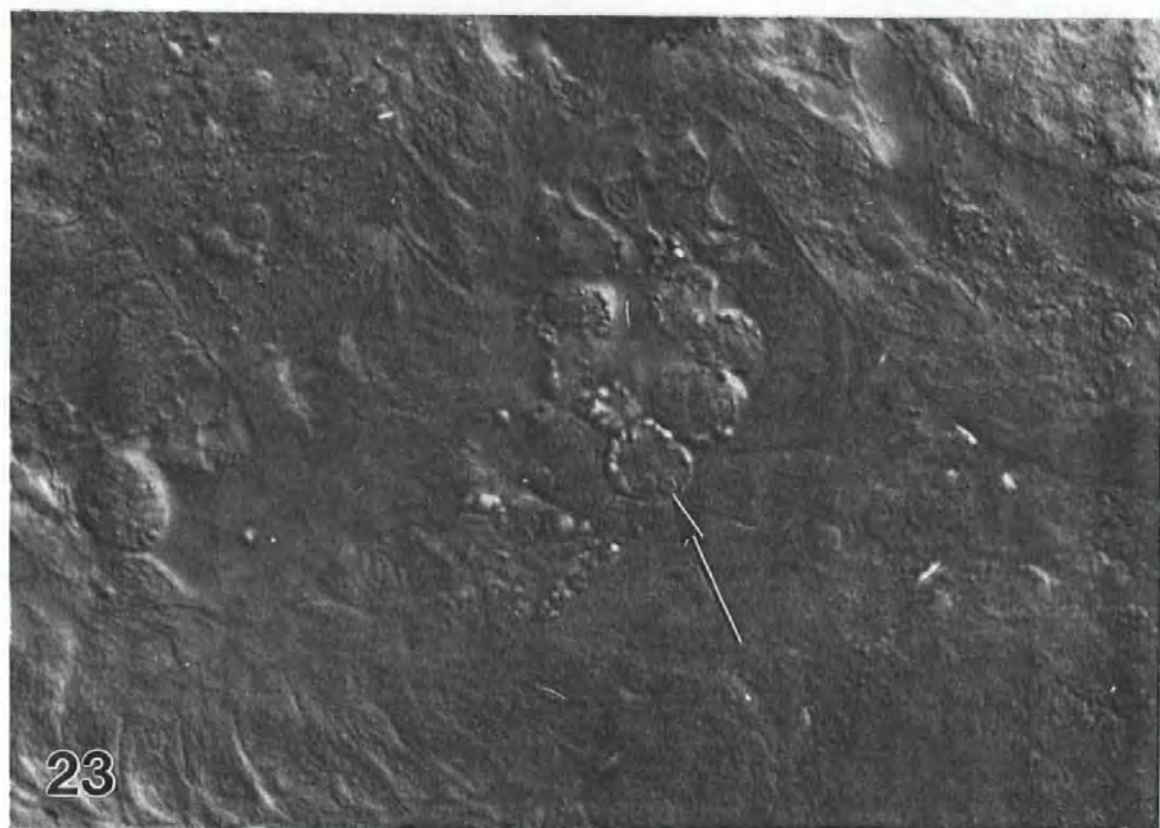
Figure 25.   A kidney impression smear from *Salmo trutta* recovering from PKD. The pseudoplasmodium is monosporous and characterised by the presence of numerous granules (appearing as vacuoles in this preparation). Developing polar capsules can be seen (arrowed). May-Grünwald Giemsa, x825.

Figure 26.   As Fig. 25. In this case, division of one of the sporoblast cells can be seen (arrow). Note that maturation of the polar capsules appears to be asynchronous. May-Grünwald Giemsa, x825.

Figure 27.   Electron micrograph of the sporoplasm cell from a developing spore from *Salmo trutta* kidney. Note the sporoplasmosomes (arrows) lack the characteristic 'bar' seen in PKX haplosporosomes. x10,500.
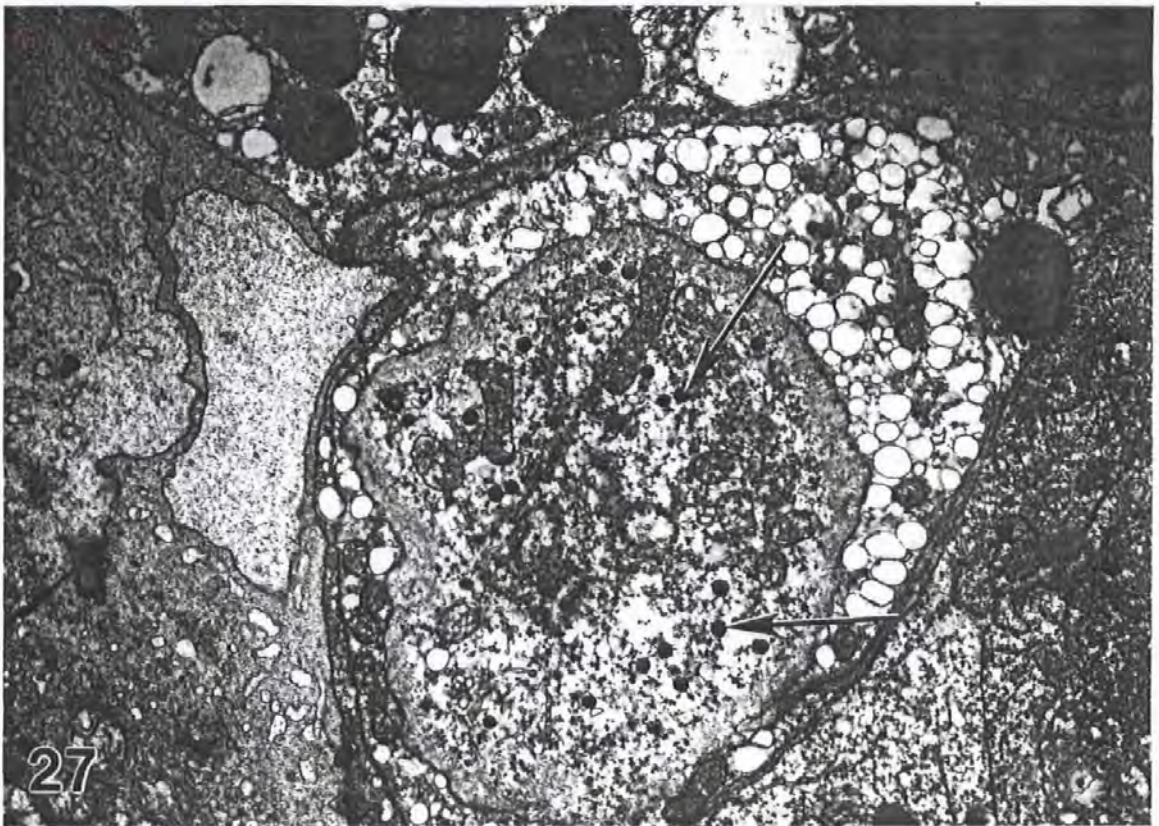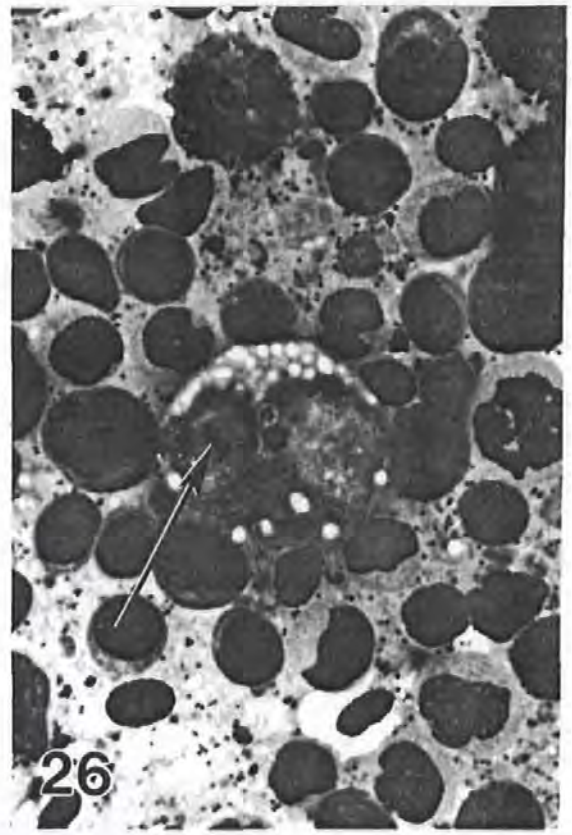
Figure 28. Electron micrograph of the capsulogenic cell from a developing spore from *Salmo trutta* kidney. The external tube (arrows), which will become the coiled polar filament of the polar capsule can be seen. x18,250.

Figure 29. An unidentified *Chloromyxum* sp. in the renal tubule lumen of *Salmo trutta* from Haweswater. Normarski interference contrast, x1,292.

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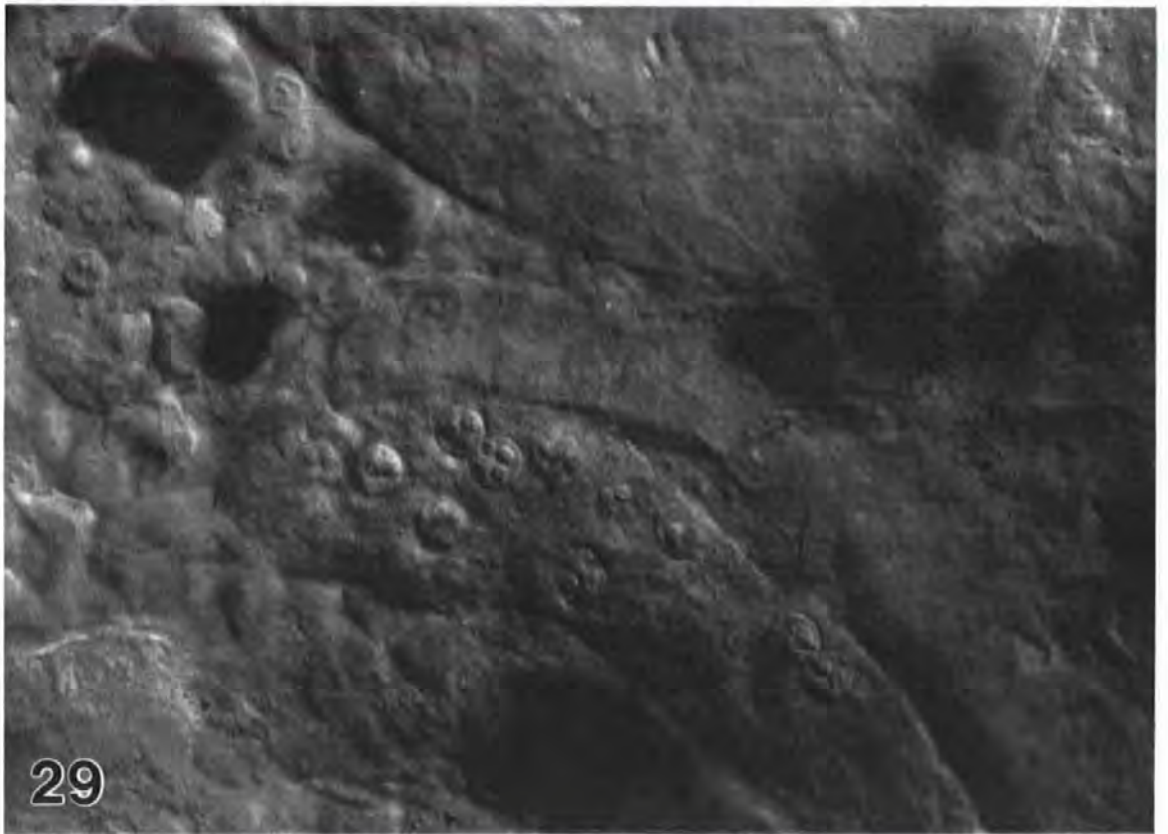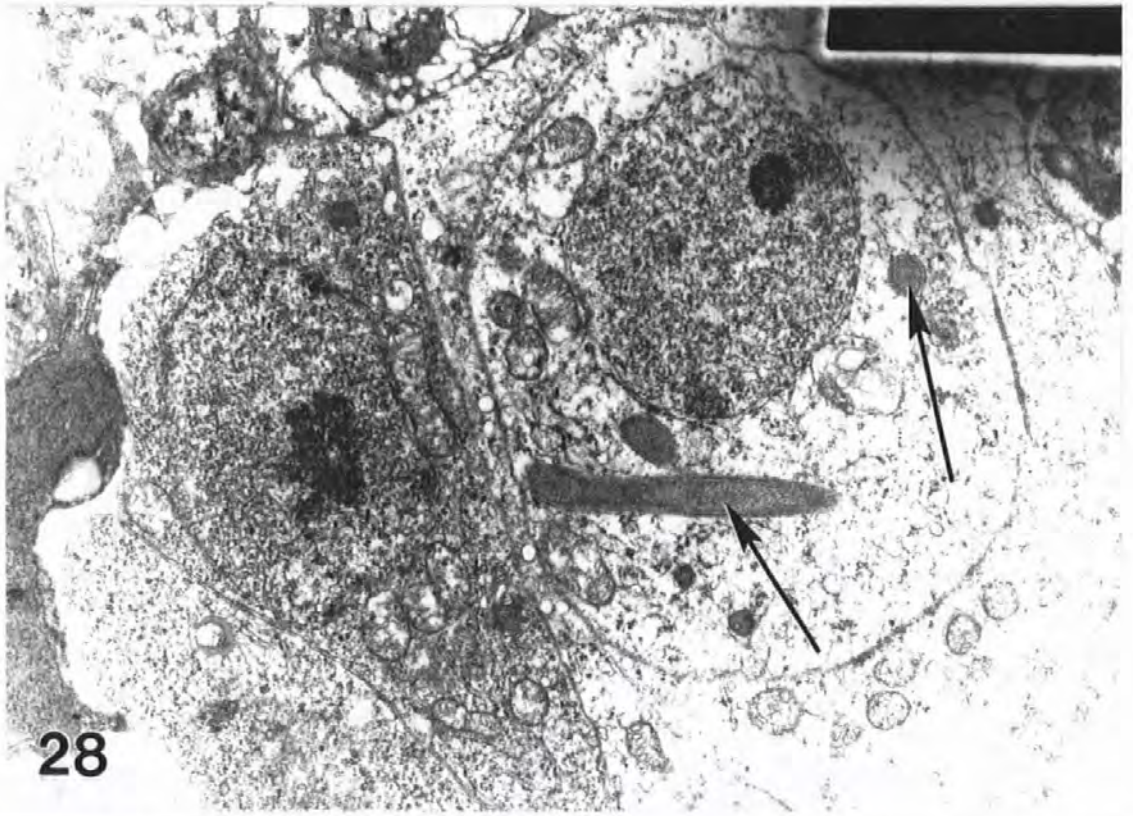
Figure 30. Focal PKD lesion in *Thymallus thymallus* kidney. H & E, x32.

Figure 31. Periphery of lesion shown in Fig. 29. Note the proliferation of haemopoietic tissue and lack of tubular elements in the affected tissue.
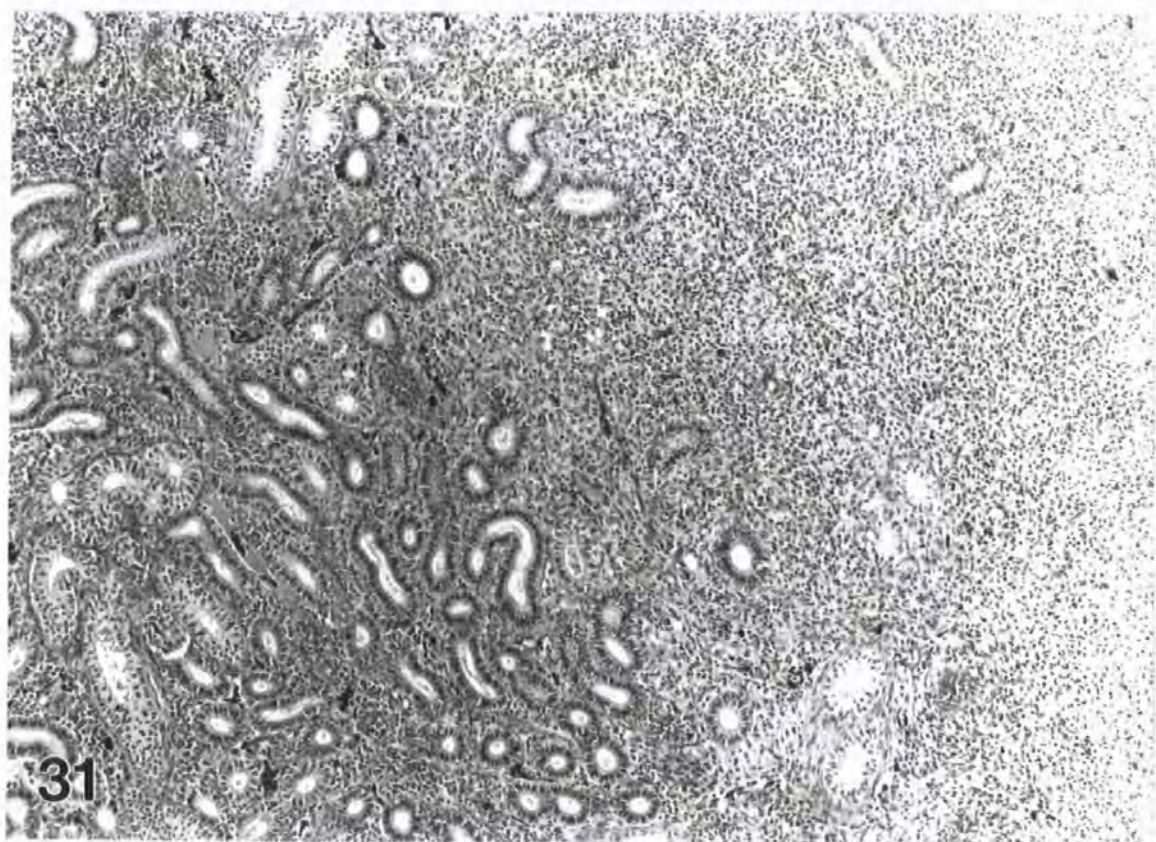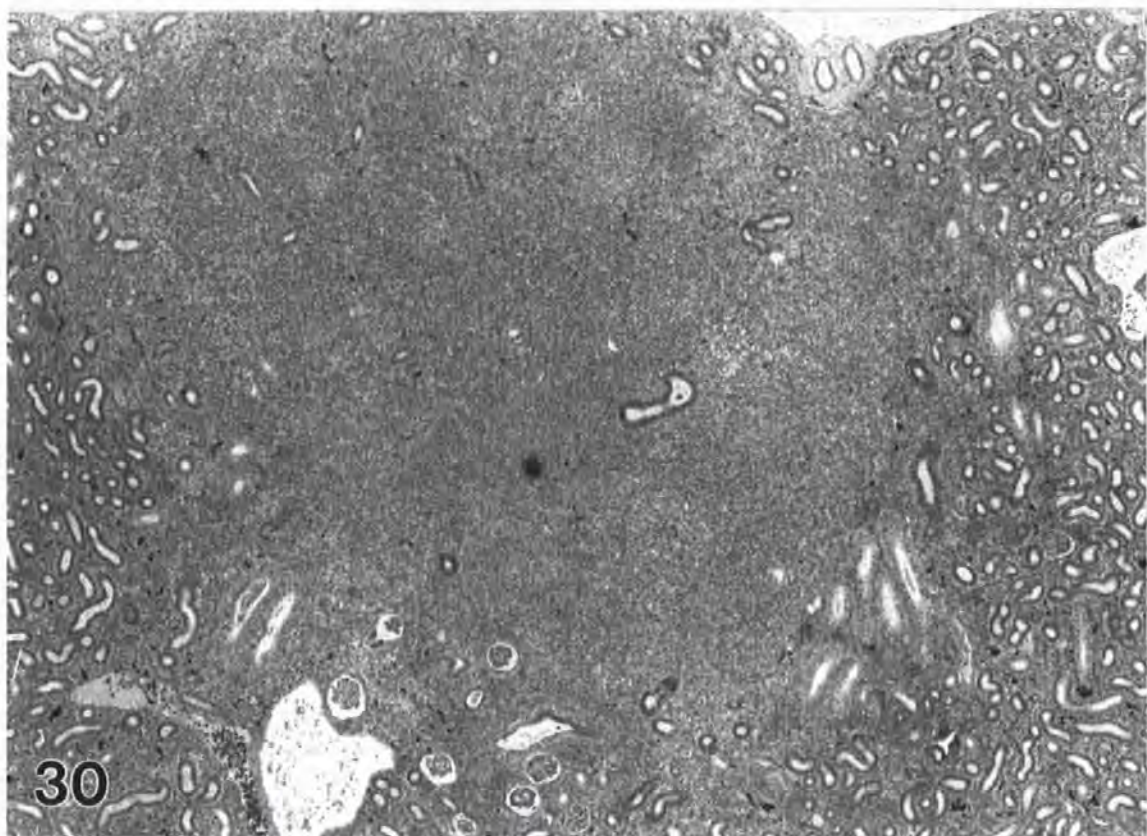
Figure 32. Proliferation of haematopoietic tissue in PKD affected *Thymallus thymallus* in response to the presence of numerous PKX cells (arrowed). H & E, x 330.

Figure 33. PKX cell from *Thymallus thymallus* kidney. Numerous haplosporosomes are present (arrowed), and a secondary (S) and tertiary (T) cell can be seen. The primary cell nucleus is absent in this section, x 10,500.

32

33
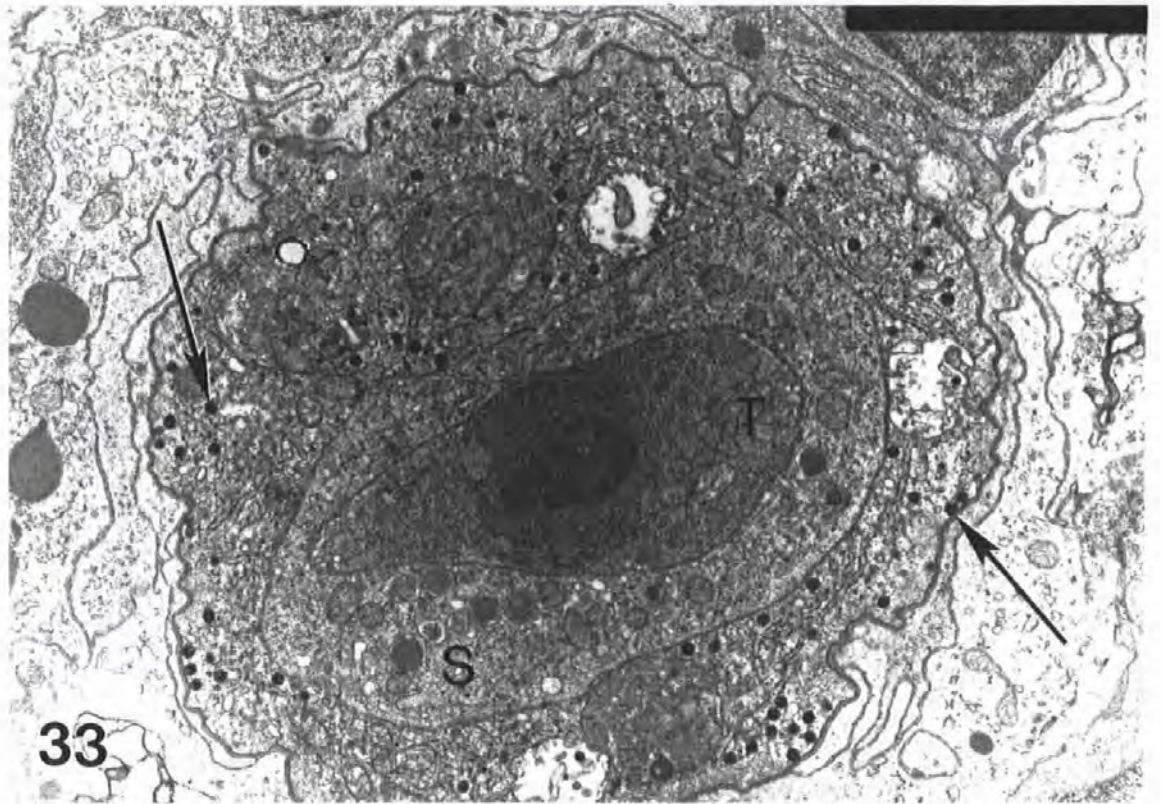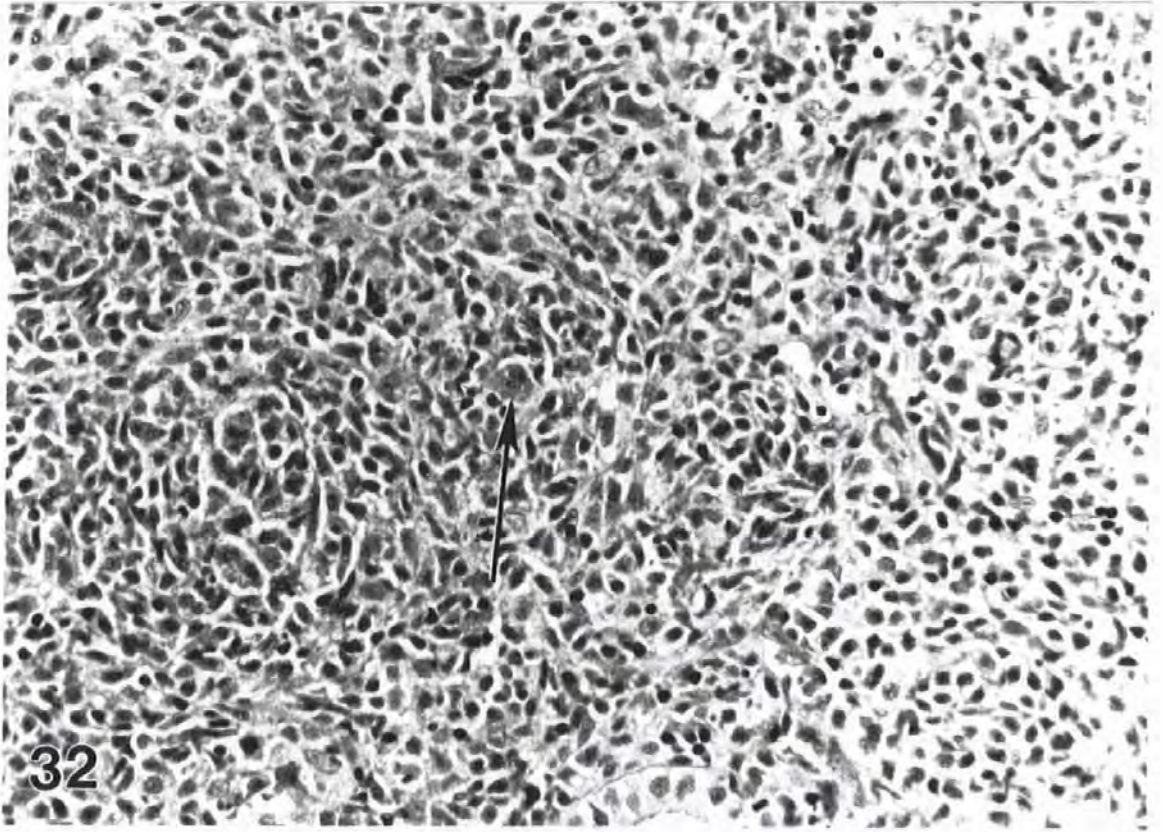
pathogenesis of naturally occuring and experimentally induced PKD in grayling is given in Chapter 6.

Pseudoplasmodia of a *Sphaerospora* sp. were found in the renal tubules of several fish, (see Table 5), and were characterised by the presence of numerous refractile granules (Fig. 35). These granules were found to be accumulations of electron-dense material and lipoid inclusions in the primary or 'mother cell' of the pseudoplasmodia (Figs. 36 & 37). Secondary or generative cells were characterised by the presence of numerous mitochondria in the cell cytoplasm. Pseudoplasmodia were attached to the tubule epithelium by cellular extensions or pseudopodia which in some cases appeared to penetrate between the epithelial cells (Fig. 37). The host response to these parasites was minimal. However, apical swelling of the epithelial cells was evident in most cases. Sporogenesis was not observed in fresh preparations, but developing spores were found in Giemsa stained impression smears from affected kidneys (Fig. 38).

3.4   DISCUSSION

Renal myxosporidiosis was recorded in all 18 species examined with the exception of *Anguilla anguilla* and *Cottus gobio*. Infection with the genus *Sphaerospora* Thélohan, 1892 was encountered most frequently, in a total of 10 species. However, infections usually lacked the presence of mature spores and only *S. elegans* in sticklebacks was positively identified. Lom *et al.*, (1985) in a survey of freshwater fishes from the Czech Republic diagnosed sphaerosporosis in 9/33 of the species examined. Mature spores were only recorded from *G. gobio*, *R. rutilus*, *Tinca tinca*, *Gymnocephalus cernua* and *Perca fluviatilis*. In addition, Lom *et al.*, (1985) reported the presence of motile myxosporean stages in the blood of *G. gobio*, *R. rutilus* and *T. tinca* which were thought to represent extrasporogonic phases of the life cycle of the *Sphaerospora* species infecting each host. Similar stages have been reported from grayling (Jurajda, 1989) and several other cyprinid fish (Baska & Molnár, 1988). Grupcheva *et al.*, (1985) demonstrated marked seasonal fluctuations in the prevalence of myxosporean bloodstream stages associated with *Sphaerospora renicola* infections in

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Table 5.   Seasonal prevalence of PKD and renal Myxosporean stages in wild grayling, *Thymallus thymallus*.

Month of sampling	Source	Number of fish examined	Incidence*			
			PKD	PKX	PS	IS
January	R. Avon	5	-	-	-	-
"	R. Test	51	-	-	-	-
February	R. Avon	16	-	-	1	-
March	"	10	-	-	-	-
"	R. Test	1	-	-	1	-
April	R. Nadder	5	-	-	2	-
"	R. Test	8	-	-	4	-
April/May	R. Avon	40	-	-	24	2
June	R. Nadder	23	-	-	15	8
July	R. Avon	1	-	-	1	-
August	NO SAMPLES OBTAINED					
September	R. Avon	5	-	-	-	-
October	"	46	11	-	30	-
"	R. Itchen	17	15	5	12	5
"	R. Test	32	2	2	1	-
November	R. Avon	10	-	-	-	1
"	R. Dove	1	-	-	-	1
December	NO SAMPLES OBTAINED					

*PKD - Proliferative kidney disease

 PKX -   "   "   "   organism 'X'

PS - Pseudoplasmodia

IS - Immature spores

Figure 34.   PKX cell from PKD affected *Thymallus thymallus* kidney. Note the close association of the two secondary cells (arrowed), x6,800.

Figure 35.   *Sphaerospora* sp. pseudoplasmodia in the renal tubule lumen of *Thymallus thymallus* . Normarski interference contrast, x830.
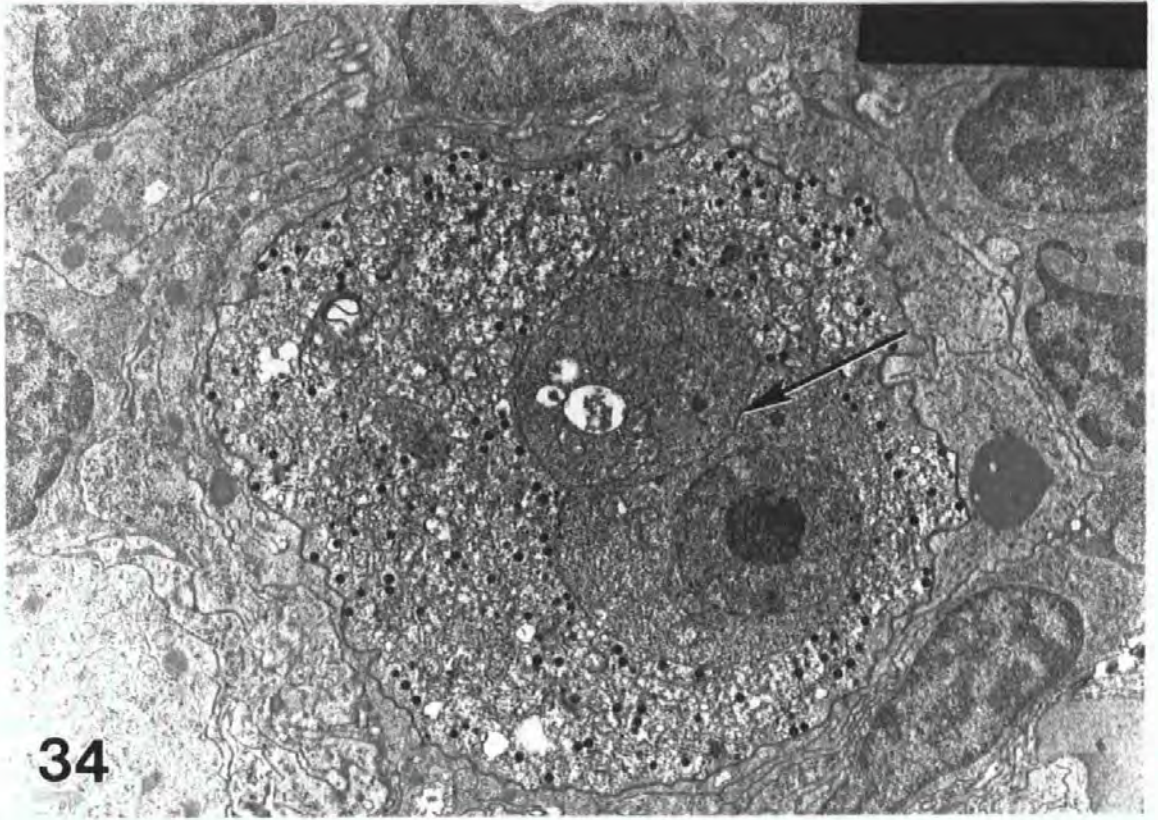
34

35

Figure 36. Electron micrograph of a luminal pseudoplasmodium as shown in Fig.35. Within the pseudoplasmodium a generative cell (G) and accumulations of electron-dense material (arrowed) can be clearly seen. x10,700.
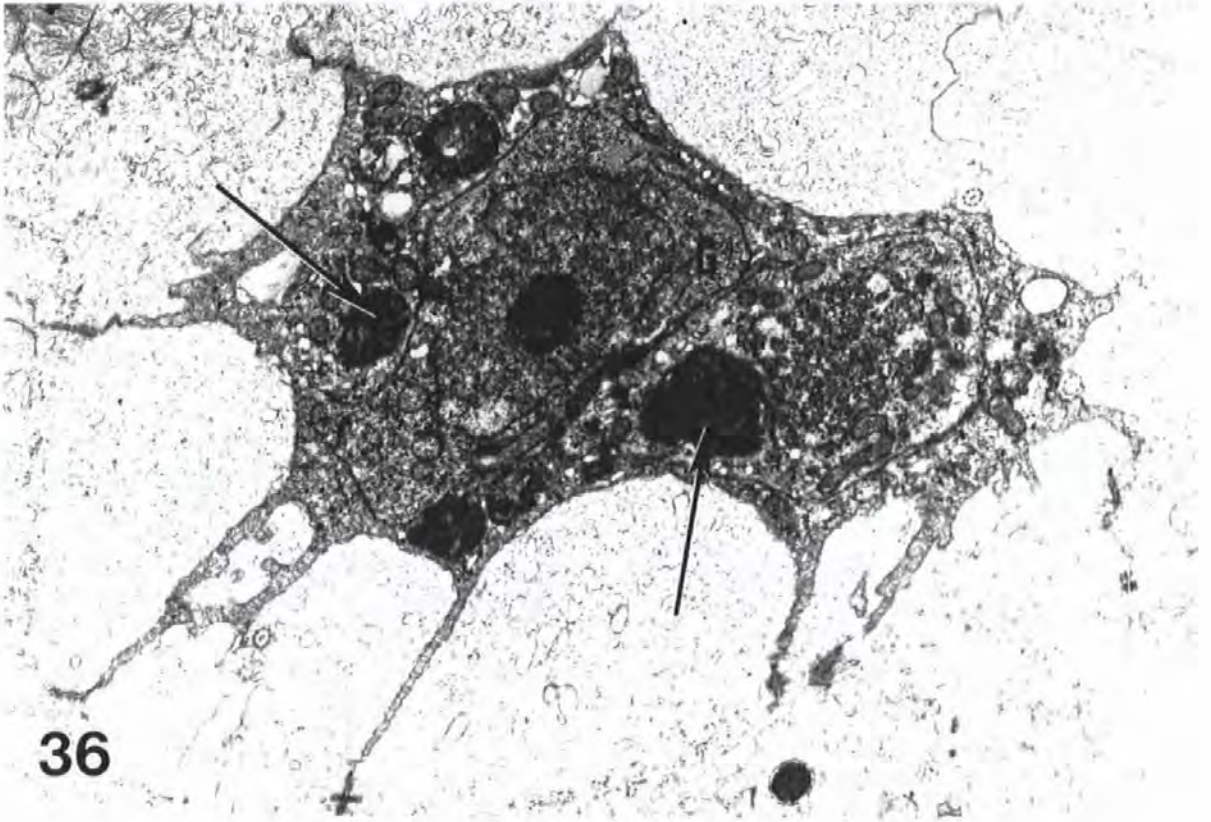
36

Figure 37. As Fig.35. In this case the pseudopodia (arrowed) of the pseudoplasmodium can be seen penetrating between the epithelial cells of the renal tubule. These cells show apical swelling (*), possibly in response to the parasite. x13,500.
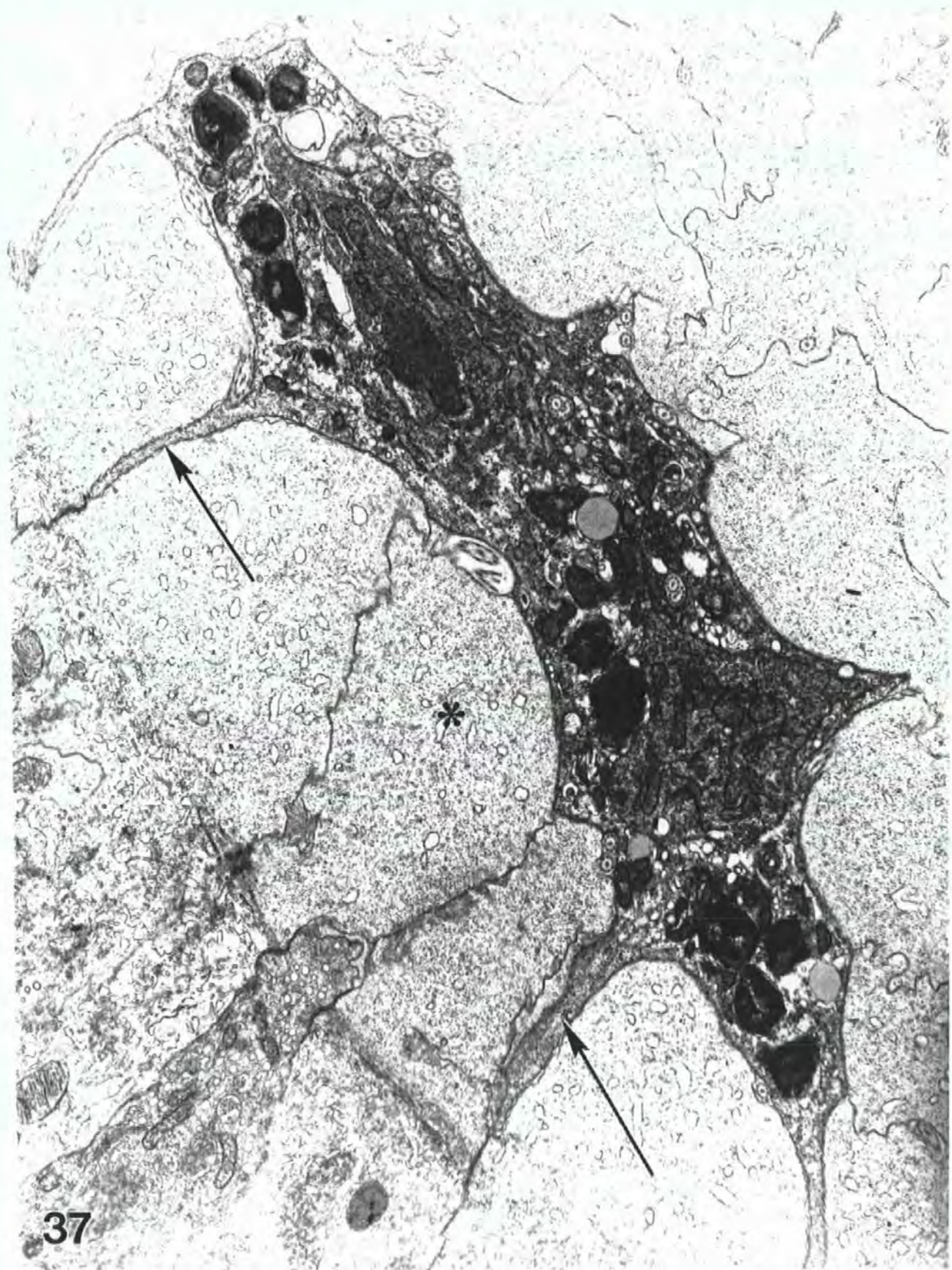
37

Figure 38.   A kidney impression smear from a wild grayling without clinical
              signs of PKD.   A developing spore, still contained within the
              pseudoplasmodium (arrow), is characterised by the presence of two
              spherical polar capsules (double arrow).   May-Grünwald Giemsa,
              x825.
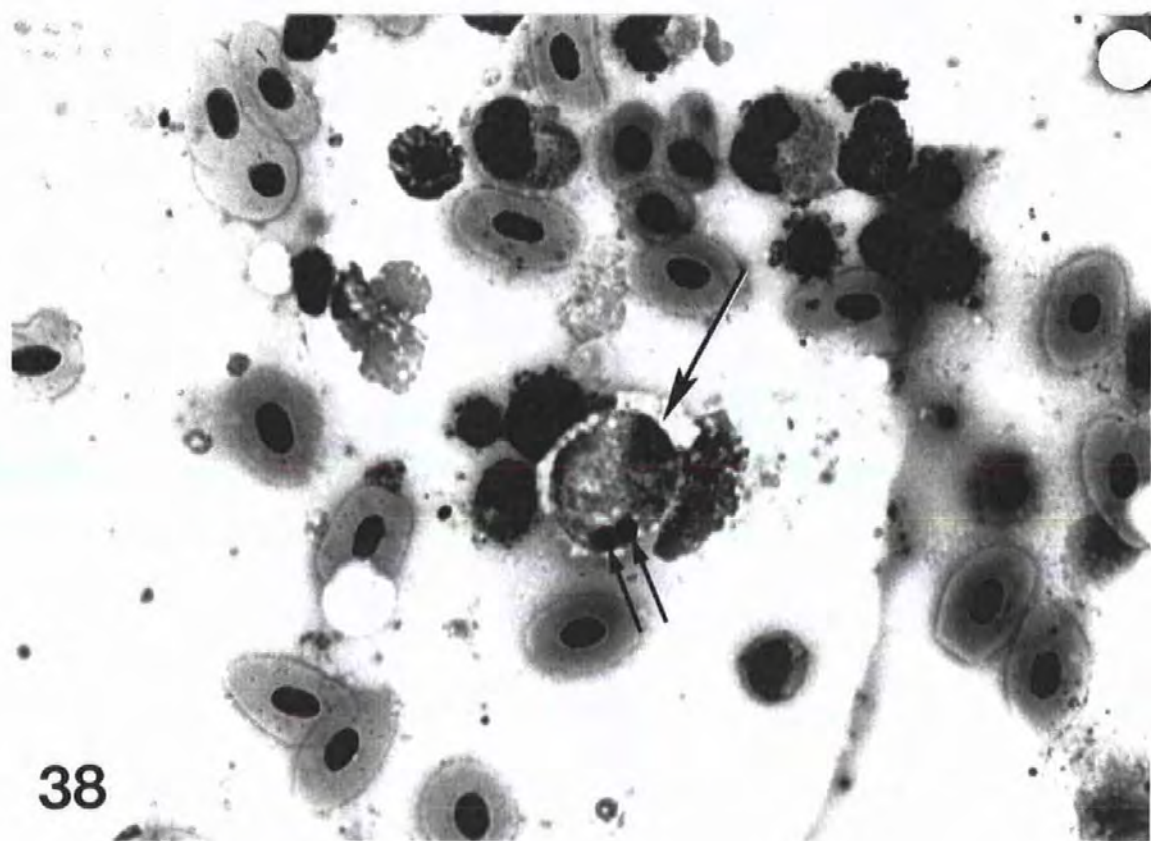
38

carp. Peak prevalence rates of up to 60% occurred during the autumn and spring months with the infection almost undetectable during the winter months. In contrast, Baska & Molnár (1988) and Jurajda (1989) detected bloodstream stages in fish sampled between the months of April and July. It is uncertain why these forms were not found during the present study since grayling were sampled throughout the year. However, in mild infections only one parasite per several hundred blood cells may be present (Jurajda, 1989). Since typically only one blood smear was taken from each fish it is possible that these infections were overlooked. The parasite enrichment technique described by Sovenyi & Molnár (1990) was published after the present investigation was completed; however its use in future studies is recommended.

