STUDIES ON MYXOZOAN PARASITES OF FRESHWATER FISH AND INVERTEBRATE HOSTS

By

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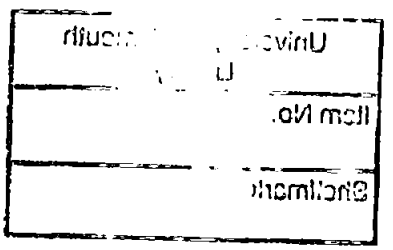

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ABSTRACT

A study of myxozoan parasites has been investigated in hosts from freshwater environments in the UK. Over 17,000 oligochaetes, almost 5,000 juvenile cyprinids representing 7 species and over 60 invertebrate species have been examined for the presence of myxozoan parasites. In addition, studies on the lifecycle of *Tetracapsuloides bryosalmonae* (the causative agent of salmonid proliferative kidney disease, PKD) and of selected cyprinid myxozoans were conducted.

A total of 21 actinospore types in seven collective groups were isolated and described from oligochaetes collected from seven different river systems in England and Wales. Twelve of the actinospores isolated appear to be new to science. Differences were noted in types of actinospores released at different sites and between seasons. Most actinospores were released from oligochaetes in spring and summer with prevalence of release ranging from 0.11% up to 5.83%. The most common actinospores were members of the collective group Echinactinomyxon with seven types identified, followed by the collective group Triactinomyxon, of which 6 types were identified. Five actinospores types were each encountered only once during the study.

In juvenile cyprinid fish, 14 identifiable species of myxozoans in the genera *Myxidium*, *Myxobolus* and *Sphaerospora* plus three developmental stages were detected by histological examination. The most common myxozoans in cyprinids were *Myxobolus pseudodispar* and *Myxobolus pfeifferi*. Roach contained the most number of myxozoan species. Only seven myxozoan species were found in chub, but

pathological responses and intensity of infections, particularly with *M. pseudodispar, M. pfeifferi* and *Myxobolus buckei* were greater when compared to other cyprinids examined. Juvenile cyprinids only appear to mount a pathological response to myxozoans once sporogony is initiated and some of those responses were considered severe enough to be detrimental to host survival.

Mathematical models were produced using parasite data and incorporating a variety of data, including fish length, year class strength and environmental data to attempt to demonstrate a population level effect of disease. Many of the models developed clearly show that parasitism by *Myxobolus* spp. and *Bucephalus polymorphus* in juvenile fish is strongly correlated with population success in selected UK rivers.

Laboratory experiments to transmit *Myxobolus* spp., *Myxidium* spp. and *Sphaerospora* spp. from selected cyprinid hosts to oligochaetes were unsuccessful. The most likely explanation is that the genetic strain of *Tubifex tubifex* used in the trials was not susceptible to infections by the myxospores selected.

Specific DNA primers for *Tetracapsuloides bryosalmonae* were used on samples of over 60 invertebrate species collected from sites enzootic for PKD and on all 21 actinospore types isolated during the current study. All PCR reactions were negative for the presence of *T. bryosalmonae* DNA.

Naïve rainbow trout exposed to *T. bryosalmonae* spores from naturally infected bryozoans by bath challenge for 10 minutes developed PKD. Intraperitoneal injection of spores failed to induce the disease. The favoured route of entry by the parasite appears to be through mucous cells in the skin epithelium.

CONTENTS

Parts of this work and allied work have been published or presented at national and international conferences. Some of these papers have been included within chapters (denoted by *):

1. **Longshaw, M.** and Feist, S.W. (1997) Oligochaetes as hosts for aquatic parasites. *European Association of Fish Pathologists, Eighth International Conference, Edinburgh, September 14-19, 1997* Poster
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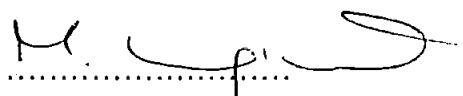
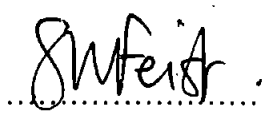
DECLARATION

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



Matthew Longshaw



Stephen W. Feist
(Director of Studies)

I would like to dedicate this thesis to my family. I couldn't have done it if you weren't all part of my life.

CHAPTER ONE

GENERAL INTRODUCTION

Myxozoans are common parasites of teleost fish and invertebrates and include a few species that also infect amphibians and reptiles. In addition, there have been reports of myxozoans in elasmobranchs (Arthur and Lom, 1985; Heupel and Bennett, 1996; Stoffregen and Anderson, 1990, Feist *et al.*, unpublished data), one in a digenean (Overstreet, 1976), a myxozoan-like parasite in European mole, *Talpa europaea* (Friedrich *et al.*, 2000) and most recently, a *Kudoa* sp. has been reported causing myoliquefaction in octopus (Yokoyama and Masuda, 2001). Additionally, there has been a report of myxozoans in inflammatory lesions in the bile ducts and hepatic parenchyma of anatid ducks held in a zoological garden (Lowenstine *et al.*, 2002). The presence of myxozoans in human stool samples appears to be incidental and due to the ingestion of infected fish (McClelland *et al.*, 1997; Lebbad and Wilcox, 1998; Boreham *et al.*, 1998; Moncada *et al.*, 2001). Most myxozoan infections are relatively innocuous causing little harm to the host, although several are serious pathogens, which have been associated with significant epizootics in wild populations and losses in aquaculture (Bucke and Andrews, 1985; Dyková *et al.*, 1987; Molnar, 1988; Dyková and Lom, 1988; Lom and Dyková, 1992; Bartholomew *et al.*, 1997; Hedrick *et al.*, 1993, 1998; Kent *et al.*, 2001). Myxozoans were classified as multicellular (=pluricellular) protistans but recent molecular evidence has shown that they are in fact highly specialised metazoans. Their phylogenetic position has been a source of contention, being placed within either the Bilateria (Smothers *et al.*, 1994; Schlegel *et al.*, 1996; Anderson *et al.*, 1998; Kim *et al.*, 1999; Zrzavý and Hypša, 2003) or the cnidarians (Siddall *et al.*, 1995; Zrzavý *et al.*, 1998, Zrzavý, 2001). Recent ultrastructural and molecular evidence on *Buddenbrockia plumatellae* has provided further proof that the myxozoans are metazoans allied to the Bilateria (Montiero *et al.*, 2002; Canning *et al.*, 2002). Over 1300 species have been described principally on spore morphology and in some cases on tissue tropism (Bahri & Marques, 1996). Spores are derived from the differentiation of three cell types comprising one to seven capsulogenic cells, which form the polar capsules, up to seven valvogenic cells enclosing the polar

capsules and the infective sporoplasm, which consists of one to twelve cells (Feist & Longshaw, 2000). Myxozoans are classed as either histozoic (within tissues) or coelozoic (inhabiting cavities).

Until the pioneering studies by Wolf and Markiw (1984) it was thought that myxospores were transmitted directly from fish to fish and that the spores needed to be "aged" in mud for a period of several months in order to be infective. However, Wolf & Markiw (1984) demonstrated that an alternate oligochaete host was an essential requirement in the development of *Myxobolus cerebralis* (the causative agent of whirling disease in salmonids). The oligochaete stage, known as an actinospore is the infective stage to fish. Subsequently a number of other studies have established that some myxozoans undergo a two-host lifecycle (El-Matbouli & Hoffmann, 1989; Kent *et al.*, 1993; Uspenskaya, 1995; Andree *et al.*, 1997; Özer and Wootten, 2000; Székely *et al.*, 2001). In addition, a polychaete has been shown to act as the alternate host for *Ceratomyxa shasta* (Bartholomew *et al.*, 1997) and bryozoans are definitive hosts for *Tetracapsuloides bryosalmonae,* the causative agent for Proliferative Kidney Disease (PKD) in salmonid hosts (Longshaw *et al.*, 1999, 2002; Feist *et al.*, 2001). However, not all myxozoan lifecycles have been elucidated and whilst there is a consensus that most will undergo a two host lifecycle, it is recognised that some, at least can transmit directly (Diamant, 1997; Redondo *et al.*, 2002, Yasuda *et al.*, 2002) and the possibility of additional invertebrate hosts has not been excluded. The establishment of myxozoan lifecycles under experimental conditions and understanding the fundamental transmission requirements of myxozoans will facilitate the development of methods for avoidance of the infective stage of the parasite and of potential therapeutants or vaccines.

PKD is one of the most serious parasitic diseases of farmed and wild salmonids. The disease is found in Europe, the USA and Canada and most species of salmonids are considered susceptible. Ultrastructure studies in the 1980s demonstrated that the agent is a member of the Myxozoa (Kent and Hedrick, 1985; Feist and Bucke, 1987; Clifton-Hadley

and Feist, 1989). This view was further supported by molecular evidence in the 1990's (Saulnier and de Kinkelin, 1997; Kent *et al.*, 1998; Saulnier *et al.*, 1999). Subsequently, the studies of Anderson *et al.* (1999a, b), Longshaw *et al.* (1999), Canning *et al.*, (1999, 2000) and Feist *et al.* (2001) unequivocally placed the parasite within the Myxozoa. The demonstration that *T. bryosalmonae* utilises bryozoans in part of its lifecycle has provided opportunities for studying the infectious stage and transmission requirements which may eventually lead to control of PKD in farmed and wild fish through laboratory studies and management practises against bryozoans.

In England and Wales, concern has been raised over the apparent declines in cyprinid populations. Data collected by the Pathology/Parasitology team at CEFAS Weymouth over a number of years has shown that cyprinid fry (mainly chub, roach, dace, minnow) can harbour significant infections of a number of myxospores, particularly in the muscle and vertebral column (Feist and Longshaw, 2000; Feist *et al.*, unpublished data). In some cases the severity of infection and host response may lead to a decline in cyprinids recruitment that may be attributed, at least in part, to these highly pathogenic parasites.

The main objectives for this study were: (1) To examine the role of oligochaetes in the lifecycle of Myxozoa, including the agent for Proliferative Kidney Disease, and examine spatial and temporal patterns of distribution, (2) to examine the pathogenicity of myxozoan infections in juvenile cyprinids in UK rivers at the individual and population level, (3) to link different life stages of myxozoans through experimental challenges and by use of molecular biology, and (4) to determine the early pathogenesis and route of entry of *T. bryosalmonae* into salmonid hosts.

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CHAPTER TWO

LITERATURE REVIEW

2.1 BACKGROUND

Although myxozoans have been studied for over a hundred years, it has only been in the last twenty years or so that our understanding of their lifecycles, development and phylogeny has begun to be better understood. The major review articles that have been written in the last ten years bear testament to the importance of these parasites both as scientific curiosities and as disease causing agents (El-Matbouli *et al.*, 1992a, Garden, 1992, Lom and Dyková, 1992a; Moser and Kent, 1994; Kent *et al.*, 2001). Following the demonstration of the link between actinospores and myxospores as two stages of the same animal, a number of other studies further demonstrated that in the freshwater at least, myxozoans alternate between a fish and oligochaete host. Although it is known that marine oligochaetes can harbour actinospores (Kent *et al.*, 2001), there remains a paucity of data on the lifecycles of marine myxozoans. To date, direct transmission of four marine myxozoans, *Myxidium* spp. and *Enteromyxum* spp. have been shown (Diamant, 1997; Yasuda *et al.*, 2002; Redondo *et al.*, 2002) and more recently Koie *et al.* (2004) have completed the lifecycle of the marine myxozoan *Ellipsomyxa gobii* through the polychaete *Nereis* sp. Feist *et al.* (2001) have also shown experimentally that *Tetracapsuloides bryosalmonae* (formerly PKX) utilises a bryozoan in at least part of its lifecycle. Major advances in molecular tools have allowed the linkage of the two stages to be demonstrated and clarified the systematics and phylogeny of the Myxozoa.

One of the benefits of the new molecular techniques has been the development of new sensitive tools for the detection and understanding of myxozoan parasites of economic concern. The use of PCR and sequencing initially allowed the alternate host of *Tetracapsuloides bryosalmonae* to be determined (Anderson *et al.*, 1999a and b; Longshaw *et al.*, 1999). Subsequent biological transmission trials were confirmed using the PCR technique (Feist *et al.*, 2001) and the routes of entry into the fish host by the parasite were demonstrated by the *in-situ* hybridisation technique (Morris *et al.*, 2000b; Longshaw *et al.*,

2002). Molecular tools, including monoclonal and polyclonal antibodies (Markiw and Wolf, 1978; Griffin and Davis, 1978; Hamilton and Canning, 1988; Bartholomew *et* al., 1989; Adams *et al.*, 1992; Saulnier and de Kinkelin, 1996; Marín de Mateo *et al.*, 1996; Clouthier *et al.*, 1997; Morris *et al.*, 1997, 2000a; Muñoz *et al.*, 1998, 1999b; Chase *et al.*, 2001; Lu *et al.*, 2002, 2003), lectins (Castagnaro *et al.*, 1991; Marín de Mateo *et al.*, 1996; Muñoz *et al.*, 1999a, 2000), and DNA based tools (Andree *et al.*, 1997, 1998, 1999a, 1999b; Bartholomew *et al.*, 1997; Hervio *et al.*, 1997; Saulnier and de Kinkelin, 1997; Kent *et al.*, 1998, 2001; Lin *et al.*, 1999; Pote *et al.*, 2000; Xiao and Desser, 2000c; Feist *et al.*, 2001; Hanson *et al.*, 2001; Eszterbauer *et al.*, 2001; Eszterbauer, 2002) have been used in studies of myxozoan biology.