Six species of myxosporean parasite were recorded here for the first time in the U.K. These were, *Z. nova* and *M. cotti* in *C. gobio*, *Chloromyxum* sp. in *S. trutta* kidney, *Sphaerospora* sp. and *H. cyprini* in *C. carpio* and *Hoferellus* sp. in *L. leuciscus*. Lom (1986) listed the known species of *Hoferellus*, which occur in crucian carp *Carassius carassius* and goldfish *C. auratus*. The presence of *Hoferellus* sp. in *L. leuciscus* may represent a new host record. However, this can only be confirmed if mature spores typical of the genus are found in affected fish. These were not located in fish examined during this investigation. Spores of *Sphaerospora renicola* have subsequently been identified from fingerling carp kidneys taken from fish from the same source as those of the present study (Feist, unpublished). Intracellular epithelial stages identified above as *H. cyprini* were also detected. These are now considered to represent an extrasporogonic phase of *S. renicola* (Lom & Dyková, 1992b).

The presence of *Chloromyxum* sp. in *S. trutta* kidney tubules has previously been reported (Lom *et al.*, 1985). Recently similar infections have been discovered in the renal tubules of Atlantic salmon *S. salar* in Scotland (Dr Baska, Hungarian Academy of Sciences, pers.comm. 1991) and in brown trout from Germany (Sedlaczek, 1991) and may involve the same species of parasite. In these cases the material was insufficient for specific diagnosis. Other *Chloromyxum* sp. have been reported from *S. trutta*. *C. truttae* Léger, 1906 is known to infect the gallbladders of *S. trutta*. *C. wardi* Kudo, 1919, *C. coregoni* Bauer, 1948 and *C. orientalis* Shulman, 1962 also infect the

gallbladders of various salmonids. Insufficient material was available for adequate comparison with the above species. However, these species have not been recorded from the kidney and appear to be unrelated to the renal *Chloromyxum* sp. described by Sedlaczek (1991).

Of the six *Henneguya* sp. known to infect *E. lucius*, three species infect the gills. *H. zschokkei* Gurley, 1894 is known to infect numerous hosts, but is primarily a parasite of salmonid fish. Spores are 10-14 μm in length with extremely long caudal processes adding a further 26-40 μm to the overall length of the spore. *H. lobosa* Cohn, 1895 has characteriatically narrow spores (width 4-8 μm) and caudal appendages up to 20 μm in length. *H. psorospermica* Thélohan, 1895 has spores of the same length range as *H. zschokkei*, but the caudal processes are shorter at 14-30 μm in length. Consequently, the *Henneguya* sp. found during this study was identified as *H. psorospermica*.

Two species of *Myxobilatus* are known to infect *G. aculeatus*. Spores of *M. gasterostei* Parisi, 1912 (Davis, 1944) are significantly larger (length 33-48 μm) than spores of *M. medius* Thélohan, 1892 (length 24-30 μm). Thus, the species reported during the present study was identified as *M. gasterostei*.

Renal sphaerosporosis in *P. phoxinus* does not appear to have been reported previously, although Bauer, (1984) lists *P. phoxinus* as a host of *S.elegans*. It was not possible from the limited material obtained during the present investigation to confirm the link with *S. elegans*. Nine *Myxobolus* sp. are known to infect *P. phoxinus* (Bauer, 1984). Only *M. muelleri* matches the dimensions and site of infection of the *Myxobolus* sp. observed in *P. phoxinus*. Spores of *M. musculi* Keysselitz, 1908 are of similar shape but its overall width (8-11μm) is greater. Consequently, the spores described in the present study were assigned to *M. muelleri*.

Campbell (1974) reported a *Henneguya* sp. from the stickleback *Gasterosteus aculeatus*. This finding was not confirmed during the present study. Since details of the parasite morphology and location in the host were not given, it is possible that the parasite recorded was in fact *Myxobilatus gasterostei* which has a very similar morphology and was frequently found in renal tubules of *G. aculeatus* during the present investigation.

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PKD was identified in four species, namely, rainbow trout, brown trout, grayling and pike, confirming previous findings (Seagrave *et al.*, 1981; Bucke, Feist & Clifton-Hadley, 1991). In each of these hosts the presence of the PKX parasite was confirmed by light and electron microscopy. The ultrastructure of PKX in infected pike tissues was examined for the first time and its general morphology was found to be identical to the PKX cell occuring in salmonids. The pike remains the only non-salmonid host known to be susceptible to PKD. Outbreaks of PKD in arctic charr and Atlantic salmon have been reported previously (Seagrave *et al.*, 1981; Bucke *et al.*, 1991) and were not sampled from waters harbouring the infectious stage of the PKX parasite during the present study. Several cyprinid species, as well as pike and grayling, harboured small myxosporean plasmodia in the lumens of renal tubuli. These were often indistingiushable, at the light microscope level, from PKX cells. In pike the luminal forms were identified by electron microscopy as stages of *Myxidium lieberkuehni*. In other hosts the presence of developing spores often allowed tentative identification of the parasites involved. Nevertheless, in many cases spores were absent and specific identification was not possible. Recently, the development of lectin-based techniques and monoclonal antibodies has allowed the accurate identification of PKX cells, even when present in very low numbers (Adams, Richards & Marin de Mateo, 1992; Marin de Mateo, Adams, Richards, Castagnaro & Hedrick, 1993). The use of these techniques is recommended to confirm infections with PKX.

In addition to the published data and the findings presented in this chapter, the Registry of Aquatic Pathology (RAP) held at the Weymouth Fish Diseases Laboratory (FDL) has a number of accessions involving myxosporean infections of freshwater fish. These were re-evaluated and confirmed as part of the present study and are shown in Table 6. A catalogue of the RAP accessions is currently being updated and prepared for publication.

Table 6. Accessions of Myxosporean infections in the Registry of Aquatic Pathology (RAP) held at the Fish Diseases Laboratory (MAFF) Weymouth.

RAP No.	Disease/ Parasite	Host	Origin	Depositor
1-A5	Proliferative kidney disease (PKD)	Rainbow trout *O. mykiss*	R.Test, UK (Hampshire)	D .Bucke
1-A19	PKD	Rainbow trout *O. mykiss*	R.Test, UK (Hampshire)	D. Bucke
1-A21	Whirling disease (WD) *M. cerebralis*	Rainbow trout *O. mykiss*	France	P. de-Kinkelin
1-A35	PKD	Rainbow trout *O. mykiss*	Farm site, Yorkshire, UK	E. Hudson
1-A36	PKD	Rainbow trout *O. mykiss*	Farm site, S England	D. Bucke
1-A43	WD, *M. cerebralis*	Rainbow trout *O. mykiss*	Farm site, SE England	D. Bucke
1-A64	PKD	Rainbow trout *O. mykiss*	Idaho, USA	C.E. Smith
1-A66	PKD plus mycosis	Rainbow trout *O. mykiss*	Fish diseases Lab. experimental site	D. Bucke
1-A69	PKD	Rainbow trout *O. mykiss*	Farm site, Isle of Man	S.W. Feist
1-A70	WD, *M. cerebralis*	Rainbow trout *O. mykiss*	Trout Hatchery	S.W. Feist
1-A76	PKD	Rainbow trout *O. mykiss*	Farm site, Wiltshire, UK	S.W. Feist
1-D6	PKD	Atlantic salmon *S. salar*	Scotland	T. O'Hara
1-E2	Milky flesh *Kudoa* sp.	Sea trout *S.trutta*	Reservoir, Wales	D.Bucke

Table 6. (continued)

RAP No.	Disease/ Parasite	Host	Origin	Depositor
1-G3	PKD	Grayling *T. thymallus*	Yorkshire, UK	E. Hudson
2-A8	*Sphaerospora* sp.	Common carp *C. carpio*	Lake in SW England	D. Bucke
2-A11	*Sphaerospora* sp.	Common carp *C. carpio*	Lake in SW England	D. Bucke
2-A13	Myxosporidiosis *Myxobolus* sp.	Common carp *C. carpio*	Imported stock	D. Bucke
2-B3	Myxosporidiosis	Grass carp *C. idella*	Wessex Water Authority	D. Bucke
2-C3	*Sphaerospora* sp.	Goldfish *C. auratus*	Imported stock	D. Bucke
2-C6	*Sphaerospora* sp. plus polycystic kidney	Goldfish *C. auratus*	Yorkshire Water Authority	D. Bucke
2-E1	*Mitraspora cyprini*	Koi carp *C. carpio*	Imported stock	D. Bucke
2-F16	*Myxidium* sp.	Roach *R. rutilus*	Pond in SW England	D. Bucke
2-H2	*Myxidium* sp.	Bream *A. brama*	Hampshire, UK	D. Bucke
2-I1	*Mitraspora cyprini*	Goldfish *C. auratus*	Pet fish dealer	D. Bucke
2-I3	*Sphaerospora* sp.	Goldfish *C. auratus*	Private owner	E. Hudson
2-K2	*Myxobolus* sp.	Chub *L. cephalus*	Yorkshire Water Authority	C. Andrews

Table 6. (continued)

RAP No.	Disease/ Parasite	Host	Origin	Depositor
3-A1	*Myxidium* sp.	Eel *A. anguilla*	Farm site, SW England	D. Bucke
3-A3	*Myxidium* sp.	Eel *A. anguilla*	Farm site, SW England	D. Bucke
3-A8	*Myxidium* sp.	Eel *A. anguilla*	Farm site, Yorkshire, UK	D. Bucke
3-A12	*Myxidium* sp.	Eel *A. anguilla*	Farm site, SW England	E. Hudson
3-B5	Myxosporidiosis	Perch *P. fluviatilis*	S England	R.A. Sweeting
3-D3	Myxopsoridiosis	Pike *E. lucius*	E Anglia, UK	D. Bucke
3-D5	*Henneguya* sp.	Pike *E. lucius*	River Test Hampshire	D. Bucke

CHAPTER 4

STRUCTURE AND DEVELOPMENT OF THE PKX CELL AND THE

CELLULAR RESPONSE TO PKD WITHIN FISH HOSTS

4.1 INTRODUCTION

The diagnosis of PKD has relied chiefly on direct observations of the PKX cell in histological sections (Ferguson & Needham, 1978; Ellis *et al.*, 1985; Kent & Hedrick, 1985a). The use of stained tissue impression smears for a more rapid diagnosis of PKD was proposed by Clifton-Hadley *et al.*, (1983). This method having the advantage of viewing whole PKX cells. Combined with the use of specific monoclonal antibody probes to PKX, these techniques now provide a sensitive diagnostic tool (Adams *et al.*, 1992). However, only limited information on the structural organisation of the parasite can be discerned at the light microscope level.

The ultrastructural anatomy of PKX was first described by Ferguson & Adair (1977) and subsequently in more detail by Ferguson & Needham, (1978); Seagrave *et al.*, (1980a & b); Smith *et al.*, (1984); Kent & Hedrick, (1985, 1986); Rafferty, (1986) and Feist & Bucke, (1987).

Cell types involved in the host response to the PKX parasite are those usually associated with a chronic inflammatory or granulomatous response and include macrophages, lymphocytes and plasma cells (Ferguson & Needham, 1978; Clifton-Hadley *et al.*, 1987; MacConnell *et al.*, 1989).

The major aims of this study were:

(a)   To confirm and supplement existing data on PKX morphology and development within the host.

(b)   To describe and identify the cell types involved in the host response to the PKX parasite.

4.2 MATERIALS AND METHODS

Rainbow trout fingerlings were obtained from a commercial fish farm on the river Avon (Wilts.) with an annual history of PKD outbreaks. Approximately 200 fish from stock exhibiting clinical signs of PKD were transported back live to the

laboratory. In addition kidneys and urinary bladders from 20 1yr+ rainbow trout that had experienced PKD the previous year were examined for the presence of myxosporean spores. Fish were held and maintained as described previously in Chapter 3. Tissue samples, including kidney and spleen, taken for histological and ultrastructural examination, were taken only from fish showing gross renal changes (Fig. 39). These were processed according to the procedures given in Chapter 3. In addition, ultrathin sections prepared for transmission EM were stained using the Thiéry method for carbohydrates (Thiéry, 1967). Terminology for the description of the various morphological forms of PKX was based on that used by Lom *et al.*, (1983). Identification of host cell types examined by electron microscopy was based on features of fish leucocytes described by Ferguson (1976); Ellis (1977); Breazile, Mass, Wollscheid & Zinn, (1982); Blaxhall (1983) and Fujimaki & Isoda (1990) and of human leucocytes (Ham & Cormack, 1979).

4.3   RESULTS

4.3.1   PKX structure and development

PKX cells were identified in histological sections of various tissues and organs including kidney, spleen, striated muscle, gills, heart, brain, liver and gonad. Although the PKX cell was not observed in renal tubule lumens, they were occasionally observed in the tubule epithelium. Neither myxosporean sporoblasts nor mature spores were observed in clinically infected rainbow trout or in 1yr+ fish that had fully recovered from PKD.

Several morphologically different stages of PKX were recorded from kidney impression smears stained with May-Grünwald Giemsa. However, a clear developmental sequence could not be established. Mononucleate cells with prominent cytoplasmic vacuoles (Fig. 40a) were the earliest stage of PKX observed in rainbow trout kidney. These were rarely observed in the tissue smears examined. Larger mononucleate primary cells, enclosing a single secondary cell, were more numerous (Fig. 40b). Secondary cells were produced by endogenous cleavage within the primary cell and also by binary fission of existing secondary cells (Figs. 40c, d & e). Nuclei

Figure 39. Dissection of a rainbow trout with clinical PKD showing gross renal swelling. Note the corrugated appearance of the renal capsule and the presence of white deposits typical of nephrocalcinosis (arrow).

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were frequently seen in mitosis (Figs. 40e & f and 41a & b). The largest forms of PKX observed in rainbow trout kidney contained several secondary cells with tertiary cells frequently present within these. Rarely, the primary cell appeared to be bi-nucleate (Fig. 40g).

Staining characteristics were variable when using May-Grünwald Giemsa. In most cases the primary cell cytoplasm stained light blue and the primary cell nucleus light pink. Secondary cell cytoplasm stained slightly darker blue than that of the primary cell and likewise the secondary cell nucleus stained a slightly darker pink (Fig. 41c). However, in several instances the secondary cells became fusiform and had similar staining characteristics to the tertiary cells the cytoplasm of which stained dark blue and the nucleus, which often occupied most of the tertiary cell, dark pink (Fig. 41c). In most cases PKX cells were surrounded by host cells (Fig. 41d), described later.

Dimensions of the PKX cell depended on the number of secondary cells present. Overall diameter varied from 7.0μm to 24.0μm. In stained impression smears the average dimension of the primary cell nucleus was 3.7μm; the secondary cell, 5.7μm; secondary cell nucleus 3.1μm; the tertiary cell 4.0μm and tertiary cell nucleus 2.5μm.

In H&E stained sections PKX cells were eosinophilic. The primary cell nucleus, often with a prominent endosome, and secondary cells were usually present. Frequently, one or more of the secondary cells appeared shrunken and surrounded by a clear halo (Figs. 42 & 43). The primary cell cytoplasm contained numerous PAS positive granules which were also argyrophilic (Fig. 42). Several examples of PKX cells exhibited constriction of the primary cell and appeared to be in the process of division by plasmotomy (Fig. 43).

The primary cell of PKX is characterised by the presence of numerous electron-dense cytoplasmic inclusion bodies or haplosporosomes (Figs. 44 & 45). These structures measured 0.15 μm to 0.2 μm in diameter and contained an electron-lucent bar. Haplosporosomes were distributed throughout the cytoplasm but were often observed in close association with the plasma membrane of the primary cell (Fig. 46). In several sections formation of haplosporosomes could be seen associated with the Golgi apparatus and rough endoplasmic reticulum (RER) (Fig. 47). When positioned at

Figures 40(a-g).   Diagrams of PKX morphology from May-Grünwald Giemsa stained kidney impression smears. N - Primary cell nucleus, S - Secondary cell, T - Tertiary cell. For full explanation see text. Bar = 10μm.

(a)
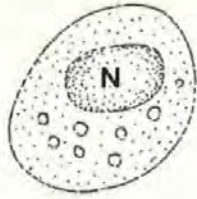
(b)
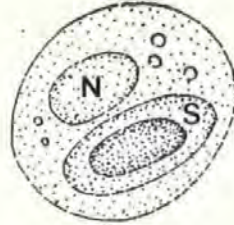
(c)
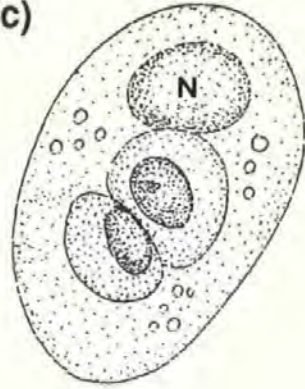
(d)
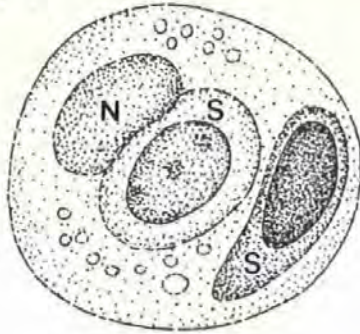
(e)
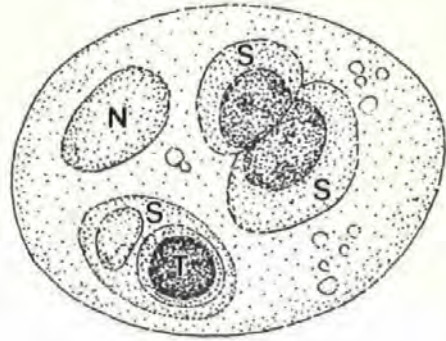
(f)
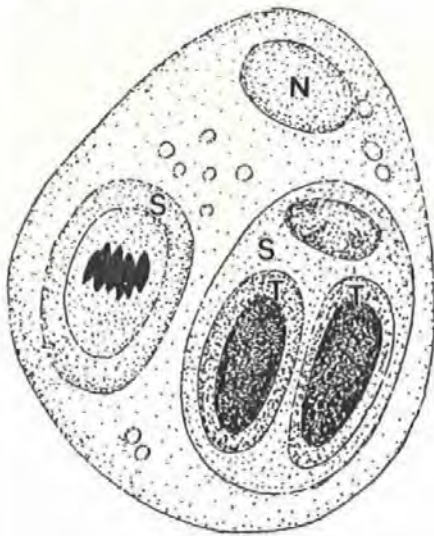
(g)
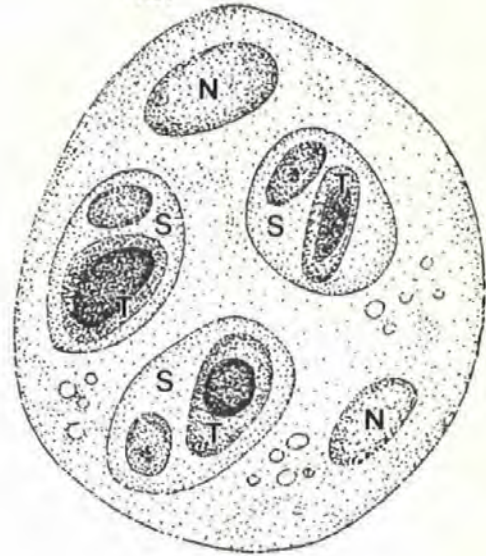
Figures 41(a & b). PKX cells from May-Grünwald Giemsa stained kidney impression smears showing typical staining characteristics. 41(a) PKX with the secondary cell nucleus at metaphase (arrow), note the typical vacuolation of the primary cell. 41(b) Shows a secondary cell at a later stage of nuclear division (arrow). May-Grünwald Giemsa, x970.

41a

41b
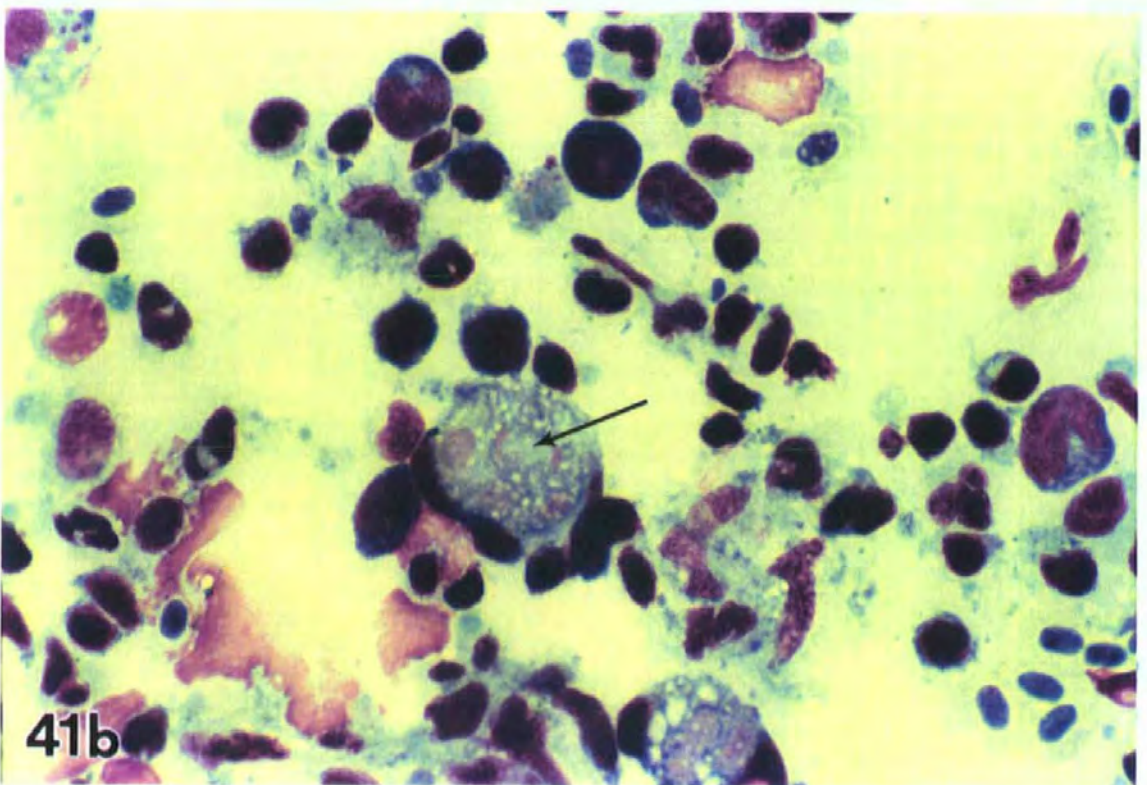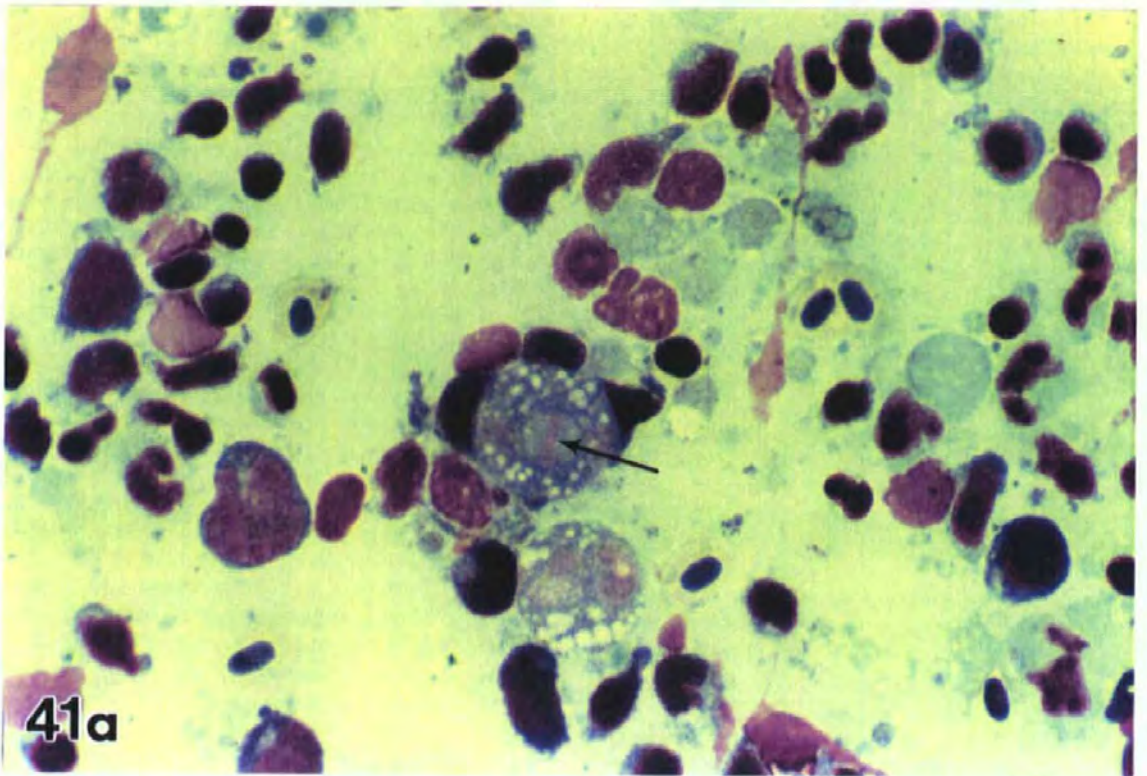
Figures 41(c & d).   41(c) PKX containing a fusiform secondary cell with dense dark blue cytoplasm (arrow) and another containing a small tertiary cell (T). 41(d) PKX with a prominent eosinophilic primary cell nucleus (N) and host phagocytes attached to the parasite (P) May-Grünwald Giemsa, x970.

41c

41d
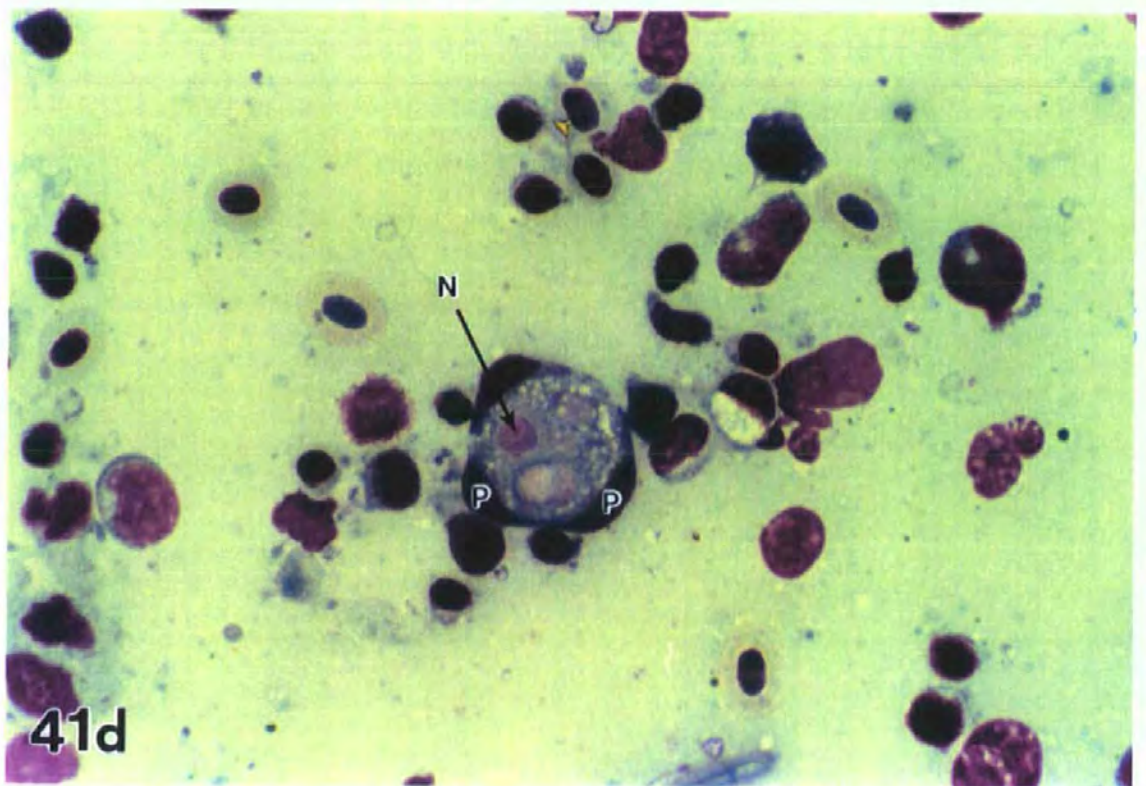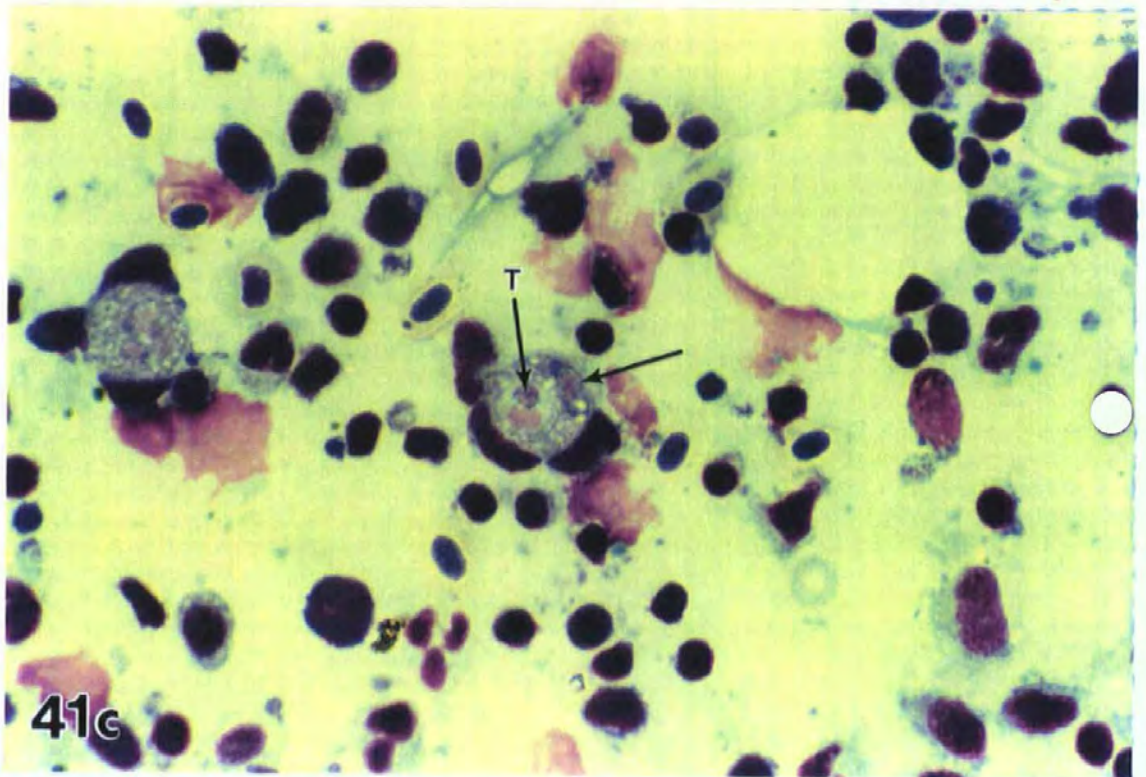
Figure 42.   Histological section showing the presence of argyrophilic granules in PKX (arrow). Note the presence of the shrunken secondary cell. Gordon and Sweet's reticulin stain, x980.

Figure 43.   PKX undergoing division by plasmotomy (arrow). H & E, x980.
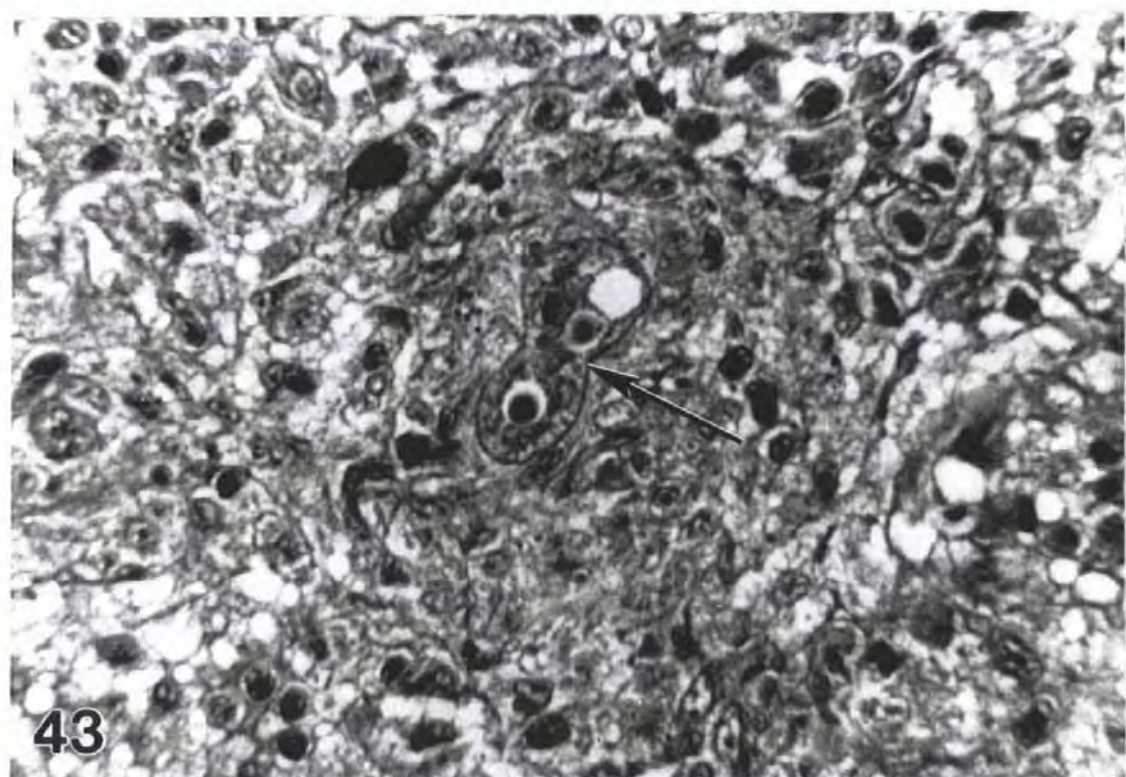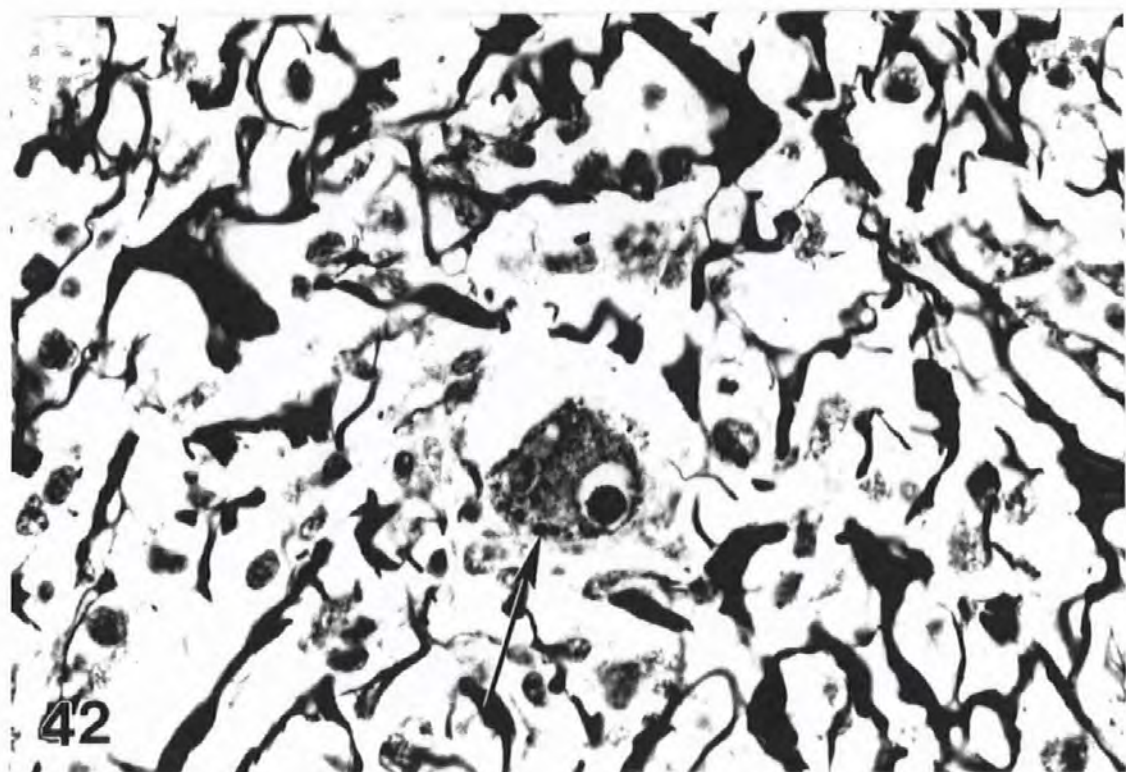
Figure 44. PKX primary cell with a prominent nucleus (N) containing a large endosome. Two secondary cells (S) are present each with its own nucleus. Numerous haplosporosomes are distributed throughout the primary cell cytoplasm (arrowed). Mitochondria (M), lipoid inclusions (L) and accumulations of membranous material (large arrow) are also present. x13,200.

44
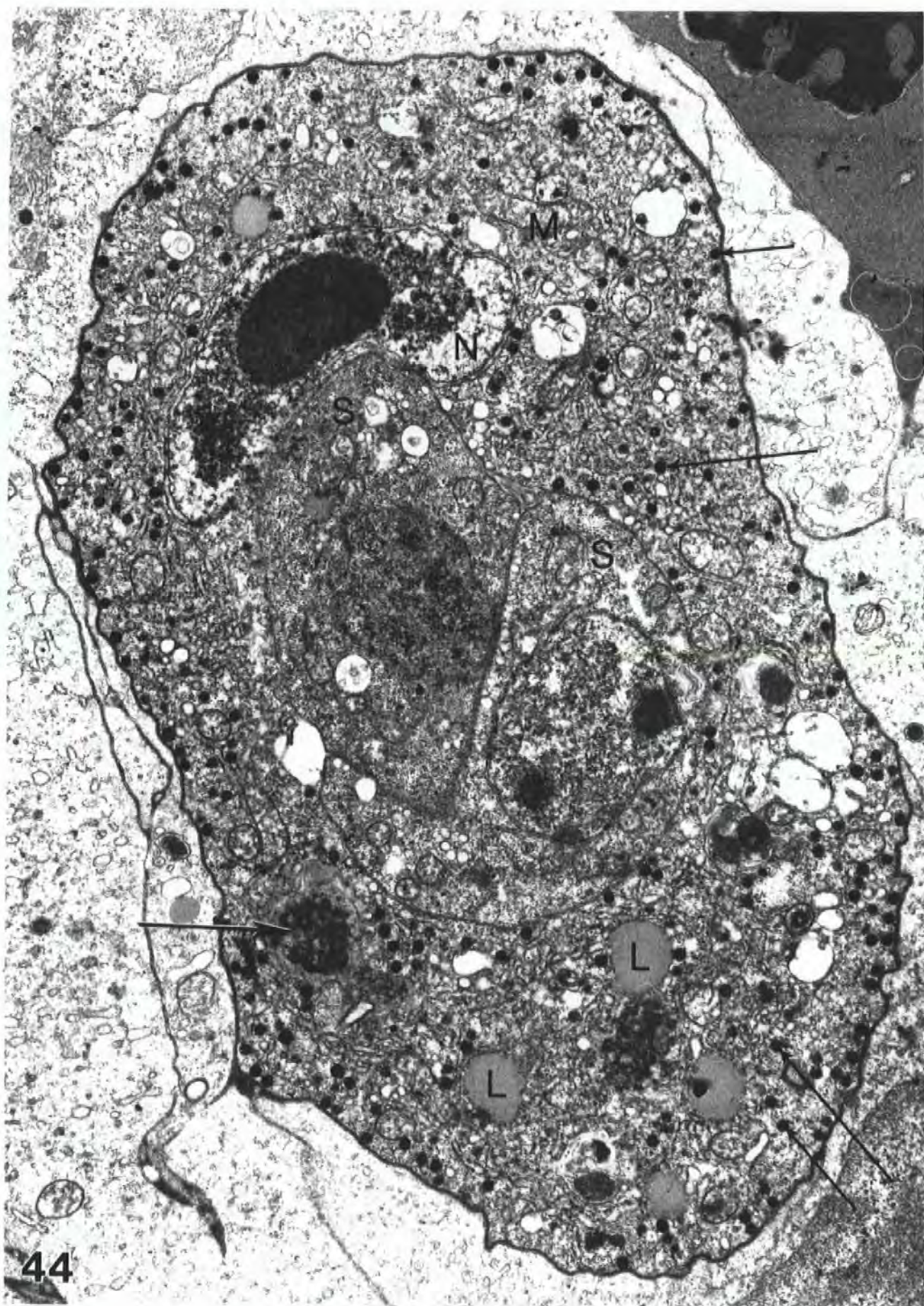
the primary cell wall the electron-lucent bar of the haplosporosomes was typically orientated perpendicular to the cell wall. The bar appeared to be formed by the invagination of the inner membrane forming a tube circular in cross section (Fig. 47). The presence of membrane fragments and a uniform electron-dense substance on the surface of the PKX cell were noted. Both haplosporosomes and the extracellular deposits were unstained by the Thiéry technique for carbohydrates.

The nucleus of the primary cell was bound by a double membrane and contained a prominent endosome with additionally one or more zones of condensed chromatin (Figs. 44 & 45). In PKX cells without secondary cells the nucleus was usually spherical. With secondary cells present the nucleus was typically larger, flattened and in contact with one secondary cell. The contact was limited to zones on the surface of the secondary cell which produced a cup shaped depression of the nuclear membrane (Fig. 45). The cytoplasm of the primary cell contained a variety of other structures including lipoid inclusions, mitochondria containing plate-like cristae, Golgi apparatus, primary and secondary lysosomes, the latter containing accumulations of membranous material, an extensive network of RER, and secondary cells, some of which contained tertiary cells. Multilaminate bodies were observed occasionally (Fig. 48).

Secondary cells were often found in close association with each other. Each bounded by a double membrane, the cells were in effect contained within a vacuole of the primary cell cytoplasm. Isolated secondary cells were occasionally observed with filose pseudopodial processes extending into the primary cell cytoplasm (Fig. 49). In these cases the secondary cell was bound by a single bi-lipid membrane. Haplosporosomes were absent in secondary cells, although spherical electron-dense structures measuring up to 0.3μm in diameter and lacking the 'bar' structure were occasionally observed. These structures appeared to be formed from Golgi apparatus (Fig. 50). Other organelles included mitochondria containing plate-like cristae, sparsely distributed strands of RER and various vesicles and lysosomes. The cytoplasm also contained numerous free ribosomes and occasional microtubules. The large accumulations of closely packed membranous material observed in the primary cell cytoplasm were absent.

Figure 45. A small PKX cell showing the primary cell nucleus (N) in contact with the secondary cell (S). x4,100.

Figure 46. Haplosporosomes (H) with the prominent electron-lucent bar aligned at the primary cell membrane. Note the double membrane of the primary cell (arrowed) and the electron-dense glycocalyx between the parasite and host cell (double arrow). x72,110.
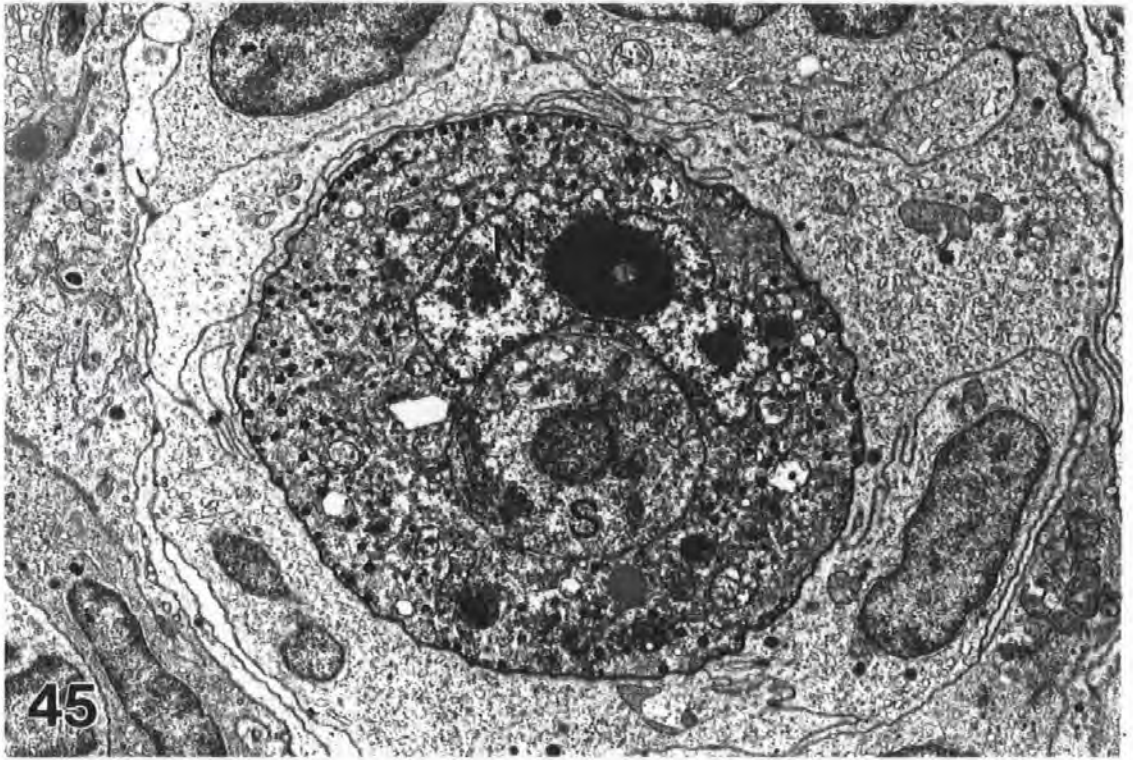
Figure 47.   Golgi apparatus (G) packaging haplosporosomes (H) with the apparent involvement of RER (arrowed). x72,110.

Figure 48.   Multilaminate body (arrow) in the cytoplasm of a PKX primary cell. x72,110.
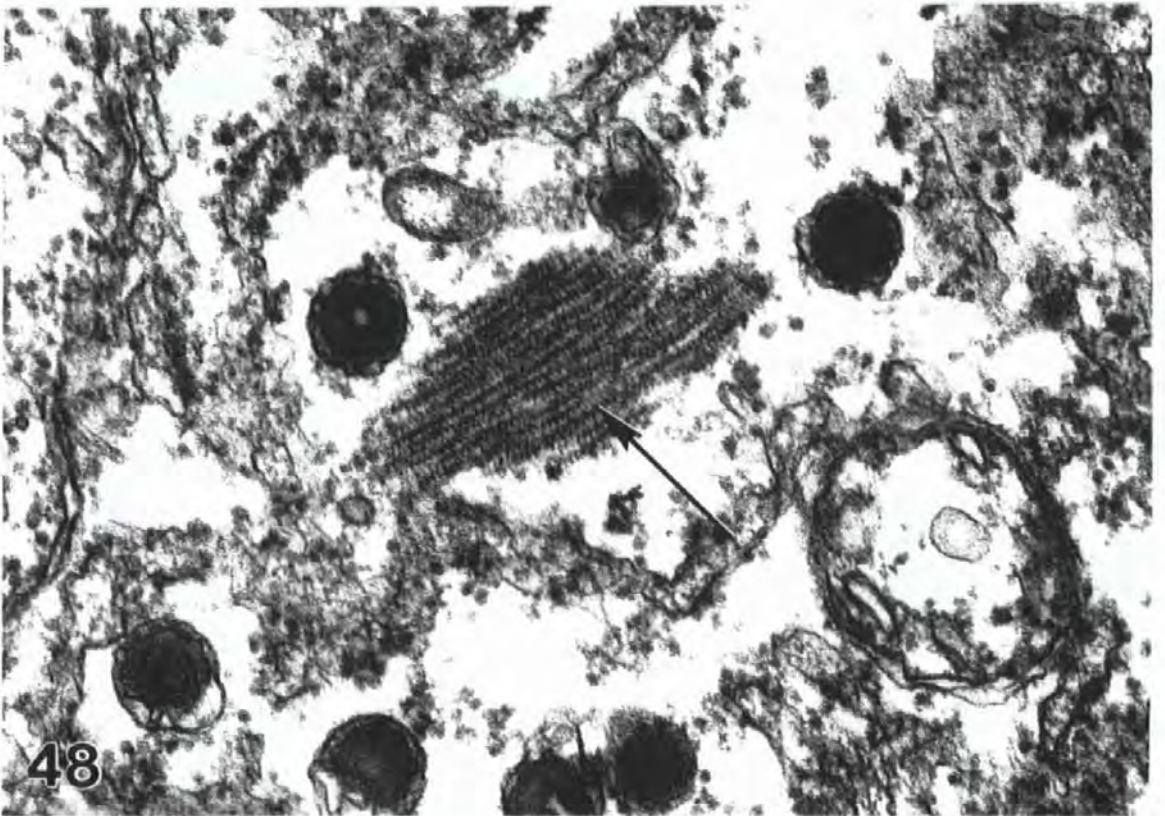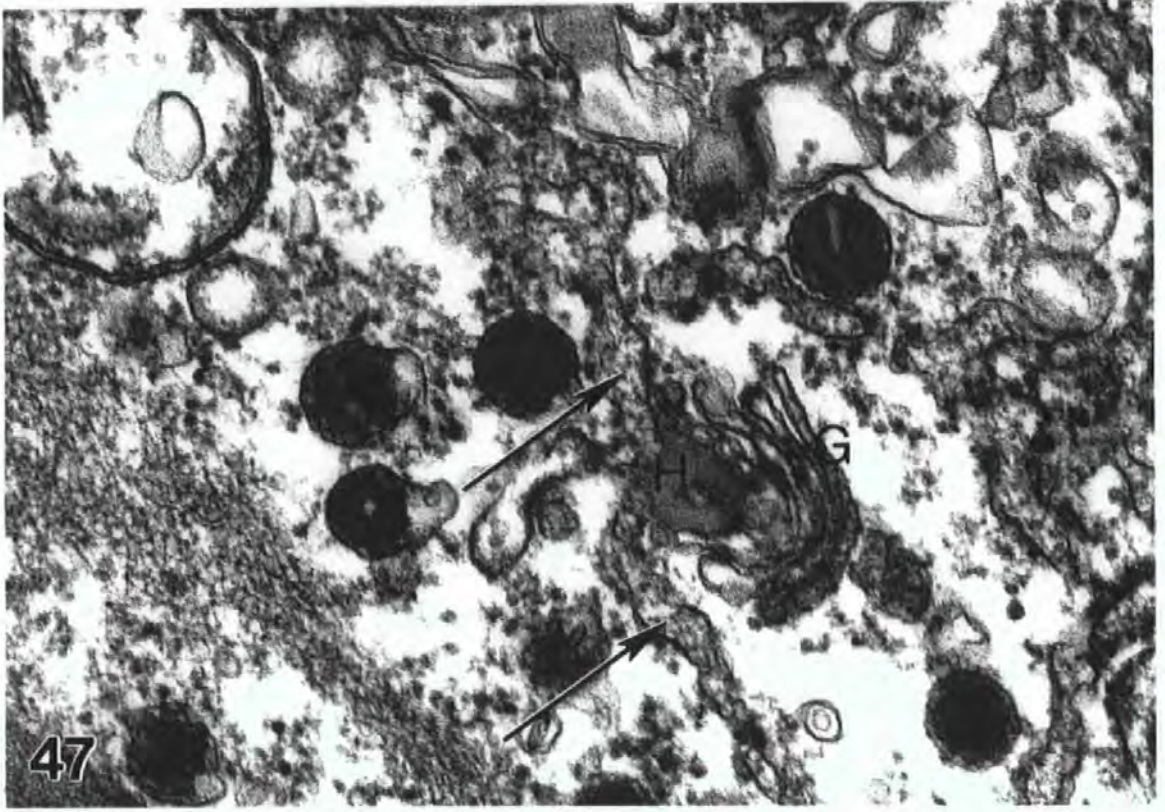
Figure 49.   A secondary cell with filose pseudopodia (F). Fine fibrillar material
within the pseudopodia can just be discerned. x26,000.

Figure 50.   Two apposed secondary cells. Cell (A), the pericyte, in the process
of enveloping cell (B). Note Golgi (G) packaging an electron-dense
inclusion body. x14,260.
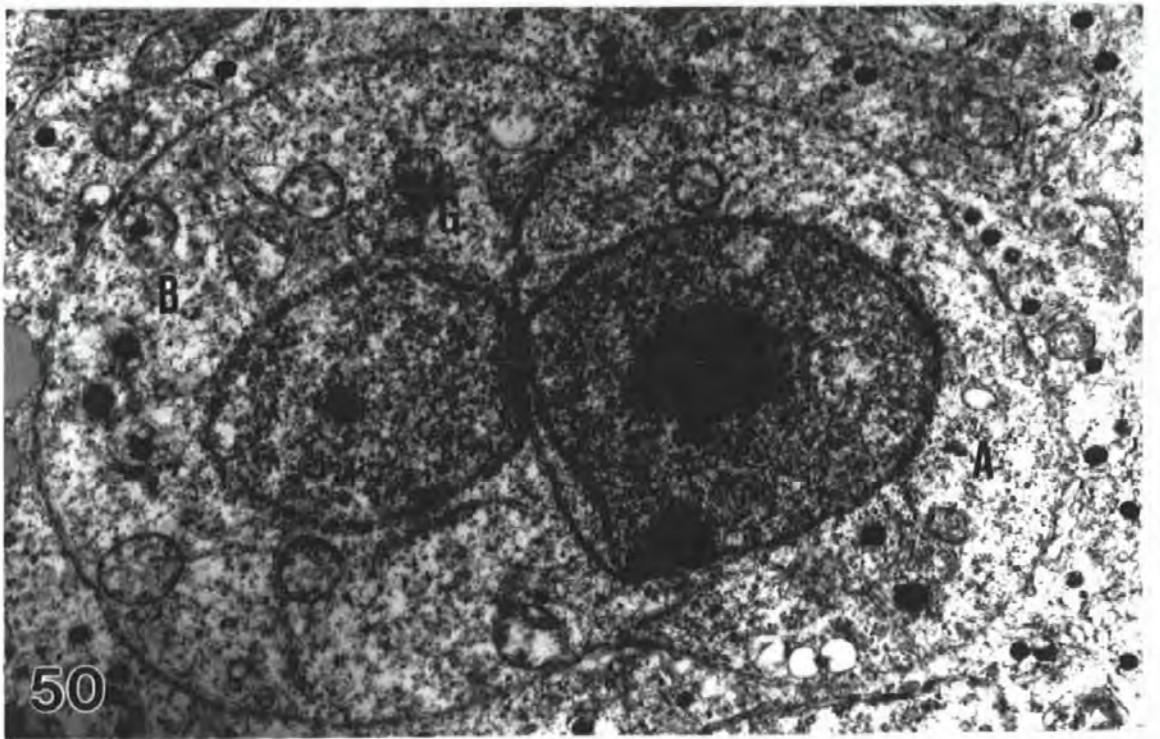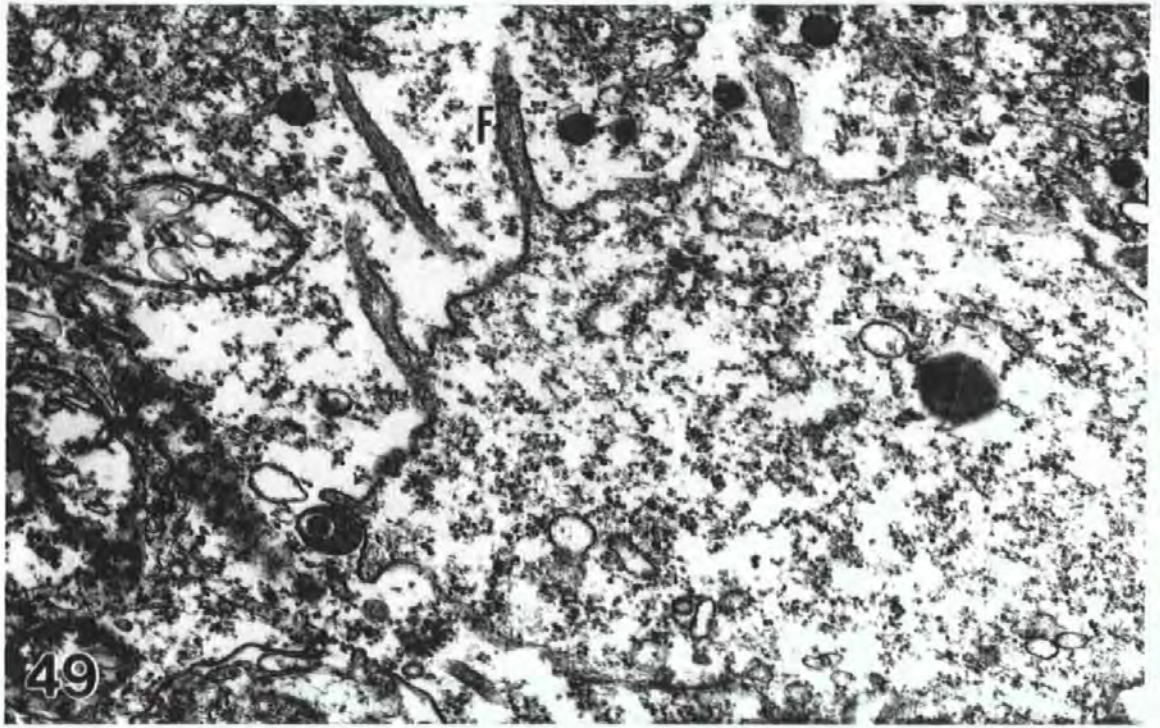
Tertiary cells were produced by the envelopment of one secondary cell by another (Figs. 50 to 53). The nucleus of the enveloping cell or pericyte was characterised by the presence of two nucleoli (Fig. 52). The enveloped or tertiary cell cytoplasm was condensed with numerous free ribosomes present. Mitochondria were abundant and of the same form as those seen in the primary and secondary cells. Bundles of microtubules were often observed in the tertiary cell cytoplasm sometimes causing an elongation of the cell producing a fusiform appearence (Fig. 54).

Occasionally, degenerate parasites were observed in both kidney and spleen. In these cases the primary cell appeared shrunken and osmiophilic with numerous vacuoles present (Fig. 55). Secondary cells did not display the marked osmiophilia of the primary cell and vacoules, although present, were smaller and less numerous. Nuclei were enlarged with clumps of condensed chromatin typically distributed along the nuclear membrane. Disrupted PKX cells were also observed (Fig. 56). Remnants of the primary cell could be seen scattered amongst the host tissue. Most parasite organelles that could be discerned were degenerate; however, haplosporosomes retained their integrity. In rainbow trout, later developmental stages of PKX, i.e. those containing more than two tertiary cells or in which sporoblasts were present, were not observed.

4.3.2   Cellular response to PKX

PKX cells in this study were always found in close association with host cells of various morphological types. PKX cells in the kidney were frequently observed within small blood vessels and capillaries (Fig. 57). Host cells adhering to the PKX parasite were predominately neutrophils and macrophages. Neutrophils were characterised by the presence of a large lobed nucleus with condensed chromatin distributed along the inner surface of the nuclear envelope (Fig. 58). The cytoplasm contained few organelles apart from mitochondria and small quantities of RER. Osmiophilic granules were usually present in small numbers. Lymphocytes were generally smaller than neutrophils and contained a large nucleus with abundant condensed chromatin which occupied most of the cell. The cytoplasm was also condensed with occasional

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Figure 51.   The pericyte, cell (A), partially enclosing cell (B). The nucleus of the pericyte is characterised by the presence of two prominent nucleoli. x10,880.

Figure 52.   Fully enveloped by the pericyte (A), cell (B) becomes a tertiary cell. Primary cell (arrowed). x10,880.

51

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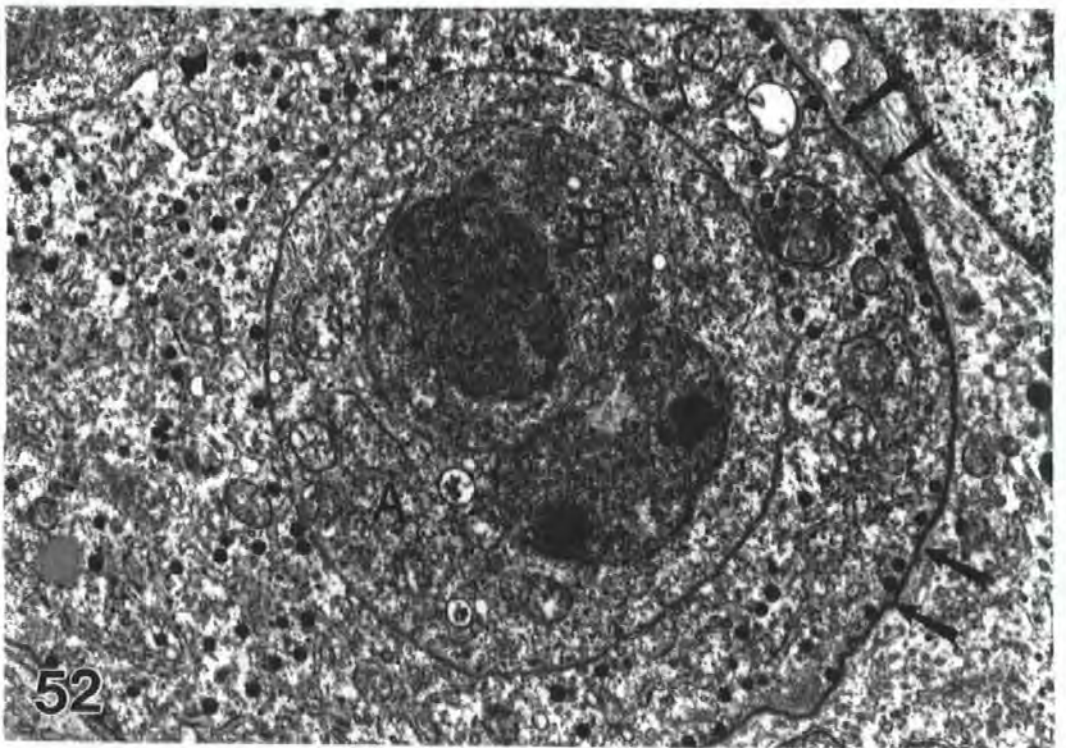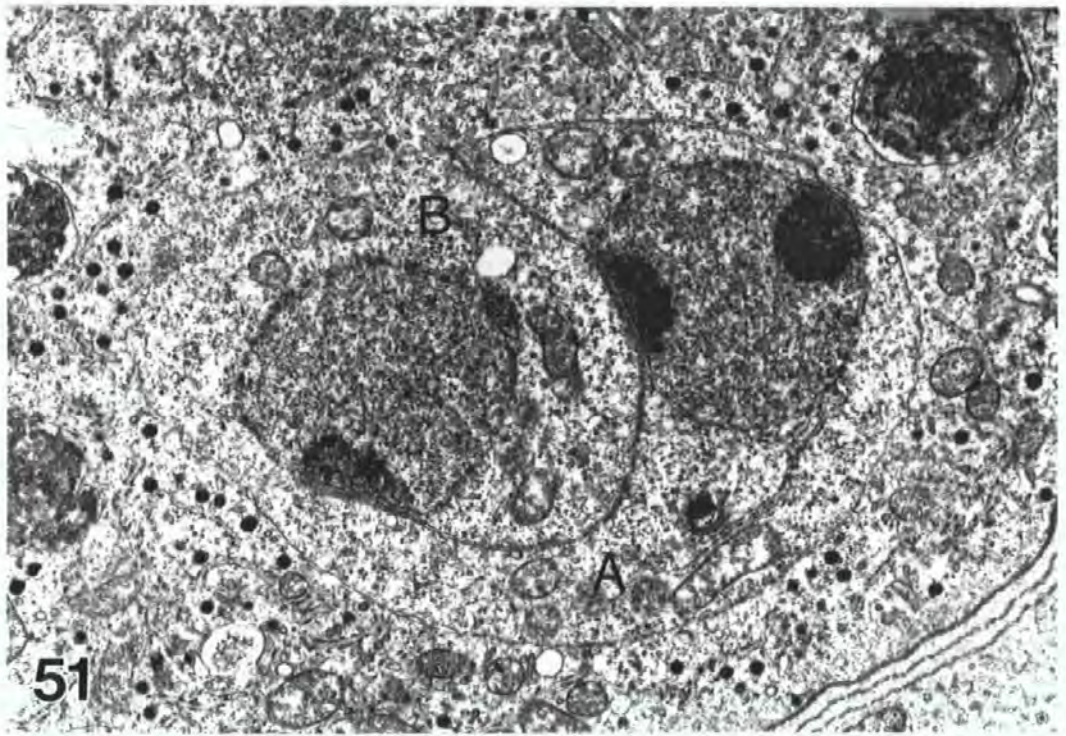
Figure 53.   PKX containing two secondary cells (S) and one tertiary cell (T). Primary cell nucleus (N). x4,980.

Figure 54.   Microtubules in tertiary cell cytoplasm (single arrow). Pericyte cell membrane (double arrow). Mitochondria (M). x20,500.

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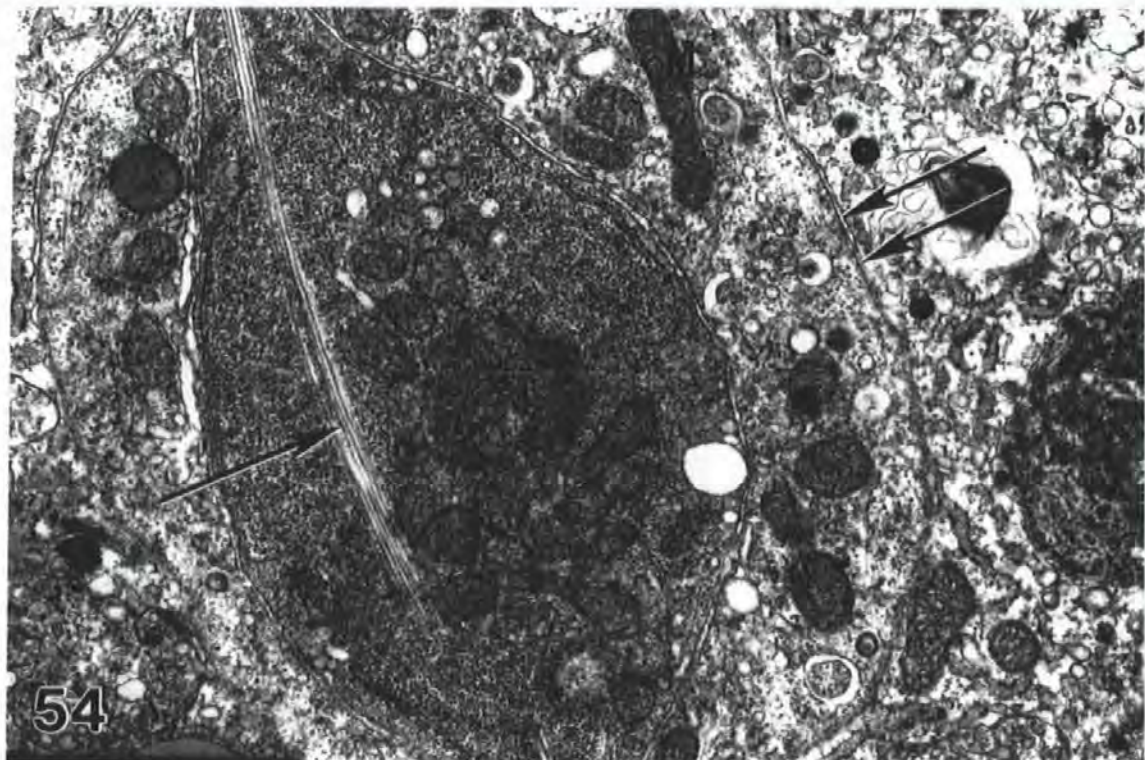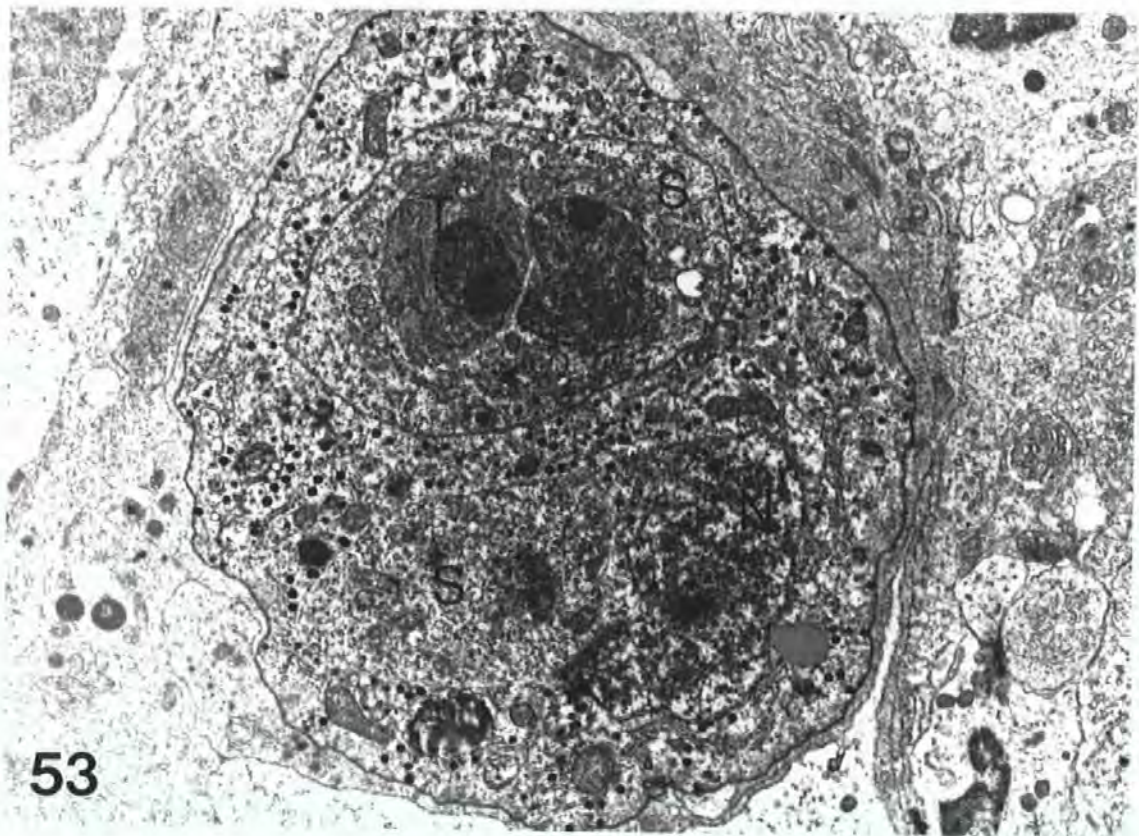
Figure 55.   Degenerate PKX (arrowed). Note the extensive vacuolation of the
             primary cell. x6,650.

Figure 56.   Disrupted PKX cell with numerous haplosporosomes scattered
             amongst host cells (arrowed). x6,650.

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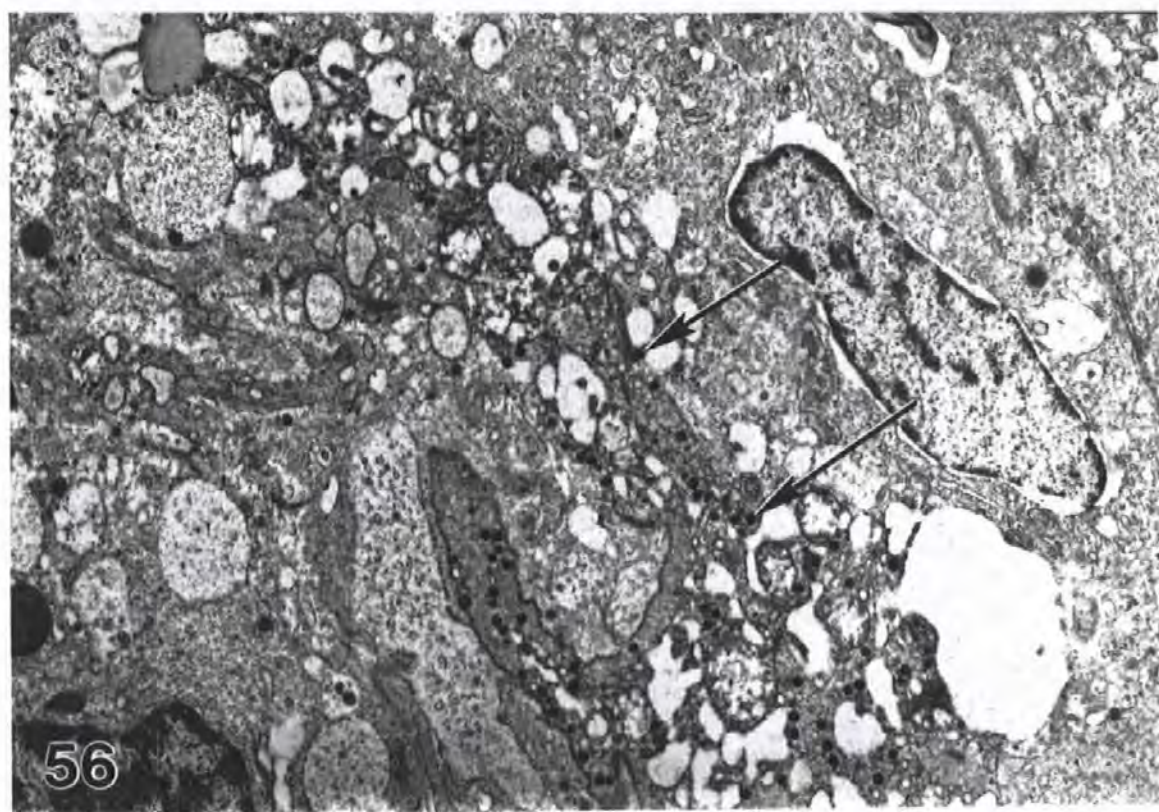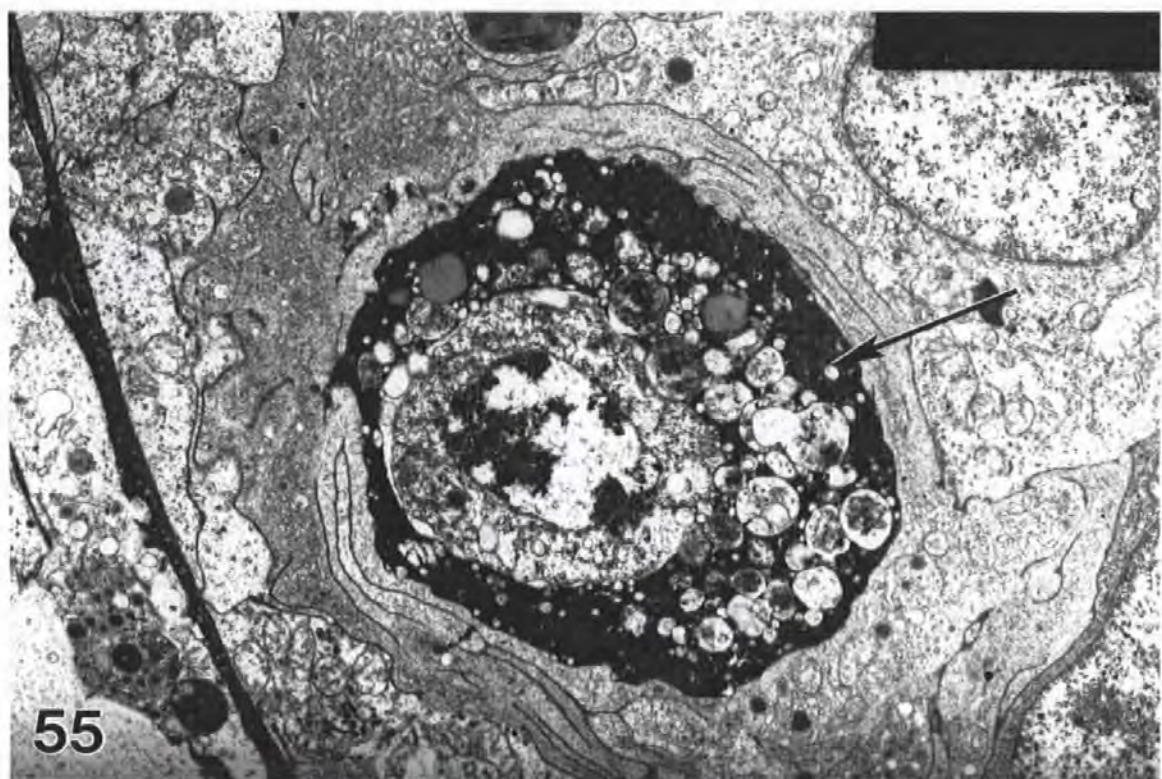
Figure 57. PKX cells, one within a capilliary (A) and the other (B) close to the endothelium (E). Cells involved in the host response include neutrophils (N), lymphocytes (L) and plasma cells (P). x3,490.

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mitochondria present (Figs. 57 & 59). Some lymphocytes possessed dilated RER and were in the process of transforming into plasma cells (Fig. 57). Fully formed plasma cells were slightly larger than un-transformed lymphocytes. The increase in size was accounted for by the large amounts of distended RER in the cytoplasm. The nucleus was indistinguishable from lymphocyte nuclei (Fig. 59).

Within the renal interstitial tissue PKX cells were invariably surrounded by neutrophils which often completely enclosed the parasite (Fig. 60). In most cases the PKX cell appeared necrotic with numerous large vacuoles and lysosomes (Fig. 61). Lymphocytes were not usually observed attached to the PKX cells but were closely associated with the neutrophils adhering to the parasites.

Rarely, PKX cells were observed penetrating the epithelium of renal tubules although none were ever seen in the lumen itself (Fig. 62). Cellular reaction to the parasite was minimal. Occasional lymphocytic infiltration accompanied the migration of PKX cells through the tubule epithelium.

In the kidneys of severely affected fish, macrophages were more abundant. These cells possessed numerous cytoplasmic pseudopodia and a large irregular nucleus with a coarse chromatin pattern (Fig. 63). Although presumed phagocytosis of PKX cells by macrophages was seen by light microscopy it was not confirmed at the ultrastructural level. Macrophages were often seen with phagocytosed crystalline material which in some cases occupied most of the cytoplasm of the cell (Fig. 64).

The splenic cellular response also involved phagocytes - predominantly neutrophil types, lymphocytes and plasma cells (Fig. 65). In addition, large numbers of thrombocytes were observed distributed throughout the spleen. These cells were slightly larger than lymphocytes and contained numerous electron-lucent cytoplasmic vesicles arranged in an array at one end of the cell (Fig. 66). Other cytoplasmic inclusions included mitochondria, golgi apparatus and small strands of RER. Nuclei were often deeply cleft and contained prominent clumps of condensed chromatin (Figs. 65 & 66). Thrombocytes were never seen in contact with PKX cells.

The spleen of some fish contained eosinophilic granulocytes (Fig. 67). At the ultrastructural level these cells were characterised by the presence of numerous

Figure 58.   Neutrophil from a PKD affected trout kidney. Note the lobed nucleus. x10,730.

Figure 59.   Plasma cell from the same kidney as above, characterised by extensive dilation of the RER. x11,310.
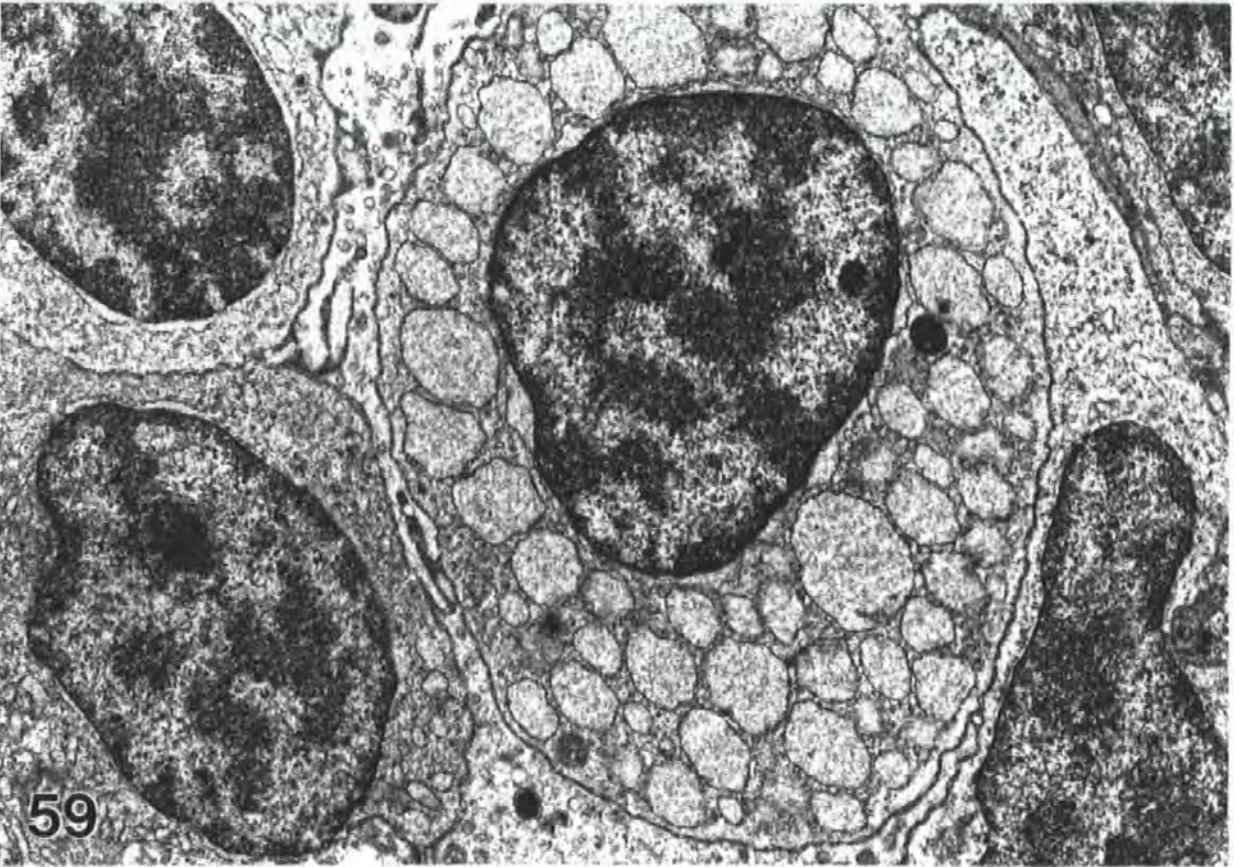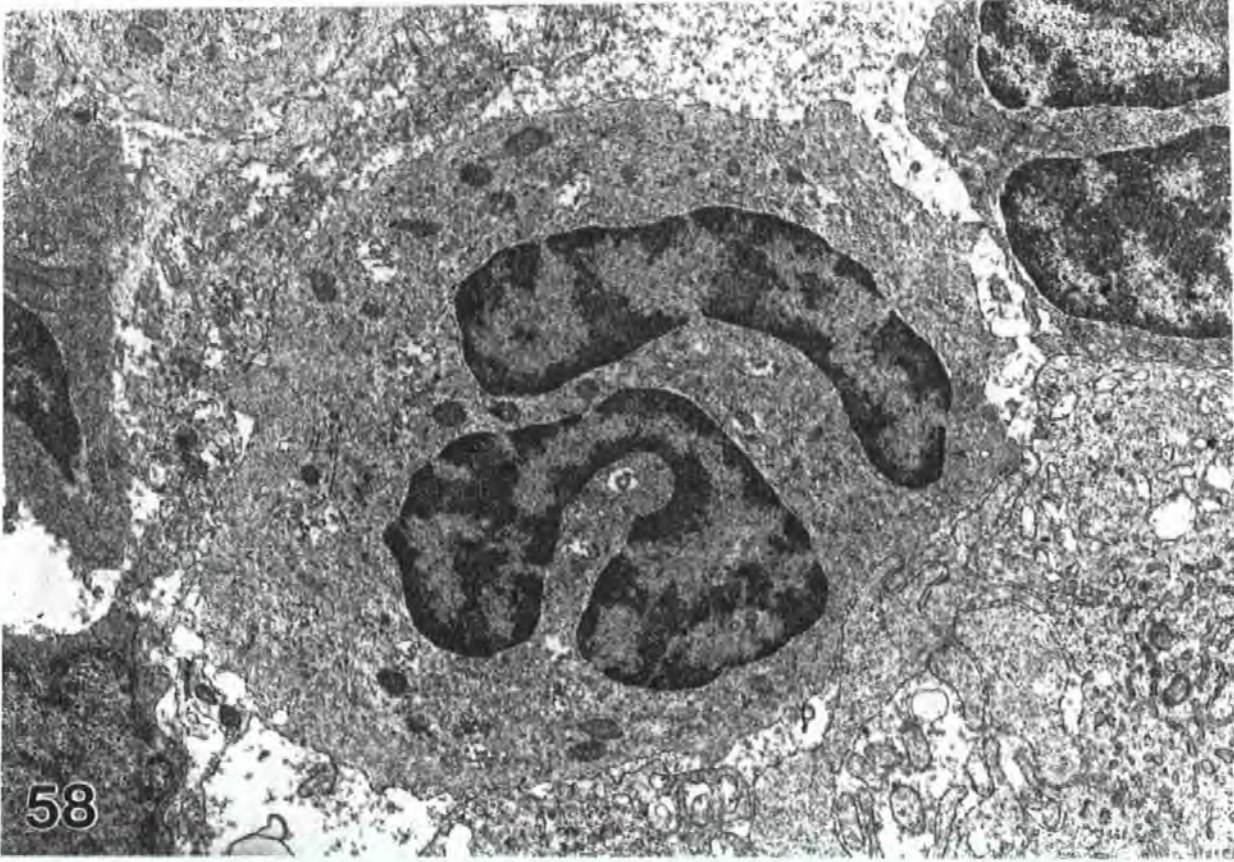
Figure 60.   A small PKX cell surrounded by a neutrophil. The PKX cell shows
no evidence of necrosis. x5,090.