Most studies involving myxozoans have considered the taxonomy or pathology of the parasite and have been concerned with spatial rather than temporal changes in the myxozoan fauna of fish. On the other hand, more recent studies of actinospore stages have examined both spatial and temporal alterations (El-Mansy *et al.*, 1998a, b; Oumouna *et al.*, 2003; Özer *et al.*, 2002; Xiao and Desser, 1998c), perhaps due to the widespread availability of oligochaetes. In addition, there are logistical problems associated with the removal of large numbers of fish from a system. Most oligochaetes are invariably only infected with one species of actinospore at any one time, unlike fish which may contain several myxospores. Therefore, the amount of time spent isolating myxozoans from oligochaetes can be considerably reduced compared with fish. Few studies have examined the role of myxozoans on determining population structure and size and unequivocally demonstrated the population level effects of myxozoan disease. These have included the studies on whirling disease (Hedrick *et al.*, 1998) and *Thelohanellus* sp. in roach (Williams, 1964).

2.2 MYXOZOAN TAXONOMY

Following the original description of myxozoans in 1881 by Bütschli, the phylum has undergone a series of taxonomic revisions. Despite early suggestions that myxozoans may be metazoans, they were classified as multicellular (=pluricellular) protistans for many years. Actinospores in oligochaetes were first described by Štolc (1899) and whilst for many years they were placed within the same phylum as myxozoans from vertebrate hosts, no attempts were made to link them biologically. As a result the phylum Myxozoa contained two classes – the Myxosporea and the Actinosporea. The class Myxosporea currently consists of the order Bivalvulida composed of three suborders and twelve families and the order Multivalvulida containing two families (see Figure 2.2.1). Following the discovery that actinospores represented an alternate stage of the Myxosporea, Corliss (1985) and Kent *et al.*, (1994) called for the suppression of the class Actinosporea and considered that all myxozoan parasites in annelid worms be relegated to *species inquirendae*. The exception to this was that the actinosporean genus *Tetractinomyxon* should be moved to the myxozoan order Multivalvulida. Subsequently, Kent and Lom (1999), in agreement with El-Matbouli and Hoffman (1998), moved *Tetractinomyxon* back into the actinospores and reduced the genus to a collective group. The suppression of the class Actinosporea was further supported by Lom *et al.*, (1997). The proposals by Lom *et al.*, (1997) and Kent *et al.*, (1994) that new forms of actinospore should not be named and referred to as e.g. collective group sp. 1, of Smith, 1995, etc were not universally accepted. Lester *et al.*, (1998) suggested that the naming of new forms of actinospores should continue. Various arguments in justification were proposed by proponents of the two positions (Lester *et al.*, 1998; Kent and Lom, 1999; Lester *et al.*, 1999) to justify their stance and currently most authors agree with Kent and Lom (1999) in publishing descriptions of new actinospore forms and referring to them as type 1, 2, etc. Following the discovery of myxozoan parasites in bryozoans, Canning *et al.*, (2000)

Figure 2.2.1 Current classification of the Phylum Myxozoa.

Phylum Myxozoa Grasse 1960

Class Myxosporea Bütschli, 1881

Order Bivalvulida Shul'man, 1959

Suborder Platysporina Kudo, 1919

Family Myxobolidae Thélohan, 1892

Suborder Sphaeromyxina Lom & Noble, 1984

Family Sphaeromyxidae Lom & Noble, 1984

Suborder Variisporina Lom & Noble, 1984

Family Alatosporidae Shul'man *et al.*, 1979

Family Auerbachiidae Evdokimova, 1973

Family Ceratomyxidae Doflein, 1899

Family Chloromyxidae Thélohan, 1892

Family Fabesporidae Naidenova & Zaika, 1969

Family Myxidiidae Thélohan, 1892

Family Ortholineidae Lom & Noble, 1984

Family Parvicapsulidae Shul'man, 1953

Family Sinuolineidae Shul'man, 1959

Family Sphaerosporidae Davis, 1917

Order Multivalvulida Shul'man, 1959

Family Kudoidae Meglitsch, 1947

Family Trilosporidae Shul'man, 1959

Class Malacosporea Canning, Curry, Feist, Longshaw & Okamura, 2000

Order Malacovalvulida Canning *et al.*, 2000

Family Saccosporidae Canning *et al.*, 1996

erected a new class and order within the phylum Myxozoa to accommodate these parasites.

The advent of molecular techniques led to a series of publications on the phylogenetic position of the Myxozoa. They have been allied either to the Bilateria (Smothers *et al.*, 1994; Schlegel *et al.*, 1996; Anderson *et al.*, 1998; Kim *et al.*, 1999; Zrzavý and Hypša, 2003) or to the cnidarians (Siddall *et al.*, 1995; Zrzavý *et al.*, 1998, Zrzavý, 2001) and, on the basis of the molecular data, led to calls for the suppression of the phylum and its transfer to the cnidarians by Siddall *et al.*, (1995). Anderson *et al.*, (1998) examined the HOX genes within the Myxozoa, which showed that the parasites clearly sat within the bilaterians. Ultrastructural characterisation of the bryozoan myxozoan *Buddenbrockia plumatellae* supported the contention that whilst myxozoans are allied to the bilaterians, the phylum Myxozoa should be retained (Montiero *et al.*, 2002; Canning *et al.*, 2002). Currently, myxozoans are considered to be highly specialised metazoans.

Myxozoan taxonomy is based on the structure of the spore stages, principally on the number of shell valves, spore shape and number and position of the polar capsules (Figure 2.2.2). The main diagnostic features are demonstrated in Figure 2.2.3. Over 1300 species have been described principally on spore morphology and in some cases on tissue tropism (Bahri & Marques, 1996). The use of molecular tools has allowed further characterisation of parasites to take place and in many cases confirmed the morphological criterion for species discrimination (Estzerbauer, 2002; Molnár *et al.*, 2002). However, it has also demonstrated in some cases that morphological criteria may not necessarily be the best method to elucidate the relationships within the group. For example, Whipps *et al.*, (2003) recently described a new species of *Kudoa* using morphological and molecular approaches. On the basis of morphological characters, the new species should have been classified within the family Septemcapsulidae as it possessed 12-13 polar capsules. Sequence information however, clearly placed the parasite within the family Kudoidae.

Figure 2.2.2 Line drawings of representative myxospore genera showing key morphological features (not to scale). A. *Ceratomyxa*, B. *Henneguya*, C. *Myxobilatus*, D. *Hoferellus*, E. *Myxobolus*, F. *Sphaerospora*, G. *Kudoa*, H. *Chloromyxum*, I. *Parvicapsula*, J. *Sphaeromyxa*, K. *Myxidium*, L. *Thelohanellus*, M. *Unicapsula*.

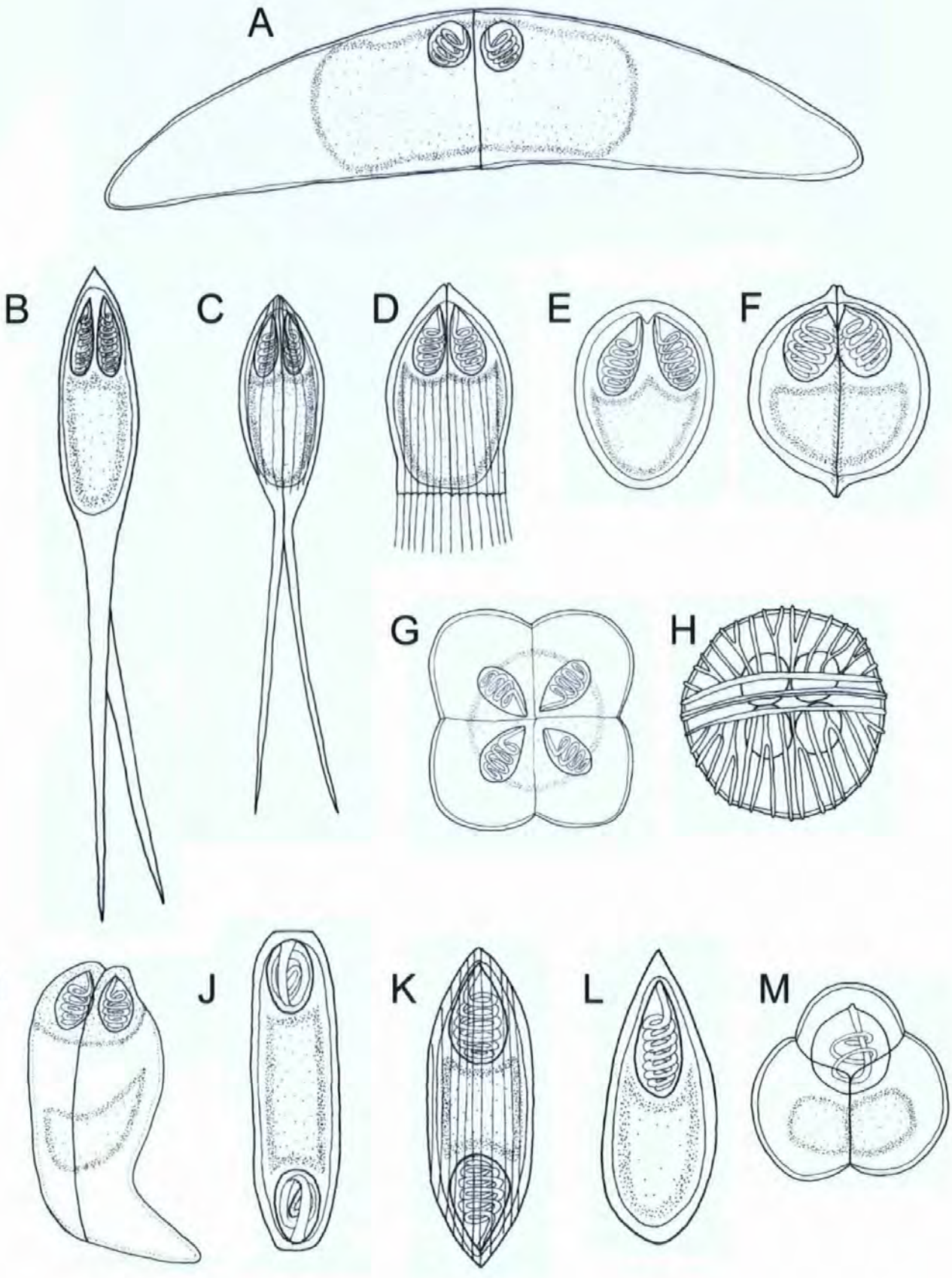
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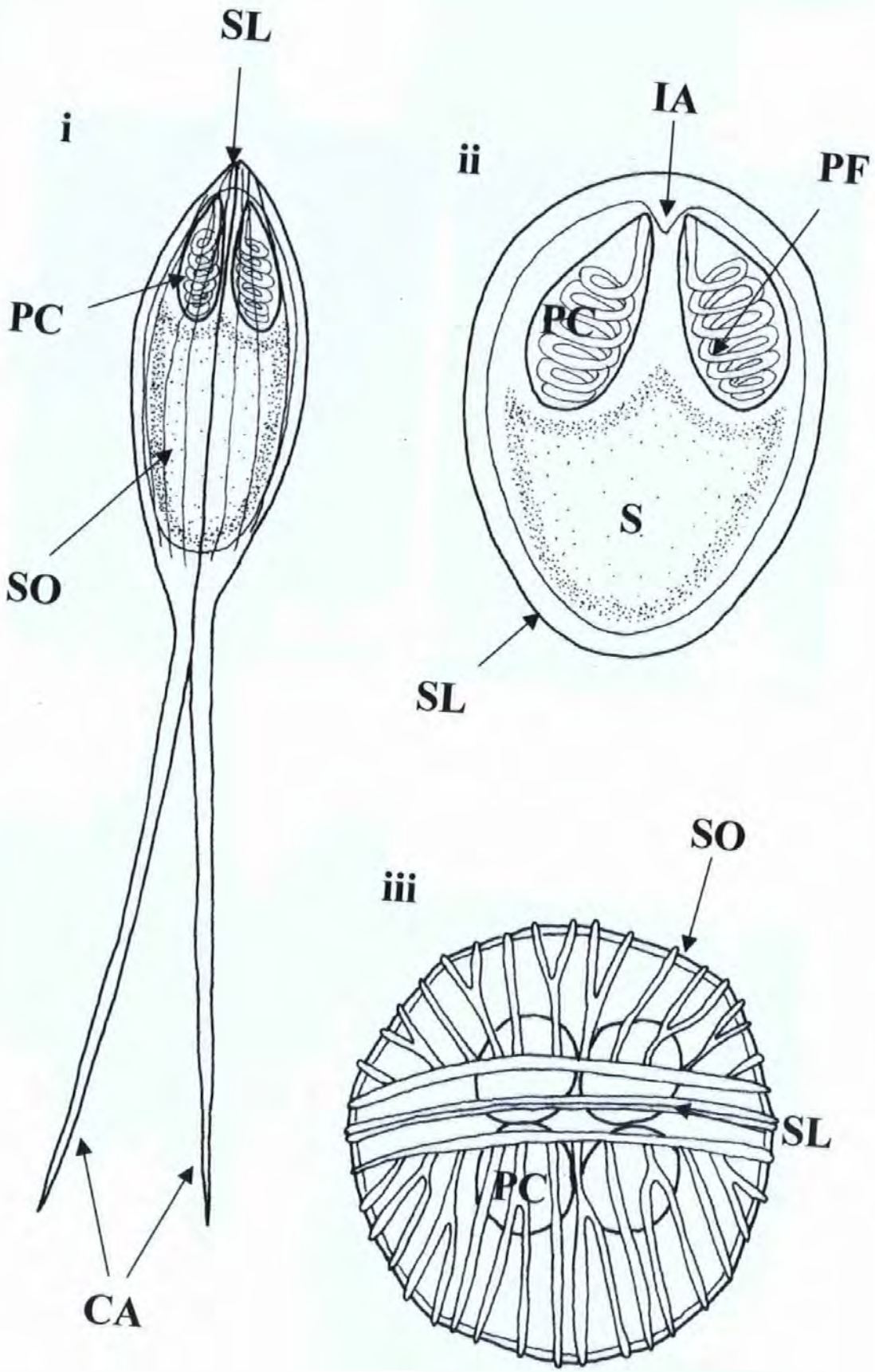
Figure 2.2.3 Diagrams of main features used in the classification of myxospores. Representative genera are (i) *Myxobilatus* (ii) *Myxobolus* and (iii) *Chloromyxum*. Where CA = caudal appendages; IA = intercapsular appendix; PC = polar capsule; PF = polar filament; S = sporoplasm; SL = sutural line; SO = spore ornamentation.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Subsequently, Whipps *et al.* (2004) suppressed the multivalvulid families Pentacapsulidae, Hexacapsulidae and Septemcapsulidae on molecular evidence so that the genera *Pentacapsula, Septemcapsula* and *Hexacapsula* become junior synonyms of *Kudoa.* Phenotypic variation in actinospores with identical sequences are also known (Hallett *et al.*, 2002). The overlapping morphological characteristics for other genera can also be problematic e.g. discrimination of *Zschokkella* and *Myxidium* and of *Leptotheca, Ceratomyxa* and *Sphaerospora.* A revision and re-evaluation of the morphological and molecular characteristics used to discriminate the myxozoans at the species, genus and family level needs to be undertaken, especially since it has been demonstrated by Kent *et al.* (2001) that taxa cluster more by tissue location and mode of development than by spore morphology.