Figure 61.   A necrotic PKX cell completely enclosed by a neutrophil. x7,980.
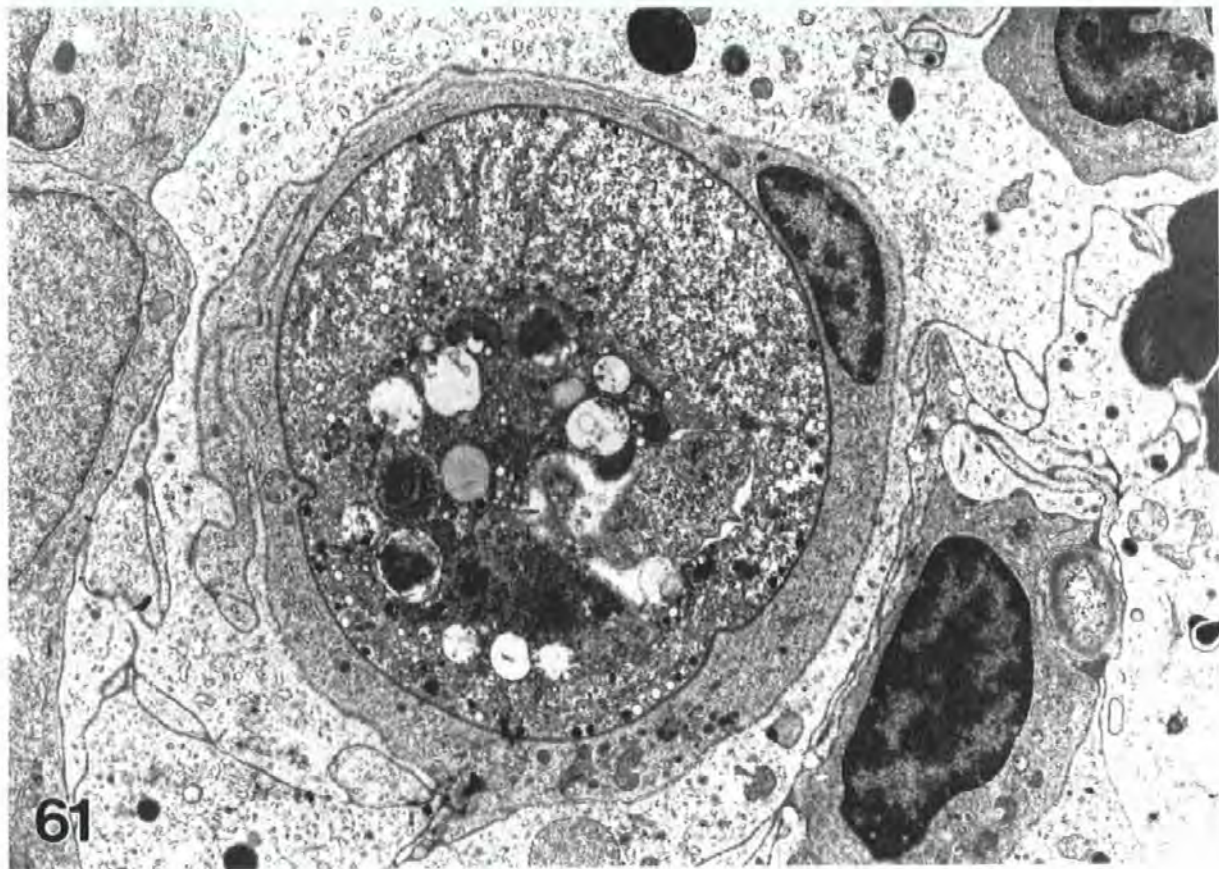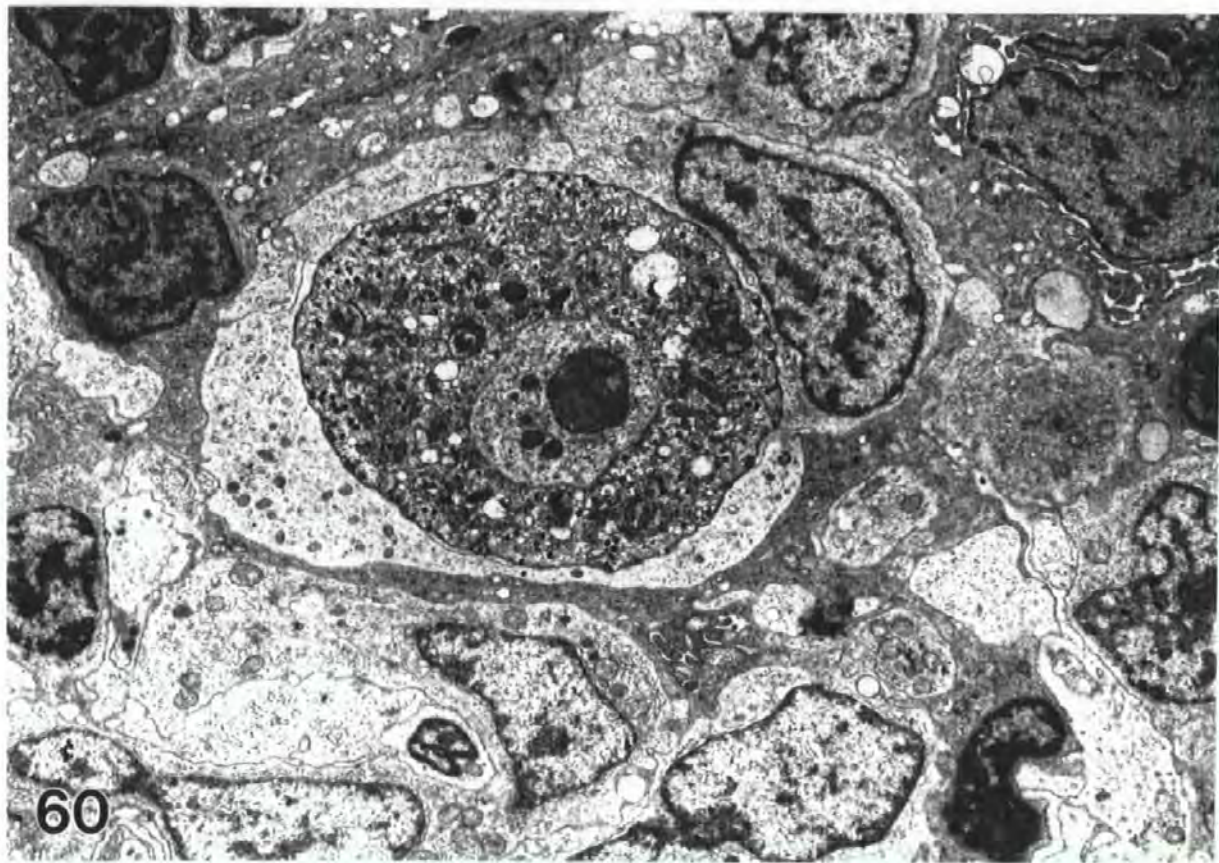
Figure 62. A PKX cell penetrating the epithelium of a renal tubule. The basement membrane of the tubule epithelium (double arrow) appears intact. The lumen of the tubule (L) contains necrotic material, possibly another PKX cell. Note the apparent lack of host response. x5,100.

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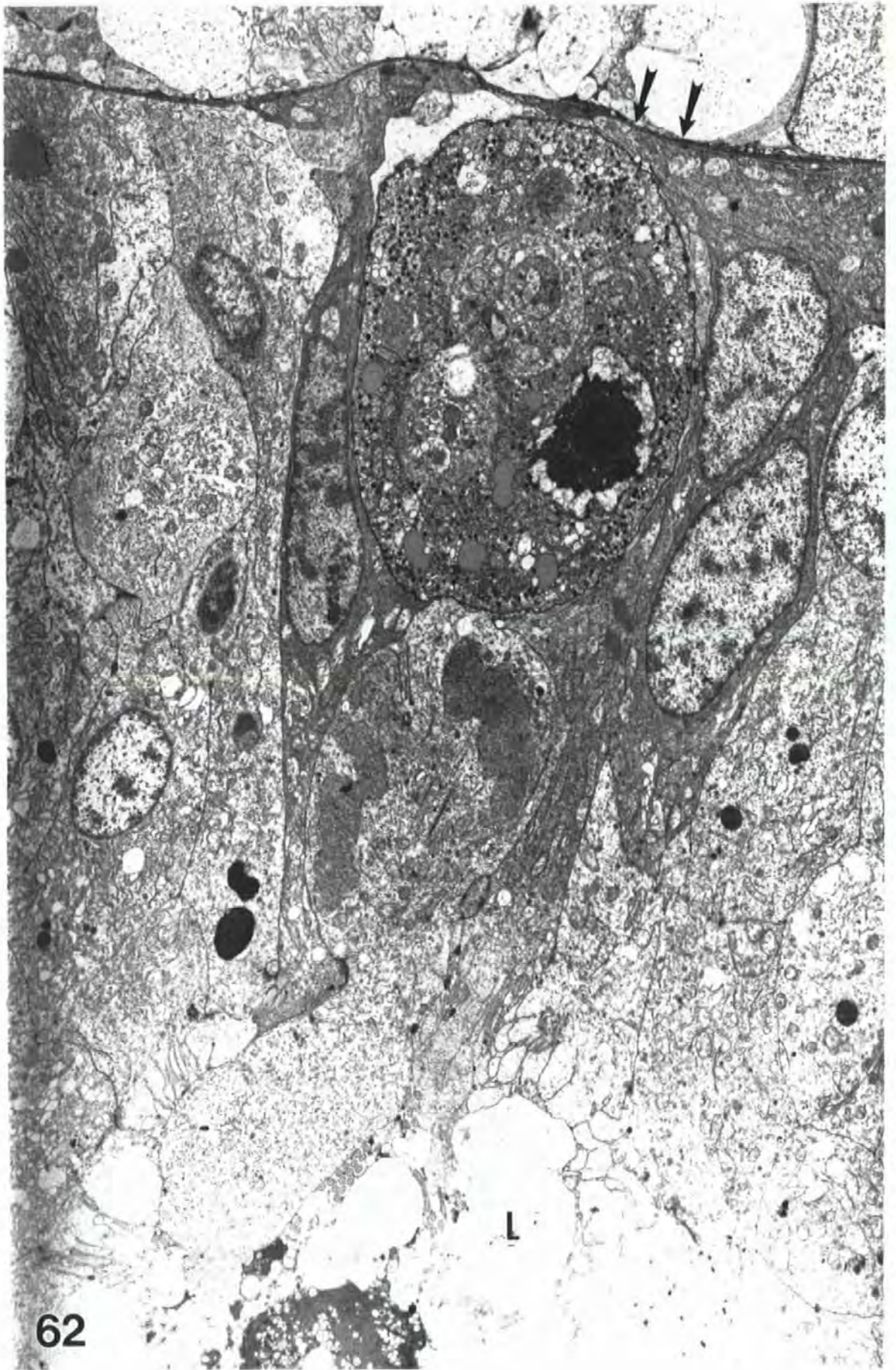
Figure 63.   Two macrophages (M) and a neutrophil (N). One of the
              macrophages contains a crystalline inclusion (arrow). x5,520.

Figure 64.   Macrophage containing several crystalline inclusions. x14,460.

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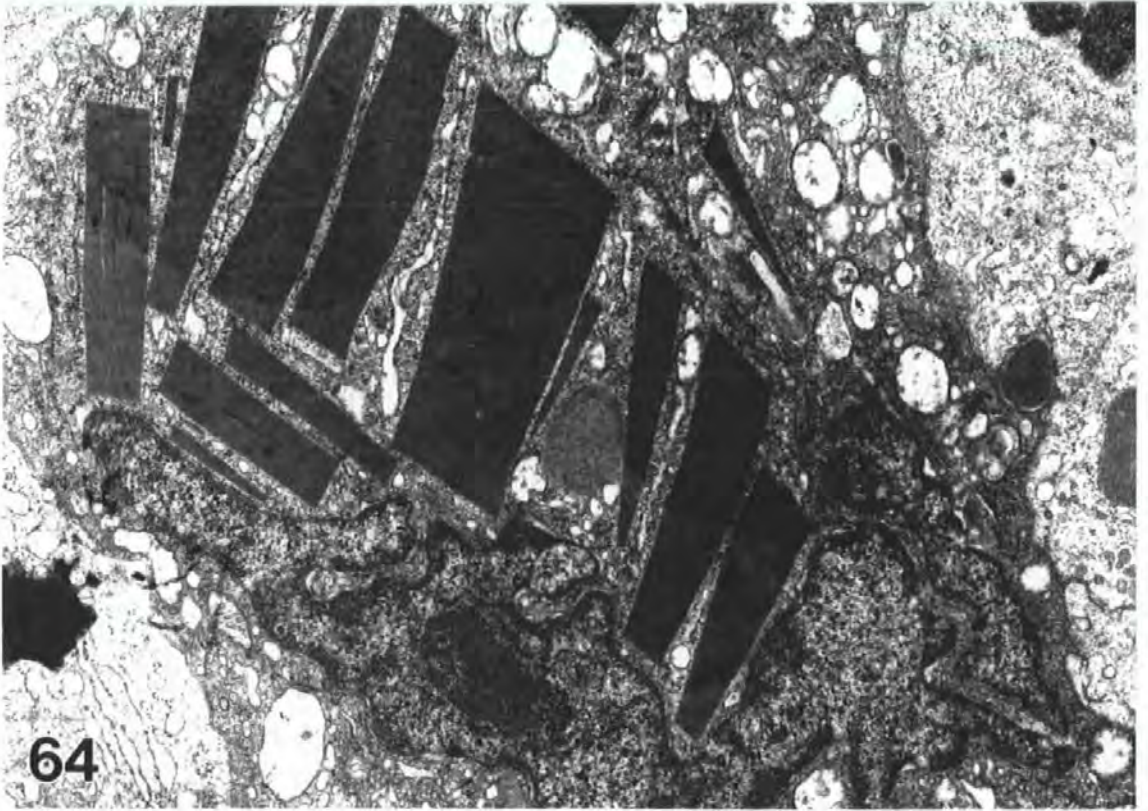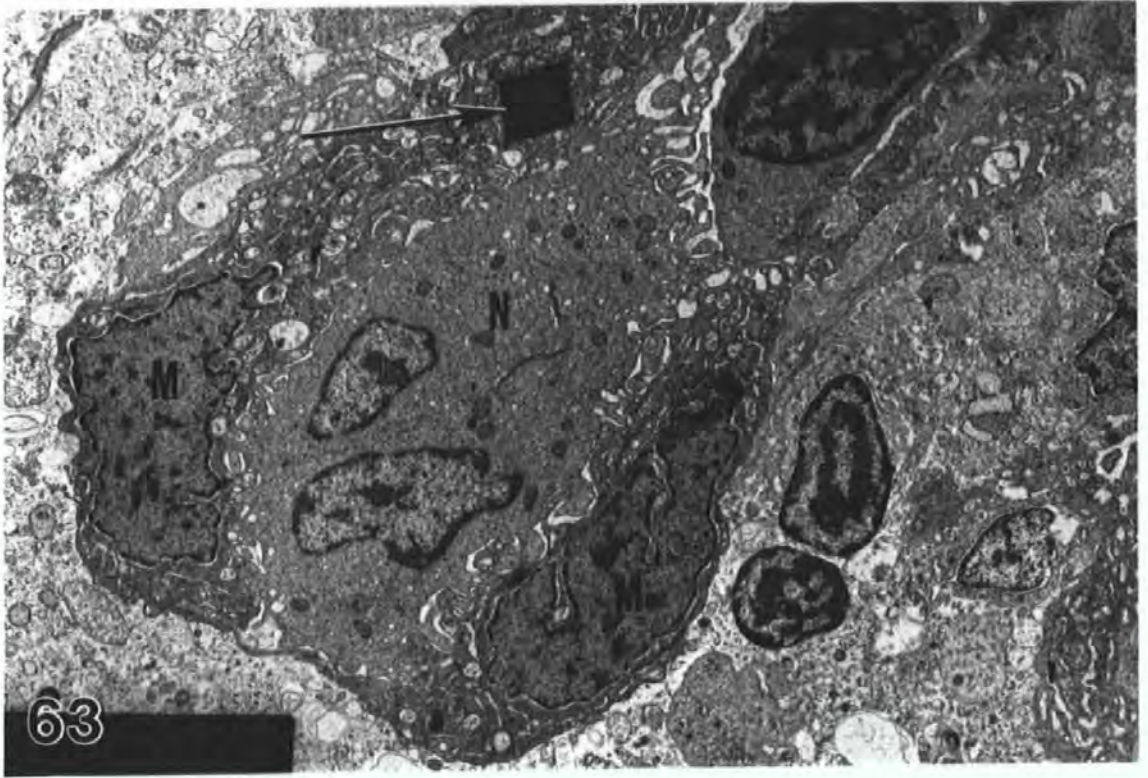
electron-dense membrane-bound granules up to 4.2μm in diameter. The cytoplasm also contained a few mitochondria and sparse RER. The nucleus was plaeomorphic but typically elongate with condensed chromatin distributed along the inner membrane of the nuclear envelope. Eosinophils were evenly distributed throughout the spleen and were never observed in direct contact with PKX parasites.

4.4   DISCUSSION

Evidence from this study confirms the myxosporean nature of the PKX parasite and verifies previous findings on the main structures of the PKX cell. However, several features of the ultrastructure are recorded here for the first time. Electron microscopy of the cells involved in the host response indicated that neutrophils rather than macrophages are primarily involved in attachment to PKX cells in both the kidney and spleen. The splenic cellular response also included lymphocytes, plasma cells, thrombocytes and eosinophil granulocytes.

The basic components of the PKX primary cell have been well documented (Ferguson & Needham, 1978; Seagrave *et al.*, 1980a & b; Kent & Hedrick, 1985, 1986) and include one or more nuclei, secondary and tertiary cells, mitochondria, RER, Golgi, vacuoles containing membranous material and the so called 'haplosporosomes' discussed in detail below. The rare multilaminate bodies first reported by Seagrave *et al.*, (1980a) were found during this study. Similar structures (chromatoid bodies) are found in axenic cultures of *Entamoeba histolytica* and other amoebae (Lowe & Maegraith, 1970) and in the plasmodia of the European flat oyster pathogen *Marteilia refringens* (Phylum Haplosporea) (Perkins, 1976). The function of chromatoid bodies which are found in trophozoite and cyst stages of *Entamoeba* is uncertain, although they are thought to consist of nucleoprotein in the form of crystalline RNA which is utilised during the formation of the infective stages within the cyst. Perkins, (1976) referred to the laminated inclusion in *M. refringens* as a "striated inclusion" and demonstrated that it consisted of tightly packed cisternae of endoplasmic reticulum (ER). The multilaminate bodies in PKX do not appear to be associated with Golgi apparatus or ER

Figure 65.   Cells from rainbow trout spleen infected with PKX. Plasma cell (P),
thrombocyte (T). Red blood cells (B) are also present. x7,470.

Figure 66.   Thrombocytes characterised by the presence of groups of
cytoplasmic vacuoles (V) and nuclei with clumps of condensed
chromatin. x7,470.

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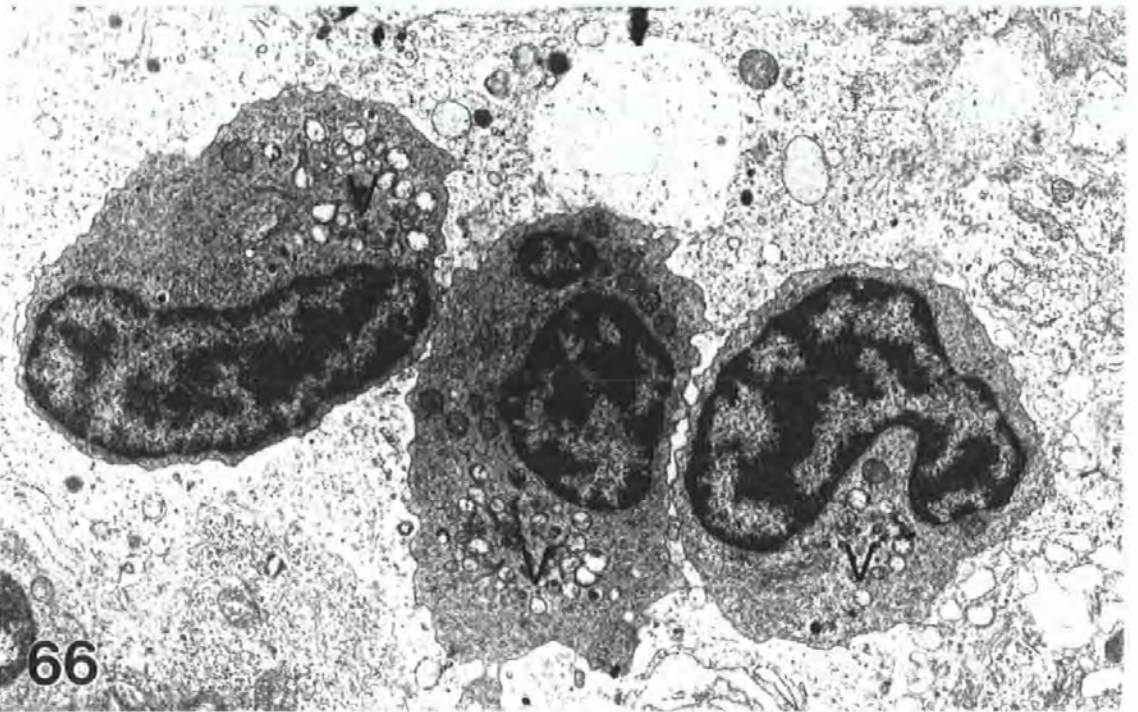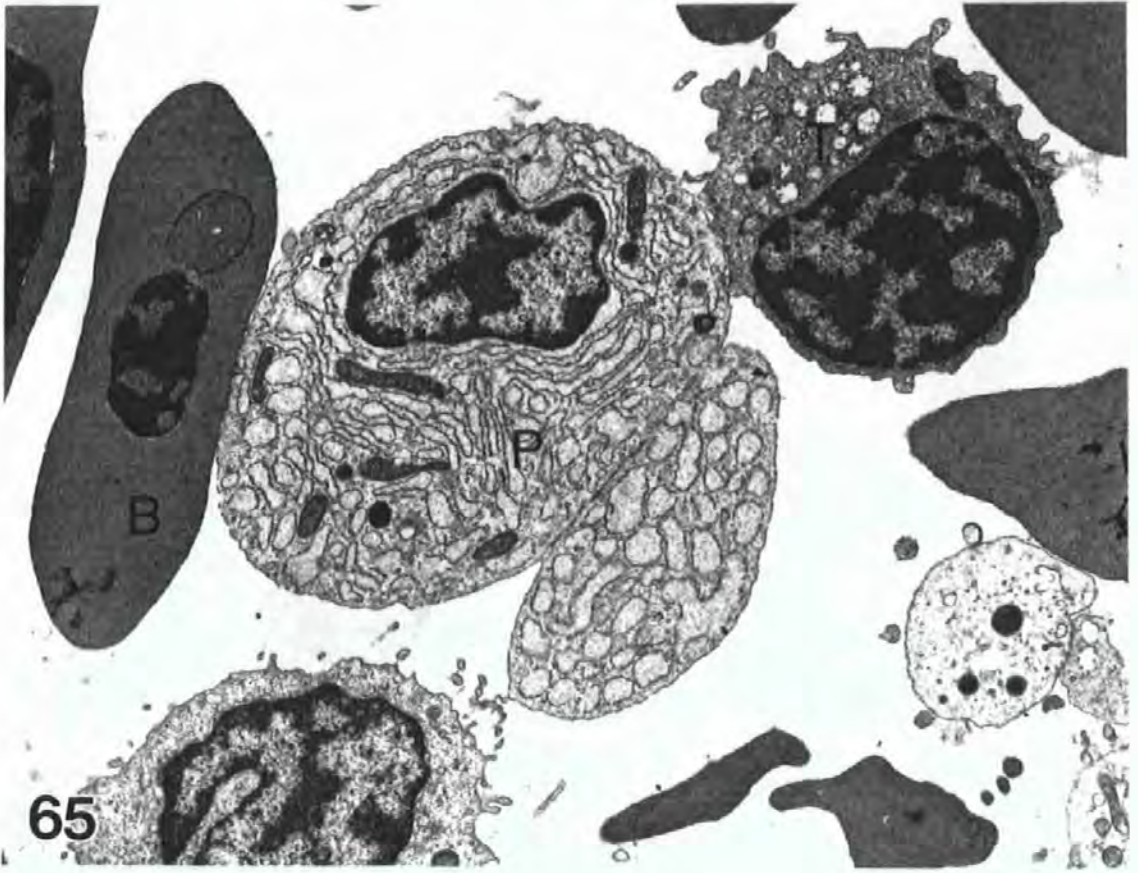
Figure 67. Eosinophil granulocyte from a PKX infected spleen. Several osmiophilic membrane-bound granules (arrows) are present. x12,950.
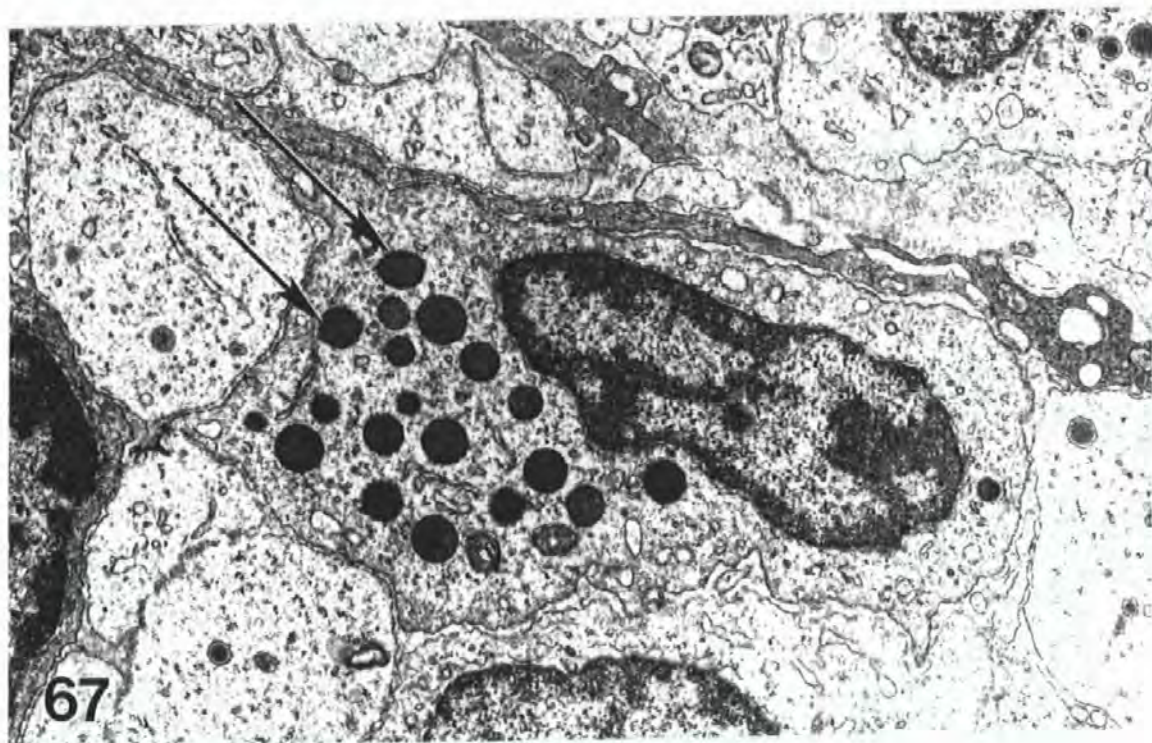
and their origin and function remain unknown. However, it is interesting to speculate that the presence of these bodies may be associated with the need for rapid development i.e. the production of secondary and tertiary cells during the proliferative stage of the parasite. The multilaminate bodies described by Kent & Hedrick (1985) differ from those described by Seagrave *et al.*, (1980a) and the present author in several respects. They were much larger and generally spherical rather than of angular appearance and in addition were membrane-bound. The contents were also more tightly packed. It is probable that the multilaminate bodies described by Kent & Hedrick (1985) are not analagous to those reported by Seagrave *et al.*, (1980a) but represent condensed vesicular structures similar to those reported by Rafferty (1986) which were interpreted as secondary lysosomes containing large amounts of membranous material.

The electron-dense bodies in the primary cell of PKX were first referred to as haplosporosomes by Seagrave *et al.*, (1980a) because of their likeness to similar structures in *M. refringens* (Perkins, 1976). This study confirms the findings of Smith *et al.*, (1985) and Rafferty, (1986) that unlike the haplosporosomes in *M. refringens* those in PKX originate from the Golgi apparatus. Multivesicular bodies described by the above authors as possible alternative sites for haplosporosome production were not identified in this study. Electron-dense inclusions are frequently found in myxosporean sporoplasm cells. Lom, Molnár & Dyková (1986) named these inclusions 'sporoplasmosomes' reflecting their location in the sporoplasm cell of sporoblasts or mature myxosporean spores. Their occurrence in PKX primary cells may represent a new location for these inclusions or indicate that the PKX primary cell is itself a sporoplasm cell. However, sporoplasmosomes have not been reported from similar extrasporogonic stages of other myxosporeans. Similar structures have been found in other phyla. In *M. refringens* haplosporosomes occur in various stages of the life cycle; in plasmodia, decreasing in number during sporogenesis and in large numbers in mature spores. Some haplosporosomes give a positive Thiéry reaction for polysaccharides (Azevedo & Corral, 1985) and others are known to contain DNA (Perkins, 1979). However, the PKX haplosporosomes tested in this study did not contain polysaccharides and Rafferty (1986) failed to demonstrate the presence of DNA. In this

study several haplosporosomes appeared to have discharged their contents outside the PKX primary cell, possibly contributing to the electron-dense layer surrounding the parasite. Taking into account the intense host reaction to the parasite it is possible that the host reacts, initially at least, to this layer. Consequently, further investigations into the chemical nature and function of haplosporosomes are required and may provide information which could be used in the development of effective treatments for PKD including vaccine production.

The features of PKX secondary cells such as prominent Golgi apparatus, double cell membranes and the presence of numerous cytoplasmic ribosomes are consistent with myxosporean generative cells (Current, 1979). The sequence of secondary cell envelopment was first recorded by Feist & Bucke, (1987) and was considered to represent the initial stage of sporogenesis. The presence of microtubules in PKX tertiary cells and to a lesser extent secondary cells had not been observed in previous studies. Cytoplasmic microtubules are also a feature of myxosporean generative and sporogonic cells and appear to be associated with the formation of the polar capsules in mature spores. Similar microtubules have also been found in secondary cells of the blood stages of *S. renicola* in carp. That they may be contractile is supported the 'twitching' motion of the parasites seen in fresh preparations (Lom *et al.*, 1983). In both the carp parasite and PKX, bundles of microtubules were often extended causing distortion of the cell membranes resulting in fusiform cells.

In myxosporean genera that form large polysporoblastic plasmodia producing large numbers of spores the sequence of secondary cell envelopment resulting in the formation of a tertiary cell has been well documented as an early stage in the formation of the pansporoblast (Lom & de Puytorac, 1965; Current, 1979; Desser, Molnar & Weller, 1983). However, the same sequence of envelopment has not been reported in those myxosporeans that produce small actively dividing trophic stages e.g. pseudoplasmodia of *Sphaerospora* or *Ceratomyxa* species (Yamomoto & Sanders, 1979; Lom, Dyková & Lhotaková, 1982). In such cases spores are formed not by division of a tertiary (sporogonic) cell within the pericyte but by the simple division of the sporogonic cell within the pseudoplasmodium until enough cells are produced to

form one or two spores (Lom, 1989; Lom & Dyková, 1986). Although sporogenesis is initiated within the PKX cell mature spores of PKX have never been confirmed. Spores of *Sphaerospora* species have occasionally been recorded in rainbow trout with current PKX infections and in fish that had recovered from PKD (Hedrick *et al.*, 1988; Odening *et al.*, 1988). However, in both reports the authors were unable to show that the spores arose from PKX cells. Kent & Hedrick (1985a, 1986) reported the presence of myxosporean sporoblasts in the lumens of renal tubules of rainbow trout with PKD and showed that these resulted from secondary and tertiary cells liberated from disintegrated PKX primary cells that had penetrated the tubule epithelium. Since the primary cell of PKX occasionally contains more than one nucleus it should not, by definition, be referred to as a pseudoplasmodium (Dyková & Lom, 1982) which in the case of PKX development should be reserved for the luminal stage since the enveloping cell is mononucleate (Kent & Hedrick, 1986). However, PKX structure is also similar to the extrasporogonic stages of *S. renicola*, a parasite of carp (*C. carpio*). These stages infecting blood and swim-bladder tissues also harbour secondary and tertiary cells which are formed by endogenous cleavage and may subsequently sporulate in the lumen of renal tubules (Baska & Molnár, 1988; Dykova, Lom & Korting, 1990; Lom, *et al.*, 1983). Thus, PKX probably represents an extrasporogonic stage of an unidentified myxosporean, most likely a species of *Sphaerospora* (Kent & Hedrick, 1986).

In the kidney the earliest cellular response to PKX is the proliferation of interstitial haematopoietic cells (Clifton-Hadley, Richards & Bucke, 1985). Subsequently, infiltration and proliferation of mononuclear cells occurs and phagocytic host cells become attached to the PKX parasite (Clifton-Hadley *et al.*,1987). Most authors have described these cells as macrophages (Ferguson & Needham, 1978; Clifton-Hadley *et al.*, 1987; MacConnell *et al.*, 1989 & Castagnaro *et al.*, 1991). During the present study two types of phagocytic cells were observed in kidneys and spleens from fish with PKD. Based on ultrastructural studies the cells adherent to PKX cells resembled neutrophils rather than macrophages which were only occasionally observed. Although both mammalian and fish neutrophils are characterised by the presence of numerous electron-dense granules (Ham & Cormack, 1979; Ferguson,

1976; Ellis, 1977 & Hine, 1992) those observed during the present study contained only few granules. The reason for this is uncertain but may be the result of de-granulation in response to the presence of PKX cells. Rafferty (1976) demonstrated cytoplasmic granules in "leucocytes" attacking PKX cells. Similarly, Sharp, Pike & Secombes (1991) studying leucocyte interactions in *Diphyllobothrium dendriticum* in rainbow trout clearly differentiated between neutrophils which contained cytoplasmic granules, macrophages and an additional unidentified leucocyte. The phagocytic properties of rainbow trout neutrophils have been demonstrated by Suzuki (1984) using bacteria and zymogen granules. There is little information on the role of rainbow trout neutrophils in parasitic infections. Studies by Sharp *et al.*, (1991) showed neutrophils to be prominent in the host response to *D. dendriticum*, and in other fish species similar non-specific cytotoxic cells have a role in the killing of protozoan parasites such as *Ichthyophthirius multifilis* (Graves, Evans & Dawe, 1985). Although macrophages may directly attack PKX cells (Rafferty, 1986), phagocytosis by neutrophils appears to have been underestimated in previous studies and in several cases neutrophils may have been mis-identified as macrophages (Ferguson & Needham, 1978; Kent & Hedrick, 1985 & MacConnell *et al.*, 1989). However, Ferguson & Needham (1978) observed the presence of neutrophils in PKX infected tissues, and Clifton-Hadley *et al.*, (1987) reported their increased numbers in rainbow trout kidneys before the onset of clinical signs of PKD. In their investigation of PKD in brown trout and Atlantic salmon Ellis *et al.*, (1985) described "large frothy cells" which were interpreted as activated macrophages. These may have been involved in the formation of the large multi-nucleated giant cells which were also observed. Alderman & Feist (1985) reported similar giant cell formation in response to *Exophiala* sp. infection in kidneys of rainbow trout recovering from PKD. Numerous giant cells were observed throughout affected kidneys and many contained fungal hyphae. However, phagocytosis of PKX cells was not recorded. Although the activated macrophages reported by Ellis *et al.*, (1985) were not seen during the present study, macrophages containing phagocytosed haemoglobin crystals were recorded, confirming the findings of Clifton-Hadley *et al.*, (1987).

In contrast to the findings of the present study Ellis *et al.*, (1985) found only a limited host response to PKX cells in the spleen and details of the cell types involved were not given. Thrombocytes and plasma cells have been noted in PKD affected kidneys Ferguson & Needham, (1978) and MacConnell *et al,.* (1989) and their presence was confirmed in the present study.

The function of eosinophilic granule cells (EGC) in fish is uncertain although they may play a role in the inflammatory response and are known to accumulate in parasitic infections (Lester & Desser, 1975; Hine, 1992). In mammals, eosinophilia is also associated with inflammation and is a prominent feature of the cellular response to macroparasites. In addition, eosinophils may also be involved in modulating hypersensitivity reactions in allergic reactions (Slauson & Cooper, 1984). The only previous report of EGC's in PKD affected fish was by Clifton-Hadley *et al.*, (1987). The authors noted their presence in large numbers in renal granulomata from fish recovering from the disease. Whether the presence of eosinophils in PKX infections is protective or not is questionable since no evidence of cell adherence was found. Ultrastructural features of the EGC's in the present study was similar to those reported by Ezeasor & Stokoe (1980) in the submucosa of the oesophagus and stomach of rainbow trout. Although populations of EGC's are particularly prominent at these sites in rainbow trout their presence has been noted at several other locations (Smith, 1975). Mammalian EGC's are known to have a limited phagocytic capability (Slauson & Cooper, 1984), however there are few reports of EGC phagocytosis in fish (Ellis, 1977) and none was observed during the present study. The significance of eosinophils in PKD remains unclear and requires further investigation.

CHAPTER 5

STRUCTURE AND DEVELOPMENT OF SELECTED SPECIES OF

MYXOSPOREANS FROM THE KIDNEYS OF FISH HOSTS

5.1 INTRODUCTION.

During the survey for myxosporean infections (Chapter 3), several species infecting renal tubules were identified. Of these *Sphaerospora elegans* (Thélohan, 1892) from the sticklebacks *Gasterosteus aculeatus* and *Pungitius pungitius*, *Myxobilatus gasterostei* (Parisi, 1912) Davis, 1944, from *G. aculeatus* and *Myxidium lieberkuehni* (Buetschli, 1882) infecting the kidney and urinary tract of pike *Esox lucius* produced trophic stages of similar size and morphology to the PKX parasite. These were investigated further to determine possible relationships with PKX.

Feist (1988) suggested a possible link between *S. elegans* and PKX in view of the wide distribution of the host and its frequent association with fish farms. In addition, the author speculated that the production of renal secretions used during nest building might also liberate spores into the environment. Although the spawning period for sticklebacks occurs several weeks before clinical signs of PKD typically appear in susceptible fish, the liberation of *S. elegans* spores approximately coincides with the time that the fish first become infected. Consequently, studies into the structure and development of *S. elegans* in the sticklebacks *G. aculeatus* and *P. pungitius* were undertaken to determine structural affinities with the PKX parasite. The results of which were described by Feist, Chilmonczyk & Pike (1991) and Lom, Pike & Feist, (1991). Supplementary information only is presented in this chapter.

M. gasterostei was also found as a double infection with *S. elegans* in *G. aculeatus* from the River Avon (Table 3). Thélohan (1890) was the first to notice the presence of myxosporean spores with long caudal appendages in the renal tubules of *G. aculeatus*. Two species, namely, *Henneguya media* and *Henneguya brevis* were subsequently named although no figures were provided (Thélohan, 1892). *H. media* was figured in Thélohan's (1895) paper but details of the spore were indistinct. *H. brevis* was not figured. A third species, *Henneguya gasterostei* was discovered by Parisi (1912). The author figured trophozoites and spores of *H. gasterostei* with polar

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capsules lying in the sutural plane as in other members of the *Myxobolidae* Thélohan, 1892. A fourth renal *Henneguya* species was named by Schuurmans Stekhoven (1920) from *G. pungiticus* (= *P. pungitius*) however, the validity of this species does not appear to have been recognised subsequently (Shulman & Shtein, 1964; Shulman, 1966; Bauer, 1984). *H. medius* and *H. gasterostei* were later transferred to the genus *Myxobilatus* by Davis, (1944). This genus was erected within the family *Sphaerosporidae* (Davis, 1917), to accommodate those species with two polar capsules opening at the anterior tip of the spore and situated in a plane perpendicular to the sutural line, rather than in the sutural plane typical of the genus *Henneguya* (family *Myxobolidae* Thélohan, 1892). Members of the genus *Myxobilatus* also possess anteriorly pointed shell valves with fine longitudinal ridges and each valve possesses a caudal appendage (Davis, 1944; Lom & Noble, 1984). *M. gasterostei* (Parisi, 1912) Davis, 1944 is currently recognised as the type species and apart from a brief description of *M. gasterostei* spores from North American *G. aculeatus* (Arthur & Margolis, 1975), infections with this and other *Myxobilatus* species have attracted relatively little attention and their potential as pathogens of fish is unknown (Lom, 1986).

M. lieberkuehni from pike *E. lucius* was one of the first myxosporeans to be described (Lieberkuehn, 1854), although the species was not named until later (Buetschli, 1882). Although plasmodia occur within the renal tubules, the main site of infection is the urinary bladder. In addition to the coelozoic plasmodia, numerous parasitic stages within hypertrophied glomerular cells were also described (Debaisieux, 1919, 1920; Weissenberg, 1922, 1923). Debaisieux regarded the parasites as stages in the development of *M. lieberkuehni*. Weissenberg, however, disagreed and considered them to belong to a new species of myxosporean parasite which he named *Nephrocystidium pickii* (Weissenberg, 1923).

During investigations to assess the impact of PKD in pike, infections with *M. lieberkuehni* were noted both in the urinary bladder and in renal tubules. Glomerular infections with *N. pickii* and extrasporogonic stages of an unidentified myxosporean in the collecting duct epithelial cells were also noted. Results of these investigation were

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provided by Lom, Dyková & Feist, (1989) who speculated that *N. pickii* represented an extrasporogonic stage of *M. lieberkuehni*. Only supplementary data will be presented in this chapter.

5.2   MATERIALS AND METHODS.

Two species of sticklebacks, *G. aculeatus* and *P. pungitius* were collected by hand or seine netting at a variety of sites (Table 7) between 1986 and 1990. A total of 42 pike, *E. lucius* were sampled by electric-fishing from the rivers Avon (Wilts.), Frome (Dorset), Itchen (Hants) and Stour (Dorset). Details of sample numbers per river and month of sampling are given in chapter 3 (Table 3). Procedures for the dissection of fish and details of the techniques employed for the examination of fresh and fixed tissues are given in chapter 3.

5.3   RESULTS.

5.3.1   *Sphaerospora elegans* (Thélohan, 1892) in *Gasterosteus aculeatus* and *Pungitius pungitius*.

Details concerning the structure and development of *S. elegans* in the above hosts have already been published by Feist *et al.*, (1991) and Lom *et al.*, (1991). Pseudoplasmodia and spores of *S. elegans* were found in the renal tubules and glomeruli of *G. aculeatus* from 5 out of the 6 sites sampled and in *P. pungitius* kidneys from both sites where fish were obtained. Numbers sampled and percentage prevalence of infection are given in Table 7.

Heavily infected renal tubules were frequently occluded with pseudoplasmodia and spores of *S. elegans* and appeared dilated with a reduction in the height of the epithelial cells (Fig. 68). However, many tubules appeared free from infection.

Pseudoplasmodia were noted within glomeruli of most fish exhibiting renal tubule infections (Fig. 69) in which site they were attached to the epithelial microvilli by small interdigitating pseudopodia (Fig. 70). In heavy infections, pseudoplasmodia were also found free in the tubule lumen.

The plasmalemma of pseudoplasmodia lacked a surface coat. Internally,

Table 7. Sample sites and percentage prevalence of *Sphaerospora elegans* in *Gasterosteus aculeatus* L. and *Pungitius pungitius* L.

Source	Nos. fish examined (percentage infected with with *S. elegans* in brackets)	
	G. aculeatus	*P. pungitius*
1. River Avon (Wiltshire) NGR SU 181236	156 (55)	26 (58)
2. The Kirkley Run, Lowestoft (Suffolk) NGR TM 545927	36 (6)	41 (37)
3. River Weaver (Devon) NGR ST 073066	202 (16)	-
4. River Erme (Ludbrook) (Devon) NGR SX 647535	34 (0)	-
5. River Hooke (Dorset) NGR ST 534005	20 (30)	-
6. The Fleet (Dorset) NGR SY 563842	22 (86)	-
	470	67

NGR = National Grid Reference.

Figure 68. Renal tubule of *Gasterosteus aculeatus* containing pseudoplasmodia and spores of *Sphaerospora elegans* (arrow). Giemsa, x350.

Figure 69. Pseudoplasmodia (arrow) within Bowman's space of the glomerulus and the proximal portion of the renal tubule. Normarski interference contrast, x1,125.

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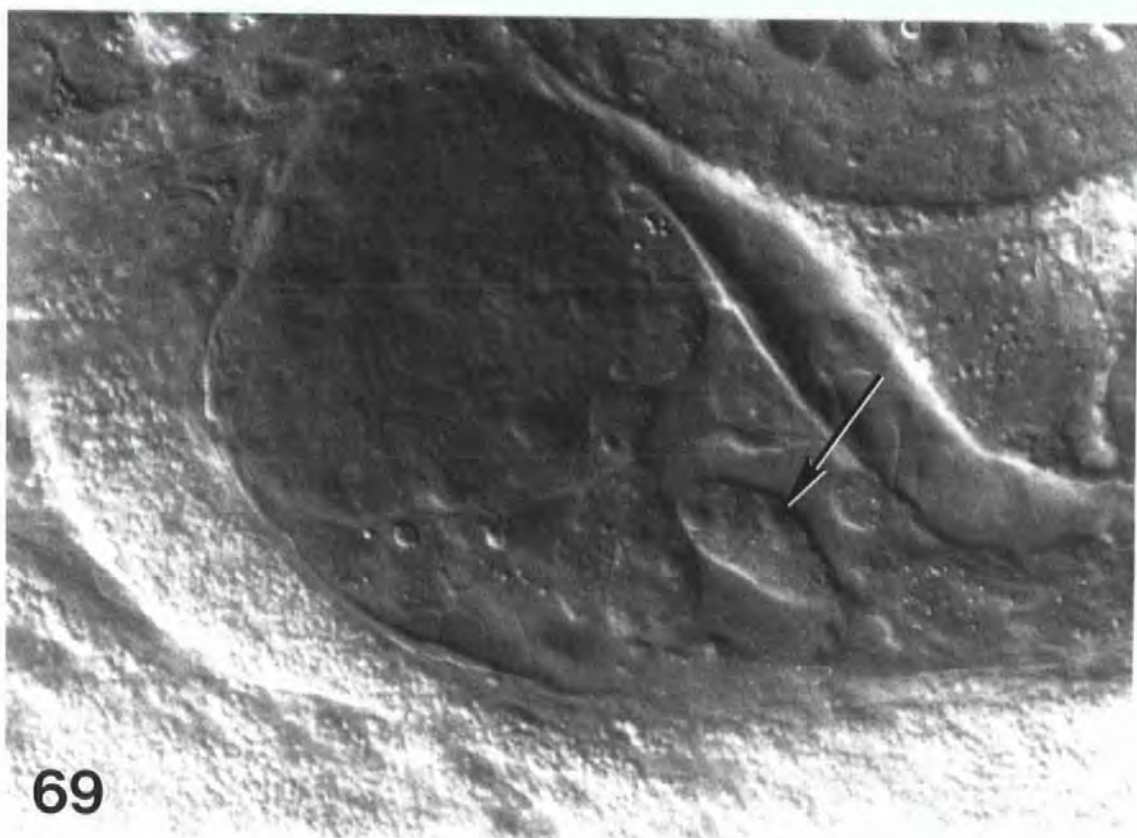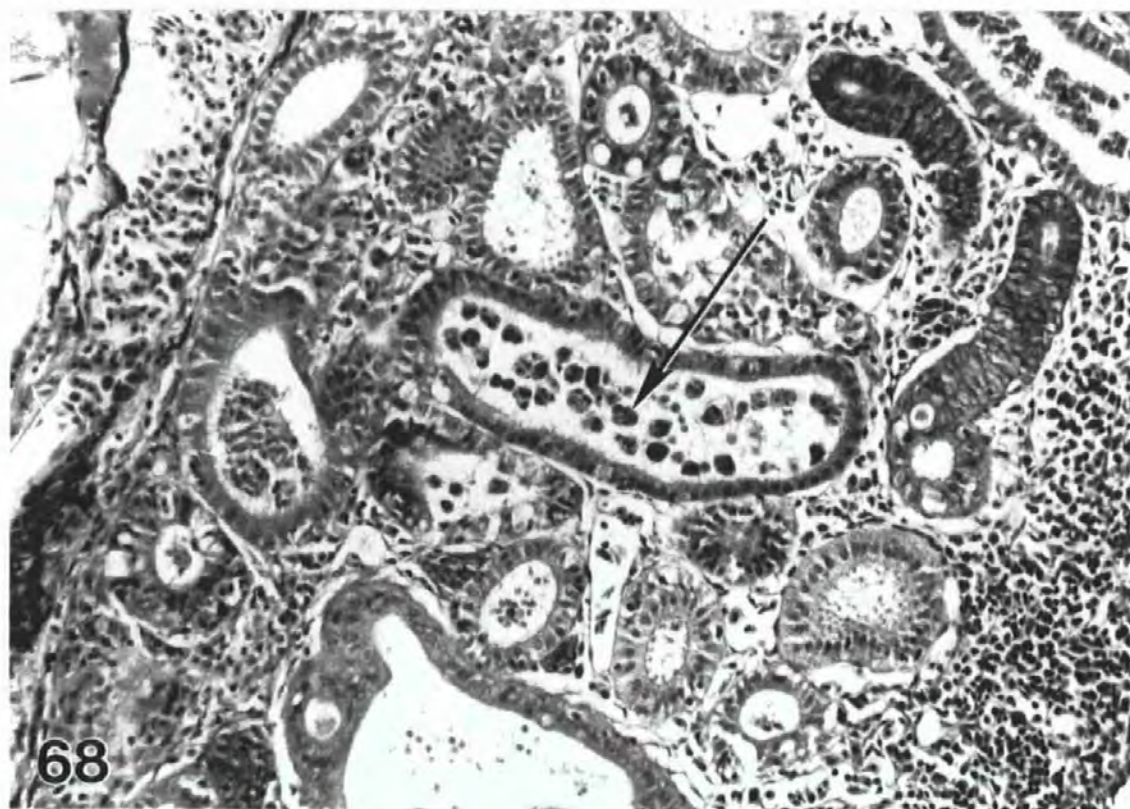
mitochondria, often with detached plate-like cristae, and a variety of small vescicles were the most numerous structures present (Fig. 71). RER and occasional Golgi bodies were also observed. Sporogonic cells were bounded by two membranes, the outer arising from the pseudoplasmodium which was closely applied to the inner membrane belonging to the sporogonic cell itself (Fig. 72). Sporogonic cells, characterised by the presence of a large nucleus and numerous rounded mitochondria, were found singly or in groups. Pairs of sporogonic cells were occasionally observed in close association. In these cases a cytoplasmic bridge between the two cells could sometimes be observed (Fig. 72).