A number of homonyms exist between myxozoans and genera in other phyla (Kingdoms). Whilst The International Commission on Zoological Nomenclature (1985) recommends that "it is preferable not to propose for a genus of animals a name already in use for a genus outside the animal kingdom" (Recommendation 1A), it does not preclude such an event. The homonyms occur within the genus group but outwith the Kingdom and are thus not covered by the code (Article 53, International Commission on Zoological Nomenclature, 1985). Three genera have been erected within the phylum Myxozoa without consideration of priority in other phyla. The myxozoan genus *Alatospora* Shulman, Kovaleva and Dubina, 1979 is a generic name that was already in use for the anamorphic fungus *Alatospora* Ingold. The same has occurred with the myxozoan genus *Renispora* erected by Kalavati *et al.* (1996), the generic name for the fungus *Renispora* Sigler *et al.* 1979. Finally, the name *Tetraspora* has been used for an algae genus and the actinospore genus by Hallett and Lester (1999). On the other hand, the myxozoan genus *Coccomyxa* Léger and Hesse (1907) has also been used for green algae (*Tetraspora* Jaag, 1933).

2.3 ACTINOSPORE BIOLOGY

Originally considered as parasites exclusively of oligochaetes, actinospores are now recognised, at least in some cases as the alternate stage of myxospores. The taxonomy of the group is based on the morphological features of the spores, which usually have a triradiate structure. However, as has been demonstrated for myxospores, recent molecular evidence has shown that the examination of the actinospore phenotype might not be sufficient to describe new actinospore forms (Hallett *et al.*, 2002). Until the early 1980's most studies focused on the taxonomy of the group. Marques (1984) reviewed the known literature of the group and carried out a number of ultrastructural studies of these parasites. In addition, a series of experimental transmission trials were set up, which failed. It is now known that the reason Marques (1984) failed to achieve transmission of actinospores between oligochaetes is that actinospores are only transmissible to vertebrate hosts. Subsequent workers have described further actinospore types, examined in more detail the ultrastructure (Lom and Dyková, 1992b, 1997; Özer and Wootten, 2001a; Alvarez-Pellitero *et al.*, 2001; Oumouna *et al.*, 2002) and carried out transmission trials to link the stages in the worm with fish stages (see table 2.4.1). The use of molecular techniques has assisted in the linking of the two stages and in actinospore phylogeny (Hallett *et al.*, 2002; Negredo *et al.*, 2003). Surveys of actinospore infection have generally been carried out on oligochaetes collected in the wild to enhance the general awareness of these parasites (e.g. El-Mansy *et al.*, 1998b). Other studies have looked at actinospore infections associated with fish farms to determine a link between actinospores and pathogenic myxozoans in farmed fish (El-Mansy *et al.*, 1998a, McGeorge *et al.*, 1997). Lowers and Bartholomew (2003) examined the actinospore fauna of oligochaetes imported from Europe into the USA and considered that the unregulated import and export of oligochaetes provided a possible route of transmission for pathogenic myxozoans to fish.

Most actinospores have been recorded in oligochaetes, predominately in the families Tubificidae and Nadidae although there are limited records of these parasites in polychaetes (Ikeda, 1912; Bartholomew *et al.*, 1997; Hallett *et al.*, 1998; Køie, 2000, 2002). With the exception of the actinospore stage of *Ceratomyxa shasta* in the freshwater polychaete *Manayunkia speciosa*, all of the records in polychaetes derive from marine species and, with the exception of Køie *et al.*, (2004), thus far none have been linked to a myxospore counterpart in a vertebrate host. Whilst Bartholomew *et al.*, (1997) also reported the presence of an Aurantiactinomyxon-type in the same host, all other records of actinospores in marine polychaetes are of the Tetractinomyxon type (Ikeda, 1912; Køie, 2000, 2002). Despite a number of publications on the actinospore fauna of marine oligochaetes, no links have been made between the stages in this host and a vertebrate counterpart (Caullery and Mesnil, 1905; Hallett *et al.*, 1995, 1997, 1998, 1999, 2001; Hallett and Lester, 1999).

Following the suppression of the class Actinosporea, all actinospores became *species inquirendae* and the generic names should have become collective group names. However, the problem with this approach is that it has, to an extent stifled the description of new forms of actinospore, with some authors forcing actinospore types into established "genera". Other workers have been less rigid in their approach and consider that the erection of new genera is both practical and feasible. This has lead to a dichotomy within the old class Actinosporea with, in some cases, the original diagnostic features of each genus becoming more plastic. For example, Lowers and Bartholomew (2003) described 7 triactinomyxon-types in oligochaetes. Their triactinomyxon type 7 was characterised by possessing six valve cells that were fused to give rise to three caudal processes. El-Mansy (2001) described a similar triactinomyxon-type from Egypt. Hallett *et al.* (2003) however, proposed that the form described by El-Mansy (2001) should be called a Pseudotriactinomyxon type. Furthermore, Koprivnikar and Desser (2002) described a new Raabeia-type from the freshwater oligochaete *Uncnais uncinata* with straight caudal

processes which gradually widen and terminate in a prominent branch perpendicular to the caudal process. The original definition of Raabeia types was that the caudal process was clearly curved and contained no branching at the tips, although some small bifurcations or extensions are known to occur within the group. Koprivnikar and Desser's new form clearly does not conform to the original description of the type. The presence of straight caudal processes is characteristic of Echinactinomyxon forms. Either the actinospore should be considered as an Echinactinomyxon-type and the generic characters of that collective group adjusted, or there should be the erection of a new group to accommodate this new form. Many actinospore types, such as triactinomyxons, aurantiactinomyxons and hexactinomyxons, can be easily placed within a collective group; others are not so easy, as exemplified by the Raabeia and Echinactinomyxon types. Unfortunately, until there is a clearer understanding of the biological reasons for the different actinospore forms and further lifecycles are completed, the arbitrary reasons for classifying actinospore as one type or another based on morphology will remain.

To assist in the future classification of actinospore types, the basic defining characters of each group are listed below along with considerations within each group of misclassifications and/or those that may need to be redescribed as belonging to a new group.

Collective group Antonactinomyxon (Figure 2.3.1)

Style-less spore with three caudal processes. Spore body less than half length of caudal process and sporoplasm completely fills spore body. Each caudal process joins to the caudal process of three neighbouring spores to form a cube shape composed of eight spores. Spores develop in groups of 8 within the pansporocyst.

Collective group Aurantiactinomyxon (Figure 2.3.2)

Style-less spore body, three equal sized, leaf shaped, short caudal processes curved downwards with pointed ends. Propeller shaped in apical view. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Xiao and Desser (1998b) considered that *Aurantiactinomyxon trifolium* sp. inq. Marques 1984 and the Aurantiactinomyxon stage of *Hoferellus carassii* reported by Troullier *et al.*, (1996) should be transferred to the collective group Guyenotia as the caudal processes are finger-like and possess rounded tips. By the same argument Aurantiactinomyxon type 2 of Negredo and Mulcahy (2001), and Aurantiactinomyxon type 5 of El-Mansy et al (1998b) should also be transferred to the collective group Guyenotia. However, as discussed in chapter 3, one cannot concur with this suggestion.

Collective group Echinactinomyxon (Figure 2.3.3)

Style-less spore with three equal, spiny, straight, rigid and pointed processes. Spore body less than half the length of the caudal processes, sporoplasm completely filling the spore body. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Endocapsa (Figure 2.3.4)

Triradiate spores. Small swellings on spore body, less than half the total length of spore, not cojoined. Swellings do not increase in size when the spore is released into the water. Three polar capsules on apical surface which are submerged. Spores develop in groups of 8 within a pansporocyst.

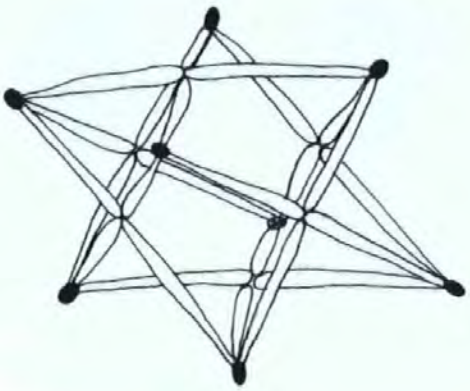
Figure 2.3.1 Antonactinomyxon (after Janiszewska, 1955)

Figure 2.3.2 Aurantiactinomyxon (Original)

Figure 2.3.3 Echinactinomyxon (Original)

Figure 2.3.4 Endocapsa (After Hallett *et al.*, 1999)

2.3.1



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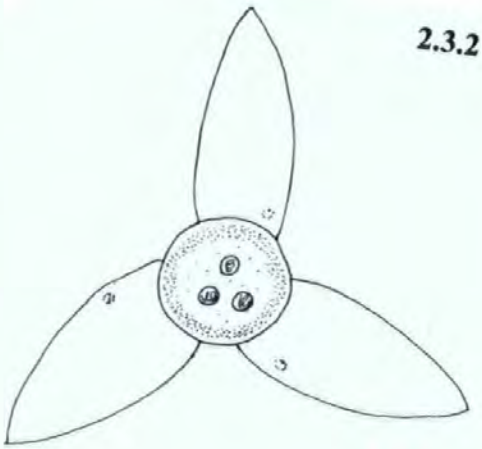

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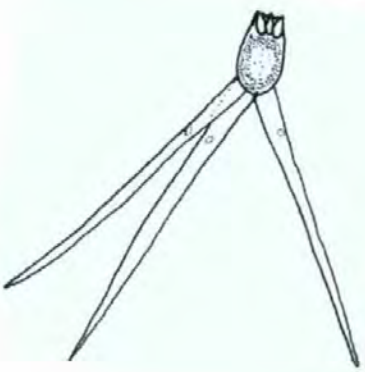

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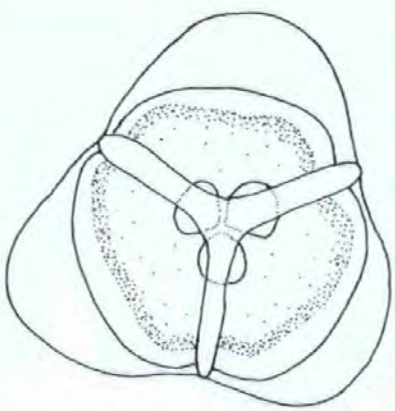


Collective group Guyenotia (Figure 2.3.5)

Triradiate spore possessing three finger shaped, long caudal processes arising below the spore body, pointed downwards. Tips of caudal processes rounded. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Hexactinomyxon (Figure 2.3.6)

Triactinomyxon like spores possessing six valve cells, partially fused at base of caudal processes which separate to form six caudal processes at tips. May contain small bifurcations at tips, tips generally pointed. Style length greater than half the length of the caudal processes. Sporoplasm does not completely fill spore body. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Neoactinomyxum (Figure 2.3.7 and 2.3.8)

Triradiate spherical valves with semicircular lobes greater than half the total length of spore, swellings increase in size when parasite is released into the water. Caudal processes joined together at edges. Three polar capsules project from spore. Spores develop in groups of 8 within a pansporocyst.

Collective group Ormieractinomyxon (Figure 2.3.9)

Triradiate spores similar to Echinactinomyxon group with style-less spore body. Length of spore body less than half length of caudal processes with sporoplasm completely filling spore body. Caudal processes straight and possessing small hooks at tips. Tips of each caudal process joined to seven other spores to form large net like structure. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Figure 2.3.5 Guyenotia (After Naville, 1930)

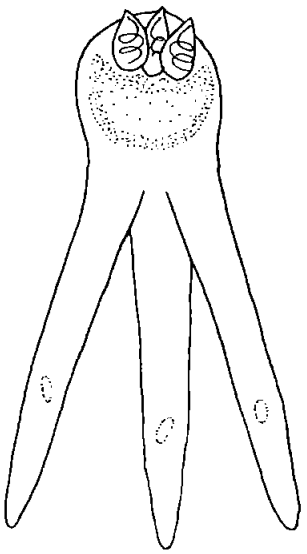
Figure 2.3.6 Hexactinomyxon (After Janiszewska, 1957)

Figure 2.3.7 Neoactinomyxum (After Ormières and Frézil, 1969)

Figure 2.3.8 Neoactinomyxum (After El-Mansy *et al.*, 1998a)

Figure 2.3.9 Ormieractinomyxon (After Marques, 1984)

Figure 2.3.10 Pseudotriactinomyxon (After Lowers and Bartholomew, 2003)



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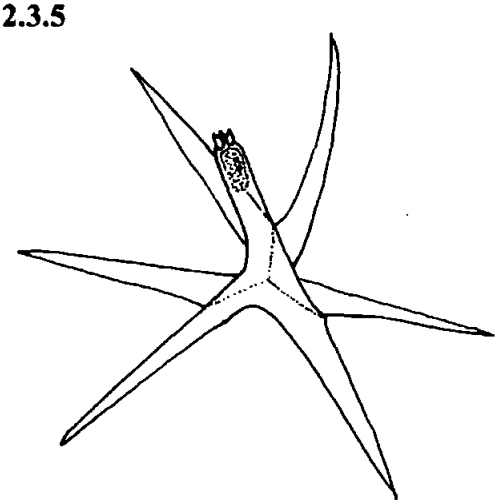

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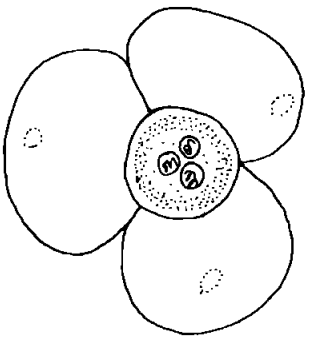

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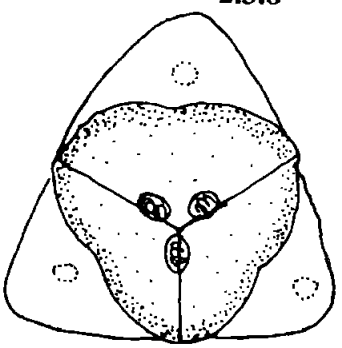

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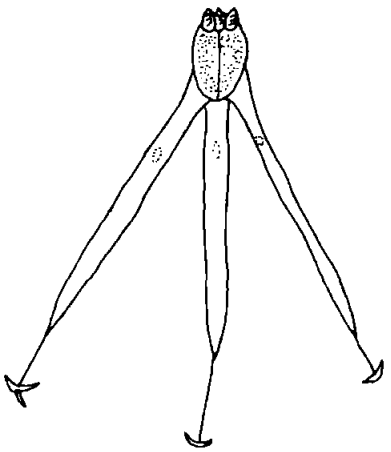

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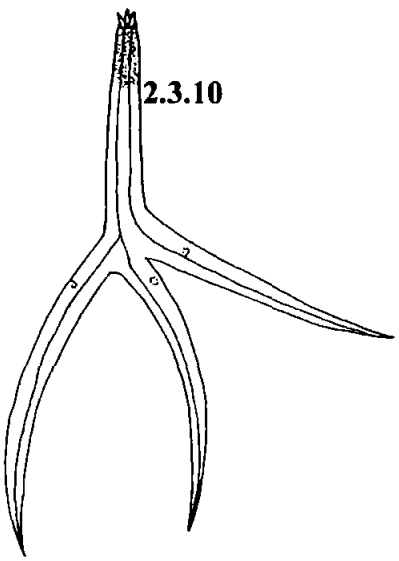


2.3.10

Collective group Pseudotriactinomyxon (Figure 2.3.10)

Triactinomyxon like spores, possessing six valve cells, fused in groups of two to form three caudal processes. Tips of caudal processes pointed. Style length greater than or equal to half the length of the caudal processes. Sporoplasm does not completely fill spore body. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Raabeia (Figure 2.3.11)

Spore possessing three long, pointed and curved processes arising from spore body. Does not possess a style. Spore body length less than half the length of the caudal processes. Sporoplasm is rounded or barrel shaped, contains numerous germ cells and completely fills spore body. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Raabeia-type of Koprivnikar and Desser (2002) should not be included within Raabeia.

Collective group Siedleckiella (Figure 2.3.12)

Triradiate spores with short style, joined in a hexahedral formation of eight spores by the tips of the caudal processes. Each caudal process attaches to one caudal process of a neighbouring spore. Spore body less than half the length of the caudal processes. Sporoplasm does not completely fill spore body. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Sphaeractinomyxon (Figure 2.3.13)

Triradiate spherical spores, triangular in apical view, valve processes absent, three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Figure 2.3.11 Raabeia (Original)

Figure 2.3.12 Siedleckiella (After Janiszewska, 1955). Inset, collection of eight spores forming net.

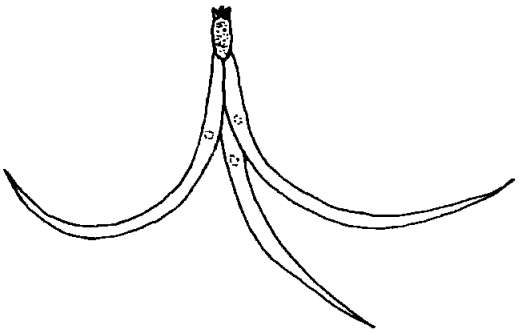
Figure 2.3.13 Sphaeractinomyxon (After Hallett *et al.*, 1999a)

Figure 2.3.14 Synactinomyxon type with two long caudal processes and one short one. Inset showing structure of an isolated spore. (After McGeorge *et al.*, 1997).

Figure 2.3.15 Synactinomyxon type with three long caudal processes (Original)

Figure 2.3.16 Tetractinomyxon (After Køie, 2000)

2.3.11



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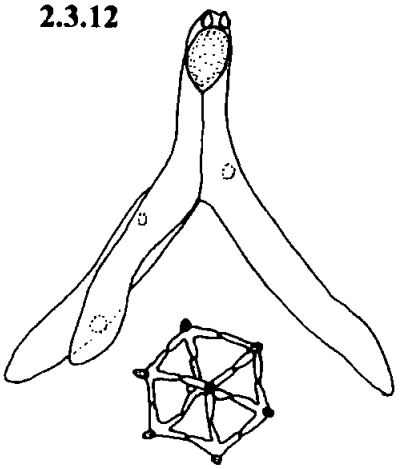

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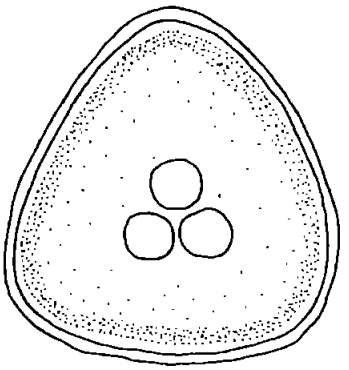
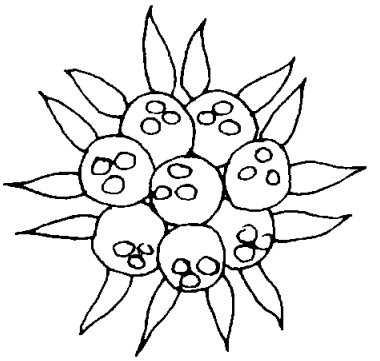
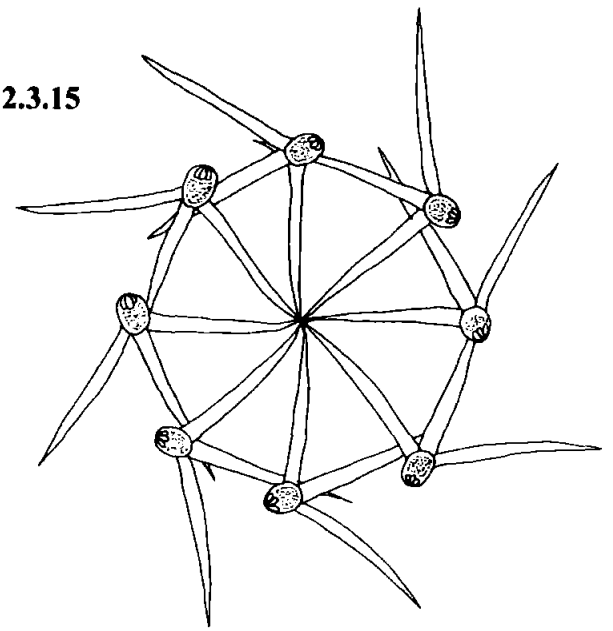




2.3.14

2.3.15



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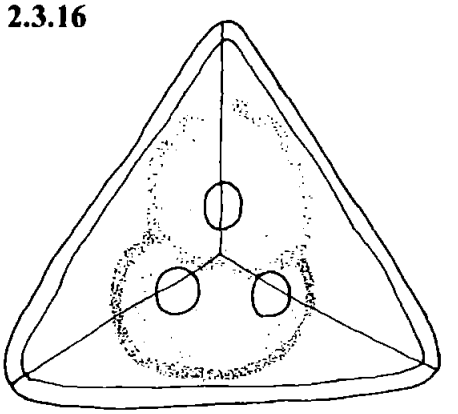


Collective group Synactinomyxon (Figure and 2.3.14 and 2.3.15)

Style-less spore possessing either one short caudal process and two longer caudal processes or three equal sized caudal processes. Joined together in groups of eight by either the short caudal process or the tip of one caudal process to form a circle. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Collective group Tetractinomyxon (Figure 2.3.16)

Tetrahedral shaped spores. Binucleate sporoplasm. No processes present. Three polar capsules. Spores develop in groups of 8 within a pansporocyst.

Tetractinomyxon sp. from *Hydroides norvegica* described by Køie (2002) should be transferred to Neoactinomyxum on the basis that the spore processes swell on contact with water.

Collective group Tetraspora (Figure 2.3.17)

Spores with characteristics of the collective group Sphaeractinomyxon (triradiate spherical spores, valve processes absent, three polar capsules). Sporoplasm can contain numerous germ cells. Spores develop in groups of four within the pansporocyst.

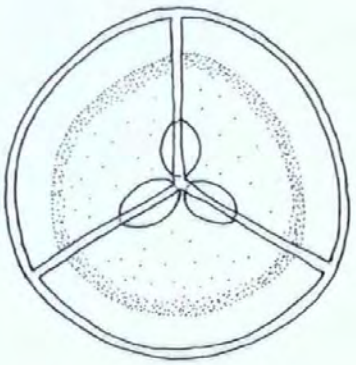
Collective group Triactinomyxon (Figure 2.3.18)

Triradiate spore possessing a style and three long, pointed and straight caudal processes. Style length greater than or equal to half the length of the caudal processes. Sporoplasm does not completely fill spore body. Three polar capsules. *Triactinomyxon astilum* sp. inq. of Janiszewska, 1964 is an Echinactinomyxon-type, Triactinomyxon type 7 of Lowers and Bartholomew (2003) should be Pseudotriactinomyxon type 1 of Lowers and Bartholomew (2003), Triactinomyxon

Figure 2.3.17 Tetraspora (After Hallett and Lester, 1999)

Figure 2.3.18 Triactinomyxon (Original)

2.3.17



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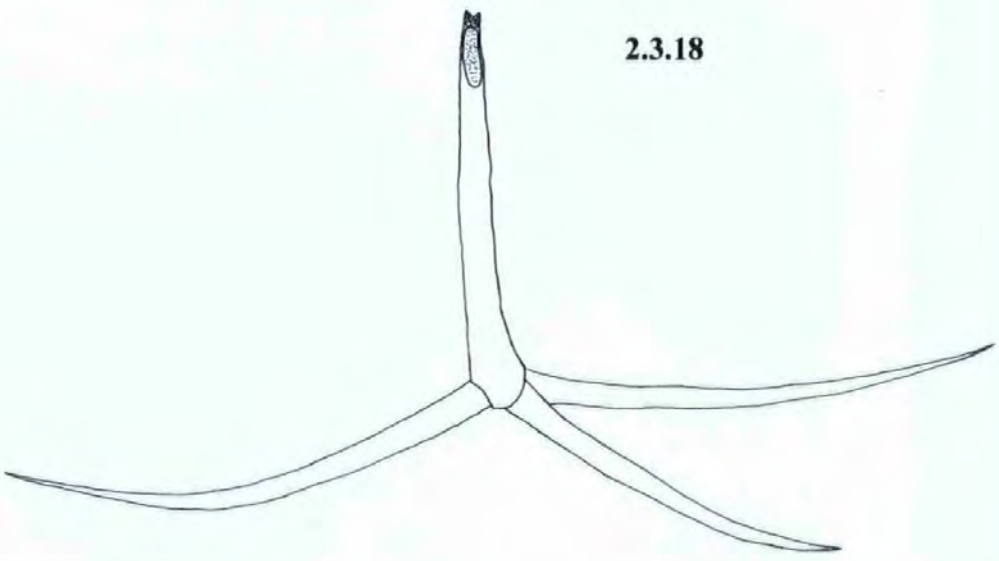


type of El-Mansy (2001) should be transferred to Pseudotriactinomyxon and Triactinomyxon F of Xiao and Desser (1998a) should be a Raabeia type.

The number of surveys of actinospore infection in oligochaetes has increased in recent years, with a concomitant increase in the numbers of actinospores described. Few of these studies however, have considered the ecology of actinospores, or the factors that determine the spatio-temporal trends observed. Most of the work has focused on the actinospore form of *Myxobolus cerebralis*, in part due to the devastating effect caused by the parasite in salmonids from the USA.

Actinospore release follows a circadian pattern in oligochaetes, with most spores being released in the late evening/early morning (Yokoyama *et al.*, 1993a; Özer and Wootten, 2001b). The maximum number of spores released daily by an individual worm can be as many as 80,000 for Echinactinomyxon-types (Özer and Wootten, 2001b). Whilst most actinospores are released during the summer months, actinospores are released from the oligochaete host throughout the year (El-Mansy *et al.*, 1998a and b; Özer *et al.*, 2002). There have been limited studies on the longevity of actinospores following release from the oligochaete, with estimates ranging from 11 through to 25 days (Yokoyama *et al.*, 1993a; Xiao and Desser, 2000a). Actinospores appear to release their sporoplasms in response to mucus from the specific fish host (Yokoyama *et al.*, 1993a, 1995a).