Spores of *S. elegans* (Fig. 73) have already been described by Feist *et al.*, (1991). The description of stages in early sporogenisis given by these authors is further supported here by micrographs of these stages from renal impression smears stained with May-Grunwald Giemsa (Fig. 74a-e). The earliest stage observed consisted of a pseudoplasmodium containing a single sporogonic cell (Fig. 74a). More advanced stages with two sporogonic cells were more frequently observed (Fig. 74b). Division of the sporogonic cells continued until the twelve cells required to form the disporoblast were present (Fig. 74c-e). Subsequent maturation of spores could not easily be followed from stained impression smears since the developing shell valves became densely staining and obscured details of internal changes.

Possible extrasporogonic stages of *S. elegans* were present in the choroidal *rete* of several kidneys of *G. aculeatus* from fish co-infected with spores of *S. elegans* and *M. gasterostei*. In fresh preparations, the stages appeared rounded with numerous refractile granules and cellular inclusions present within the cytoplasm (Fig. 75). Ultrastructural features of the parasite were described by Lom *et al.*, (1991). Although the basic structure was similar to the PKX parasite, the characteristic haplosporosomes were not observed. Histologically, a cellular host response was absent (Fig. 76), the parasites lodging themselves within the capillaries without any specific form of attachment.

Figure 70.   Pseudoplasmodium of *Sphaerospora elegans* (P) attached to the renal tubule epithelium (E) by cytoplasmic pseudopodia (arrow). x10,640.

Figure 71.   A pseudoplasmodium containing three sporogonic cells (S). The primary cell cytoplasm contains numerous mitochondria (arrow) and a variety of vacuoles and vesicles. x10,000.

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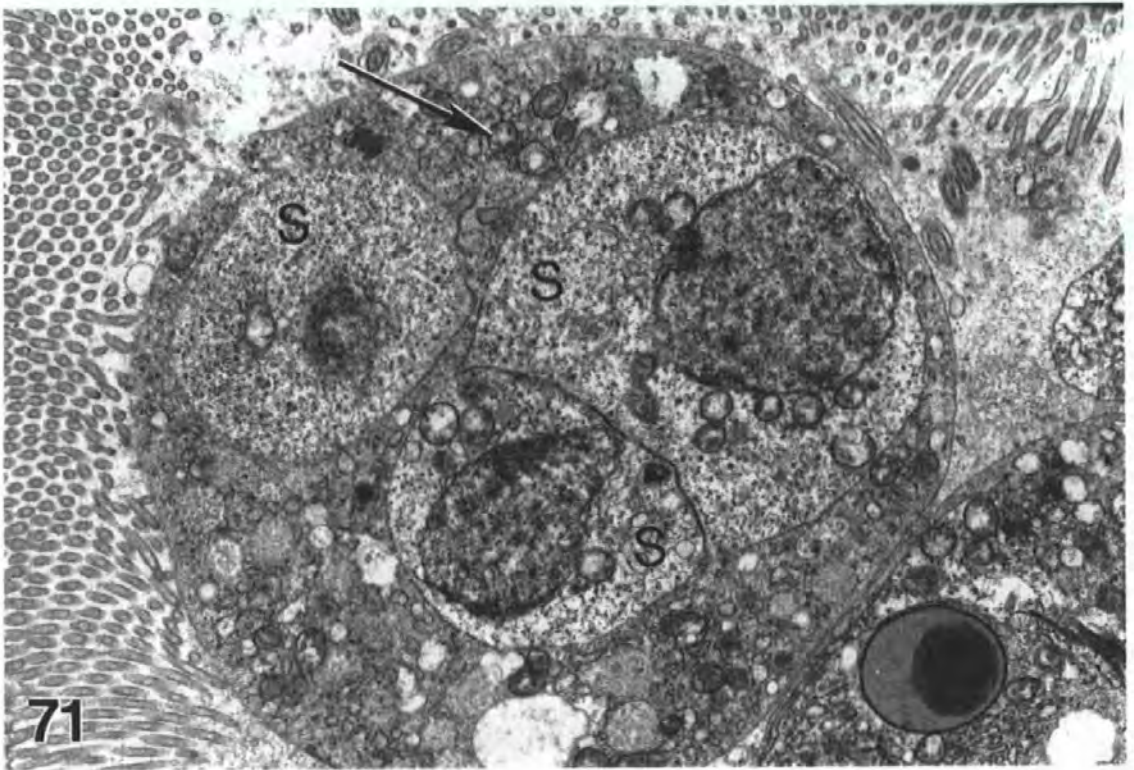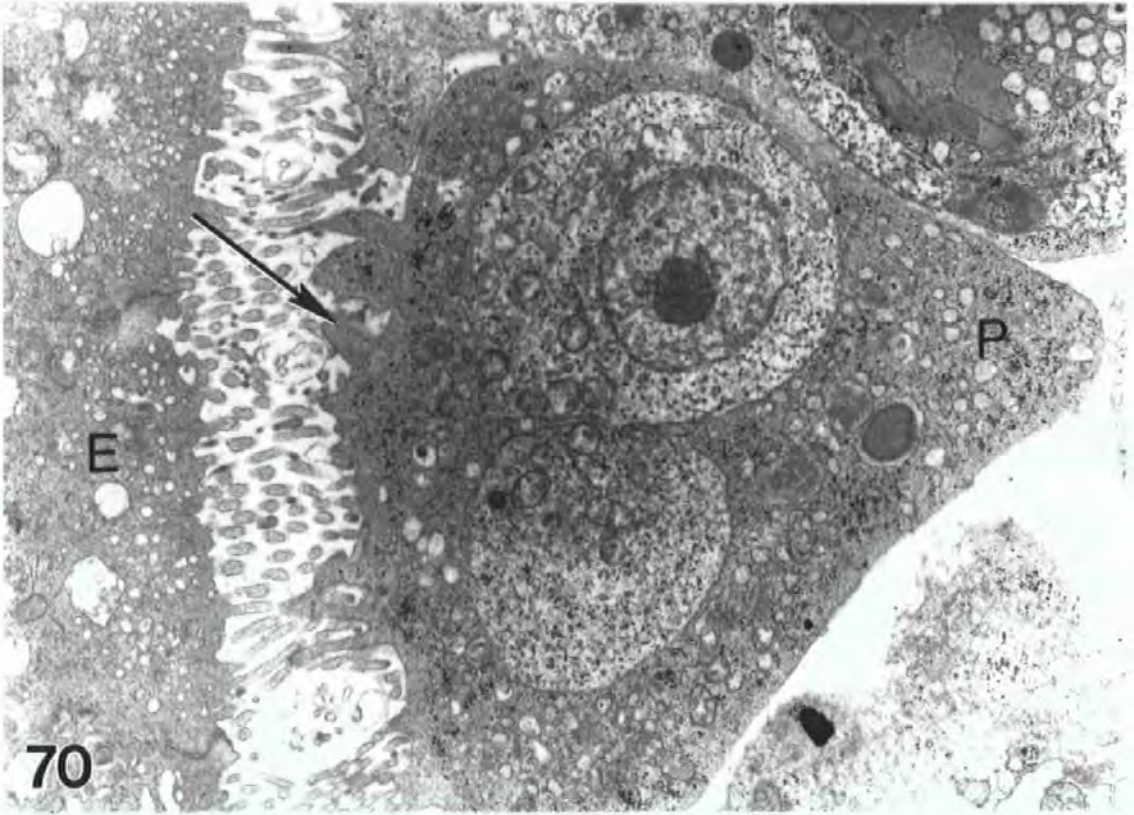
Figure 72.   Detail of sporogonic cells showing the presence of a cytoplasmic bridge between two of the cells (arrow) and the close association of the sporogonic cell membranes with the vacuole membrane of the pseudoplasmodium (*). Sporogonic cell nucleus (N), mitochondria (M). x20,000.

Figure 73.   Mature spores of *Sphaerospora elegans*. Normarski interference contrast, x1,275.

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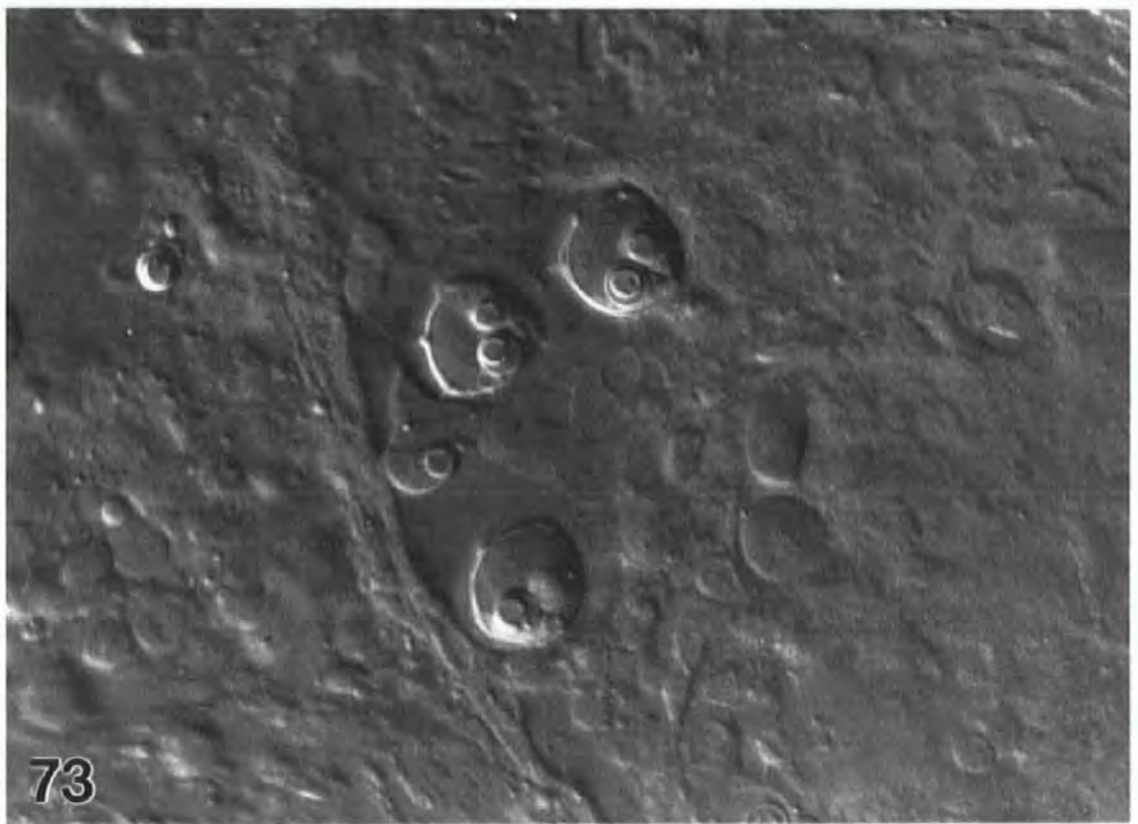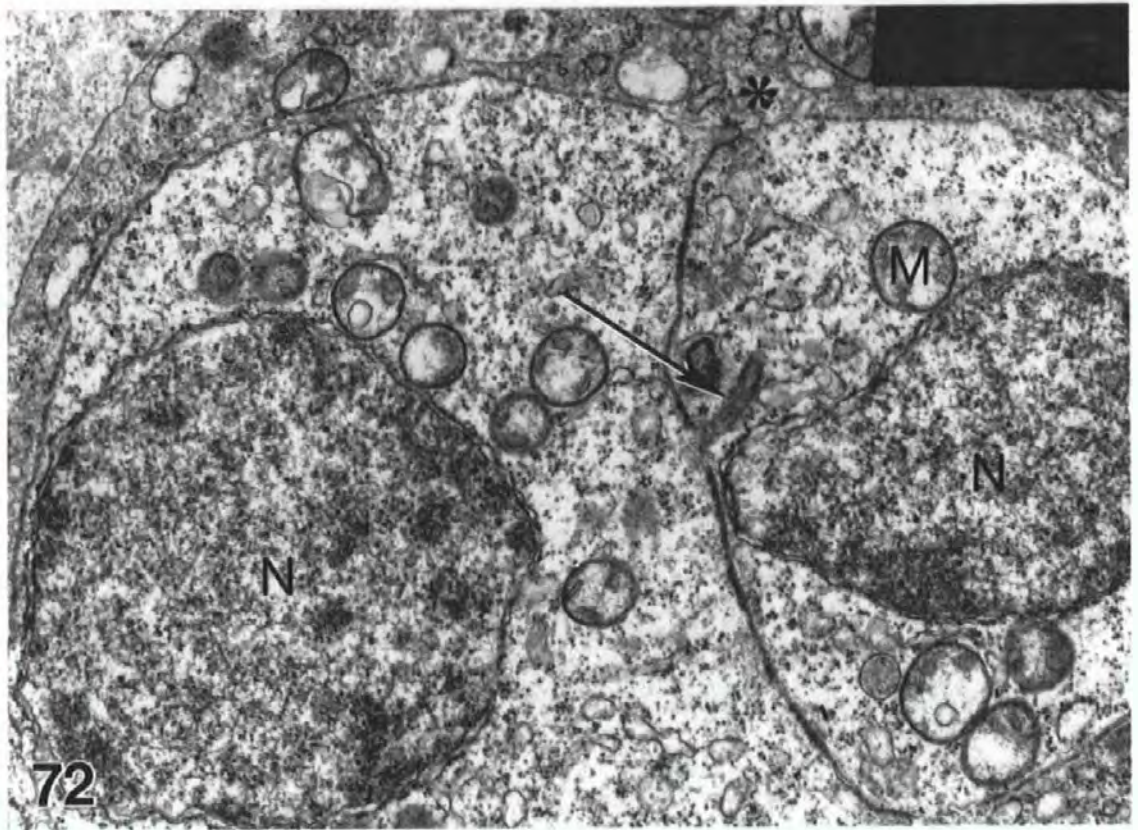
Figure 74(a-e). (a) A small pseudoplasmodia (arrow) containing a single sporogonic cell, (b) pseudoplasmodium containing two sporogonic cells undergoing synchronous division (arrows), (c) a more advanced stage with four sporogonic cells, note the presence of cytoplasmic vacuoles (arrow), (d) a disporic pseudoplasmodium containing the twelve cells required to form two spores. Two capsulogenic cells with capsular primordia present are visible (arrow), (e) pseudoplasmodium with two almost mature spores, valvogenic (v), capsulogenic (c) and sporopasm cells (s) are clearly visible. May-Grunwald Giemsa, x1,150.

Figure 75. Possible extrasporogonic stage of *Sphaerospora elegans* in the choroidal *rete*, refractile granules (G) and secondary cells (S) can be seen. Normarski interference contrast, x1,250.

Figure 76. Extrasporogonic stages within capillaries of the choroidal *rete* (arrow), note the lack of host response. Giemsa, x410.

74a

b

c

d

e

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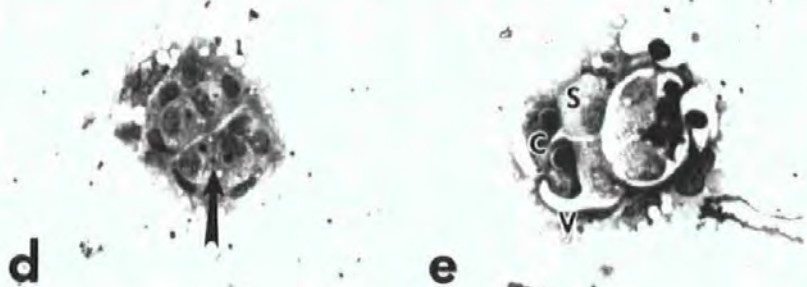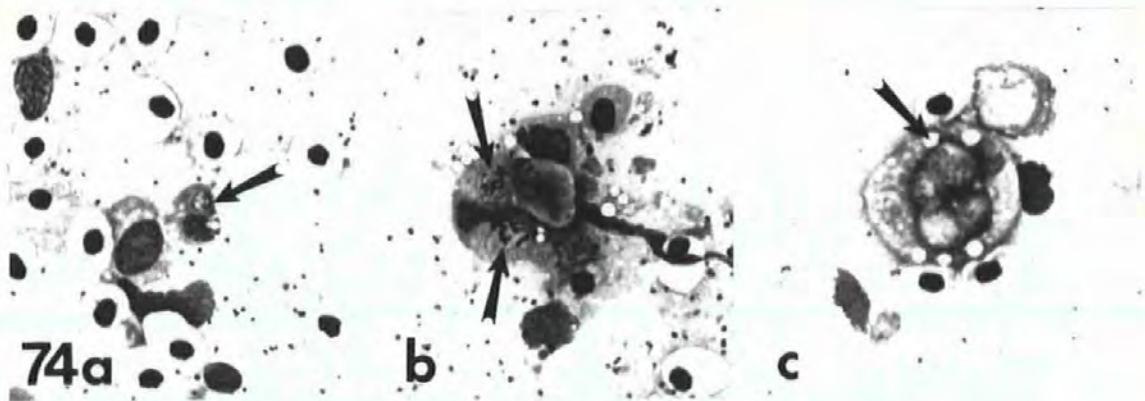
5.3.2 *Myxobilatus gasterostei* (Parisi, 1912) Davis, 1944 in *Gasterosteus aculeatus*.

Spores of *M. gasterostei* were found in the kidneys of 59% *G. aculeatus* examined from the River Avon (Wilts.) and 39% of the fish sampled from The Kirkley Run, Lowestoft (Suffolk) (Table 3). The renal tubules were frequently occluded with spores and developmental stages (Fig. 77) both of which were also observed within Bowman's space in the glomerular capsule (Fig. 78) and occasionally in the urinary bladder. Most cases occured as a co-infection with *S. elegans*, and low level infections with *M. gasterostei* were frequently obscured by large numbers of spores and pseudoplasmodia of *S. elegans*, a species frequently co-inhabiting the same host.

Plasmodia of *M. gasterostei*, measuring up to 45μm in diameter, contained numerous refractile granules and at least two spores, which were frequently observed protruding through the plasmodial wall (Fig. 79). Generative cells and vegetative nuclei were detected within luminal plasmodia. Although sequential events in early sporogenisis were not observed, pansporoblast formation by the envelopment of a sporogonic cell by a pericyte was confirmed (Fig. 80). Disporic sporoblasts were formed within a vacuole of the plasmodium (Fig. 81). The association of valvogenic, capsulogenic and sporoplasm cells followed the general pattern seen in other bivalvulid myxosporeans, the valvogenic cells enveloping the capsulogenic cells and the single binucleate sporoplasm cell which contained numerous spherical osmiophilic sporoplasmosomes measuring 200nm in diameter (Fig. 82). Mature spores of *M. gasterostei* were fusiform, anteriorly pointed with several fine longitudinal ridges running the length of the spore terminating at the caudal appendages (Figs. 83 & 84). Spores were 40.6(36-45)μm (n=15) in length including the caudal appendages. The spore body measured 18.3(17-20)μm in length with an additional 22.3(19-25)μm for the caudal appendages. In valvular view spores measured 7.1(7-8)μm in width and the two unequal polar capsules 10.3(9.5-11)μm and 8.85(8-9)μm in length.

Infections with *M. gasterostei* were frequently heavy and it was usual to observe renal tubules filled with plasmodia and spores. In these cases the tubules became dilated and epithelial cells became flattened. In severe infections the tubule epithelium ruptured allowing spores and plasmodia to infiltrate the interstitial renal tissues. The

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Figure 77. Plasmodia and spores of *Myxobilatus gasterostei* (arrow) within a renal tubule of *Gasterosteus aculeatus*. Normarski interference contrast, x375.

Figure 78. Plasmodia of *Myxobilatus gasterostei* with conspicuous refractile granules (arrow) within Bowman's space of a glomerulus. Two spores of *M. gasterostei* can also be seen (S). Normarski interference contrast, x1,275.

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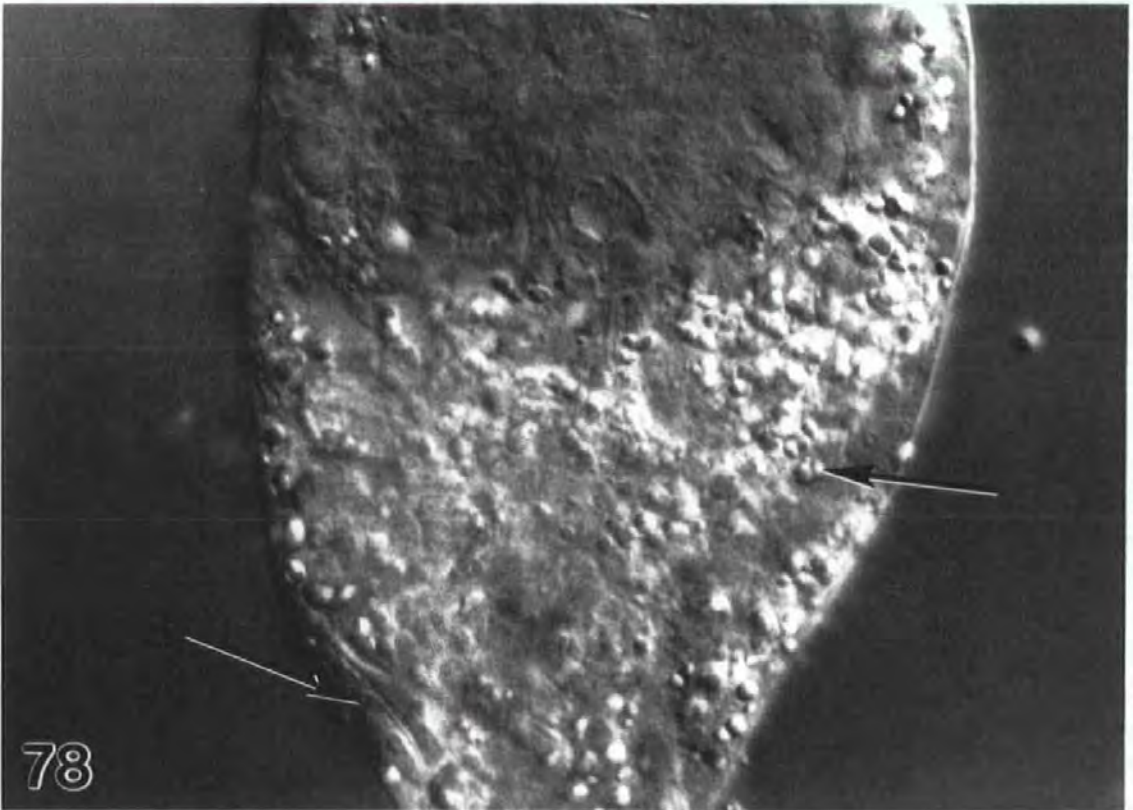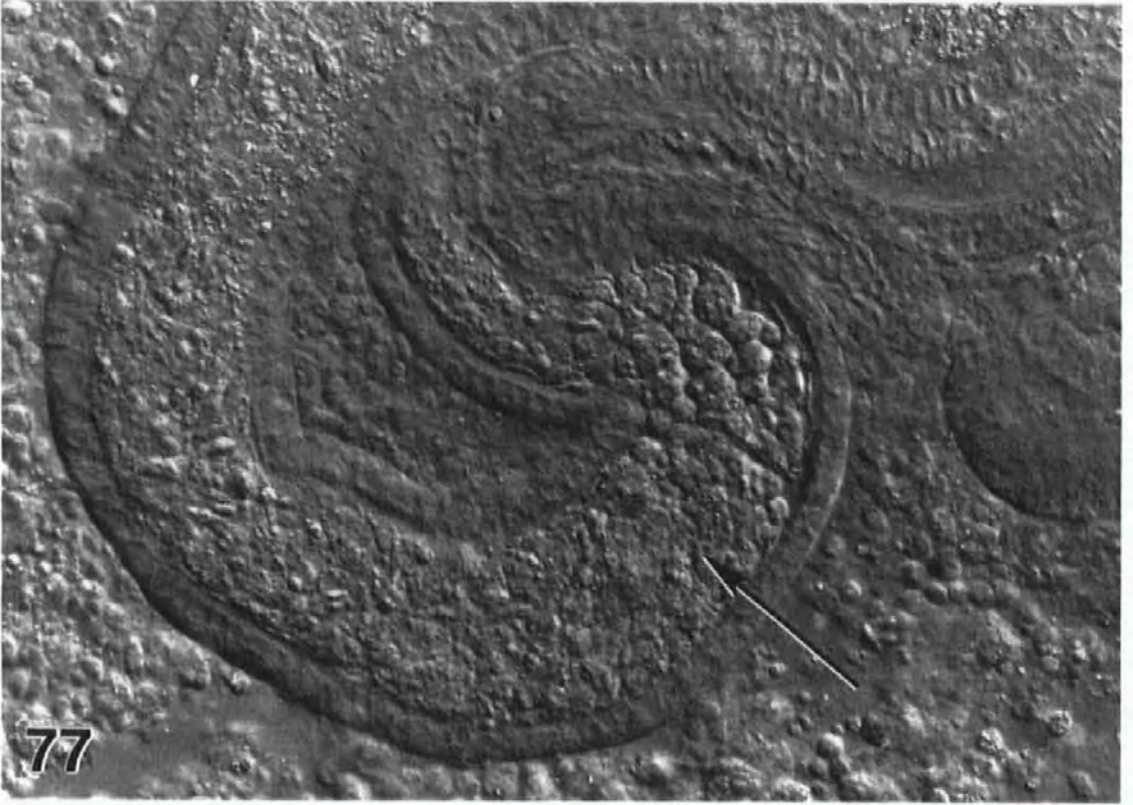
Figure 79. Disporic plasmodia of *Myxobilatus gasterostei.* Developing spores (arrowed) and numerous cytoplasmic granules can be seen. Normarski interference contrast, x1,275.

Figure 80. Plasmodium of *Myxobilatus gasterostei* (arrowed). Plasmodial nucleus (N), pericyte (P), sporogonic cell (S) and several generative cells (G) are present. Host epithelium (E). x14,180.

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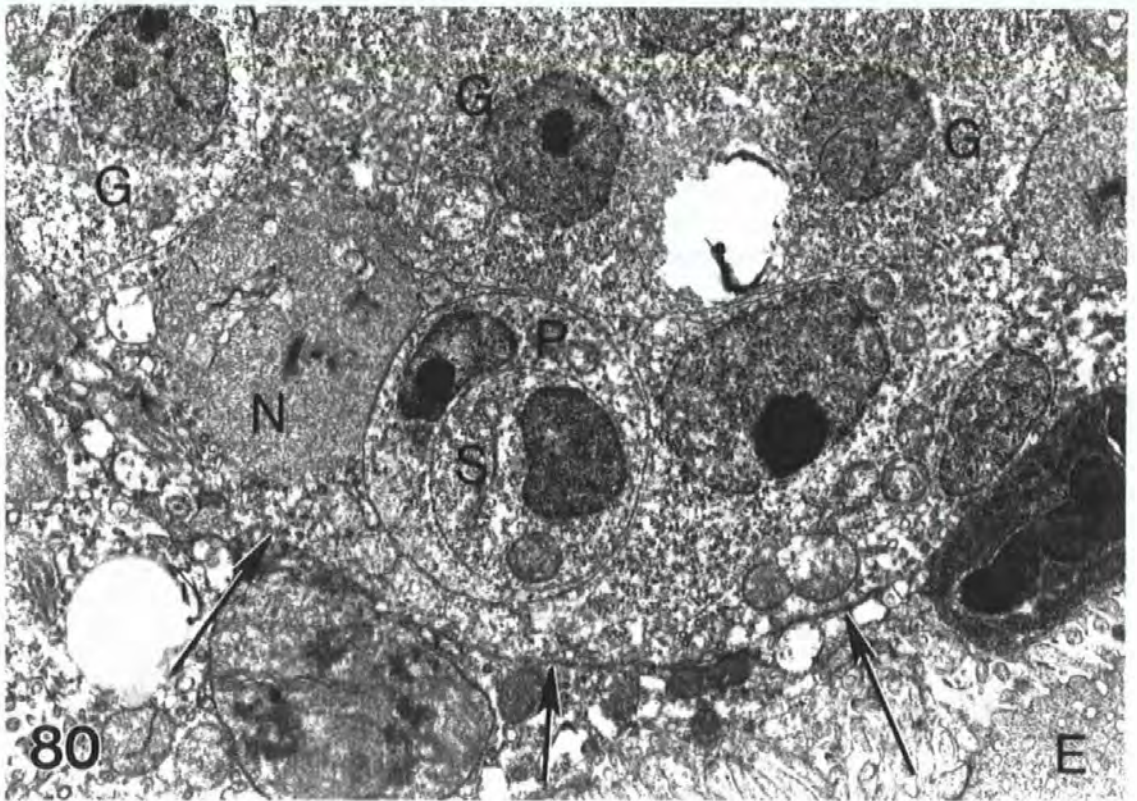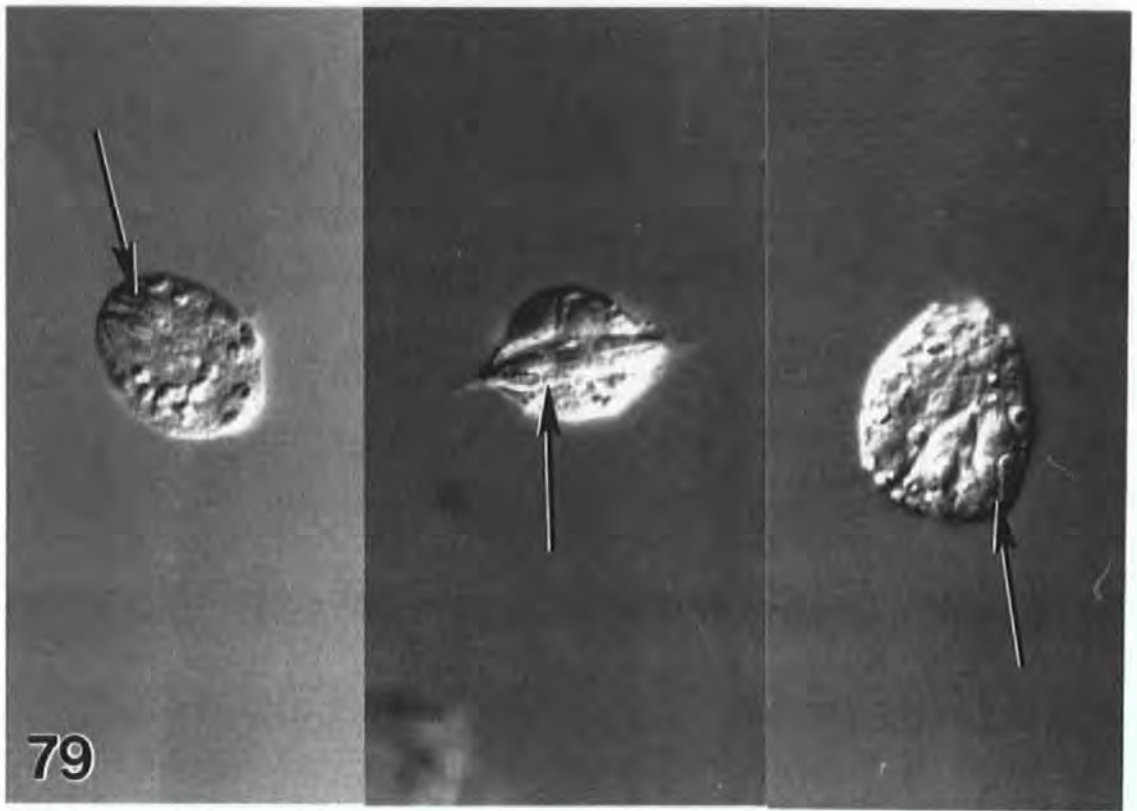
Figure 81.   Disporic sporoblast of *Myxobilatus gasterostei* contained within a
              vacuole of the plasmodium (arrowed).   Sporoplasm cell (S),
              capsulogenic cell (C), valvogenic cell (V). x6,700.

Figure 82.   Transverse section through two spores of *Myxobilatus gasterostei*.
              Sporoplasmosomes (arrows) are conspicuous within the binucleate
              (N) sporoplasm cells. Characteristic ridges of the valvogenic cells
              (V) and the sutures (S) are also visible. x14,040.
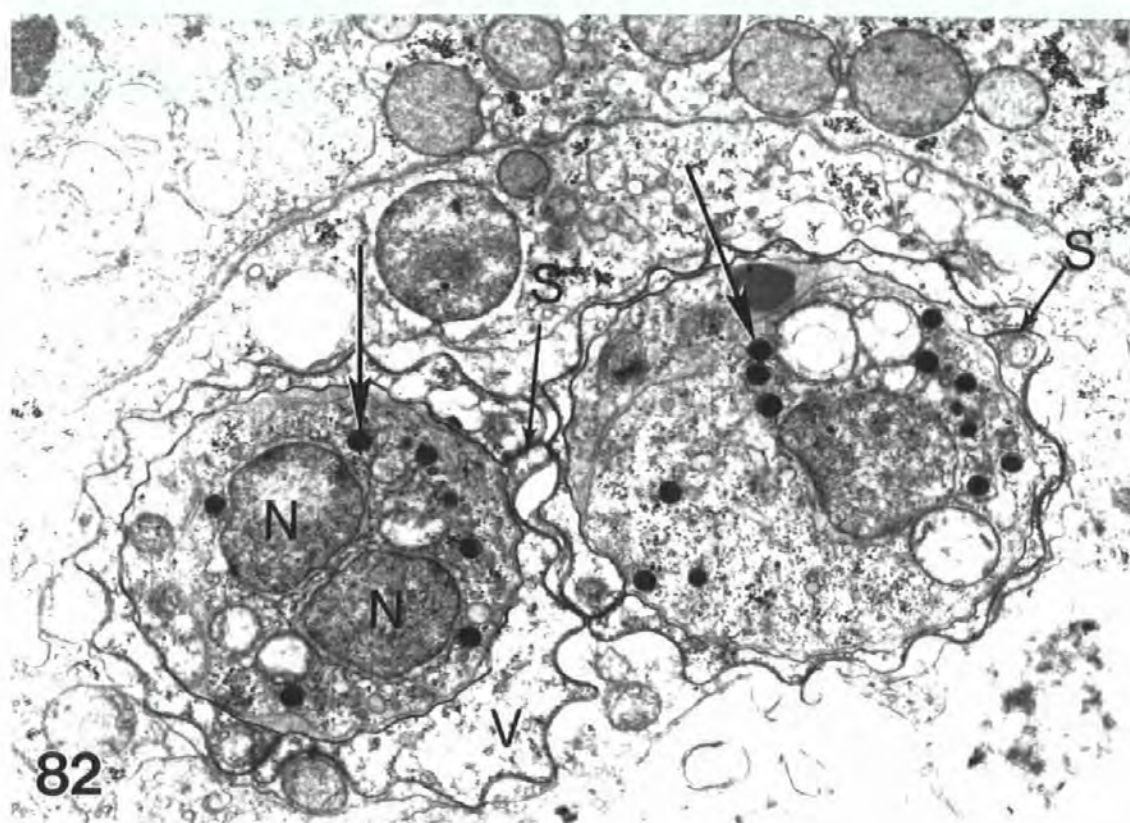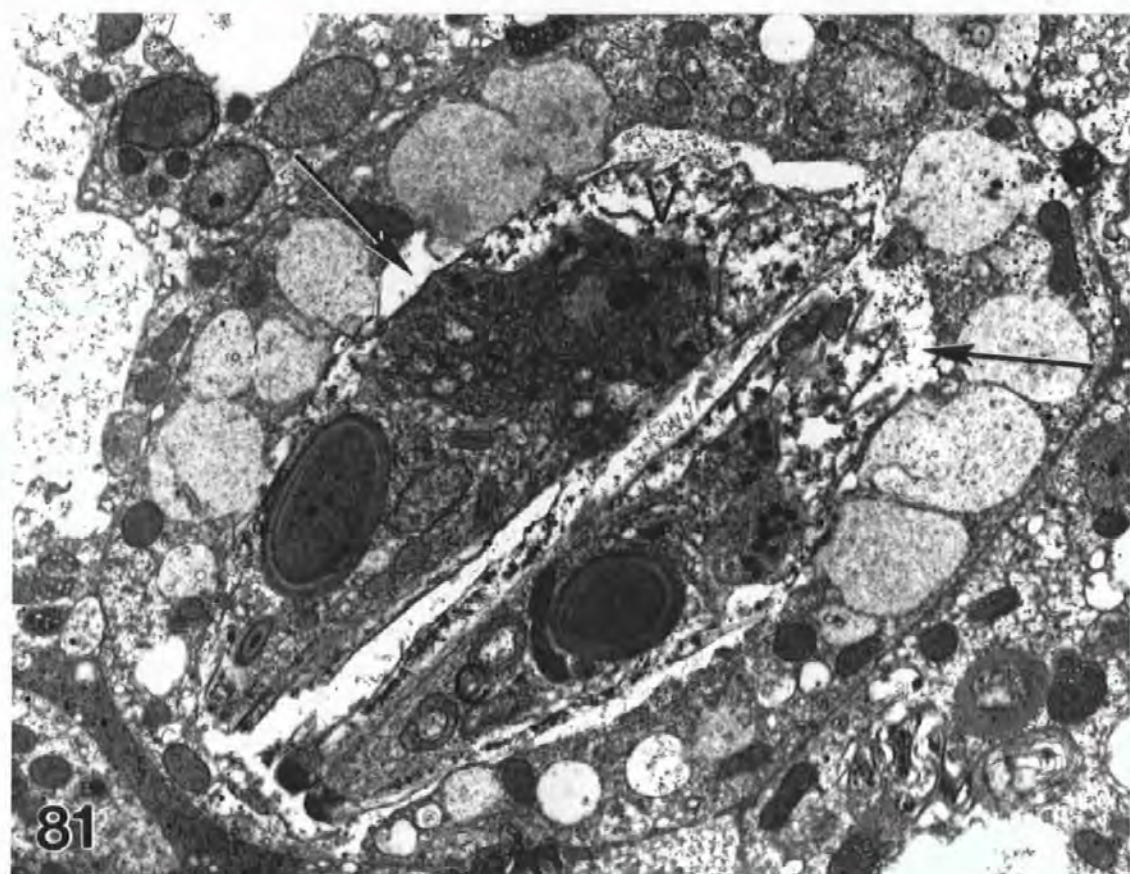
Figure 83.   Mature spores of *Myxobilatus gasterostei*. Normarski interference contrast, x1,250.

Figure 84   See Page 103.

Figure 85.   Granulomatous response to the presence of spores of *Myxobilatus gasterostei* (arrowed) released into the renal interstitium of *Gasterosteus aculeatus*. Giemsa, x380.

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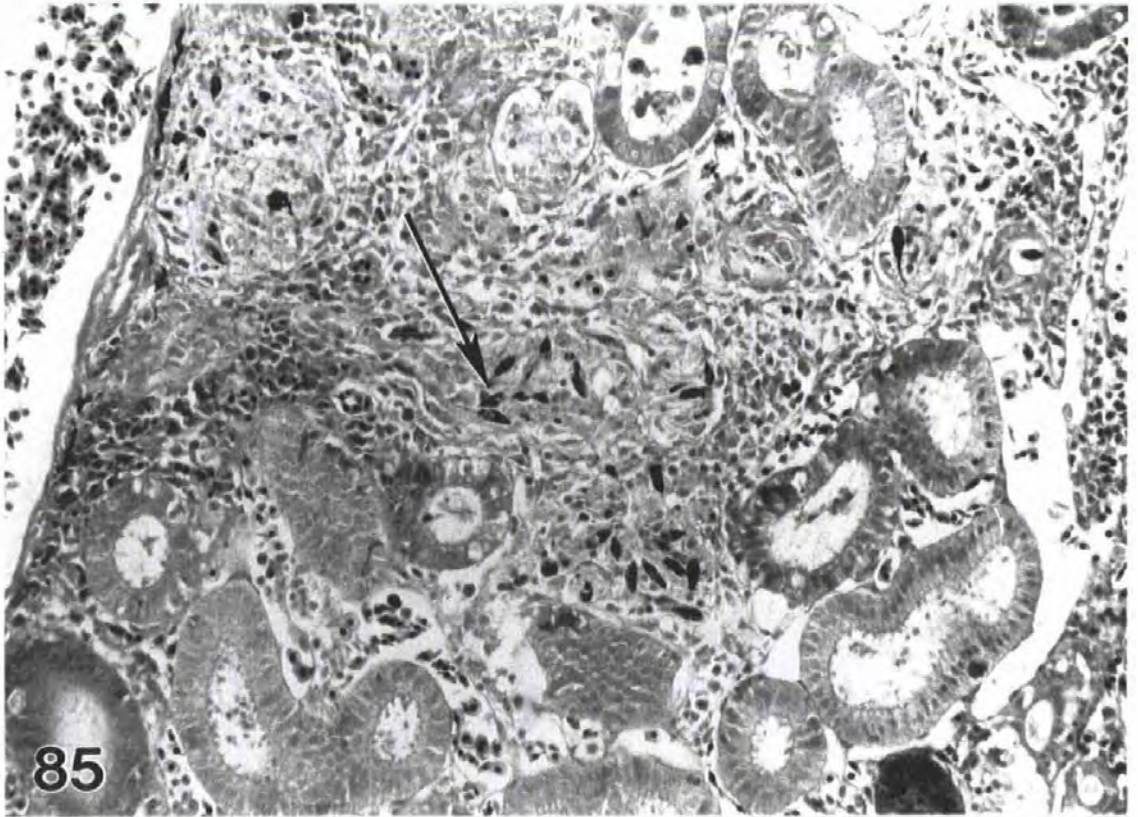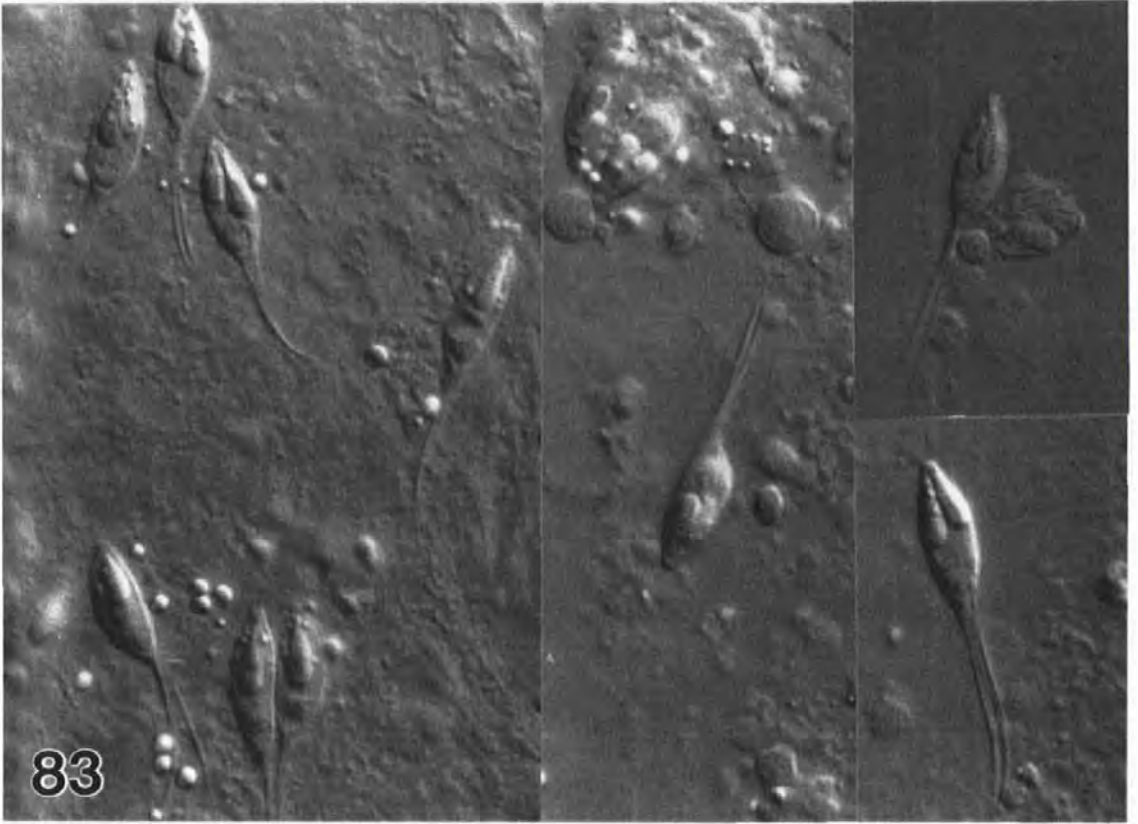
Figure 84.   Diagram of spores of *Myxobilatus gasterostei* in valvular (A) and sutural view (B).