The general paucity of data on actinospore biology contrasts markedly with that gained on the actinospore form of *Myxobolus cerebralis*. In addition to determining the susceptibility of different genetic strains of *Tubifex tubifex* to *M. cerebralis* myxospores (Beauchamp *et al.*, 2002), the factors that influence the distribution of actinospore infections in oligochaetes have been determined (Allen and Bergersen, 2002). The *M. cerebralis* actinospore develops optimally at 15°C, and at temperatures above 25°C, developmental stages of the parasite within the oligochaete degenerate (El-Matbouli *et al.*, 1999a). The effect of different chemicals and salinity on the viability of the

Triactinomyxon stage have also been conducted (Wagner *et al.*, 2003). Whilst it is recognised, and expected, that sediment type has an impact on oligochaete distribution (Xiao and Desser, 1998c), Blazer *et al.*, (2003) showed that the substrate type affected the number and duration of release of *M. cerebralis* actinospores. Actinospore release was greatest in oligochaetes maintained in mud and sand, and least in a leaf litter substrate (Blazer *et al.*, 2003). Interestingly, Stevens *et al.*, (2001) examined the effect of myxospore dose on the oligochaete host. They found that the initial myxospore dose affected the number of actinospores produced and that the parasite reduced the biomass, abundance and individual weights of oligochaetes. There is a need to conduct more wide ranging studies on actinospore biology and ecology in order to ascertain whether the patterns that have become apparent in the studies of the *M. cerebralis* actinospore are generic for all actinospores.

2.4 MYXOZOAN LIFECYCLES IN INVERTEBRATE AND FISH HOSTS

It is now accepted that at least some myxozoans undergo a two-host lifecycle. Since the pioneering discovery by Wolf and Markiw (1984) that demonstrated that *Tubifex tubifex* is the alternate host for *Myxobolus cerebralis,* a number of laboratory based studies have confirmed the initial finding and demonstrated that other myxozoans undergo a two host lifecycle in oligochaetes (see Table 2.4.1). These include representatives of the genera *Myxidium*, *Sphaerospora*, *Hoferellus*, *Myxobolus*, *Thelohanellus* and *Zschokkella.* In general, *Myxobolus* spp. appear to produce Triactinomyxon-type actinospores, with the exception of *Myxobolus cultus* which produces Raabeia-type actinospores (Yokoyama *et al.*, 1995b) and *M. pavlovskii* which produces Hexactinomyxon-type actinospores (Ruidisch *et al.*, 1991). Aurantiactinomyxon-type actinospores have been demonstrated to be the alternate stage for *Myxidium giardi* (Benajiba and Marques, 1993), *Hoferellus carassii* and *H. cyprini* (El-Matbouli *et al.*, 1992b; Grossheider and Körting, 1992)

Table 2.4.1 List of known myxozoan lifecycles

Myxozoan species	Fish host	Actinospore type	Invertebrate host	Reference
Ceratomyxa shasta	*Oncorhynchus mykiss*	Tetractinomyxon	*Manayunkia speciosa*	Bartholomew *et al.*, (1997)
Ellipsomyxa gobii	*Pomatoschistus microps*	Tetractinomyxon	*Nereis diversicolor N. succinea*	Køie *et al.*, (2004)
Enteromyxum leei	*Sparus aurata*	None – direct transmission	None	Diamant (1997)
Enteromyxum scophthalmi	*Scophthalmus maximus*	None - direct transmission	None	Redondo *et al.*, (2002)
Henneguya exilis[1]	*Ictalurus punctatus*	Aurantiactinomyxon	*Dero digitata*	Lin *et al.*, (1999)
Henneguya ictaluri	*Ictalurus punctatus*	Aurantiactinomyxon	*D. digitata*	Pote *et al.*, (2000)
Hoferellus carassii	*Carassius auratus*	Aurantiactinomyxon	*Nais* cf. *elingius*	Troullier *et al.*, (1996)
Hoferellus carassii	*Carassius auratus*	Neoactinomyxum	*Branchiura sowerbyi*	Yokoyama *et al.*, (1993b)
Hoferellus cyprini	*Cyprinus carpio*	Aurantiactinomyxon	*Nais* sp.	Grossheider and Körting (1992)
Kudoa ovivora[2]	*Thalassoma bifasciatum*	None – direct transmission?	Via infected host eggs?	Swearer and Robertson (1999)
Myxidium fugu	*Takifugu rubripes*	None - direct transmission	None	Yasuda *et al.*, (2002)
Myxidium giardi	*Anguilla anguilla*	Aurantiactinomyxon	*Tubifex tubifex*	Benajiba and Marques, (1993)
Myxidium sp. TP	*Takifugu rubripes*	None – direct transmission	None	Yasuda *et al.*, (2002)
Myxobolus arcticus	*Oncorhynchus nerka*	Triactinomyxon	*Stylodrilus heringianus*	Kent *et al.*, (1993)
Myxobolus arcticus	*Oncorhynchus masu*	Triactinomyxon	*Lumbriculus variegatus*	Urawa (1994)
Myxobolus bramae	*Abramis brama*	Triactinomyxon	*T. tubifex*	Eszterbauer *et al.*, (2000)
Myxobolus carassii	*Leuciscus idus*	Triactinomyxon	*T. tubifex*	El-Matbouli and Hoffman (1993)
Myxobolus cerebralis	*Oncorhynchus mykiss*	Triactinomyxon	*T. tubifex*	Wolf and Markiw (1984) El-Matbouli *et al.*, (1999b)
Myxobolus cotti	*Cottus gobio*	Triactinomyxon	"tubifex"	El-Matbouli and Hoffman (1989)
Myxobolus cultus	*Carassius auratus*	Raabeia	*B. sowerbyi*	Yokoyama *et al.*, (1995b)
Myxobolus dispar	*Cyprinus carpio*	Raabeia	*T. tubifex*	Molnár *et al.*, (1999a)
Myxobolus drjagini	*Hypophthalmichthys molitrix*	Triactinomyxon	*T. tubifex Limnodrilus hoffmeisteri*	El-Mansy and Molnár (1997a)
Myxobolus hungaricus	*Abramis abramis*	Triactinomyxon	*T. tubifex, L. hoffmeisteri*	El-Mansy and Molnár (1997b)
Myxobolus intimus	*Rutilus rutilus*	Triactinomyxon	*T. tubifex*	Rácz *et al.* (2004)
Myxobolus macrocapsularis	*Abramis brama*	Triactinomyxon	*T. tubifex*	Székely *et al.*, (2002)
Myxobolus pavlovskii	*Hypophthalmichthys molitrix*	Hexactinomyxon	*T. tubifex*	El-Matbouli and Hoffman (1991a)
Myxobolus portucalensis	*Anguilla anguilla*	Triactinomyxon	*T. tubifex*	El-Mansy *et al.*, (1998c)
Myxobolus pseudodispar	*Rutilus rutilus*	Triactinomyxon	*T. tubifex, L. hoffmeisteri*	Székely *et al.*, (1999)
Sphaerospora renicola	*Cyprinus carpio*	Neoactinomyxum	*B. sowerbyi, T. tubifex*	Molnár *et al.*, (1999b)
Sphaerospora truttae	*Salmo trutta*	Echinactinomyxon	*L. variegatus, T. tubifex*	Özer and Wootten (2000)
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	None	Bryozoans	Feist *et al.*, (2001)
Thelohanellus hovorkai	*Cyprinus carpio*	Aurantiactinomyxon	*B. sowerbyi*	Yokoyama (1997); Székely *et al.*, (1998)
Thelohanellus nikolskii	*Cyprinus carpio*	Aurantiactinomyxon	*T. tubifex*	Székely *et al.*, (1998)
Zschokkella sp.	*Carassius auratus*	Echinactinomyxon	*B. sowerbyi*	Yokoyama *et al.*, (1991)
Zschokkella nova	*Carassius carassius*	Siedleckiella	*T. tubifex*	Uspenskaya (1995)

[1]Lin *et al.* (1999) used sequence information to link the actinospore stage with the myxospore stage – no experimental transmission trials have confirmed this finding.

[2]This report needs confirming as Swearer and Robertson (1999) used fish collected from the field for both control and challenge fish.

and *Thelohanellus hovorkai* and *T. nikolskii* (Yokoyama, 1997; Székely *et al.*, 1998). Other actinospores types that have been shown to be the alternate stage of myxospores include Siedleckiella (*Zschokkella nova*) and Neoactinomyxum (*Sphaerospora renicola*). However, the study of myxozoan lifecycles has been, in some instances, problematic. Thus far, only two lifecycles have been replicated consistently in laboratory studies, *M. cerebralis* (El-Matbouli *et al.*, 1999b) and *M. pseudodispar* (Székely *et al.*, 2001). At least one study in different laboratories around the world has produced conflicting results in the types of actinospores produced for known myxospore type. El-Matbouli *et al.*, (1992b) reported that the actinospore stage of *Hoferellus carassii* was an Aurantiactinomyxon-type, whereas Yokoyama *et al.*, (1993b) considered that the actinospore form was a Neoactinomyxum type. Whilst the myxospore forms used in those studies were considered to be morphologically identical, it is possible that they represented two different species. However, no adequate studies on the genetic relatedness of the forms has been carried out to confirm any differences. In the two studies, different oligochaete hosts were found to be involved in the production of actinospore types and it therefore remains possible that the oligochaete itself has an impact on the type of actinospore produced. Xiao and Desser (2000b) used a cladistic approach to examine the relatedness of the two myxozoan life stages and found that the conventional classification of the actinospore stage at the supraspecific level was not supported. Indeed, there was a lack of congruence between the two stages, which is apparent from the studies on myxozoan lifecycles carried out to date. They suggested that the different life-stages were subjected to different environmental selection pressures thus accounting for the observed morphological differences observed. A greater understanding of the morphological adaptations and the ecology of the actinospore stages will assist in elucidating the reasons for the different phenotypes observed.

Although actinospores have been recorded in a number of different genera of Tubificidae, Lumbriculidae and Nadidae, only representatives of the genera *Stylodrilus,*

Tubifex, Limnodrilus, Lumbriculus, Branchiura, Dero and *Nais* have been demonstrated to be alternate hosts. In addition, the polychaete *Manayunkia speciosa* has been shown to act as an alternate host for *Ceratomyxa shasta* (Bartholomew *et al.*, 1997), the polychate *Nereis* sp. as an alternate host for *Ellipsomyxa gobii* (Køie *et al.*, 2004) and freshwater bryozoans as hosts for *Tetracapsuloides* spp. and *Buddenbrockia* sp. (Anderson *et al*, 1999a, b; Longshaw *et al.*, 1999; Canning *et al.*, 2000, 2002; Feist *et al.*, 2001). There are a few reports that suggest that some myxozoans may transmit directly fish-to-fish. Diamant (1997) used co-habitation to demonstrate that *Enteromyxum* (=*Myxidium*) *leei* could transmit to uninfected fish and Redondo *et al.*, (2002) have shown that *Enteromyxum scophthalmi* also has a direct lifecycle. Yasuda *et al.*, (2002) demonstrated that *Myxidium fugu* and *Myxidium* sp. TP, parasitic in cultured tiger puffer fish, were both capable of fish-to-fish transmission through the faeces. They suggested that although an alternate host may be available in the natural environment, the parasites were able to transmit from fish-to-fish via trophozoites passed out through the faeces.

Due to a lack of knowledge concerning the development of myxozoans in both the fish and invertebrate hosts, there has been a general reluctance to describe either stage as the final host in which the sexual process takes place. El-Matbouli *et al.* (1995) and El-Matbouli and Hoffman (1998), followed the development of *Myxobolus cerebralis* in both the fish and oligochaete host respectively. From their studies, they concluded that sexual reproduction occurred in the oligochaete host and thus that host should be considered as the final host. Whilst this is correct, descriptions of some myxozoan lifecycles continue to refer to oligochaetes as alternate hosts. Until further detailed studies of myxozoan development in oligochaetes are carried out of the same quality and scale of El-Matbouli *et al.*, (1995) and El-Matbouli and Hoffman (1998) that demonstrate a similar development, oligochaetes will be no doubt be considered as alternate hosts by many workers.

The intra-oligochaete development of myxozoan lifecycles undergoes three phases – schizogony, or proliferative stage, gametogamy and sporogony (Figure 2.4.1). The