A   B

10μm
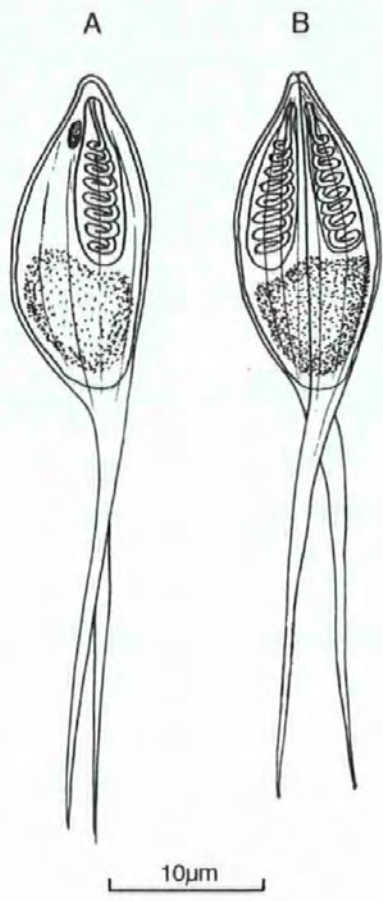
host reaction involved focal inflammation with an increase in the numbers of phagocytic cells in the affected areas. This initial response was followed by phagocytosis of the parasites and formation of melano-macrophage centres within which numerous spores could be seen. In extreme cases, the presence of numerous parasites provoked a granulomatous response (Fig. 85). In each of these cases most of the glomeruli and excretory tubules remained intact.

5.3.3 *Myxidium lieberkuehni* (Buetschli, 1882) in *Esox lucius*.

A total of 86% of pike examined during this study were infected with *M. lieberkuehni* (Table 3). Large polysporic plasmodia measuring up to 280µm by 150µm in size were found attached to the epithelium of the urinary bladder or within the lumen. Heavy infections with *M. lieberkuehni* imparted a reddish/orange appearance to the urinary bladder due to the presence of pigment granules within the plasmodia. At the light microscope level plasmodia were characterised by the presence of numerous microvilli and a clear ectoplasmic layer surrounding the developing spores (Fig. 86). Smaller plasmodia, measuring up to 30µm in diameter, were observed in renal tubule lumens which in heavy infections were occluded with parasites (Fig. 87). These plasmodia lacked microvilli and contained large amounts of PAS positive material (Fig. 88). Numerous vegetative and generative cell nuclei were detected with the aid of the Feulgen technique for DNA (Fig. 89).

Spores were fusiform 19.2(18-21)µm (n=20) in length, with attenuated ends and 5.7(4-6.5)µm in width. Shell valves bore several longitudinal striations. Polar capsules were pyriform in shape and 4.7(4.5-5.5)µm (n=30) in length (Fig. 90). Host response to the renal tubule plasmodia was minimal. In a few cases the tubules became dilated and the tubule epithelial cells became flattened. There was no evidence of an inflammatory response (Fig. 91).

In kidney impression smears the renal tubule plasmodia were visualised as flattened structures of varying size. Numerous generative cells with dark blue staining cytoplasm, vegetative nuclei and large vacuoles were detected on staining with the May-Grunwald technique (Fig. 92a-d). Accumulations of intensely osmiophilic

Figure 86. Plasmodia of *Myxidium lieberkuehni* from the urinary bladder of *Esox lucius*. Note the presence of the clear ectoplasmic layer within the plasmodia (arrowed). Normarski interference contrast, x390.

Figure 87. Plasmodia and spores of *Myxidium lieberkuehni* within a renal tubule of *Esox lucius* (arrow). Host epithelium (E). Normarski interference contrast, x370.

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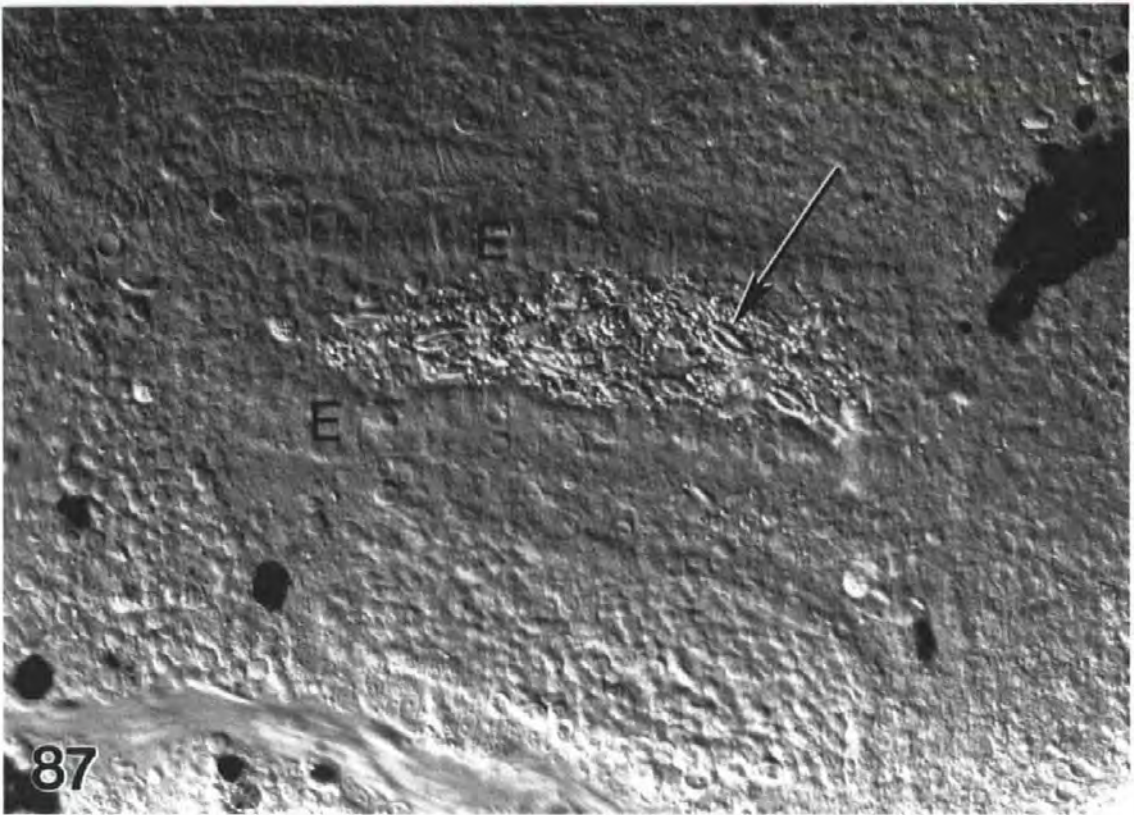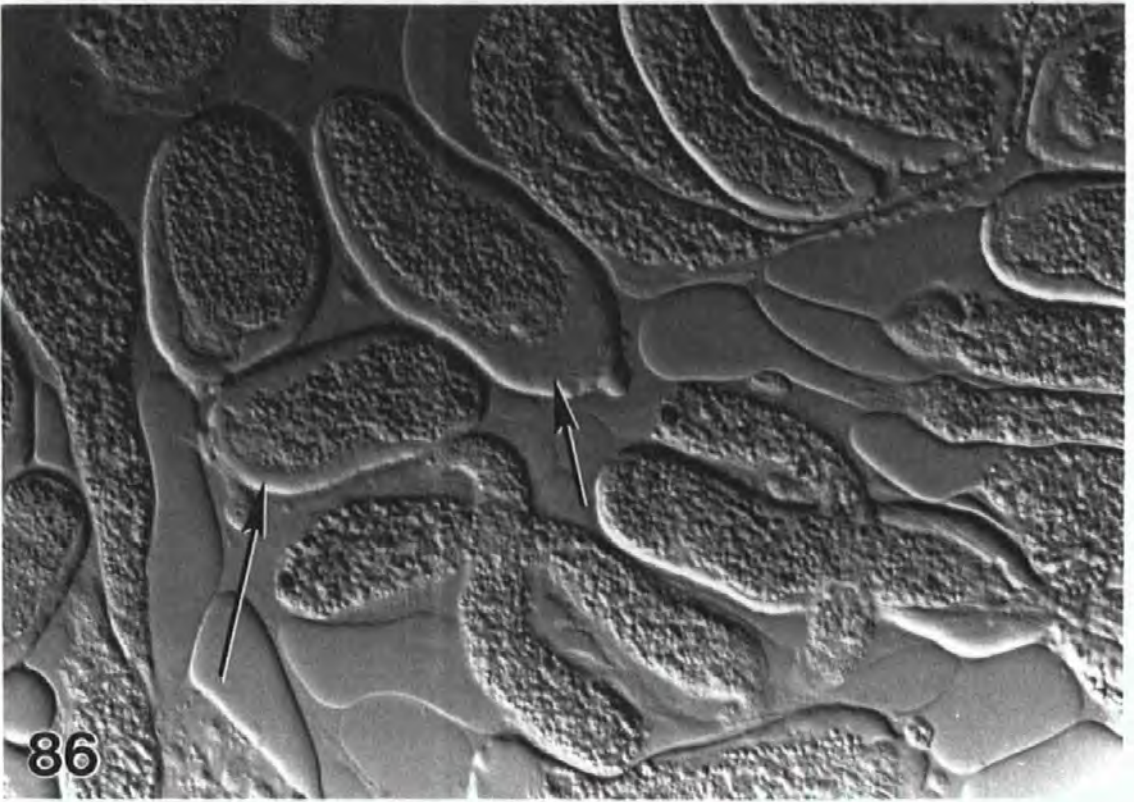
Figure 88. Plasmodia of *Myxidium lieberkuehni* (arrow).   Large amounts of PAS positive material is present in the primary cell cytoplasm. PAS, x900.

Figure 89. Vegetative and generative cell nuclei within *Myxidium lieberkuehni* plasmodia (arrow), demonstrated by the Feulgen technique for DNA. Within a glomerular xenoma cell nuclei of extrasporogonic stages are also demonstrated (*). Hypertrophied cell nucleus (N). Feulgen, x925.

Figure 90. Mature spores of *Myxidium lieberkuehni* (arrow) within a plasmodium.   Numerous refractile granules are also present. Normarski interference contrast, x1,250.
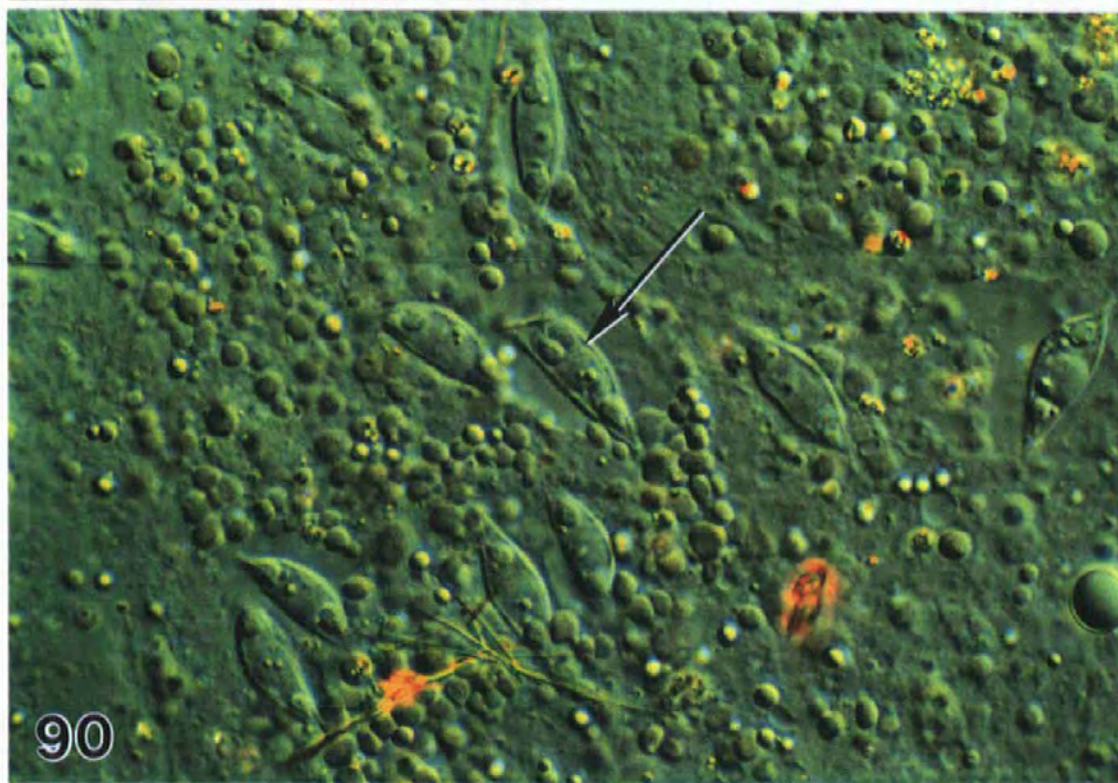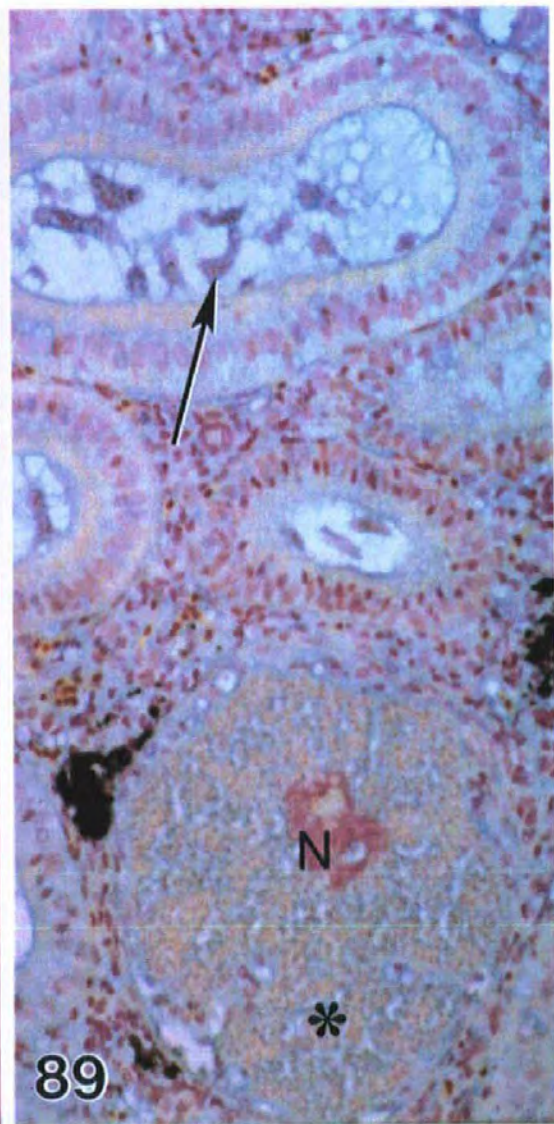
Figure 91.	Plasmodia and spores of *Myxidium lieberkuehni* within renal tubules of *Esox lucius* (*). Note the increased luminal diameter and reduction in the height of infected tubules (arrow). Normal tubule epithelium (E). Giemsa, x375.

Figure 92(a-d).	Stages in the growth and development of plasmodia of *Myxidium lieberkuehni*; (a) a young stage containing two generative cells (arrow), (b) a more advanced plasmodium with three generative cells (arrows), (c) during plasmodial growth numerous vacuoles become apparant (arrow), (d) as (c), generative cells are darkly stained (arrow), vegetative nuclei are also clearly seen (V). Renal impression smear, May-Grünwald Giemsa, x1,275.
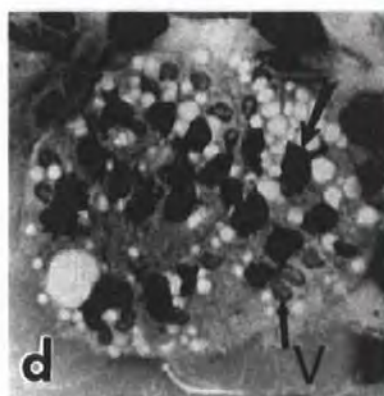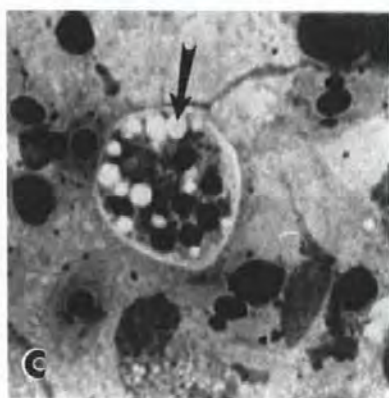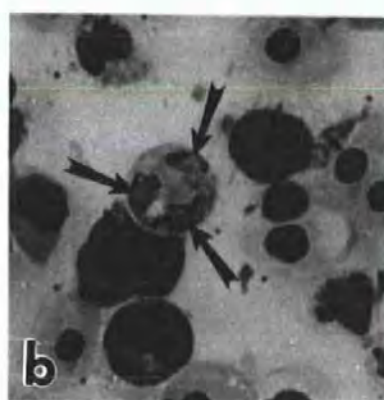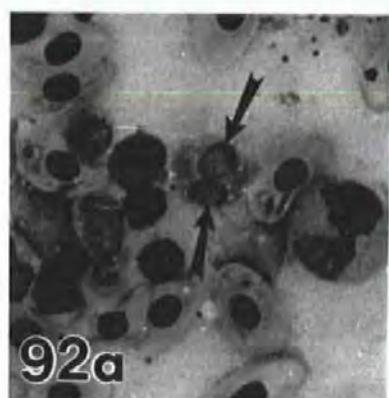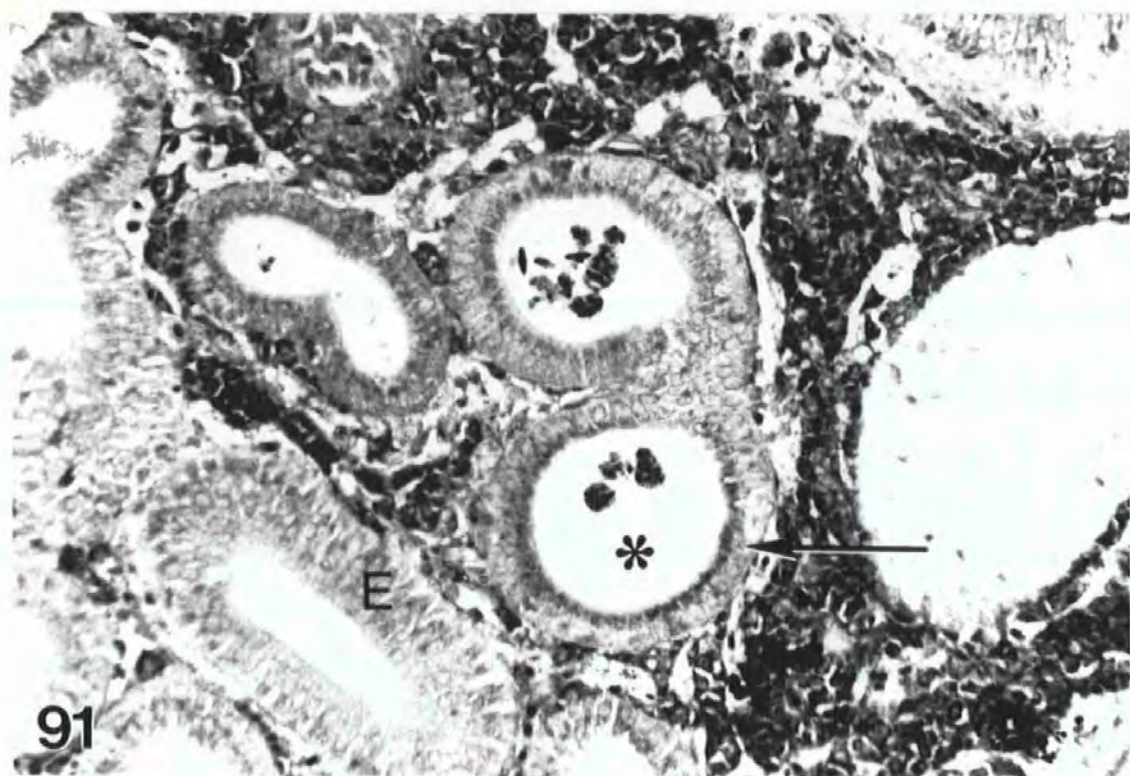
material were detected within the vacuoles (Fig. 93). Glycogen deposits, lipoid inclusions and mitochondria were also seen to be distributed throughout the plasmodial cytoplasm (Fig. 94). Generative cells contained large eccentrically-placed nuclei, each with a prominent nucleolus, and several sub-spherical mitochondria. Microtubule bundles and strands of RER were only occasionally seen. Although pericyte formation was only rarely observed, generative cells were frequently seen closely apposed to one another (Fig. 95). Most plasmodia rested directly on the microvilli of the renal tubule epithelium without any form of direct attachment. However, in a few instances plasmodia were attached by thin pseudopodia which were insinuated between individual epithelial cells without penetrating the cell wall (Fig. 94). Host cells showed no evidence of necrosis and microvilli of the brush border remained intact.

Glomerular infections with extrasporogonic stages of *M. lieberkuehni* were always associated with the presence of sporogonic plasmodia in the renal tubules and urinary bladder. Infected glomerular cells were grossly hypertrophied and contained numerous parasitic cells, the whole complex constituting a parasitic xenoma (Figs. 96 & 97). The sequential development of the xenoma has been described in detail by Lom *et al.*, (1989). The cytoplasm of the parasite primary cell contained numerous small vesicles, Golgi bodies, small strands of RER and mitochondria with plate-like cristae. Aggregates of glycogen granules were also present but were more prominent in the secondary cell cytoplasm (Figs. 98, 99 & 100). Details of secondary and tertiary cell ultrastructure have been described by Lom *et al.*, (1989). No evidence of sporogenesis was observed in any of the samples examined. In the latter stages of infection the xenomas became infiltrated with host phagocytes and most parasites degenerated and were phagocytosed. Cells from ruptured xenoma were observed within the neck segment of the proximal tubule. In most cases these cells increased in size as they progressed down the tubule, accumulating refractile granules and becoming indistinguishable from small sporogonic plasmodia.

In addition to the extrasporogonic stages located in the glomeruli, further intracellular parasites were observed within the epithelial cells of renal collecting ducts from a total of eight pike, all from the river Avon (Wilts). In each case the kidney and

Figure 93.   Plasmodium of *Myxidium lieberkuehni* free within a renal tubule. Numerous aggregates of osmiophilic material are present within the cytoplasm (arrow).   A generative cell (G), pericyte (P) and sporogonic cell (S) can also be seen.   Note the lack of microvilli on the surface of the plasmodium. x11,980.

Figure 94.   Plasmodium of *Myxidium lieberkuehni* attached to the epithelium of a renal tubule by thin pseudopodia (arrow).   Microvilli of epithelial cells covered by the plasmodium remain intact. x10,600.

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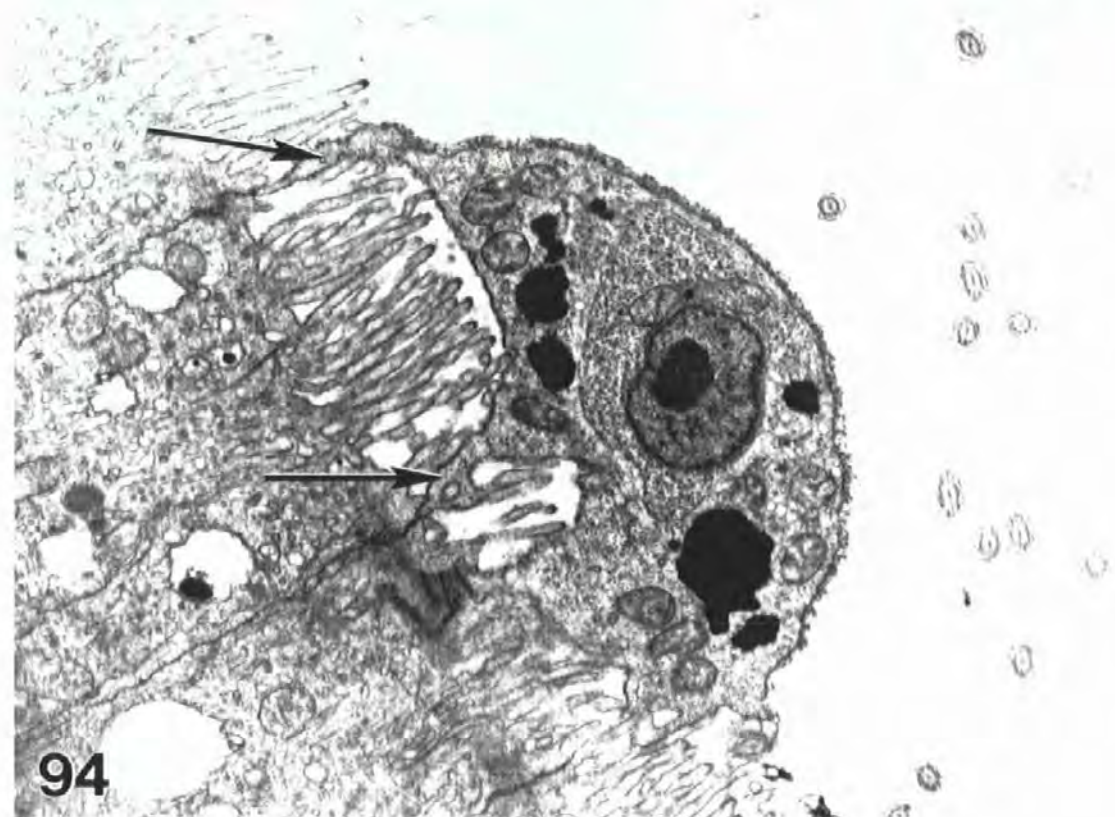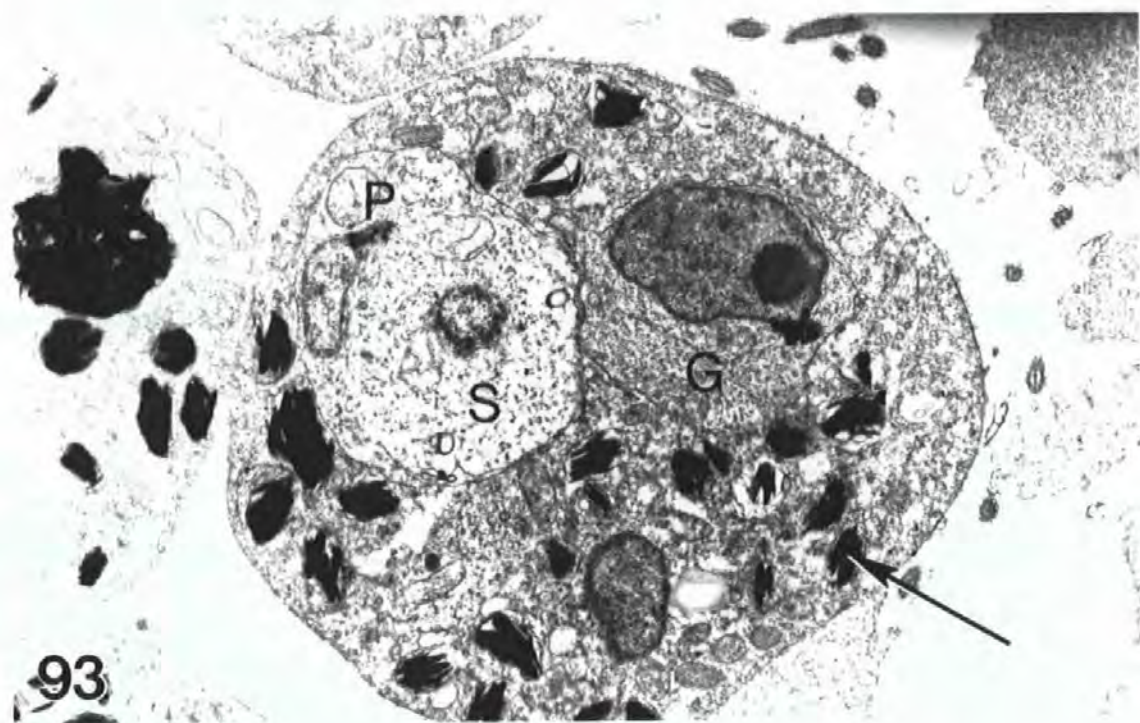
Figure 95.   Renal tubule of *Esox lucius* fully occluded with plasmodia of
              Myxidium lieberkuehni. Host epithelium (E), glycogen deposits (G),
              transverse section through a developing spore (S). x7,750.
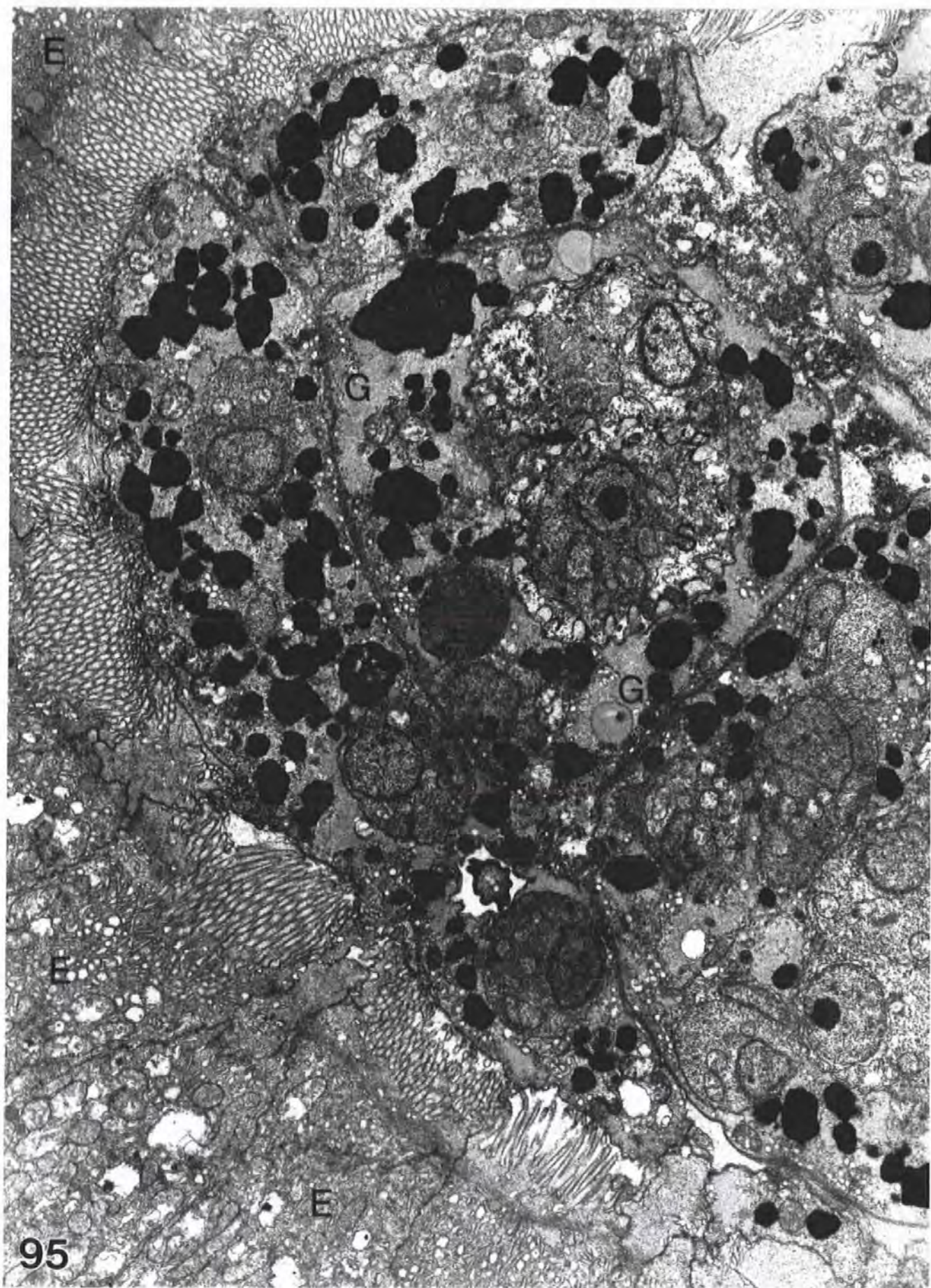
Figure 96.   Glomerulus (G) infected with extrasporogonic stages of *M. lieberkuehni* (*). Hypertrophied cell nuclei (N). H&E, x375.

Figure 97.   Xenoma of *M. lieberkuehni* extrasporogonic stages (*) still contained within a glomerulus (G), a thin peripheral layer of the xenoma is devoid of cells (arrow). Ruptured host cell nucleus (N). H&E, x375.

Figure 98.   Stage from an advanced xenoma. Two tertiary cells (T) with intertwining cellular processes (arrow) are contained within a secondary cell (S) which is itself within a primary cell. Nuclei of primary, secondary and tertiary cells (N1, N2 and N3 respectively). x13,250.
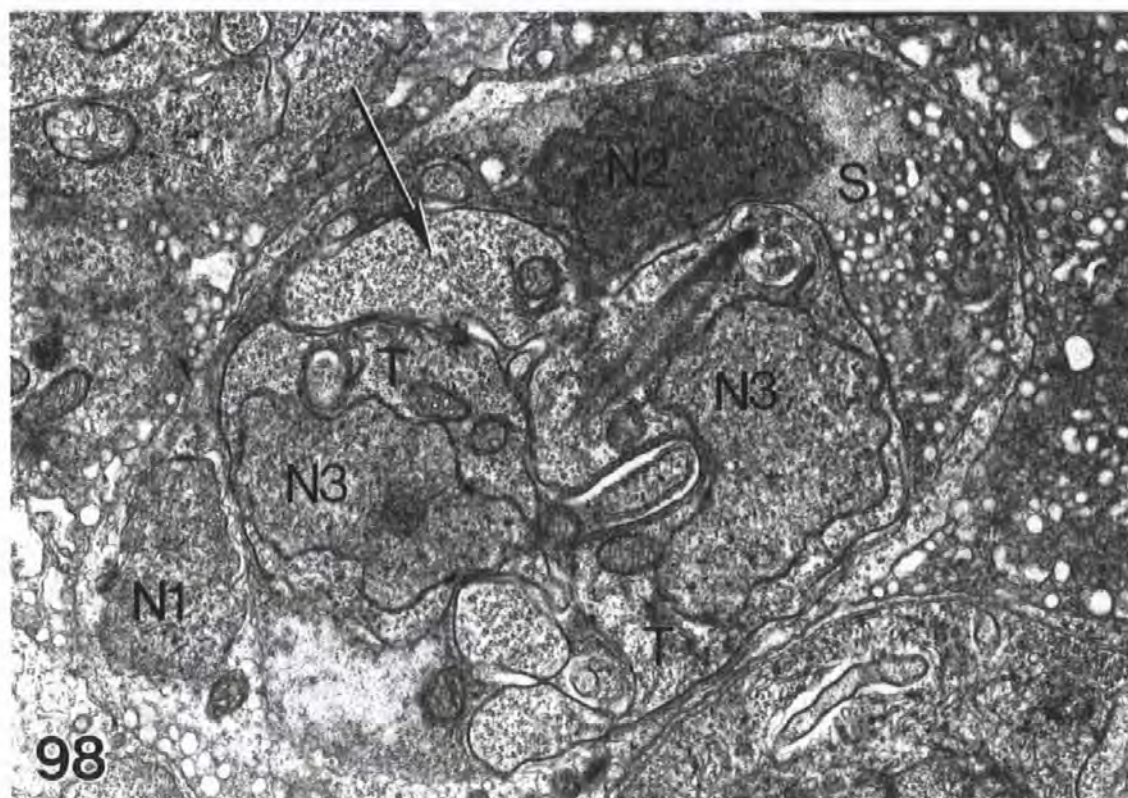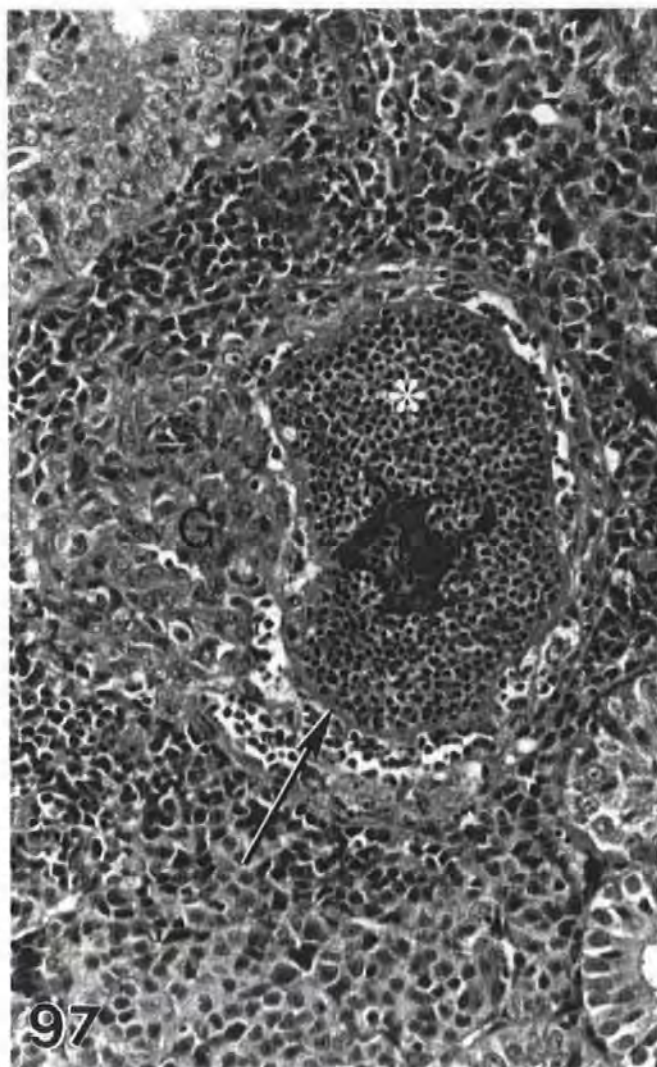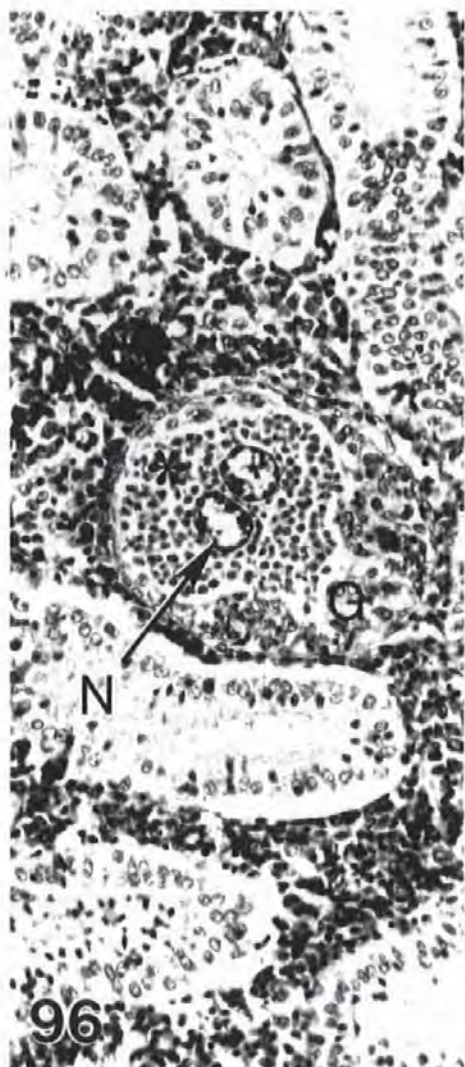
Figure 99. Section through an immature xenoma containing several stages with secondary (S) and tertiary cells (T) enclosed within primary cells (P). Tertiary cell cytoplasm frequently contains bundles of microtubules (arrow). x10,250.

99
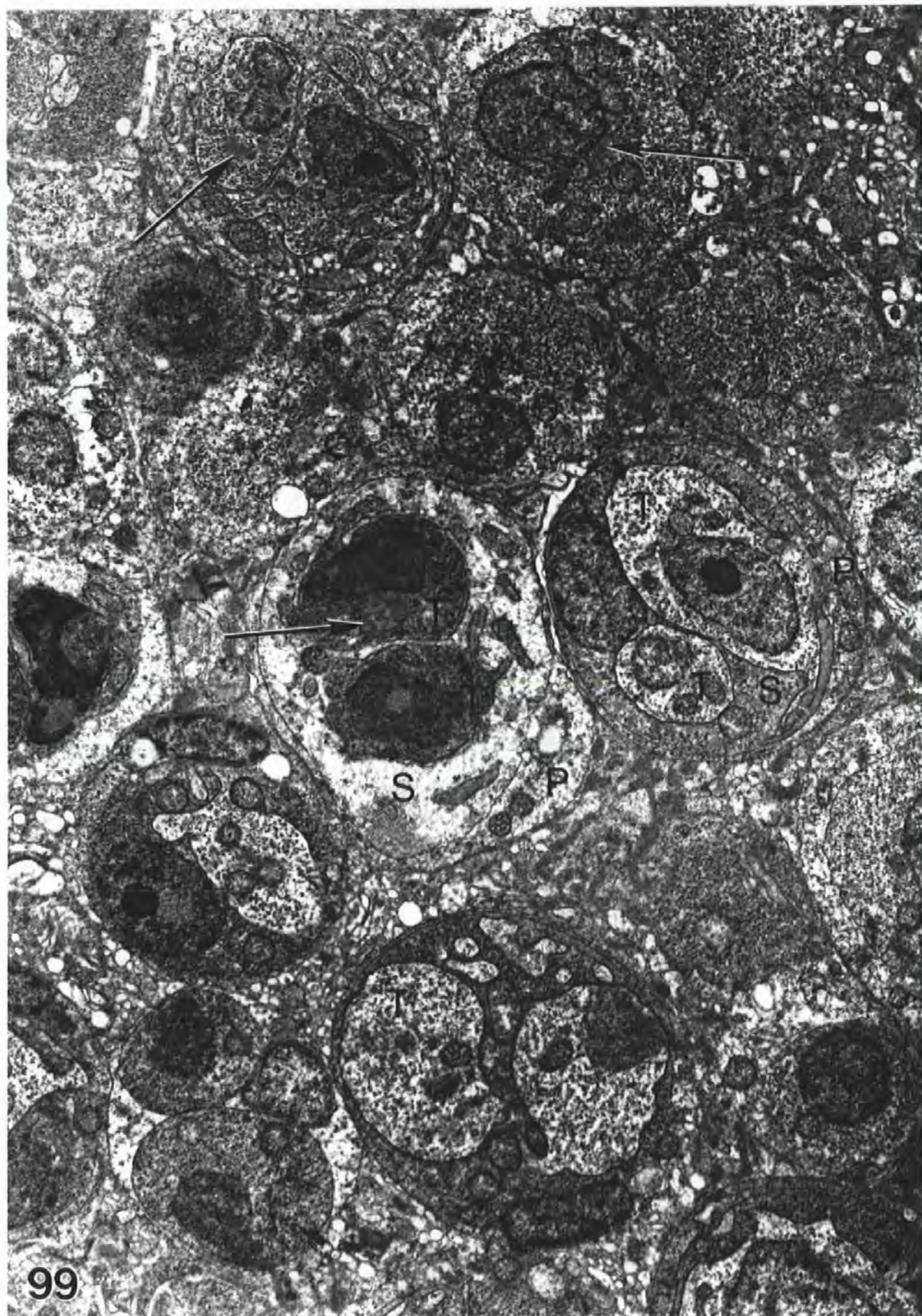
urinary bladder also harboured *M. lieberkuehni* plasmodia and spores. Infected collecting ducts were frequently markedly dilated and the epithelium thrown into papillomatous folds (Fig. 101). In a single juvenile pike, approximately 15cm in length, sampled from the river Avon in May 1987, the renal pathology was severe enough to produce gross changes. The kidney in this case was hypertrophied and the surface appeared mottled with grey and white patches. The lumen of affected ducts contained cellular debris and plasmodia of *M. lieberkuehni*, many of which were attached to the affected epithelium (Fig. 102). No spores or developmental stages of any other myxosporean were identified in the urinary tract.

Infected epithelial cells were hypertrophied, elongated and contained up to three parasitic cells bounded by a single membrane. Most cells were heavily vacuolated and accumulations of electron-dense material, either within lysozomes or free within the cytoplasm, were also observed (Figs. 103 & 104). Nuclei were irregular in outline and contained homogenous chromatin (Fig. 104).

The primary cell of the parasite was round to oval in shape. The cytoplasm was pale staining and contained few organelles. Rounded to elongate mitochondria were distributed throughout the cytoplasm and contained a characteristic osmiophilic matrix and several plate-like cristae. Small vesicles, larger vacuoles, some containing lipoid material or cellular debris, Golgi bodies and sparse strands of RER were also present (Fig. 105 & 106). Primary cell nuclei were also round to oval in shape and were bound by a double membrane, occasionally with a cup-shaped depression in which Golgi bodies were observed (Fig. 105). The nucleoplasm was uniformly dense with a prominent nucleolus approximately 0.75μm in diameter (Fig. 107).

Secondary cells were approximately 2.5μm in diameter and were contained within a double membrane, the outer belonging to the primary cell and the inner to the secondary cell. The cytoplasm appeared electron-dense due to the presence of numerous free ribosomes. Mitochondria of the same form as those in the primary cell cytoplasm were also present and were often observed clustered around the nucleus (Fig. 108 & 109). Bundles of microtubules, strands of RER, Golgi bodies and small vesicles were also observed. In addition, an unidentified ring-shaped structure was present in

Figure 100.   Deposits of glycogen granules (arrows) within the secondary cell. Thiery technique, x10,830.

Figure 101.   Distended collecting ducts in *Esox lucius* kidney.   Epithelium infected with intracellular parasites is thrown into papillomatous folds (arrow). Uninfected epithelium (E). H&E, x95.
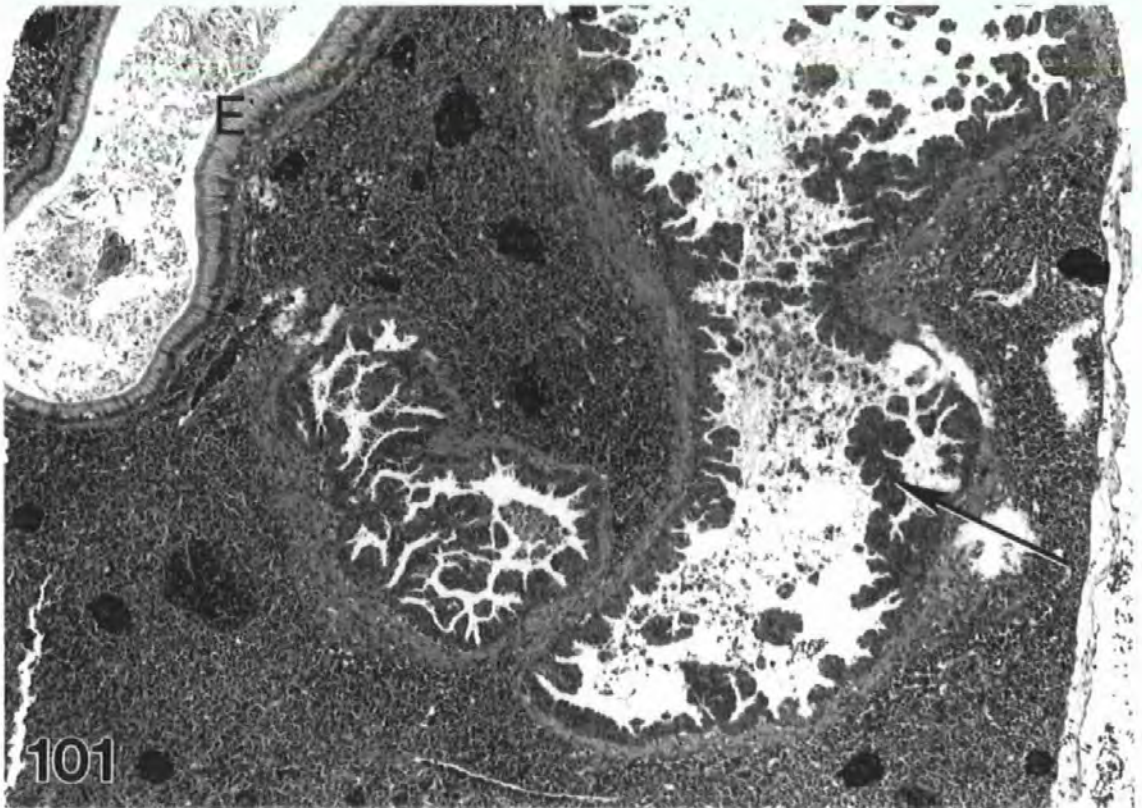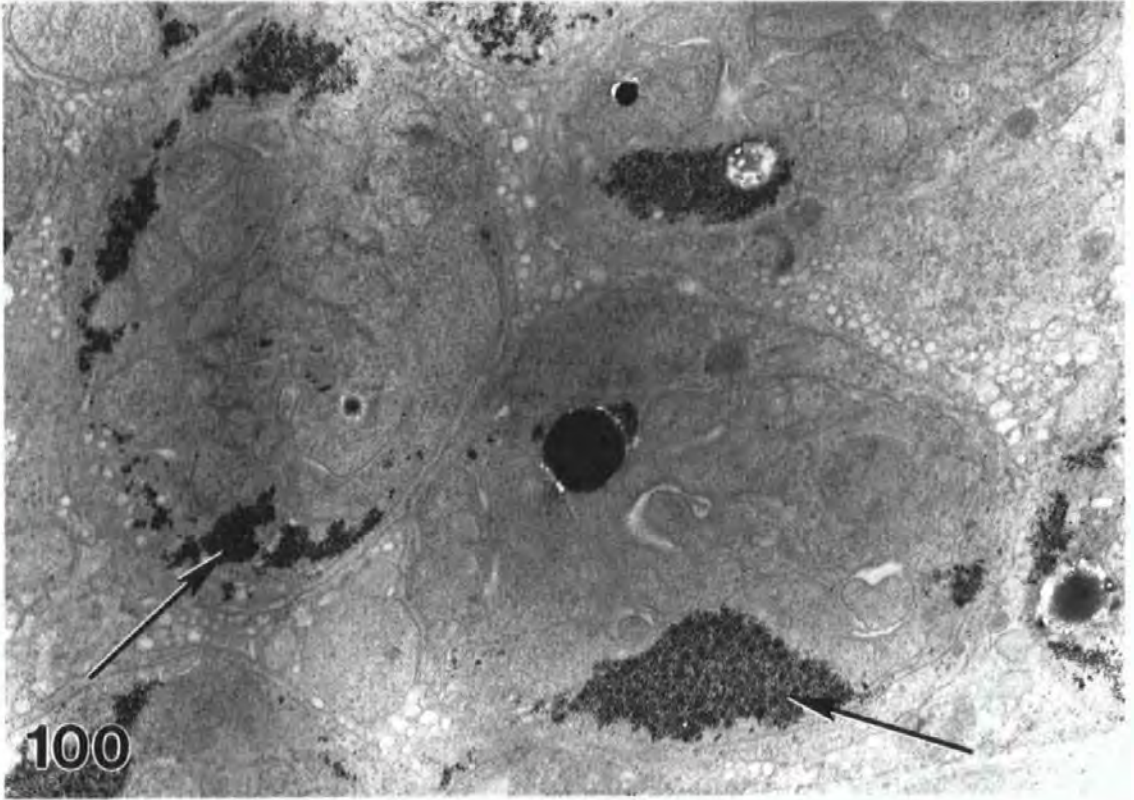
Figure 102. Detail of epithelium infected with intracellular parasites (arrow). Epithelial cells are elongate and hypertrophied. Thickening of the basal connective tissue layer (CT) is also apparant. The lumen of the collecting duct contains cellular debris and plasmodia of *Myxidium lieberkuehni* (P) may be attached to the epithelium. H&E, x390.

Figure 103. Epithelial cells from a section of infected epithelium harbouring one or two parasitic stages (arrows). x7,220.

102

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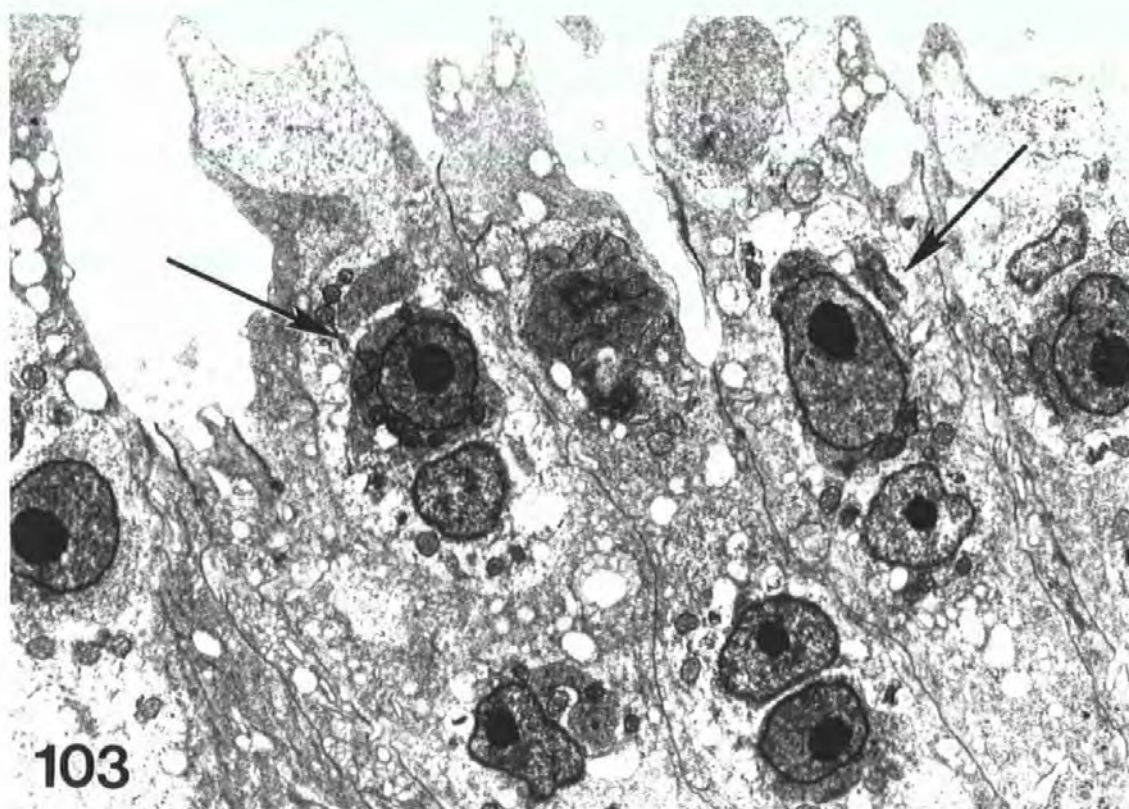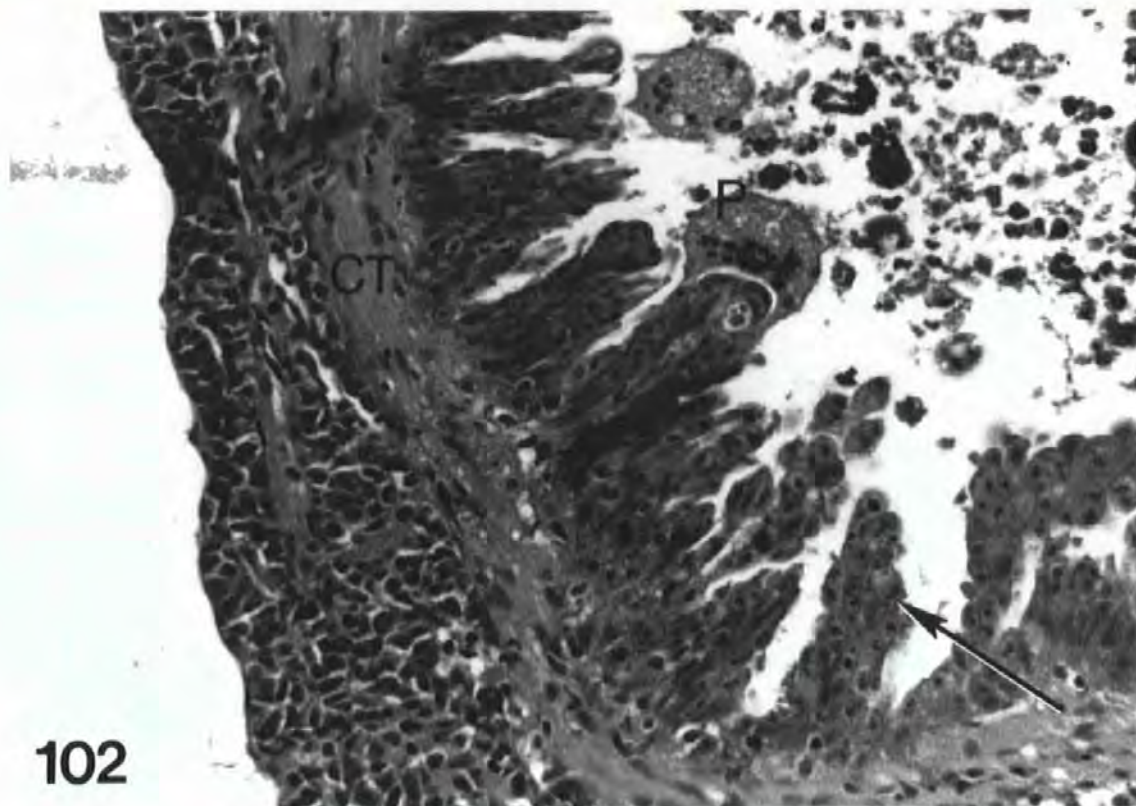
Figure 104. Infected epithelial cells with numerous cytoplasmic vacuoles (arrows) and irregular shaped nuclei (N). x7,240.

Figure 105. Primary cell of the parasite with secondary cells (S) and mitochondria with electron-dense matrices (M). The primary cell nucleus (N) is indented, with a Golgi body (arrow) positioned within the depression. x14,440.

104

105
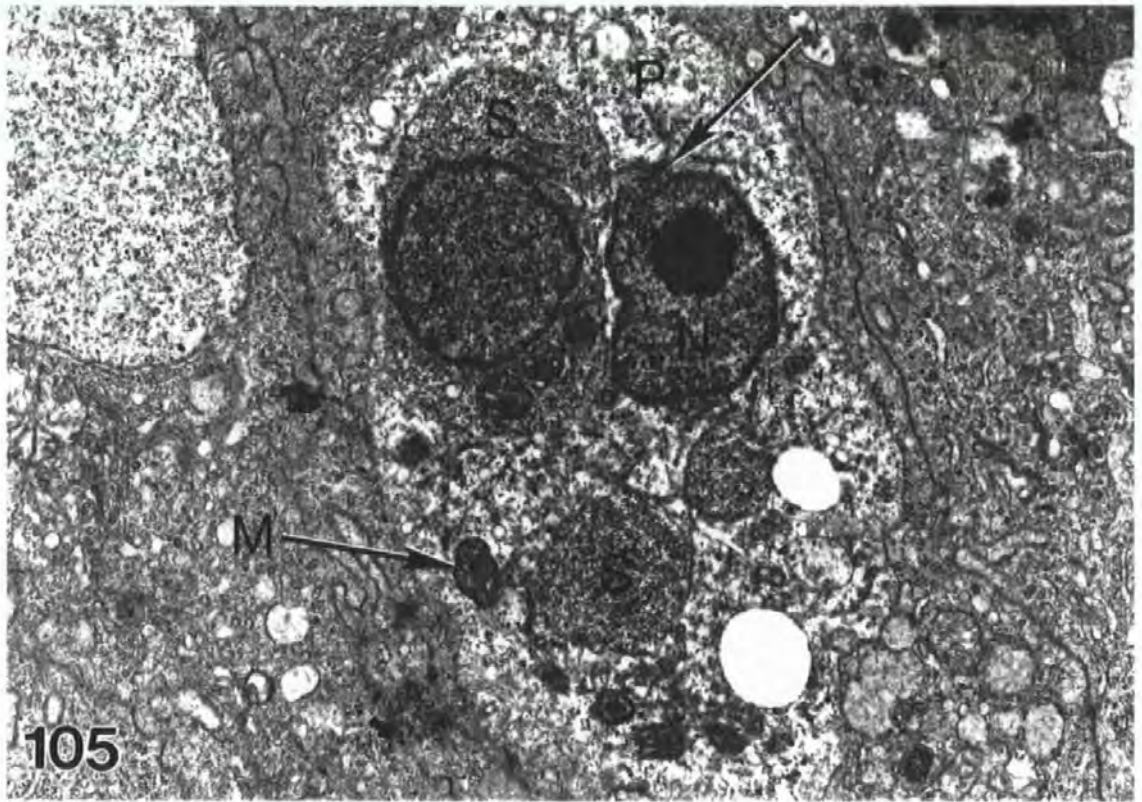
the cytoplasm of some secondary cells. This consisted of an electron-dense outer ring 0.4μm in diameter, surrounding a pale staining zone and an electron-dense core (Figs. 106 & 108). The nuclei of secondary cells were spherical or sub-spherical in shape and of the same size as those in primary cells; however, the nucleoplasm and nucleoli were more densely staining. Rarely observed tertiary cells possessed all of the features seen in secondary cells (Fig. 108). Sporogonic stages of the epithelial cell parasites were not observed.

5.4 DISCUSSION.

The basic structure and development of *S. elegans* agrees with that now established for other *Sphaerospora* species (Desser, Lom & Dyková, 1986; Dyková & Lom, 1982; Hedrick, McDowell & Groff, 1990; Supamattaya, Fischer-Scherl, Hoffmann & Boonyaratpalin, 1991; El-Matbouli & Hoffmann, 1992; Sitja-Bobadilla & Alvarez-Pellitero, 1990, 1992). Glomerular infections which were frequently observed in the present study have also been reported from other *Sphaerospora* infections. Desser *et al.*, (1986) reported infections with *Sphareospora ohlmacheri* in Bowman's space in bullfrog tadpoles (*Rana catesbeiana*), Supamattaya *et al.* (1990, 1991) observed sporogonic stages of *Sphaerospora epinepheli* in the glomerular capillaries of cage cultured grouper (*Epinephelus malabaricus*) and Sitja-Bobadilla, Franco-Sierra & Alvarez-Pellitero (1992) described pathological changes in the glomeruli of cultured gilt head sea bream (*Sparus aurata*). In addition, glomerular infections with *Sphaerospora cristata* in *Lota lota* and *Sphaerospora truttae* in *Salmo trutta* have also been observed (I. Dyková and J. Lom, pers. comm., 1990). It was not possible to determine if the infection in *G. aculeatus* was initiated in the glomerulus with a subsequent passage of developmental stages and spores down the proximal tubule. Whether intracellular extrasporogonic stages occur in *S. elegans* was not determined here, although El-Matbouli *et al.*, (1992) reported their presence in rudd *Scardinius erythrophthalmus* infected with *Sphaerospora scardinii*. Uninucleate stages were observed in the epithelial cells of the ureter and multicellular stages were seen in the lumen of the ureter. The only other published report of intracellular stages were those

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Figure 106. Diagram showing the main features of the epithelial cell parasites in *Esox lucius* collecting ducts.

PC - Primary cell.

PCN - Primary cell nucleus.

M   - Mitochondria.

G   - Golgi body.

CV - Cytoplasmic vacuole.

ER - Endoplasmic reticulum.

AV - Autophagic vacuole.

SC - Secondary cell.

SCN - Secondary cell nucleus.

MT - Microtubule bundle.

'X' - Ring structure of unknown function.

PC

PCN

CV

M

ER

G

'X'

AV

MT

SCN

SC
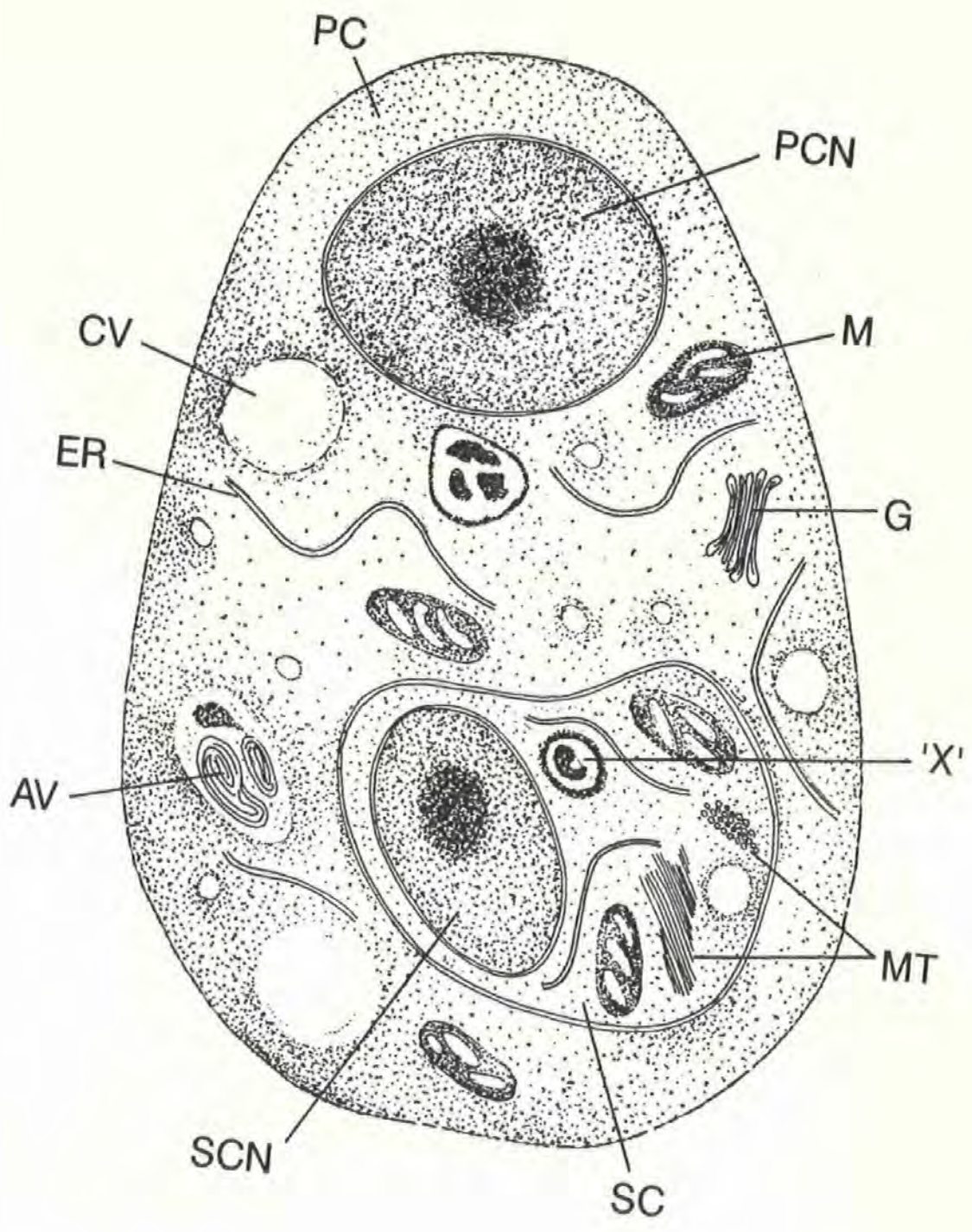
Figure 107. Epithelial stage containing a single secondary cell (S). The primary cell contains few organelles including a Golgi body (G), mitochondria (M) and a single nucleus which is bound by a double membrane (arrow). x14,800.

Figure 108. Parasitic stage infecting a mucous cell. Primary (P), secondary (S) and tertiary cells (T) are visible, the latter containing a bundle of microtubules (MT) and the 'ring-shaped' structure (arrow) seen in secondary cells. Accumulation of mucous (M). x14,140.

107

108
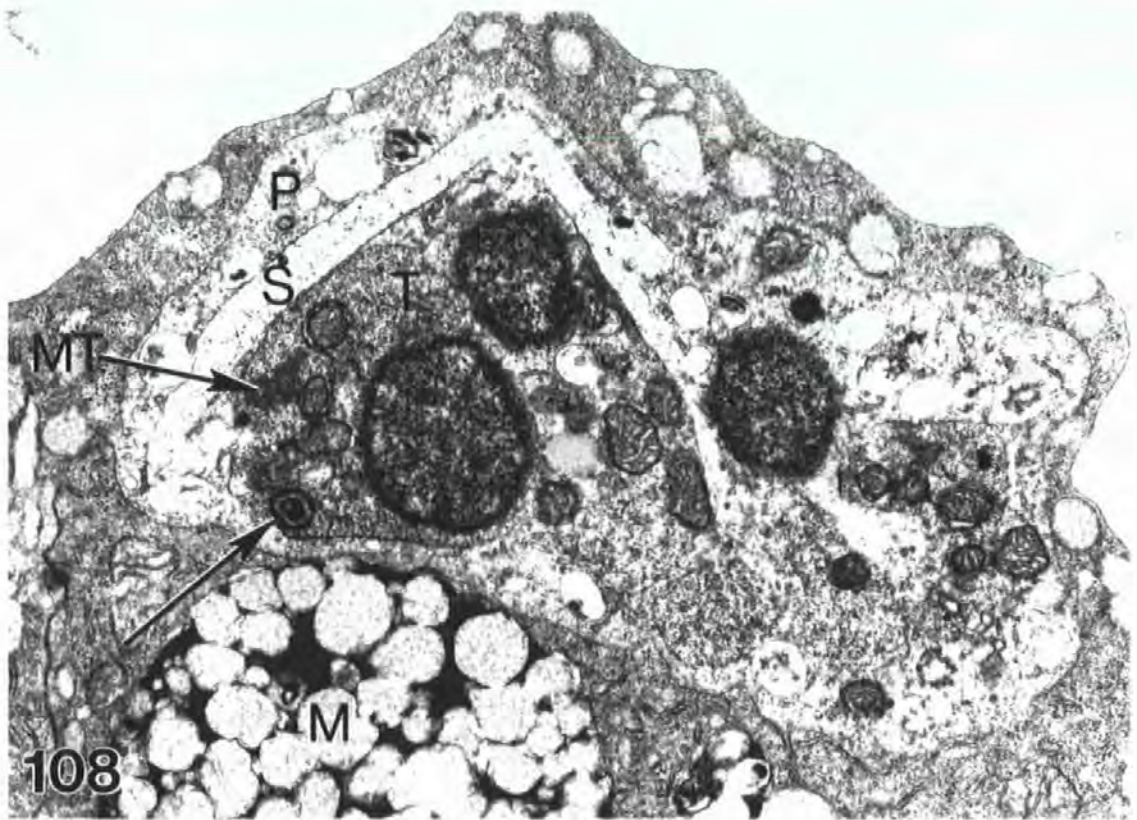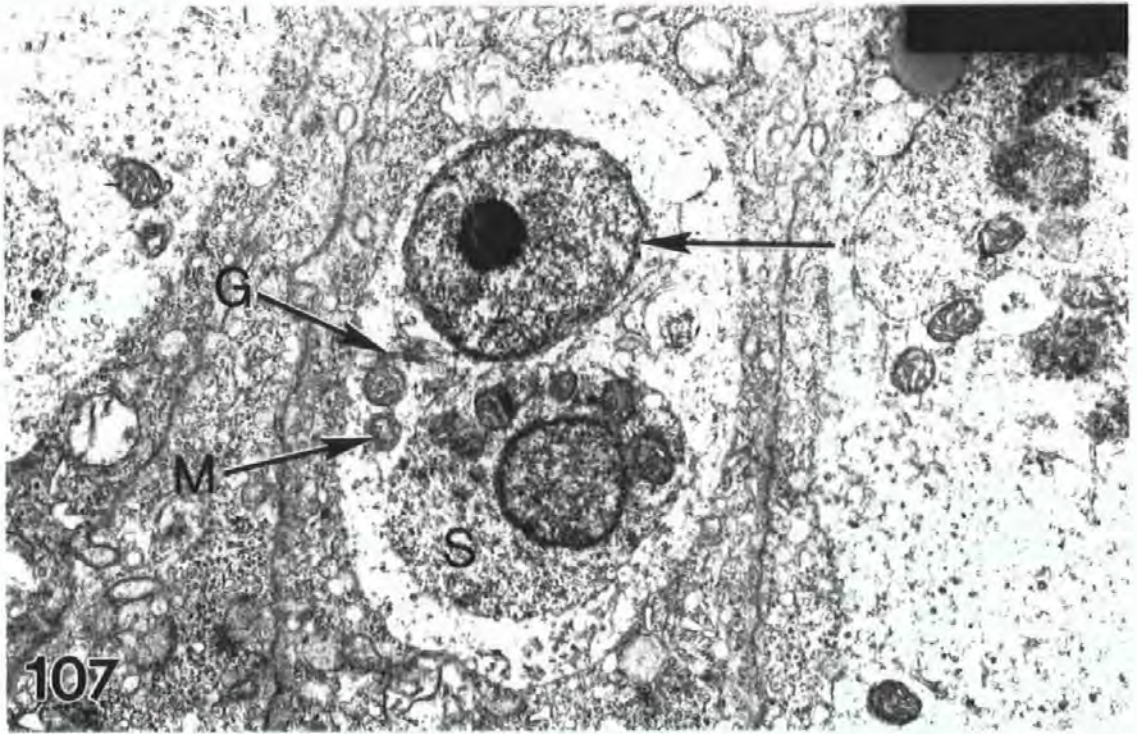
Figure 109.   Parasite primary cell (P) containing two recently divided secondary cells (S) each bound by two membranes, the inner belonging to the secondary cell and the outer to the primary cell. Mitochondria (M) cluster around the secondary cell nuclei. Lipoid inclusion (L). x26,450. Inset. Bundle of microtubules (arrow) within secondary cell cytoplasm. x33,900.

associated with *Sphaerospora renicola* infections in carp *C. carpio* (Dyková & Lom, 1982). The significance of these stages is unknown.

The presence of surface pits on the spore of *S. elegans* has not been reported previously although similar ornamentation has been described in species from other myxosporean genera such as *Myxobolus* and *Chloromyxum* (Lom & Arthur, 1989; Lom, Dyková & Kepr, 1988). Most members of the genus *Sphaerospora* possess either smooth shell valves or valves with anterior ridges (Lom & Noble, 1984). Although topographical features can assist in identification (Lom & Arthur, 1989), their presence can be influenced by the maturity of the spore and individual variation within a species can be marked, consequently their use in myxosporean taxonomy is limited.

The ultrastructure of *S. elegans* was similar to previously described members of the genus *Sphaerospora* (*S. renicola*: Lom *et al.*, 1982, Desser *et al.*, 1983; *S. molnari*: Desser *et al.*, 1983; *S. tincae* and *S. galinae*: Lom *et al.*, 1985; *S. epinepheli*: Supamattaya *et al.*, 1991). The observation of cytoplasmic bridges between sporogonic cells confirms similar findings by Desser *et al.*, (1983) in *Thelohanellus nikolskii* and in the various proliferative and sporogonic stages of *S. renicola* (Dyková *et al.*, 1990). Their presence in these parasites has been interpreted as an indication of the terminal phase of cytokinesis (Dyková *et al.*, 1990). The involvement of microtubular bundles in the formation of the cytoplasmic bridge between generative cells in *T. nikolskkii* was not observed in the present study and also appeared to be absent in the *S. renicola* stages. The reason for this is unknown but may reflect differences between myxosporean genera.

Possible extrasporogonic stages of *S. elegans* were located in the renal interstitial tissue and in the choroidal *rete* of the eye. Lom *et al.*, (1991) were unable to find evidence that the *rete* stages were related to the PKX parasite, although the possibility was not ruled out. Hedrick *et al.*, (1988) also found extrasporogonic myxosporean stages in the blood vessels of *G. aculeatus* infected with *S. elegans*, but could not confirm that they belonged to the *S. elegans* life cycle. Similarly, in the present study, neither the renal interstitial nor the *rete* stages could be conclusively identified as belonging to *S. elegans* since infected fish were invariably co-infected with

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M. gasterostei. Although extrasporogonic stages of the genus *Myxobilatus* have not been reported, the possibility of their occurrence in this genus or of any other myxosporean cannot be excluded. However, several members of the genus *Sphaerospora* are known to possess extrasporogonic cycles. Consequently, it is probable that the stages identified in the present study belong to *S. elegans* (Feist *et al.*, 1991; Lom *et al.*, 1991). Extrasporogonic blood stages were not observed despite careful screening of stained smears. Baska and Molnár (1988) pointed out that similar stages, although widespread in freshwater fish, were occasionally rare and in certain cases could only be detected in one out of ten blood smears from the same fish. Subsequently, Sovenyi and Molnár (1990) described an enrichment technique for the detection of myxosporean blood stages. Details of this technique were published after the practical work for the present investigation was completed. Its use in future studies of myxosporean extrasporogonic bloodstages is recommended.

Results from the current investigation have provided the first ultrastructural data on plasmodia and spores of *M. gasterostei* and also on its pathogenic potential within the host kidney. The overall structure of the sporoblast and spore agrees with that established for other myxosporean species. The presence of a binucleate sporoplasm cell was confirmed and cytoplasmic sporoplasmosomes were observed in the genus *Myxobilatus* for the first time. Booker and Current (1981), in the only other ultrastructural investigation of a *Myxobilatus* species, described the surface topography of *M. mictospora* plasmodia in the largemouth bass *Micropterus salmoides*. Three distinct forms of plasmodia were found in the urinary bladder, according to the time of year. Sheet-like plasmodia, attached to the urinary bladder epithelium, were present during the winter months and these gave rise to freely-floating sporogonic plasmodia during the summer months when water temperatures were higher. Seasonal variations in the prevalence of *M. gasterostei* infections were not apparent in the present study and may have been overlooked since most fish were sampled during the summer months (Table 3). Plasmodia were only rarely observed in the urinary bladder of *G. aculeatus* and further research is required to determine whether the renal tubule plasmodia of *M. gasterostei* consistently reach the urinary bladder and whether changes in plasmodial

morphology occur between the two sites. The absence of microvilli on the surface of sporogonic *M. mictaspora* and *M. gasterostei* plasmodia may reflect a stage in the maturation of the plasmodium. As sporogenesis nears completion, nutritional demands reduce, and microvilli are resorbed into the plasmodium before final degeneration and release of spores occurs. A similar phenomonen has been observed in *Myxidium gadi* in pollack (*Pollachius pollachius*) (Feist, unpublished) and probably occurs in other coelozoic myxosporeans exhibiting seasonality of infection.

Intracellular stages of *M. gasterostei* were not observed in the current study. Molnár (1988) reported intracellular trophozoites of *Myxobilatus legeri* in the epithelial cells of cyprinid renal tubules and ureters. Affected cells were hypertrophied and deformed, with nuclei compressed by the trophozoites. The only other report of pathology associated with *Myxobilatus* infections involved *M. accessobranchialis* in the accessory breathing organ of cultured catfish (*Heterobranchus bidorsalis*) (Obiekezie and Okaeme, 1987). Coelozoic infections with *M. gasterostei* provoked little host response; however, rupture of infected tubules resulted in an inflammatory, and eventually, a granulomatous response. The capacity for coelozoic myxosporeans to produce sometimes severe pathological responses has been well documented (Lom, 1989), indeed it is likely that the PKX cell of PKD is a stage of a normally coelozoic myxosporean that induces the dramatic host response in salmonid fish because of its histozoic location.

The light and ultrastructural studies of *Myxidium lieberkuehni* confirm previous findings concerning the presence of the parasite within the urinary bladder (Buetschli, 1882; Laveran and Mesnil, 1902; Bauer, 1922; Lom and de Puytorac, 1965; Uspenskaya, 1982). In addition to the large plasmodia found in the urinary bladder, smaller plasmodia containing only a few spores were regularly found in the renal tubules. These had been noticed in previous investigations but were not examined in detail (Bauer, 1984; Lom and Dyková, 1986, 1989). Overall, morphology of the tubule plasmodia were similar with those of the urinary bladder except that the clear ectoplasmic layer and surface microvilli were not present. Presumably, the smaller overall size facilitates osmotrophic nutrition without the need for the additional surface

area provided by microvilli. The glomerular infections were studied and discussed in detail by Lom *et al.*, (1989). Although circumstantial evidence that the parasitic stages present belonged to an extrasporogonic phase of *M. lieberkuehni* was provided, additional observations made subsequently have demonstrated that cells, on reaching the renal tubules, are capable of transforming into *M. lieberkuehni* plasmodia, thus confirming the view of Debaisieux (1920). Consequently, *N. pickii* (Weissenberg, 1923) becomes a junior synonym.

The stages within the epithelial cells of renal collecting ducts were noted by Lom *et al.*, (1989) and since sporogenesis was not observed, were tentatively regarded as a further extrasporogonic phase of *M. lieberkuehni*. The ultrastructural features of these stages were reminiscent of the extrasporogonic renal forms of *S. renicola* in carp *C. carpio*. However, unlike in *S. renicola*, quaternary cells were not observed. The relationship between the xenoma and intracellular epithelial stages is unclear. Their overall structure was similar, both containing secondary and tertiary cells. However, the xenoma secondary cells were characterised by the presence of numerous pseudopodia which were absent in the epithelial forms, and the latter possessed the curious 'ring-shaped' organelle which was never seen in xenoma cells. Although evidence that the intracellular epithelial stages pass into the lumen of the collecting duct to initiate sporogony has not been supplied, this appears likely. Infected host cells exhibited degenerative changes and large amounts of cellular debris and *M. lieberkuehni* plasmodia were consistently observed within the lumen of infected collecting ducts. Additionally, there was no evidence to suggest non-viability of the parasitic stages within the tubular epithelium (Lom *et al.*, 1989). It is interesting to note the differing host cell response to the presence of the extrasporogonic stages. In the glomerulus, infected host cells, thought by Lom *et al.*, (1989) to be endothelial cells, become greatly hypertrophied to form a xenoma. Infected epithelial cells are also hypertrophied, but to a lesser extent and the primary response is one of cellular proliferation resulting in massive dilation of the infected tubule or duct. Further investigations to resolve the reasons for these differences are necessary. Additional research is also required to elucidate the relationship between the intracellular stages

and *M. lieberkuehni* plasmodia and xenoma cells. In particular, to determine whether there is a seasonal influence in their prevalence.

CHAPTER 6

TRANSMISSION OF MYXOSPORIDIOSIS

6.1 INTRODUCTION.

Based on the assumption of a direct life cycle most early attempts at experimental transmission of myxosporeans involved challenge, by various methods, of fish hosts to spores. Several authors had observed the exit of the sporoplasm from spores held under different conditions outside the host (Lieberkuehn, 1854; Pfieffer, 1890); however, Thélohan (1895) was the first to investigate the effect of digestive fluids on spores within the host. In a series of experiments he introduced myxosporeans into the digestive tract of several fish species; by feeding infected tissues and by injection. He also introduced spores of *Myxobolus ellipsoides* into the stomach of tench using balls of cotton-wool, impregnated with spores. The results of all of these experiments showed that the shell valves of some spores split and the amoeboid sporoplasms were released. Similar experiments to the above were carried out by Auerbach (1909, 1910) and others (cited by Walliker, 1967), which confirmed Thélohan's findings, but did not provide proof of successful experimental transmission since the fish used in experiments, including those that became infected, were not known to be parasite-free.

Successful infection experiments using myxosporean spores directly have been reported by several authors (Auerbach, 1910; Erdmann, 1917; Bond, 1939) but have not been repeated in recent years. Since the discovery of the requirement for an intermediate host in the transmission of *Myxobolus cerebralis* and the transformation of the myxosporean spore into an actinosporean spore in the oligochaete *Tubifex tubifex* (Markiw & Wolf, 1983; Wolf & Markiw, 1984), several other reports of myxosporean infections involving an actinosporean phase and intermediate hosts have been published (El-Matbouli & Hoffmann, 1989, 1991; Kent, Whitacker & Margolis, 1990; Ruidisch, El-Matbouli & Hoffmann, 1991; Yokoyama, Ogawa & Wakabayashi, 1991; Grossheider & Körting, 1992; El-Matbouli, Fischer-Scherl & Hoffman, 1992). However, until further experimental proof becomes available it is unclear whether transformation between myxosporean and actinosporean phases is a general rule or

whether such indirect transmission can alternate with a direct one (Lom & Dyková, 1992a).

A mature spore stage, the exact mode of transmission and nature of the infective stage of PKX are still unknown. Attempts to induce the disease by feeding with infected kidney tissue, by cohabiting susceptible and infected fish or by exposure to sediments and the associated fauna from infected sites in the laboratory have failed (D'Silva, Mulcahy & de-Kinkelin, 1984; Rafferty, 1986). Successful transmission of PKX/PKD has only been achieved by exposure of fish to water enzootic for the disease or by intraperitoneal or subcutaneous injection with PKX-infected tissues or blood (Clifton-Hadley, Richards & Bucke, 1984; D'Silva et al., 1984; Kent & Hedrick, 1985b; Rafferty, 1986; Clifton-Hadley & Feist, 1989; Arkush & Hedrick, 1990). Similar techniques were used in the successful transmission of extrasporogonic swimbladder and blood stages of S. renicola in carp (Molnár, 1984; Molnár & Kovács-Gayer, 1986; Körting, Kruse & Steinhagen, 1989). In these studies the authors were able to conclusively demonstrate that the extrasporogonic swimbladder stages were capable of reaching the kidney and there undergo sporogony.

The involvement of an intermediate host and an actinosporean phase in the PKX life cycle must be considered potential requirements for transmission. However, that a fully formed spore stage of PKX has yet to be identified with certainty rules out any immediate hope of cycling this parasite under laboratory conditions. In an attempt to explain the apparent lack of sporogony in salmonid hosts Feist (1988) suggested that the three-spined stickleback G. aculeatus might be the normal host for PKX, in which sporogony is completed, and its renal parasite S. elegans to be synonymous with PKX. Various experiments, described below, were undertaken to test this hypothesis.

During the period of this research an indoor recirculation unit at the experimental fish installation, Institute National de Recherche Agronomique, Jouy-en Josas, France, was brought to the authors attention. Within this system, susceptible fish consistently and rapidly became infected with PKD. Following a site visit in December 1988, the invertebrate fauna associated with the filtration system and settling tanks was examined for the presence of myxosporean and actinosporean parasites with negative

results. Subsequently the system was examined in detail by Chilmonczyk, Thomas & de-Kinkelin (1989). The authors were able to show that PKX-free rainbow trout rapidly became infected when exposed to sediments from the decantation tank. More recently, Hedrick, Monge & de-Kinkelin (1992) have suggested that an intermediate or alternate host, greater than 500μm in size and present in the sediments, is involved and that it liberates a smaller infective stage, less than 50μm in size, which is found in the water.

A total of 29 field and laboratory experiments to investigate the mode of transmission of PKX, the susceptibility of various species to the parasite, and the possible role of the stickleback parasites *S. elegans* and *M. gasterostei* in the transmission of PKD to salmonids were conducted (Table. 8).

6.2   MATERIALS AND METHODS.

6.2.1   Sources of fish.

Rainbow trout fingerlings and carp were obtained from commercial fish farms known to be free of PKD. Grayling and brook trout fingerlings (average weight 4 g, length 100 mm, and weight 3 g, length 80 mm, respectively) were supplied by the Yorkshire Water Authority (YWA) (now part of the National Rivers Authority). Grayling were artificially spawned from eggs and milt collected from wild fish and the brook trout spawned from YWA's own brood stock. Fish were reared on commercial trout foods and held in tanks fed by spring water at the Authority's hatchery, a site with no previous history of PKD. Wild fish were collected from a variety of sources as described in chapter 3.

Mixed populations of tubificid worms containing predominantly *Tubifex tubifex* were purchased from a local petshop.