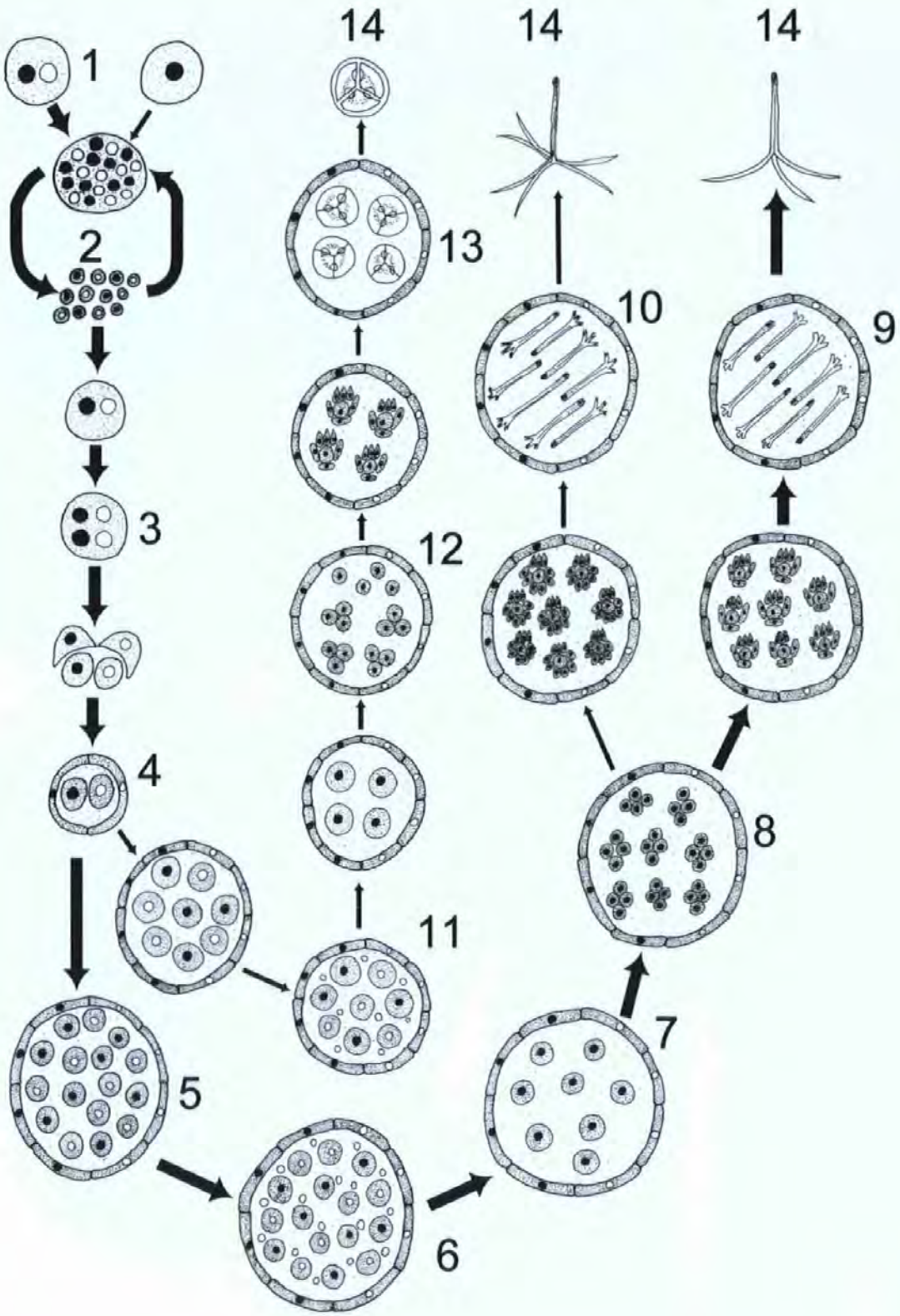
oligochaete phase is initiated by the release of myxospores from the fish host, either over the lifetime of the host or on death and subsequent decomposition of the host. This decomposition may occur either naturally or, as in the case of *Kudoa* infections, may occur more rapidly due to the release of proteolytic enzymes (Moran *et al.*, 1999). Myxospore stages are highly resilient and El-Matbouli and Hoffman (1991b) demonstrated that *Myxobolus cerebralis* spores can retain infectivity after ageing in mud for 4 months, freezing at -20°C for two months and passage through the alimentary canal of predatory birds and fish.

Myxospores are ingested by oligochaetes and, on contact with the oligochaete intestinal epithelium, the polar filaments are released anchoring the parasite to the host. The spore valves separate along the sutural line and the amoeboid sporoplasm is released, penetrating between the intestinal epithelial cells. In *M. cerebralis*, the binucleate sporoplasm undergoes a proliferative stage to produce a multinucleate cell within the oligochaete. It is presumed that a similar pattern of development occurs in myxospores with two uninucleate sporoplasms, in that each will produce a multinucleate cell, rather than fuse to form a bi-nucleate cell. The multinucleate cell undergoes plasmotomy to produce numerous uninucleate cells that invade other intercellular spaces. These cells then either undergo another schizogonic phase giving rise to more multinucleate cells leading to more uninucleate cells or fuse by plasmogamy to produce binucleate stages. Subsequent development of actinospores usually occurs within intestinal epithelial cells.

The next phase in the development is gametogony and is initiated by a binucleate stage producing a four-nuclei stage by karyogamy. This stage produces four uninucleate cells by plasmotomy, two of which become enveloping somatic cells and the other two becoming generative cells, designated as α- and β- cells. In this early pansporocyst, the two enveloping cells undergo mitosis twice to produce eight somatic cells. In pansporocysts ultimately containing 8 actinospores, the α- and β- cells undergo three

Figure 2.4.1 Schematic showing the intra-oligochaete myxozoan development (actinospore phase). Where heavy arrow = development of *M. cerebralis* demonstrated experimentally by El-Matbouli and Hoffman (1995); light arrow = development for other actinospores.

A binucleate or uninucleate sporoplasm (1) released from a myxospore following ingestion by the oligochaete undergoes a series of nuclear divisions to produce a multinucleate cell. Following plasmotomy, the cells either undergo further nuclear divisions to produce more multinucleate stages (2) or produce a two and then four nuclei cell (3). Plasmotomy follows to produce four cells, two of which envelop the other two cells (4). For all actinospore types, the outer cells undergo two mitotic divisions to produce a pansporocyst wall containing eight cells. For *M. cerebralis* and other actinospores producing eight actinospores per pansporocyst, division of the inner cells produce 16 diploid cells via three mitotic divisions (5). Following one meiotic division, 16 haploid cells are produced (6). Fusion of the α and β cells, the sexual phase, producing an early pansporocyst containing 8 cells (7) which each undergo two mitotic division to produce eight 4-cell stages (8). For *M. cerebralis*, three of these cells will undergo one mitotic division to produce the capsulogenic and valvogenic cells. Subsequent development will produce a pansporocyst containing eight actinospores (9). For actinospores with six caudal processes (e.g. *Hexactinomyxon*-type), following production of eight 4-cell stages (8), three of the cells undergo one mitotic division. Three of these new cells will undergo a further mitotic division giving rise to six valvogenic, three capsulogenic and one sporoplasmogenic cell, and develop into eight actinospores (10). For *Tetraspora*, following the division of the early developmental stage into a two cell early pansporocyst (4), the inner cells undergo two mitotic divisions and one meiotic division to produce 8 haploid cells (11). Fusion of the α and β cells produces an early pansporocyst containing 4 cells. Two mitotic divisions produce four 4-cell stages within the pansporocyst (12). Within each of these a mitotic division of 3 of the cells leads to the production of capsulogenic and valvogenic primordia. Subsequent development leads to the production of a pansporocyst containing 4 actinospores (13). Release of actinospore stages into the water allows the lifecycle to continue (14).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mitotic divisions to produce 16 diploid gametocytes composed of eight α-cells and eight β-cells. One meiotic division produces 16 haploid gametocytes and 16 polar bodies. Each α-cell fuses with a β-cell to produce a pansporocyst consisting of eight zygotes surrounded by eight somatic cells. For Tetraspora, which only contains four actinospores, the generative α- and β-cells in the early pansporocyst presumably undergo two mitotic divisions to produce 8 diploid gametocytes consisting of four α-cells and four β-cells. One meiotic division leads to the production of 8 haploid gametocytes and 8 polar bodies. The fusion of each α-cell with a corresponding β- cells produces a pansporocyst containing 4 zygotes surrounded by eight somatic cells in Tetraspora.

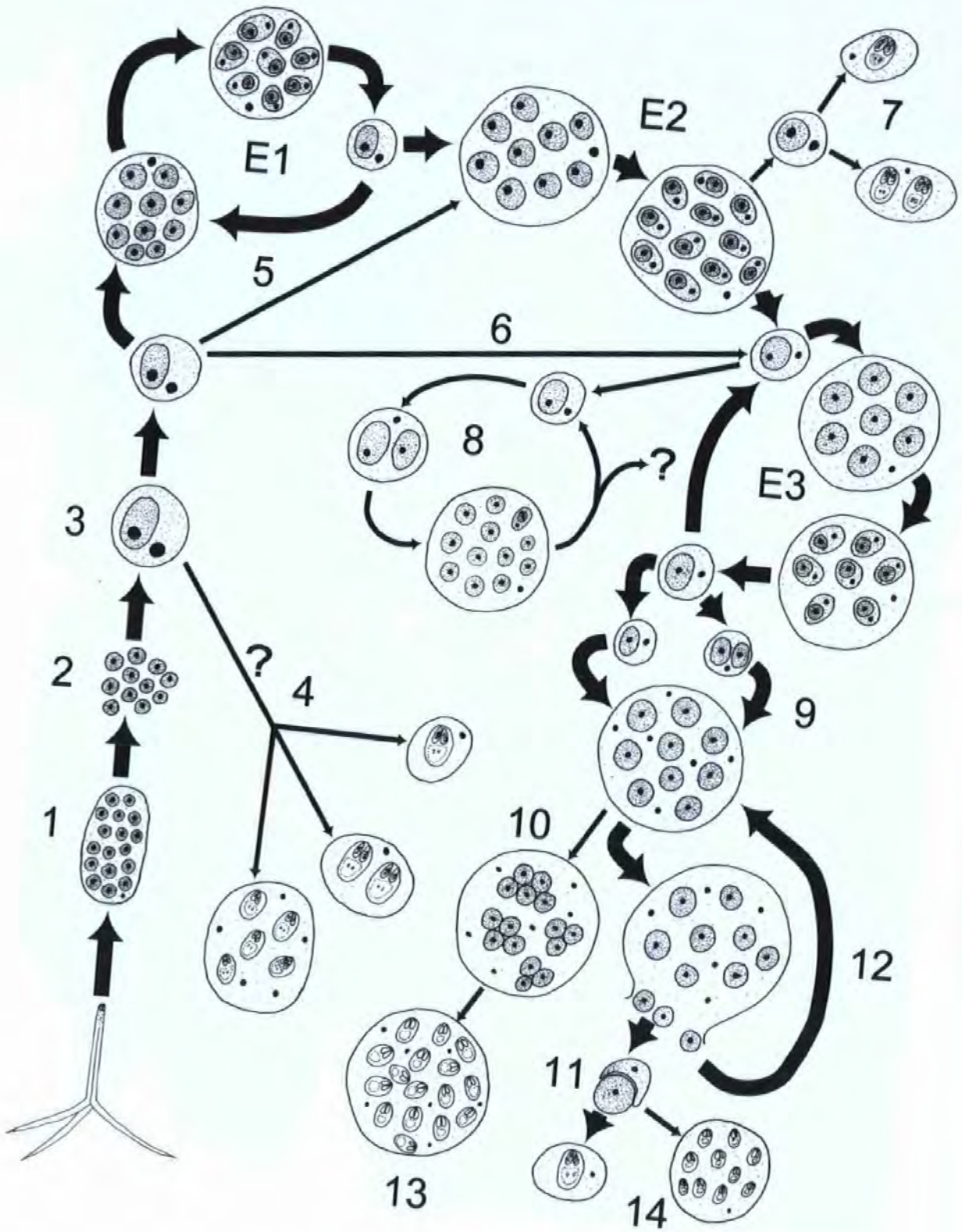
The fusion of the α- and β- cells is the sexual phase of development demonstrating that oligochaetes are the definitive or final hosts, i.e. hosts in which fusion of gametes takes place. Sporogony is the final phase of development. This begins with two mitotic divisions of the zygotes leading to a four-cell stage so that sporocysts contain 32 cells in eight groups of four cells. The exception to this is Tetraspora pansporocyst, which contains a total of 16 cells in four groups of four cells. For actinospores containing three valves, such as *M. cerebralis* and Tetraspora, three of these cells surround the fourth and divide by mitosis, leading to six cells. Three of these become capsulogenic cells, the other three becoming valvogenic cells. The three valvogenic cell primordia of actinospores containing six valve cells, such as Pseudotriactinomyxon and Hexactinomyxon, undergo a further mitotic division to produce six valvogenic cells. The fourth cell in the four-cell stage becomes the sporoplasm cell, initially dividing by internal cleavage leading to a generative cell surrounded by a somatic cell. The generative cell divides by mitosis to produce infective germ cells in various numbers, normally 2, 4, 8, 16, 32, 64, 128 and so on. The number of infective germ cells is species dependant. Further development of the capsulogenic and valvogenic cells leads to the formation of polar capsules and valve cells respectively. Polar capsule ontogeny in actinospores follows a similar pattern as in myxospores, with the polar filament being formed by the inversion of an external tube.

The exit pore for the polar capsule is covered by a "plug" of moderately dense material, topped by a cone which is then covered by the cell membrane of the capsulogenic cell (Lom and Dyková, 1997). At maturity, each pansporocyst contains eight (or four in Tetraspora) actinospores. Within the pansporocyst, the shell valves are usually folded up or concertinaed which expand on contact with water. The exception to this are members of the collective group Endocapsa whose caudal processes do not expand on contact with water. The mature actinospores are either released into the lumen of the oligochaete and passed into the environment by defecation or released on death of the oligochaete host to initiate the infection in a fish host.

On expulsion into the water column, the actinospore stage is infective to a fish host. On contact with a suitable host, the polar filaments are extruded from the actinospore to anchor it to the host. The actinospore sporoplasm penetrates the epidermis of the host, and evidence suggests that a favoured entry point is via mucus cells (Yokoyama and Urawa, 1997; El-Matbouli *et al.*, 1999b). Malacospores of *T. bryosalmonae* released from the bryozoan also enter the fish host via the mucus cells (Morris *et al.*, 2000b; Longshaw *et al.*, 2002). In the initial intercellular stage, the cell wall surrounding the actinospore sporoplasm disintegrates and the sporoplasm cells (infective germ cells) invade surrounding tissues (Figure 2.4.2). Endogenous budding gives rise to an enveloping primary cell and an internal secondary cell. At this point there are several options open to the sporoplasm cells, ranging from the production of uninucleate pseudoplasmodia through to complex extrasporogonic proliferative stages prior to spore formation. The type of intra-piscine development appears to be species dependent and varies between genera. It is probable that most myxozoans will undergo at least one proliferative stage prior to spore formation in order to maximise potential spore production. The formation of pseudoplasmodia directly from individual actinospore sporoplasm cells appears unlikely.

Figure 2.4.2 Schematic showing the intra-piscine myxozoan development (myxospore phase) for those species undergoing a two host lifecycle. Where heavy arrow = development of *M. cerebralis* demonstrated experimentally by El-Matbouli *et al.* (1999); light arrow = postulated development for other myxospores.

On contact with a suitable host, the sporoplasm is released from an actinospore and invades the epidermis (1). The cell wall of the sporoplasm disintegrates and the inner secondary cells invade surrounding host cells (2). Endogenous budding produces a secondary cell surrounded by a primary cell (3). Several routes of development are possible for the myxospore following this stage. Production of mono-, di- or slightly polysporic pseudoplasmodia without a proliferative stage may be possible (4). The primary cell containing a secondary cell can undergo one (E3), two (E2 and E3) or three (E1, E2 and E3) extrasporogonic proliferative stages in which endogenous budding gives rise to numerous secondary cells containing tertiary cells. 5 – development route with two proliferative stages (E2 and E3); 6 – development route in which only one proliferative stage occurs (E3). Production of mono- or di-sporic pseudoplasmodia following one or two proliferative stages (7). In the proliferative stage of *Sphaerospora renicola* occurring in the swimbladder, secondary and tertiary cells become trapped within the swimbladder wall (8). At the site of sporogony, endogenous cleavage and nuclear division leads to the production of a plasmodium containing numerous nuclei and secondary cells (9). Sporogony is either initiated by the aggregation of generative cells (10) or by the production of a pansporoblast derived from a pericyte enclosing a sporogonic cell (11). In plasmodia containing pansporoblasts, it is possible for a final proliferative stage to occur (12). Sporogony is completed by formation of spores after aggregation of valvogenic, capsulogenic and sporoplasmogenic cells in a plasmodia (13) or pansporoblast (14). Release of the spores into the water over the lifetime, or on death and decay of the host allows the lifecycle to continue in the invertebrate host.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Extrasporogonic cycles, which occur in sites other than those in which sporogony takes place, have been reported from several genera including *Sphaerospora, Hoferellus, Myxidium, Kudoa* and *Myxobolus*. In the extrasporogonic phase, the secondary cell within the primary cell undergoes a series of mitotic divisions to produce numerous secondary cells. Endogenous budding of the secondary cells gives rise to tertiary cell(s) within each secondary cell. In *M. cerebralis*, this first extrasporogonic phase takes place intraepithelially in the dermis and within the blood for *S. renicola*. Rupture of the primary cell releases the secondary cells, into the host cytoplasm or into the blood system. *M. cerebralis* undergoes a further two proliferative stages, one intercellularly in the subcutis and a second in the nervous tissues. For *S. renicola,* one of the proliferative stages occurs within the swimbladder wall. Molnár (1994) considered that this stage was equivalent to a blood stage as proliferation occurs within the capillaries of the swimbladder. Extrasporogonic stages have also been reported in the *rete mirabile* of sticklebacks co-infected with *Sphaerospora elegans* and *Myxobilatus gasterostei*, and in the renal tubule or collecting duct epithelium and glomeruli in infections with *S. renicola*. At the site in which sporogony occurs, the parasite develops into a plasmodium (trophozoite) or a pseudoplasmodium, in which sporogony takes place. Pseudoplasmodia can be monosporic, disporic or slightly polysporic (Lom and Dyková, 1992a), but in all cases the cytoplasm will contain one vegetative nucleus. Plasmodia on the other hand can contain several nuclei. Spores in pseudoplasmodia and in plasmodia are either formed within a pansporoblast, which can be monosporic, disporic or polysporic, or by the aggregation of generative cells. Pansporoblast formation is initiated by the envelopment of a generative cell by a pericyte. Division of the enveloped cell (sporogonic cell) produces the required number of capsulogenic, valvogenic and sporoplasmogenic cells required for the formation of the spore. Alternatively, spore formation may occur by aggregation of generative cells, which differentiate into the requisite cell types. Polar filaments within the polar capsules are formed by the inversion of an external tube. On completion of polar filament

development, the exit pore of the polar capsules is sealed with a plug-like structure, usually lacking a cap typically reported in actinospores. Myxospores within the fish host will possess one to several uni- or binucleate sporoplasms, which initiate the new infection in the invertebrate host.

2.5 PKD REVIEW

PKD is a serious disease of wild and farmed salmonids in Europe, the USA and Canada. The disease was first described by Plehn (1924) from rainbow and brook trout in Germany and subsequently, a number of studies have elucidated the pathogenesis, aetiology and taxonomic affinities of parasite responsible. The disease was first named as proliferative kidney disease (PKD) by Roberts and Shepherd (1974) and the term PKX cells proposed by Seagrave *et al.*, (1980a, b). Until recently, the alternate host was unknown, although research had been directed towards examination of oligochaetes for a presumptive actinospore stage, as it was known that the parasite had affinities to the Myxozoa. The disease has been extensively reviewed by Clifton-Hadley *et al.*, (1984b), Hedrick *et al.*, (1993) and Feist (1993).

2.5.1 Taxonomy

Plehn (1924) originally described the parasite as "amöbeninfektion der niere" believing it to be an amoeba. Subsequently, the disease agent was variously classified as an amoeba, a member of the Haplosporidia (Seagrave *et al.*, 1980a, b) and was also considered to be a chronic form of viral haemorrhagic septicaemia (VHS) based on experimental transmission trials with parasite infected kidney material. Hedrick *et al.*, (1984) and Kent and Hedrick (1985b) demonstrated, using transmission electron microscopy, that the parasite was a myxozoan as showm by the presence of intraluminal

sporogonic stages. Kent and Hedrick (1985a) further confirmed their observations by experimentally transmitting PKD to naïve fish via blood and spleen from infected rainbow trout. Electron microscopy studies of PKD-affected *Salmo trutta* and *Oncorhynchus mykiss* by Clifton-Hadley and Feist (1989) and Feist and Bucke (1987) respectively further confirmed the myxozoan aetiology of the parasite. Based on observations in wild caught salmonids, the parasite was believed to be a developmental stage of *Sphaerospora* (*S. oncorhynchi*) or *Parvicapsula* (*P. minibicornis*) (Kent *et al.*, 1995; 1997). With the advent of molecular biology techniques, it became possible to determine the taxonomic affinities of the parasite and Kent *et al.*, (1998) conclusively demonstrated that the parasite was a myxozoan and that it had diverged early in the evolution of the group. In addition, they also showed that the parasite was only very distantly related to *Sphaerospora*.

A myxozoan parasite of the freshwater bryozoan *Cristatella mucedo* was described Canning *et al.*, (1996), which they named *Tetracapsula bryozoides*. The 18S rDNA of *T. bryozoides* was sequenced and when compared to the sequence of PKX from rainbow trout kidneys, there was around 80% homology between the two parasites (Anderson *et al.*, 1999a, b). On the basis of light microscopy, electron microscopy and 18S rDNA sequencing, Canning *et al.*, (1999), named the PKX parasite as *Tetracapsula bryosalmonae*. Experimental exposure of rainbow trout to *T. bryosalmonae* spores from infected bryozoans led to the induction of PKD in naïve fish, confirming the sequence and morphological data (Feist *et al.*, 2001). As a result of the information gained on parasite development and on sequence information, Canning *et al.*, (2000) erected the class Malacosporea and the order Malacovalvulida for the clade of myxozoans infecting bryozoans. However, Monteiro *et al.*, (2002) showed, using sequence information, that *T. bryozoides* was a non-motile stage of *Buddenbrockia plumatellae*, a parasite that had previously been described by Schröder (1910, 1912). Under the rules of nomenclature, *T. bryozoides* became a junior synonym of *Buddenbrockia plumatellae*, as did the genus *Tetracapsula*. *T. bryosalmonae* was transferred to the new genus *Tetracapsuloides* on the

basis of the different development of the parasite and sequence information. The causative agent of salmonid PKD is now named *Tetracapsuloides bryosalmonae* (Figure 2.5.1).

2.5.2 Distribution

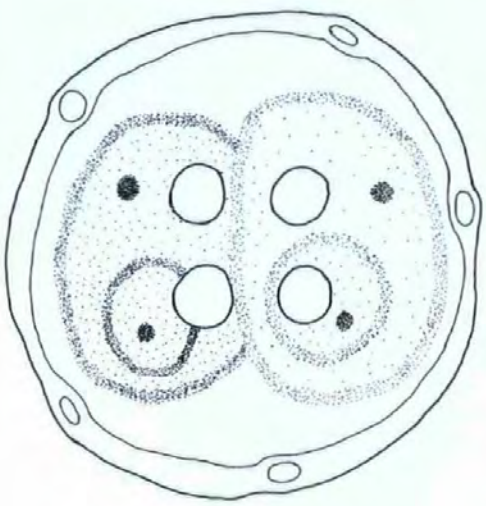
PKD was originally reported in Germany and is now recognised to occur in the United Kingdom, Ireland, Norway, Sweden, Denmark, France, Spain, Italy, the Czech Republic, Poland, Switzerland, Canada and the United States of America (Ferguson and Ball, 1979; Ellis *et al.*, 1982; Hedrick *et al.*, 1984; Chilmonczyk and de Kinkelin, 1989; Feist and Bucke, 1993; Kent *et al.*, 1995; Schmidt-Posthaus *et al.*, 2001; Prost, 2002). With the advent of better diagnostic techniques and the rapid expansion of salmonid aquaculture, it is likely that reports of the disease from countries so far reported as negative for PKD may increase.

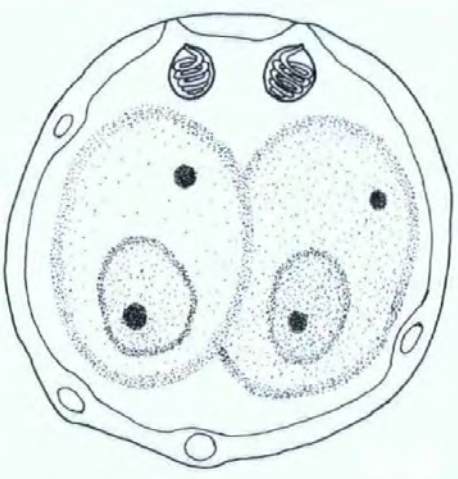
2.5.3 Host susceptibility/survey/epidemiology

The host range for PKD is restricted to members of the Salmonidae, with the exception of two reports of PKX-like cells in the kidney and gills of pike and common carp respectively (see Table 2.5.1). It is possible that the PKX-like cells in these two hosts represent other, as yet undescribed malacosporeans or possibly, stages of *Buddenbrockia plumatellae*. Susceptibility to infections can vary between species and between different individuals and populations of the same species (Feist *et al.*, 2002). Whilst it is likely that most *Oncorhynchus* spp. are susceptible to PKD, within the genus *Salvelinus*, *S. alpinus* is susceptible whereas *S. fontinalis* appears to be refractory to the disease, despite Plehn's 1924 report of the disease in this species. *Oncorhynchus* spp. appear to be most susceptible to the disease, followed by brown trout and then salmon which appear to be

Figure 2.5.1 (a) Spores of *Tetracapsuloides bryosalmonae* released from the bryozoan *Fredericella sultana* collected at a fish farm site enzootic for PKD. Note the presence of two sporoplasms and four polar capsules.

Figure 2.5.1 (b) Line drawings of *T. bryosalmonae* spores in apical and side view respectively.





amongst the least susceptible species. The factors which predispose hosts to the disease or that confer resistance remain unknown.

Due to the inherent nature of fish farming, the distribution of the disease in farms is generally well understood and it would be expected that at least some wild fish populations on the same water course as affected farms would be prone to PKD. However, whilst in fish farms disease prevalences can reach 100%, the distribution patterns in wild fish populations are less well understood. Feist *et al.* (2002) examined wild caught 0+ salmon and brown trout from 23 locations on 16 rivers in England and Wales and found that prevalence in brown trout ranged from 11 to 43% in only 5 rivers. Infected salmon were only caught in 2 rivers at prevalences ranging from 3 to 33%. A number of factors may be responsible for the variable prevalences reported in wild caught fish including innate host resistance, behavioural aspects of the fish host, distribution of the bryozoan alternate host, water temperature and flow rate and water quality. Alternatively, the sampling methods employed might only collect survivors of the disease as any mortalities would be quickly scavenged in a natural situation. Thus, the true prevalence in wild fish may be far greater than currently believed.

In the UK, fish generally become infected around April, with PKX cells appearing in the kidneys approximately four weeks later. Within two months, classical symptoms of the disease appear, including renal hypertrophy and by the end of the summer, most fish begin to show signs of recovery. The progression of the disease is exacerbated by temperature and low oxygen levels. The patterns of infection noted in farmed fish and described in detail by Clifton-Hadley *et al.* (1987b) have been demonstrated experimentally by Feist *et al.* (2001) and Longshaw *et al.* (2002).

Table 2.5.1 List of PKD susceptible hosts by common name and scientific name

Common name	Scientific name
Cutthroat trout	*Oncorhynchus clarki*
Coho salmon	*Oncorhynchus kisutch*
Rainbow trout	*Oncorhynchus mykiss*
Steelhead trout	*Oncorhynchus mykiss*
Golden trout	*Oncorhynchus mykiss aquabonita*
Kokanee salmon	*Oncorhynchus nerka*
Sockeye salmon	*Oncorhynchus nerka*
Chinook salmon	*Oncorhynchus tshawytscha*
Brown trout	*Salmo trutta*
Atlantic salmon	*Salmo salar*
Arctic char	*Salvelinus alpinus*
Grayling	*Thymallus thymallus*
Common carp[1]	*Cyprinus carpio*
Pike[2]	*Esox lucius*

[1] Voronin (1993) and Voronin and Chernysheva (1993) reported the presence of PKX-like cells in the gills of common carp. Based on transmission electron microscopy studies only, these cells were similar to *Tetracapsuloides bryosalmonae*

[2] Reported by Seagrave *et al.* (1981) and Bucke *et al.* (1991) in single wild caught pike. This has not been reported since, although Morris *et al.* (2000a) reported that monoclonal antibodies bound specifically with this form, thus confirming pike as a host for *T. bryosalmonae.*

2.5.4 Pathology

A number of studies have examined the pathology associated with the disease in salmonid hosts, either in hosts infected by field exposures or experimentally via kidney homogenates. Feist *et al.* (2001) and Longshaw *et al.* (2002) confirmed the results of previous studies by exposing fish to disrupted bryozoans under experimental conditions. It is accepted that PKD is an extremely debilitating disease where morbidity can reach 100% and mortality can be as high as 90% in farm situations (D. Butterworth, pers. comm., Burkhardt-Holm, 2002, Hedrick *et al.* 1993). The severity of the disease has been shown to be exacerbated by poor water quality (El-Matbouli *et al.*, 2002) and mortality rates may be increased as a result of this and other factors such as secondary infections.