6.2.2   Extraction of myxosporeans from fish kidney.

Kidneys of rainbow trout fingerlings, naturally infected with PKX cells and exhibiting clinical PKD, were removed aseptically and placed individually in sterile universals containing 10 cm3 chilled phosphate buffered saline (PBS), or in PBS

Table 8. Summary of transmission experiments undertaken.

Experiment number	Date commenced	Host species	Donor material	Route
1	3.8.87	*S. trutta*	*O. mykiss* PKX cells	i.p.[1]
2	"	*C. carpio*	"	"
3	"	*P. phoxinus*	"	"
4	"	*S. trutta*	"	"
5	"	*O. mykiss*	"	"
6	7.8.87	*E. lucius*	"	"
7	"	*T. thymallus*	"	"
8	4.9.87	*G. aculeatus*	"	"
9	"	"	"	immersion exposure
10	15.9.87	*O. mykiss*	*S. elegans* and *M. gasterostei*	gavage
11	"	"	"	i.p.
12	18.9.87	*G. aculeatus*	*O. mykiss* PKX cells	"
13	"	*O. mykiss*	"	"
14	29.9.87	*O. mykiss*	*G. aculeatus*	cohabitation
15	27.2.88	*O. mykiss*	"	exposure to tank effluent
16	30.3.88	"	*T. tubifex*	"
17	23.5.88	"	"	"
18	18.8.88	"	*T. tubifex* and *G. aculeatus*	"
19	17.8.88	*T. thymallus*	*O. mykiss* PKX cells	i.p.

Table 8. (continued)

Experiment number	Date commenced	Host species	Donor material	Route
20	17.8.88	*G. aculeatus*	*O. mykiss* PKX cells	i.p.
21	"	*O. mykiss*	*O. mykiss* PKX cells	i.p.
22	26.7.88	"	*S. elegans* and *M. gasterostei*	"
23	"	"	"	gavage
24	11.8.88	*T. Thymallus*	PKX	natural infection
25	"	"	"	"
26	26.6.89	*S. fontinalis*	"	"
27	"	*T. thymallus*	"	"
28	13.7.89	"	*O. mykiss* PKX cells	i.p.
29	"	*S. fontinalis*	"	"

[1] Intraperitoneal injection

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containing 10 ppm oxolinic acid to suppress possible bacterial contamination. Using sterile forceps, the tissue was disrupted manually. Relative numbers of PKX parasites present per kidney were determined by examination using phase or interference contrast microscopy of one drop of suspension from each kidney preparation placed between a slide and coverslip. Those with most PKX cells present were selected for experimental challenges.

Stickleback kidneys containing spores and developmental stages of *S. elegans* and *M. gasterostei* were prepared in the same way as indicated above. Because of the size difference, 5 stickleback kidneys were used per 10 cm3 PBS. Microscopic examination as above was used to confirm the presence or absence of *S. elegans* and *M. gasterostei* parasites prior to inoculation into test fish. It was not possible to test the viability of extracted material prior to challenge. To avoid possible deterioration of the parasites, procedures were completed within 30 minutes.

6.2.3   Introduction of myxosporean isolates into fish.

A total of three methods were employed.

6.2.3.1 Direct passage via intraperitoneal injection.

PKX cells and kidney homogenates from sticklebacks containing *S. elegans* and *M. gasterostei* were prepared as described above and introduced into various fish species by intraperitoneal injection using 21G or 25G needle size. Prior to challenge fish were anaesthetised using a solution of 0.1 g/l MS222 (Sandoz, Basle, Switzerland). The amount of homogenate injected varied according to the size of the test fish used, 0.02 cm3 for injection into sticklebacks and up to 0.5 cm3 per fish in rainbow trout or grayling fingerlings. During initial trials passage of PKX to rainbow trout naive to PKX was found to be particularly reliable and was used as a positive control in subsequent experiments to confirm that the experimental challenges had been successful.

A total of 8 fish species were challenged with kidney homogenates containing PKX cells prepared as described above.

Prior to the start of each experiment at least 10 fish of each species were examined for the presence of PKD, PKX and other myxosporean parasites. Following challenge, fish were maintained in rectangular fibreglass tanks supplied with dechlorinated aerated water at 16°C. Fish were sacrificed between 5 and 15 weeks post challenge (Table. 9) and sequentially in experiments 28 (Table. 10) and 29 (Table. 11).

All major organs and tissues, including kidney, spleen, liver, gills, muscle and intestine were sampled histologically for the presence of PKD, PKX cells and other myxosporean parasites using the procedures described in chapter three. Additional samples from stickleback kidneys were examined fresh using Normarski interference contrast microscopy.

6.2.3.2 Introduction of homogenates by gavage.

0.5 cm3 aliquots of kidney homogenate from sticklebacks containing *S. elegans* and *M. gasterostei* were administered by gavage into anaesthetised rainbow trout fingerlings, using the flexible tube and 'Luer' fitting from an intravenous cannula set (Portex Ltd.). Samples were evaluated histologically as described above.

6.2.3.3 Immersion exposure.

Sticklebacks were exposed to 1 litre of dilute rainbow trout kidney homogenate containing PKX cells prepared as indicated above for 1 hour. Fish were then removed and held at 16°C for 7 weeks. Samples were evaluated histologically as described above.

6.2.4 Exposure of rainbow trout to renal myxosporidiosis by cohabitation with naturally-infected hosts.

These trials utilised flow-through tank systems in which test fish (rainbow trout) were exposed to effluents received from tanks containing parasitised and non-parasitised sticklebacks, tubificid worms or a combination of these. Sixty-eight and 172 litre troughs containing heat-sterilised substrate, either washed Weymouth Bay sand or

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finer sediment from Chafey's Lake (NGR SY 669802), were used for holding the sticklebacks and tubificids. Samples were evaluated histologically as described above.

6.2.5 Field exposure of fish to PKX in river water

Natural challenge with PKX was arranged at a farm site on the River Avon (Wilts.), which has a known history of annual PKD outbreaks. Approximately 200 grayling (experiments 24, 25 and 27) and a similar number of brook trout (experiment 26) were held in seperate circular concrete tanks supplied directly with river water at ambient temperatures. Fish were fed medicated food treated with oxolinic acid (10 mg/kg of fish per day) to combat possible outbreaks of furunculosis caused by the bacterium *Aeromonas salmonicida*. Remaining fish were maintained in the aquarium system at the FDL as negative controls. All tissues and major organs, including the urinary bladder from fish were examined histologically for the presence of myxosporean infections.

6.3 RESULTS.

Experiments are conveniently considered under the following headings:

(a) Direct passage with PKX.

(b) Direct passage with *S. elegans* and *M. gasterostei*.

(c) Concurrent exposure of rainbow trout with *S. elegans* infected sticklebacks and with infected sticklebacks together with invertebrates.

(d) Induction of PKD in hatchery reared grayling and brook trout by exposure to naturally infected river water.

6.3.1 Direct passage with PKX.

6.3.1.1 Transfer of PKX to fish by intraperitoneal injection.

The results are given in Tables 9, 10 & 11. Successful passage of PKX was achieved in brown trout, rainbow trout (used as positive controls for each experiment), grayling and brook trout. Clinical signs of the disease were observed in each species except brook trout. Successful passage was not achieved in carp, minnows, pike or in

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Table 9.   Results of challenge experiments using rainbow trout PKX cells.

Experiment number	Date commenced	Host species and nos.( )	Wks. post challenge & no. exam.( )	Infection with PKX
1	3.8.87	*S. trutta* (60)*	15(10)	+
2	"	*C. carpio* (10)	7(10)	-
3	"	*P. phoxinus* (10)	6(10)	-
4	"	*S. trutta* (10)	8(10)	+
5	"	*O. mykiss* (10)	8(10)	+
6	7.8.87	*E. lucius* (5)	Accidental loss	
7	"	*T. thymallus* (2)	"	
8	4.9.87	*G. aculeatus* (25)	5(7) 7(1)	- -
12	18.9.87	*G. aculeatus* (30)	5(5) 8(8)	- -
13	"	*O. mykiss* (10)	9(10)	+
19	17.8.88	*T. thymallus* (30)	5(5) 8(25)	+ +
20	"	*G. aculeatus* (25)	Mortalities	-
21	"	*O. mykiss* (10)	13(10)	+
28	13.7.89	*T. thymallus* (50)	See Table 10.	
29	"	*S. fontinalis* (30)	See Table 11.	

* jointly with Dr Clifton-Hadley, investigating the pathogenesis and development of PKX in brown trout (Clifton-Hadley & Feist, 1989).

three-spined sticklebacks.

Since initial experiments showed grayling to be susceptible to infection with PKX cells subsequent experiments using grayling (Experiment 28) and brook trout (Experiment 29) were designed to investigate the pathogenesis of PKD in these hosts.

Grayling injected with rainbow trout PKX cells (Experiment 28).

Infection with PKX was seen in 50% of the fish sampled two weeks post-injection rising to 100% during the period between weeks 6 and 12 (Table. 10). The infection continued at a high prevalence until week 19. PKX cells in the spleen were detected in most samples up to week 32. Renal pseudoplasmodia were first noted at week 5 post-injection in one fish and were seen in up to 100% of samples until the end of the experiment at week 40. Myxosporean sporoblasts were detected in renal tubuli from week 12. Mature myxosporean spores were not found in any of the samples examined.

Pathological changes in the spleen, macroscopically recognised as splenomegaly, first became evident at week 8 and affected most fish by week 12, by which time renal hypertrophy was also apparent in most fish (Table. 10). Numbers of fish exhibiting clinical signs decreased between weeks 12 and 19, with splenomegaly and renal hypertrophy absent from fish sampled at week 19. Clinical signs then reappeared and splenomegaly remained until the end of the experiment.

The splenic serosal lining was frequently hyperplastic and also contained PKX parasites. Renal pathology consisted primarily of generalised haematopoietic hyperplasia and dilation of capillaries and renal tubules. Several other tissues were found to be infected with PKX cells from week 6. These included gonad, liver, swimbladder, the serosal layer of the gallbladder and the adipose tissue associated with the pyloric caecae. Control rainbow trout became infected with PKX and exhibited similar pathological signs. Splenic changes were less severe and renal pseudoplasmodia were not detected.

Table 10. Relationship between clinical signs of PKD and the incidence of PKX in the kidney and spleen and renal pseudoplasmodia in grayling injected with PKX cells.

Date	Weeks post-exposure	n	Kidney			Spleen	
			Renal hyper-trophy (%)	PKX cells present (%)	Pseudo-plasmodia present (%)	Spleno-megaly (%)	PKX cells present (%)
13/7/89	2	2	0	50	0	0	0
	6	5	0	100	20	0	80
	8	5	0	100	40	20	100
	12	5	80	100	100	80	100
	15	5	20	40	40	0	100
	19	5	0	60	80	0	80
	24	5	20	40	100	60	100
	32	5	20	20	80	60	60
24/4/90	40	11	0	0	81	18	0

Experiment terminated

Brook trout injected with rainbow trout PKX cells (Experiment 29).

None of the fish sampled exhibited clinical signs of PKD and, in addition, PKX cells were not detected (Table. 11). Myxosporean pseudoplasmodia were observed in renal tubule lumens from week 9 and sporoblasts from week 13. Mature myxosporean spores were not found.

Sticklebacks injected with rainbow trout PKX cells (Experiments 8, 12 & 20).

These experiments were initiated to test the hypothesis that PKX may be an extrasporogonic stage of *S. elegans*. The stock of wild sticklebacks used were monitored before and during the experiments and were initially found to be free of *S. elegans*. However, a latent infection was present in the sticklebacks used in Experiment 8 and sphaerosporosis became evident at a low level (10%) in the control fish. PKX-injected fish were also found to be infected with *S. elegans* at five weeks post injection, at a slightly elevated rate (14%). Similarly, in a repeat trial (Experiment 12), using sticklebacks from the same source, 10.5% of control fish and up to 25% of fish challenged with PKX cells became infected with *S. elegans* pseudoplasmodia and spores. No evidence of infection with PKX was found in either trial.

Sticklebacks used in Experiment 20 and stock control fish remained free from infection. Mortalities were experienced from the first week post injection and only two fish survived to the end of the experiment at week 13. All fish were examined for the presence of *S. elegans* and/or PKX cells and were found to be negative.

6.3.1.2 Transfer of PKX to sticklebacks by immersion exposure to a dilute suspension of PKX cells.

Following a 1 hr exposure to PKX cells, sticklebacks were removed to a clean rectangular fibreglass tank with a constant supply of dechlorinated aerated water at 16°C (Experiment 9). Unexplained mortalities occurred during the first week and several fish were lost. After 7 weeks the three survivors were sacrificed. One was found to be mildly infected with *S. elegans*. Rainbow trout controls remained free of infection.

Table 11. Prevalence of renal pseudoplasmodia in brook trout (*Salvelinus fontinalis*) injected with rainbow trout PKX cells.

Date	Weeks post-injection	*n*	Kidney			Spleen	
			Renal hyper-trophy (%)	PKX cells present (%)	Pseudo-plasmodia present (%)	Spleno-megaly (%)	PKX cells present (%)
13/8/90	4	5	0	0	0	0	0
	9	5	0	0	40	0	0
	13	5	0	0	40	0	0
20/3/91	33	14	0	0	14	0	0
Experiment terminated							

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6.3.2 Direct passage with *S. elegans* and *M. gasterostei*.

Four experiments were conducted to determine whether spores and developmental stages of *S. elegans* were capable of inducing PKD in fingerling rainbow trout. Kidney homogenates from *G. aculeatus* (ex River Avon) heavily parasitised with *S. elegans* and *M. gasterostei* were prepared as indicated previously.

6.3.2.1 Transfer of *S. elegans* and *M. gasterostei* to rainbow trout by intraperitoneal injection.

0.5 cm3 aliquots of kidney homogenate from sticklebacks infected with *S. elegans* and *M. gasterostei* were injected into each of the test fish. Fish were maintained as described above for 12 weeks (Experiment 11) and 16 weeks (Experiment 22). The trout were then sacrificed and examined for the presence of myxosporeans, including PKX. The results of both trials were negative with no evidence of infection by *Sphaerospora, Myxobilatus* or PKX.

6.3.2.2 Transfer of *S. elegans* and *M. gasterostei* to rainbow trout by gavage.

In Experiment 10, 0.5 cm3 aliquots of the stickleback kidney homogenate was administered into 10 rainbow trout fingerlings by gavage using a modified intravenous cannula set (Portex Ltd.). Fish were maintained as described previously for 12 weeks. At necropsy no evidence of infection with the above parasites was found.

In a repeat trial (Experiment 23) fingerling rainbow trout were administered with *S. elegans* and *M. gasterostei* as above. Following challenge, fish were maintained for 16 weeks. At necropsy no evidence of infection with the above parasites was found.

6.3.3 Concurrent exposure of rainbow trout with *S. elegans* infected sticklebacks and with infected sticklebacks together with invertebrates.

A total of 5 experiments to investigate the possible role of *Sphaerospora-*infected sticklebacks and tubificid worms in the transmission of PKD/sphaerosporosis to rainbow trout were conducted.

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1: Rainbow trout and stickleback cohabitation (Experiment 14).

Thirty five three-spined sticklebacks from a stock known to be heavily parasitised with *S. elegans* and *M. gasterostei* were placed in one half of a 323 litre rectangular fibreglass tank divided by a fine mesh plastic screen. Ten fingerling rainbow trout from a source known to be free from PKD were placed in the other half of the tank. The tank was supplied with a constant flow of dechlorinated aerated water at 16°C. Fish were maintained for 14 weeks. At necropsy no evidence of myxosporidiosis was observed in the rainbow trout. Histological evaluation of selected tissues including kidney, liver and spleen confirmed the result.

2: Exposure of rainbow trout to the effluent from a tank containing *S. elegans* parasitised sticklebacks (Experiment 15).

Thirty rainbow trout were exposed to the effluent of a 172 litre trough containing 20 female and 10 male sticklebacks from a stock heavily infected with *S. elegans* but not *M. gasterostei*. Sticklebacks were maintained in tanks containing heat-sterilised sand at 16°C. After 16 weeks exposure the rainbow trout were sacrificed and were found to be free from myxosporean infection.

3: Exposure of rainbow trout to the effluent from a tank containing tubificid worms harbouring actinosporean parasites (Experiment 16).

A mixed culture of several thousand tubificid worms obtained from a commercial source were placed on a substrate of washed and heat-sterilised Weymouth Bay sand in a 172 litre fibreglass trough supplied with dechlorinated aerated water at 16°C. Fresh squash examination of 200 worms revealed the presence of of an infection with a *Triactinomyxon* species at a 3% prevalence rate. Ten fingerling rainbow trout were placed in a side tank receiving effluent from the trough containing the tubificids. The trout were maintained for 11 weeks at which time they were sacrificed and examined for the presence of myxosporidiosis. No evidence of myxosporean infection was found in any of the tissues and organs examined. These included transverse sections of the head just posterior to the orbit, taken to detect possible infections with

M. cerebralis which is now known to be initiated by an actinosporean of the genus *Triactinomyxon*.

4: Exposure of rainbow trout to the effluent from a tank containing actinosporean-free tubificid worms (Experiment 17).

A second mixed culture of several thousand tubificid worms was purchased. For this experiment Chafey's Lake sediment was used as the substrate and was treated as indicated above. This sediment was selected for its finer particle size which was found to be more suitable for the maintenance of the tubificid worms than Weymouth Bay sand. Two hundred worms were checked for the presence of actinosporeans and were found to be free from infection. The remainder were equally divided into two troughs and 25 rainbow trout were placed in each of the respective side tanks. Fish were maintained for 12 weeks at ambient temperatures. During the experimental period 24 trout were lost due to a breakdown in the water supply. All the remaining fish were sacrificed and a range of tissues and organs were sampled for histological examination for the presence of myxosporidiosis. No evidence of infection was found.

5: Exposure of rainbow trout to effluent from a tank containing tubificid worms and *S. elegans/M. gasterostei* infected sticklebacks (Experiment 18).

This experiment was set up at the same time as Experiment 17 (which acted as a negative control) and was designed to investigate the possible involvement of tubificid worms in the transmission of sphaerosporosis to rainbow trout. A further mixed culture of tubificid worms was purchased as before. Two hundred, checked for the presence of actinosporeans were found to be free from infection. The remainder were evenly distributed on Chafey's Lake sediment, treated as indicated earlier, in a trough supplied with dechlorinated and aerated water at ambient temperature . The trough was divided using fine plastic mesh netting. Six male and 15 juvenille sticklebacks from a stock known to be heavily parasitised with *S. elegans* and *M. gasterostei* were placed in the half containing the water inflow pipe. Kidneys of a further 10 sticklebacks were removed, disrupted with forceps and added to the half containing only tubificids.

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Fifteen rainbow trout were placed in a side tank receiving effluent from the trough, and were maintained for 18 weeks. Specimens were taken and examined as indicated above and were found to be free of myxosporean infections. A further 10 trout were added to the side-tank and sampled after an additional 28 and 32 weeks. These fish were also free from infection. No evidence of actinosporean infection was found in tubificids sampled at the end of the experiment.

6.3.4 Induction of PKD in hatchery reared grayling (*Thymallus thymallus*) and brook trout (*Salvelinus fontinalis*) by exposure to naturally infected river water.

Approximately 200 hatchery reared grayling fry were purchased from the YWA. Of these 150 were maintained in a circular concrete tank supplied with river water at a field site with a known history of annual outbreaks of PKD. The remainder were retained at the laboratory as negative controls in a 323 litre fibreglass tank supplied with a constant flow of dechlorinated aerated water at ambient temperature. Prior to transport to the experimental site, 15 fish were examined for the presence of myxosporidiosis and were found to be free of infection. Exposed fish were returned to the laboratory as two groups. The first after 1 week exposure (Experiment 24) and the second after 3 weeks exposure (Experiment 25). Each group was held seperately as described above. Five fish from each group were sacrificed at weekly intervals for the first 10 weeks and thereafter at weeks 12, 14 and 15. Control fish were examined at week 15 and were found to be free of myxosporidiosis.

Renal hypertrophy due to PKD was observed in Experiments 24 and 25 at 8 weeks from first exposure. At this time PKX cells were readily identified in renal impression smears. Fish sacrificed during weeks 10-14 exhibited grossly swollen kidneys, abdominal ascites and anaemia. These symptoms were associated with mortalities in both groups during this period. The bacterium *Aeromonas salmonicida* was isolated from moribund fish and may have been a contributory factor. The mortalities resulted in premature termination of the experiment which was repeated the following year.

In two further experiments, 200 brook trout (Experiment 26) and 200 grayling (Experiment 27) fingerlings were held in seperate circular concrete tanks supplied directly with river water at ambient temperature as described above. Both grayling and brook trout were sequentially sampled as shown in Table. 12 and examined histologically for the presence of PKD and myxosporean parasites. No evidence of PKX or myxosporidiosis was found in fish prior to challenge.

Field exposure of brook trout (Experiment 26).

Brook trout were found to be refractory to PKD. No evidence of infection with PKX cells or other myxosporean parasites were found. Histological changes observed in the kidneys of up to 90% of fish throughout the study were attributable to nephrocalcinosis. Clinical features were minimal and comprised of slight renal swelling with occasional pale deposits of calcified material in the ureters, visible to the naked eye.

Histologically, over the period of the trial, renal tubules became dilated, with associated degenerative epithelial and luminal calcitosis. The nature of the material was confirmed using the chloranilic acid technique for calcium on histological sections. In several cases, proliferation of the interstitial tissues contributed to the renal swelling. The formation of giant cells was frequently noted. In severe cases, granuloma were formed and other tissues, such as the choroidal *rete* and endocrine pancreas contained areas of calcification. In a few fish the muscularis of the swimbladder was thickened.

Field exposure of grayling (Experiment 27).

The first signs of the disease, associated with PKX cells, were detected 5 weeks after introduction to the site (Table. 12). Between weeks 5 and 9, only the kidneys and spleens were affected.

Early renal histopathology (weeks 5 and 6) included dilation of capillaries and renal tubules, and an increase of Bowman's space in the glomerulus. Intertubular serous exudate was also present. By week 7 minimal tubular degeneration and occasional PKX cells migrating through the tubular epithelium were observed. Thickening of the

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glomerular basement membrane and generalised haematopoietic hyperplasia with PKX cells predominantly located in the mesonephros was observed in most fish at this time. At week 7, the first clinical signs were noted. These consisted of mild renal and splenic swelling. Myxosporean pseudoplasmodia were detected in renal tubules in half of the fish sampled. At week 9, all fish were clinically infected with PKD. Renal hypertrophy was slight; however, all spleens exhibited marked splenomegaly and occasional pale colouration, the latter as a result of the proliferation of serosal cells. Large numbers of PKX cells were noted in all spleens, with associated haematopoietic hyperplasia and diffuse inflammation.

During weeks 9 to 14, most fish exhibited gross signs of hypertrophied kidney or spleen, usually associated with pale gills and the presence of abdominal ascitic fluid. PKX cells were noted in other organs, although associated lesions were typically focal (multi-focal in the liver). The severity of PKD was at its maximum during this period, but mortalities were minimal and fish continued to feed normally.

From week 10, kidneys in a few fish showed a chronic diffuse inflammatory response, characterised by 'whorling' of inflammatory cells, usually around PKX parasites. This was also seen in some spleens. Renal tubular degeneration, associated with infiltration of the epithelium by lymphocytes and phagocytes, became apparent at week 10 and continued at a much reduced level to the end of the experiment. During this period, regeneration of renal tubules was noted.

Between weeks 12 and 31, the degree of gross renal and splenic hypertrophy reduced (apart from a dramatic increase in splenomegaly at week 21) (Table. 12). By weeks 31 and 39, there were no pathological changes and PKX cells were absent. Control fish remained free of the disease throughout the duration of the experiment.

The prevalence of renal pseudoplasmodia reached a peak at week 12, the numbers declined to week 21, then there was a second higher peak at week 31, after which the numbers declined again. Although sporoblasts were observed in renal tubuli as early as week 12, fully formed myxosporean spores were not detected in the kidneys or urinary bladders of any of the fish examined.

Table 12. Relationship between clinical signs of PKD and the incidence of PKX in the kidney and spleen and renal pseudoplasmodia in grayling naturally infected with PKX.

Date	Weeks post-exposure	n	Kidney			Spleen	
			Renal hyper-trophy (%)	PKX cells present (%)	Pseudo-plasmodia present (%)	Spleno-megaly (%)	PKX cells present (%)
25/7/89	4	10	0	0	0	0	0
	5	10	0	30	0	0	20
	6	10	0	20	0	0	20
	7	10	0	40	10	20	50
	8	10	30	70	50	30	70
	9	10	60	100	60	100	100
	10	10	100	100	60	90	100
	12	10	90	100	70	100	100
	14	10	60	100	40	60	90
	17[1]	10	40	90	50	60	100
	21	10	10	30	10	90	80
	26	10	40	40	50	10	40
	31	5	0	0	80	0	0
24/4/90	39	14	0	0	38	20	0
Experiment terminated							

[1] All fish were brought back to the laboratory.

6.4   DISCUSSION.

In the present study experimental PKD was established in rainbow trout, brown trout and grayling following either intraperitoneal injection with PKX infected renal tissue or exposure to river water harbouring the infective stage. The results thus confirm those of D'Silva et al., (1984); Clifton-Hadley et al., (1984); Kent & Hedrick, (1985); Rafferty (1986); Clifton-Hadley & Feist, (1989) and Arkush & Hedrick (1990).

Attempts to induce PKD in carp, minnows, pike and sticklebacks failed. Unfortunately, it was not possible to repeat trials using pike as artificial spawning was unsuccessful and an alternative source of experimental fish was not found. Repeat experiments would be desirable to ascertain whether pike can become infected with rainbow trout PKX cells under experimental conditions since this species is the only non-salmonid fish known to be susceptible to PKD (Seagrave et al., 1981; Bucke, Feist & Clifton-Hadley, 1991).

Experiments using sticklebacks were inconclusive since test fish were harbouring low level infections with S. elegans prior to injection with PKX cells. Consequently, possible transformation of PKX cells to sporogonic stages of S. elegans could not be confirmed. Hedrick et al., (1992) provided strong circumstantial evidence to support the hypothesis that a second or intermediate host, possibly the oligochaete Stylaria lacustris, and a smaller infective (actinosporean?) stage are involved in the transmission of the PKX myxosporean. Consequently, the failure to produce an infection in rainbow trout by challenge with S. elegans or M. gasterostei in the present study is not surprising. Since attempts to infect tubificid worms with these parasites also failed, it was not possible to determine whether either S. elegans or M. gasterostei have an actinosporean phase, or whether such a phase might be involved in the transmission of PKD to salmonids. Based on the above results and on the available structural data on PKX and S. elegans (Feist & Bucke, 1987; Feist et al., 1991; Lom et al., 1991) the postulated link between PKX and S. elegans (Feist, 1988) seems unlikely.

That grayling are highly susceptible to PKD is shown here for the first time. Further, although they can be severly affected by the disease at the height of infection they are also capable of full recovery. Of wild fish species examined to date, grayling

appear the most susceptible with the possible exception of brown trout in certain circumstances (Wooten & McVicar, 1982). The susceptibility of wild stocks of Atlantic salmon and Arctic charr to PKD is unknown, although the effects of the disease on cultured fish have been established (Ellis *et al.*, 1985; Bucke *et al.*, 1991). In general, the sequential pathology of the sub-acute and acute phases of the disease in grayling closely followed that seen in affected rainbow trout (Clifton-Hadley *et al.*, 1987), although the relative severity of the disease in grayling was reduced. In addition, the chronic phase of the disease was extended and recovery protracted when compared with the same phases in rainbow trout (Clifton-Hadley *et al.*, 1987).

Mortalities in grayling were minimal, but it is usually secondary infections, i.e. furunculosis, which kill rainbow trout in farm situations, and in these experiments fish were fed antibiotic-treated food to prevent such infection occuring. Nevertheless, morbidity reached 100%. Free-living grayling could be expected to suffer from secondary effects of PKD. In addition, affected fish are probably debilitated and at increased risk from predation. It is therefore hypothesised that mass mortalities in grayling populations resulting from infections with PKD are unlikely, and to date none have been reported. It seems more likely that the majority of PKD-affected fish make a full recovery. PKX parasites in the renal tubules may persist for several years and do not appear to induce a cellular response from the host. These stages may even contribute to immunity to PKD in subsequent years, although there is no data to support this hypothesis.

Whether or not grayling are natural hosts for the PKX parasite is debatable. Grayling, despite belonging to the family *Thymallidae*, are related to the salmonids, family *Salmonidae*, since they possess an adipose fin. The species has a wide distribution throughout Europe, naturally inhabiting cool, fast-flowing, gravel-bottomed streams. However, since the begining of the nineteenth century grayling were introduced into several of the slower moving chalk streams of southern England and are now particularly common in the rivers Test and Itchen (Hampshire) and the river Avon (Wiltshire) (Mackie, 1989; Hal Thirlaway, Piscatorial Society, pers. comm. 1989). Many trout farms are located on these rivers and PKD is a severe problem during the

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summer months. It is interesting to speculate that since grayling, like rainbow trout, are an 'introduced species' to these rivers they may have had insufficient time to evolve an effective defence against PKD. However, this may not explain the susceptibility of brown trout and salmon to PKD since these species are probably indigenous to these rivers. The apparent spread and increasing severity of PKD outbreaks since the early 1970's may be related to the rapid increase in fish farming since then, combined with a greater awareness of the disease. In addition, the huge increase in numbers of susceptible fish (on fish farms) may also have contributed to a build up of infection by releasing infective stages into the environment. However, fully developed spores of PKX have never been conclusively identified from rainbow trout (Hedrick *et al.*, 1988) and it remains a possibility that another fish host, in which sporogony is completed, is involved in the transmission of PKD to salmonids.

Brook trout exposed to waters enzootic for PKD showed no evidence of the disease and the PKX parasite was not detected. However, fish injected with PKX cells harboured renal myxosporean pseudoplasmodia, identical (at the light microscope level) to those found in grayling and brown trout which were similarly challenged. This would seem to indicate that brook trout can become infected and that the PKX cell is able to reach the renal tubules, develop further into pseudoplasmodia and undergo the early stages of sporogenisis. The apparent resistance of brook trout to the PKX parasite with no clinical disease occurring was interesting, since Arctic charr (*Salvelinus alpinus*), a member of the same genus, are known to be susceptible to PKD (Bucke *et al.*, 1991; Brown, Thonney, Holwell & Wilson, 1991). The reason for this difference is unknown but could be related to unidentified factors governing the degree of parasite proliferation within the host. The severity of the host response in turn being related to the numbers of parasites present with disease symptoms only becoming apparent during heavy infections.

The mechanism by which injected PKX cells migrate throughout the host is uncertain. In the early stages of natural infections, PKX cells appear to be able to move in an amoeboid fashion and are frequently found migrating from blood vessels into the kidney and other tissues (Clifton-Hadley *et al.*, 1987). Presumably, injected cells are

also able to penetrate the capillaries of the peritoneal cavity and thus disseminate throughout the body. A similar process was suggested by Molnár & Kovács-Gayer (1986) to explain how, in carp, injected swimbladder stages of *S. renicola* reached the kidneys. Rafferty (1986) proposed that injected PKX cells were phagocytosed by the reticulo-endothelial system and were subsequently transported to the melano-macrophage centres of the kidney. Evidence for this was not found during the present study.

Although PKD and infections with *Sphaerospora* species have not been reported from brook trout, Cone & Cusack (1991) described extrasporogonic myxosporean stages in brook trout kidneys which bore an overall resemblance to PKX and which were similar to extrasporogonic stages described from *Sphaerospora* infections in other hosts (Lom *et al.*, 1985; Baska & Molnár, 1988; Supamattaya *et al.*, 1991). Similar extrasporogonic stages associated with *Chloromyxum* infections were first noted by Lom, Dyková & Kepr, (1988) and it is possible that the brook trout parasite reported by Cone & Cusack (1991) represents a previously undescribed extrasporogonic stage of the *Chloromyxum* species which was also present in some of the fish sampled. It is unlikely that PKX is synonymous with a *Chloromyxum* species since infections with these species have never been recorded in fish suffering or recovering from PKD

The evidence presented in this study shows that, to a greater extent than in rainbow trout in the UK, the PKX parasite in grayling, brook trout and brown trout is able to reach the renal tubule lumens and undergo the early stages of sporogony. The absence of fully developed spores in fish examined during this study does not preclude the possibility that they can occur in wild grayling and brown trout. There are several possible reasons why mature spores were not detected:

(i)   The duration of the experiments (33 to 40 weeks) were not sufficiently long for full development to occur.

(ii)   Maturation may occur outside the fish, possibly involving an intermediate invertebrate host, in which infective stages to the fish are produced.

(iii)   The sampling procedure may have been inadequate.

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Members of the genus *Sphaerospora* appear to complete sporogony well within the experimental period used in the current study (Molnár & Kovács-Gayer, 1986; Odening *et al.*, 1988). Maturation outside the fish host is unlikely since immature spores are not fully protected by spore valves and would be unlikely to survive outside the fish host for any significant length of time. It is likely that, if an intermediate host were involved in parasite transmission, it would only be susceptible to mature and infective spores. Samples were carefully selected to include, where possible, the urinary bladder (the majority of fish), this being the most likely site for the detection of mature spores. In addition to histological examinations, the contents of several urinary bladders were examined separately. These examinations were made near the end of the experimental period, and again spores were not detected.

Mature sphaerospores associated with PKX infections have rarely been found in rainbow trout (Hedrick *et al.*, 1988; Odening *et al.*, 1988) and have not yet been reported from fish in the UK. From the evidence presented in this study the PKX parasite is able to mature further in grayling, brown trout and brook trout than in rainbow trout, and it seems likely that PKX, although able to infect several fish species, is not able to fully mature in each. If the presence of mature spores of the PKX parasite are confirmed in wild grayling or in brown trout, then either of these species may contribute to PKD outbreaks in the UK.

Several myxosporean/actinosporean life cycles have now been demonstrated and involve a variety of oligochaete genera including, *Eclipidrilus* (Kent *et al.*, 1990), *Nais* (Grossheider & Körting, 1992), *Branchiura* (Yokoyama *et al.*, 1990) and *Tubifex* (Markiw & Wolf, 1983; El-Matbouli & Hoffmann, 1989, 1991; Ruidisch *et al.*, 1991). These involving members of the myxosporean genera *Myxobolus* and *Hoferellus* only. An actinosporean stage of a *Sphaerospora* species has yet to be conclusively identified. However, Burtle, Harrison & Styer (1991) have described an actinosporean from oligochaetes sampled from ponds containing channel catfish *Ictalurus punctatus* with proliferative gill disease, which is possibly caused by *Sphaerospora ictaluri* (Hedrick *et al.*, 1990). The recently developed monoclonal antibodies to PKX (Adams, Richards & Marin de Mateo, 1992) may provide a valuable tool for the identification of currently

undetected life cycle stages of PKX, including possible actinosporean stages, both in the fish and invertebrate hosts. The confirmation of a second or intermediate host involved in the transmission of PKD to salmonids may offer opportunities for the development of disease control methods based on changes in husbandry and/or the removal of the invertebrate host from infected sites rather than chemical treatment of infected fish.

CHAPTER 7

GENERAL DISCUSSION

Although myxosporeans have been recorded in several parasite surveys (Andrews, 1979; Chubb, 1963, 1965; Mishra, 1966; Mishra & Chubb, 1969; Rizvi, 1964) their general distribution in the UK is poorly known. Results from the present investigation have shown myxosporean infections to be widespread in wild freshwater fish in the UK and in addition, the diversity of myxosporean species was found to be greater than previously reported (Kennedy, 1974). This finding reflects the lack of attention these parasites have received in the UK rather than a paucity of the fauna, which has probably increased in recent years through the importation of parasitised fish from abroad (Crashaw & Sweeting, 1986). Several studies have focussed on rare epizootics of myxosporidiosis with mass mortalities (Williams, 1964) or observable pathological features such as skeletal deformities (Bucke & Andrews, 1985; Lom, Pike & Dyková, 1991). These occurrences underline the potential importance of these organisms as disease agents of fish. However, since only non-salmonid species were affected, they caused little concern to the predominantly salmonid aquaculture industry in this country. However, on a global scale over recent decades, both carp and salmonid farming have developed and expanded. Although viral and bacterial infections are the main cause of disease, myxosporean infections are increasingly diagnosed as causal agents. In the UK, outbreaks of whirling disease during the early 1980's resulted in significant loss of stock and the imposition of movement restrictions (Bucke et al., 1983) and currently PKD is regarded by the industry as the most serious disease affecting trout production (Clifton-Hadley et al., 1984).

In eastern European countries where carp farming is extensive, myxosporean infections have become a major threat to the industry (Dyková & Lom, 1988). Since Molnár (1980) first drew attention to the pathogenicity of S. renicola (Dyková & Lom, 1982), in cultured carp, several other species of Sphaerospora have been identified as important pathogens in a wide variety of fish species (Lom, Körting & Dyková, 1985; Sitja-Bobadilla & Alvarez-Pellitero, 1990; Supamattaya et al., 1991). During the present study Sphaerospora species were frequently found within the renal tubules of

several fish species examined, although fully developed spores were often absent. Lom *et al.*, (1985) during a survey of *Sphaerospora* species also found that, in many cases only pseudoplasmodia were present within renal tubules. Similarly, the lack of spore formation in PKD infections, both in fish recovering from clinical disease and in older fish that had suffered the disease the previous year was puzzling. The possibility that PKX only completes sporogony in older fish should be considered. This could explain why mature spores associated with PKX infections have only rarely been recorded (Hedrick *et al.*, 1988; Odening *et al.*, 1988) since most hatchery reared rainbow trout experiencing a PKD outbreak will be in their first year. Older fish, in which sporogony may have been completed, are generally not retained on fish farms and samples that were obtained in the present study did not harbour spores in the kidneys or urinary bladders. Similarly, the present survey of wild fish identified PKX in brown trout, pike and grayling, but in none of these species were fully developed spores detected, even in mature fish. There is difficulty therefore, in explaining the source of PKD infections. The hypothesis that the three-spined stickleback might be the normal host for the PKX parasite, in which sporogony is completed (Feist, 1988), could not be confirmed in the present study. The possibility remains therefore, that sporogony occurs within another, as yet unidentified fish host. Alternatively, PKX may complete sporulation in the urinary bladder, a site not routinely examined in previous studies. If confirmed, wild salmonids would be implicated as the source of infection to farmed fish.

Although the results of this and other investigations have confirmed PKX to be a myxosporean, the source of infection has yet to be identified (Kent & Hedrick,1985a; Feist & Bucke, 1987). Strong circumstantial evidence that an aquatic oligochaete acts as a second or intermediate host for PKX (Hedrick, Monge & de Kinkelin, 1992), may offer possibilities for the control of PKD by the selective eradication of these organisms in affected sites. However, in order to achieve this, the use of chemicals is equally likely to harm other organisms in the environment such as insect larvae and molluscs and in addition, add to concerns over contamination of potable water supplies. Consequently, biological control methods to regulate the numbers of oligochaetes could

be employed. The identification of specific pathogens or predators, such as leeches, will be pivotal to the development of such techniques.

The results of the transmission studies have for the first time provided an assessment of the impact of PKD in grayling. That wild grayling can exhibit marked pathological changes has been noted previously (Seagrave *et al.*, 1981) and was confirmed during the present study. The control of PKD infections in populations of wild fish is currently impossible and would depend on the removal of the infective agent from the river. It was interesting that brook trout showed no signs of clinical PKD after challenge with PKX cells or exposure to infected river water and this requires further investigation. If brook trout are generally resistant to PKD, this species may offer possibilities to determine the genetic factors responsible. Once identified, these factors, through the application of genetic manipulation, could possibly be used to confer resistance to susceptible stocks or brood fish.

In general, myxosporean infections have been regarded as untreatable and control has depended on the avoidance of infected water or the clearance of diseased stock followed by pond disinfection and re-stocking. Nevertheless several drugs were found to be partially successful for the treatment of whirling disease (Scolari, 1954; Taylor, Coh & Juneh, 1973; Alderman, 1986) although none have come into regular use. Recent investigations using Triazinone (Toltrazuril) were successful in treating *Myxobolus* sp. and *Henneguya* sp. infections (Schmahl, Mehlhorn & Taraschewski, 1989; Schmahl, Sénaud & Mehlhorn, 1991). Following treatment, plasmodia located in the gills of the hosts exhibited degenerative changes but spores were not affected. The resistance of spore stages to the effects of Toltrazuril may explain the limited success of previous studies. It is likely that the spore valves limit entry of the drug, the enclosed sporoplasm remaining unaffected. Consequently, for any chemotherapeutant to be effective it should be applied either as a prophylactic treatment or during the early stages of infection before sporogenesis has commenced. Since the PKX cell during clinical PKD does not undergo sporogony and remains separated from the host tissues by only a single cell membrane (Ferguson & Needham, 1978; Smith *et al.*, 1984; Kent

& Hedrick, 1986; Feist & Bucke, 1987), the apparent vunerability of the parasite within the host may allow effective control of PKD using Toltrazuril.

A knowledge of the life cycle of myxosporean parasites would generally improve the prospect of disease control. In this respect it is now possible to interrupt transmission of *M. cerebralis* by eradicating the tubificid host. That only fingerlings are susceptible to whirling disease provides further scope for disease management by delaying transfers of fish to high risk waters until ossification of the cranial cartilage is well advanced. Similar techniques are ineffectual in preventing outbreaks of PKD, since all ages of fish are susceptible on first exposure. Control has depended instead on the use of malachite green (Clifton-Hadley & Alderman, 1987; Alderman & Clifton-Hadley, 1988) or the antibiotic Fumagillin DCH (Hedrick *et al.*, 1988; Wishkovsky *et al.*, 1990). However, both of the above treatments are associated with toxic side effects and the long withdrawal times required post-treatment with malachite green will make licensing of these compounds for fish use difficult (Gerundo, Alderman, Clifton-Hadley & Feist, 1991; Hedrick, Monge, Kazanji, Pery, Marin & de Kinkelin, 1992).

The development of effective vaccines against PKD and other myxosporeans will be dependant upon a sound knowledge of the immune response of fish to these pathogens and the identification of protective antigens. The development of monoclonal antibody probes to PKX will provide valuable tools in this respect (Adams *et al.*, 1992), although the nature of surface antigens is yet to be determined. The results of the current ultrastructural examination of PKX clearly demonstrated the presence of a thin amorphous extracellular deposit surrounding the parasite. This appeared to be produced by the secretion of haplosporosome contents. Further studies are required to test whether the deposit reacts with currently available monoclonal probes and to determine the nature of the deposit in order to understand its function in relation to the marked host response to the PKX parasite. This information may also assist in vaccine development.

Alternative control strategies include the removal or destruction of infective stages. The use of ultraviolet irradiation of water supplies containing the actinosporean stage of *M. cerebralis* has been shown to be effective in reducing water-borne

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infectivity, especially when used in conjunction with sand-charcoal filtration to remove suspended material (Hoffman, 1975). Control of *Ceratomyxa shasta* infections in N. American trout hatcheries is usually by avoidance of water supply known to harbour the infective stage. However, where this is not possible, treatment of hatchery supplies with ozone (Tipping, 1983), microfiltration followed by ultraviolet irradiation (Bower & Margolis, 1985), or chlorination (Sanders, Fryer & Leith, 1972) have been found to have a significant effect in reducing mortalities due to *C. shasta*. Although ultraviolet treatment has also been found to be effective in removing the infective stage of PKD (Hedrick, Kent & Smith, 1986) the technique has not come into general use. In the hatchery situation, PKD could perhaps be controlled by any of the above techniques since the volume of water passing through is generally small. However, the use of spring or bore-hole water precludes the need for further treatment in most hatcheries. Main farm sites however, require a much greater flow of water and costs of implementing treatment schemes are prohibitive.

In summary, the results of this study show that myxosporean parasites are widespread in freshwater fish in the UK and that renal infections with *Sphaerospora* species occurred in most species examined. PKD was confirmed in wild grayling, pike and brown trout. The structure of PKX within the fish host was investigated and compared with already documented myxosporeans. Several new features were described and although synonomy with other kidney infecting species was not confirmed, the organisation of early sporogonic stages strongly suggest affinities with the genus *Sphaerospora*. In addition, studies on *M. gasterostei* and *M. lieberkuehni* have provided new data on the pathogenicity of these species within the host kidney. Transmission studies demonstrated for the first time the pathogenesis of PKD in grayling. The absence of clinical PKD in brook trout exposed to the infective stage requires further investigation. The hypothesis that sticklebacks and *S. elegans* might be involved in the transmission of PKD was tested but could not be confirmed. The re-description of *S. elegans* (type species for the genus) provided new data on the surface morphology of the spore and ultrastructural features were described for the first time. Preliminary attempts to ascertain the involvement of invertebrates in PKD transmission

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were inconclusive and further studies to locate the source of infection within river systems are required before strategies to avoid outbreaks of PKD on fish farms can be developed.

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I hereby certify that the contents of the permanently bound thesis are identical to the version submitted for examination (*except where amendments have been made to meet the requirements of the examiners).

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INTER-RELATIONSHIPS OF MYXOSPOREANS, INCLUDING PKX WITH CERTAIN FRESHWATER FISH.

by

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DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work has been performed by myself, and that all sources of information have been specifically acknowledged.

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