2.5.4.1 Clinical signs

Clinical signs of the disease are dependant on the severity of the infection. During the early phases of the disease, no symptoms are present. As the disease progresses, a number of non-specific signs are apparent including exophthamia, melanosis, abdominal distension, ascites and pale gills. Internally, fish affected by PKD can show nephromegaly (Figure 2.5.2) and splenomegaly. Clifton-Hadley *et al.* (1987b) proposed a six point grading system to describe the macroscopical changes apparent throughout the course of a PKD infection ranging from 0 (no apparent lesions) through to H (healing). It is known that *T. bryosalmonae* cells can occur within the musculature and Fernández-de-Luco *et al.* (1997) reported a further manifestation of the disease involving a granulomatous response within the muscle of infected rainbow and brown trout.

Figure 2.5.2 Photograph of the kidney of cultured rainbow trout demonstrating mild nephromegaly and white patches on kidney surface.

2.5.4.2 Pathogenesis

During the initial phases of infection, there are no apparent pathological effects due to the parasite (Longshaw *et al.*, 2002). Clifton-Hadley *et al.* (1987b) described in detail the pathogenesis of the disease. During the first four weeks, limited changes occur within rainbow trout. As the number of PKX cells increase within the kidneys, cellular aggregations of host origin attach to blood vessel walls with a concomitant loss of endothelial definition. Within four weeks hyperplastic changes in the kidney become more apparent, with an increase in the interstitial tissues and a corresponding decrease in the numbers of renal tubules. As the disease progresses, the changes within the kidney become more prominent and PKX cells can become encapsulated within areas of diffuse chronic inflammation. At the height of the disease, degenerative changes in the kidney consisting of loss of renal tubules and a massive inflammatory response in the kidney interstitium are apparent. Within 20 weeks post-exposure, the fish can show complete recovery from the disease. A similar pattern of infection is noted within the spleen beginning with hyperplasia of the haematopoetic tissues followed by diffuse inflammation around PKX cells and the eventual complete regeneration of infected tissues.

2.5.5 Lifecycle/Development

It has now established that bryozoans are involved in at least part of the lifecycle of *T. bryosalmonae* as demonstrated by a combination of molecular (Anderson *et al.*, 1999a and b) and electron microscopy (Canning *et al.*, 2000) techniques as well as experimental transmission trials (Feist *et al.*, 2001; Longshaw *et al.*, 2002). The route of entry into and the initial early development of the parasite within the bryozoan host have not yet been elucidated. In *T. bryosalmonae*, the wall of the sac in which spores develop consists of a single layer of flattened (mural) cells. Proliferation of these cells leads to the formation of

the sac wall and production of sporogonic cells within the sac. These sporogonic cells take one of two forms – either sporoplasmogenic (pale) cells or denser, stellate cells. The sporoplasmogenic cells undergo a series of meiotic divisions, which has not been observed in the stellate cells. Division of the sporoplasmogenic cells leads to the production of two sporoplasms that are enveloped by a group of stellate cells which differentiate to form four valve cells and four capsulogenic cells. Synchronous maturation of the spores occurs within the sac which can contain several hundred spores. Feist *et al.* (2001) induced PKD in naïve rainbow trout fish exposed to the bryozoan stages of the parasite. The study was further refined by Longshaw *et al.* (2002) who used *in-situ* hybridisation to demonstrate that the parasite entered the fish via the mucous cells of the skin epithelium. This conflicts with the results of Morris *et al.* (2000b) who considered that the parasite entered the fish via the gill epithelium. Whilst it is possible that the parasite is non-specific in its portal of entry, studies have shown that the parasite will not transmit via the gavage route, indicating that it is, at least in part, tissue specific during the entry phase. Entry of the parasite into the fish is apparently rapid, with *T. bryosalmonae* cells being detected in the skin of rainbow trout 1 minute post-exposure (Longshaw *et al.*, 2002). Parasites are transported around the body via the blood, during which time they may undergo a series of multiplications (Kent and Hedrick, 1985a). Extrasporogonic stages then undergo further development primarily within the kidney, but can also be localised within the spleen, kidney and gills. The earliest identifiable stage of *T. bryosalmonae* (PKX cells) in the fish host tissues is a mononucleate primary cell containing prominent cytoplasmic granules. Following endogenous cleavage, secondary cells are produced within the primary cell. Secondary cells may also be produced by binary fission of existing secondary cells. Following on from this, tertiary cells may be produced within the secondary cells. In rainbow trout cultured within the UK, this is most often the final stage observed in fish. *T. bryosalmonae* can undergo a limited form of sporogony in some hosts, particularly in salmonids from the USA (Kent and Hedrick, 1985a). The spores are characterised as being

ovoid with indistinct valves and two polar capsules (Kent *et al.*, 2000). The complete lifecycle of *T. bryosalmonae* has not yet been completed. A diagram of the hypothetical lifecycle is attached as figure 2.5.3.

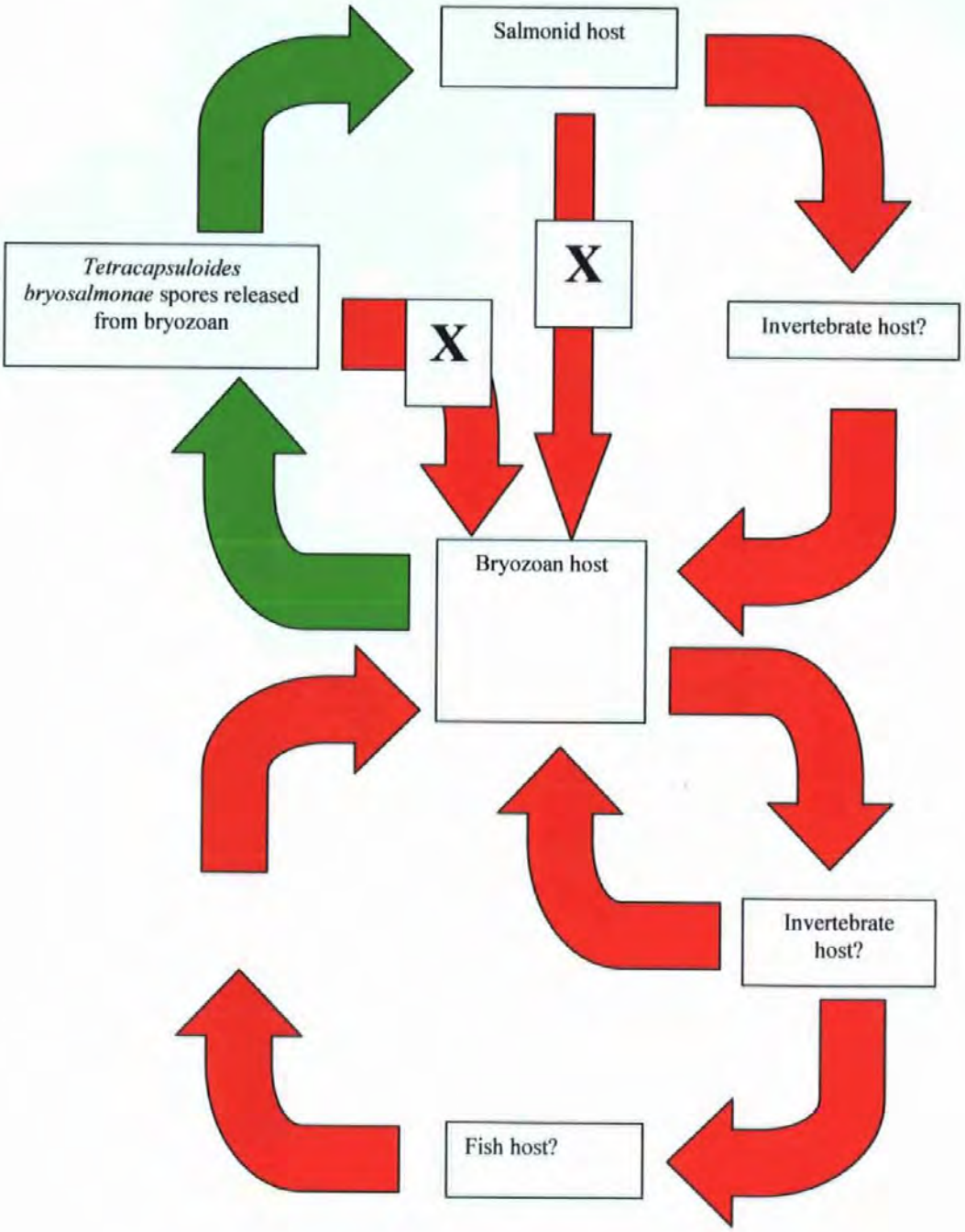
2.5.6 Diagnosis

A number of routine diagnostics and research tools have been developed and applied to assist in the identification and diagnosis of *T. bryosalmonae*. These tools have also provided the means to classify the organism and to elucidate the route of entry into the fish host by the parasite (Morris *et al.*, 2000b; Longshaw *et al.*, 2002). In addition to routine histology and transmission electron microscopy they include May-Grünwald-Giemsa of kidney imprints (Clifton-Hadley *et al.*, 1983), monoclonal antibodies (Adams *et al.*, 1992; Saulnier and de Kinkelin, 1996), immunohistochemistry (Morris *et al.*, 1997) lectins (Castagnaro *et al.*, 1991), PCR (Saulnier and de Kinkelin, 1997; Kent *et al.*, 1998) and *in-situ* hybridisation (Morris *et al.*, 1999; Longshaw *et al.*, 2002).

2.5.7 Treatment/management

In spite of the economic and ecological impact of PKD, no chemical compounds are currently licensed for treatment of PKD. Clifton-Hadley and Alderman (1987a) and Alderman and Clifton-Hadley (1988) demonstrated that repeat treatment of sub-clinical fish with malachite green controlled the development of the disease and the presence of the extrasporogonic parasites in susceptible rainbow trout. However, it was recognised that repeated doses of malachite green lead to pathological alterations of livers and gills in exposed fish (Gerundo *et al.*, 1991). Changes within the liver included sinusoidal congestion, focal coagulative necrosis, diffuse degenerative changes and cytoplasmic vacuolation. In the gills, secondary lamellae became vacuolated and epithelial cells

Figure 2.5.3. Established lifecycle of *T. bryosalmonae* is shown by the green arrows. The remainder of the lifecycle has not yet been demonstrated and several possible stages may exist as denoted by the red arrows. Lifecycle components demonstrated not to be involved are denoted by red arrows with crosses.


Salmonid host
X
X
Tetracapsuloides bryosalmonae spores released from bryozoan
Invertebrate host?
Bryozoan host
Invertebrate host?
Fish host?

deformed. The use of malachite green in food fish is however banned due to the accumulation of the chemical in fish tissues and its potential carcinogenic properties. Despite calls by Roberts (2002), malachite green remains a banned substance and monitoring of residues in fish tissues destined for food continues.

The oral administration of Fumagillin DCH at levels of 0.5g/kg diet fed to fish on a 1% body weight per day ration has been shown to be effective at controlling, but not eliminating PKD in Chinook salmon (Hedrick *et al.*, 1988). However, as with malachite green, toxic effects have been reported in fish exposed to higher doses of Fumagillin, including reduced growth rates, direct mortality and reductions in the size of kidney and spleen (Wishkovsky *et al.*, 1990). These results were further confirmed by le Gouvello *et al.* (1999) who showed that whilst a single treatment of Fumagillin administered at 3mg kg body weight^{-1} day^{-1} was sufficient to reduce the impact of PKD on rainbow trout farms, it lead to a loss of appetite in treated fish. They did not find evidence of pathological changes associated with a toxic effect due to Fumagillin, but did report the possibility that Fumagillin, at least in the early post-medication phase, may in fact depress the immune system leading to an increase in stress and bacterial infections. Higgins and Kent (1998) tested the efficacy of the Fumagillin analogue TNP-470 and found that even at a low dose (30 times less than the dose required for Fumagillin to be effective) of 0.1mg TNP-470 kg body weight^{-1}, the parasite was eliminated from sockeye salmon. No pathological alterations associated with TNP-470 were reported by Higgins and Kent (1998). In contrast, Morris *et al.* (2003) demonstrated that in rainbow trout TNP-470 had a similar pathological mechanism to Fumagillin. They suggested that whilst a treatment regime of 1.0mg TNP-470 kg^{-1} fish day^{-1} for 6 days was successful at removing parasites from the host, there was conflicting evidence regarding the mortality effects of the drug and further work would be required to test the effects of the drug on different host species.

A non-drug related method to controlling the disease was proposed by de Kinkelin and Loriot (2001) who showed that when fish were exposed to infective stages of the

parasite and then held at temperatures between 10° and 11°C for up to four months prior to an increase in temperature permissive for full development of PKD, an effective immune response was instigated that provided protection against PKD. Due to the inherent nature of fish farming, it is impossible in many farms to control the temperature of the inflowing water and thus this approach has limited value. However, many farmers are able to manage the disease by a controlled exposure programme involving the exposure of salmonids to infective stages in the late autumn and overwintering the fish. By the following summer most fish are deemed immune to PKD (Longshaw *et al.*, 2002).

Perhaps the best method for control may lie in the development of vaccines to the disease. The strong and apparently long-lived immune response to the infection provide evidence that vaccine development would prove fruitful.

2.5.8 Gaps in our knowledge

Whilst there have been many advances in our understanding of PKD in the last few years, including the classification of the pathogen, understanding of the pathogenesis of the disease, treatment and management options and elucidation of at least part of the lifecycle, there are a number of aspects of the disease that remain unresolved. The parasite has been shown to use bryozoans as a host, but successful transmission from fish hosts back to bryozoans has not been achieved. Using *T. bryosalmonae* specific primers, Feist *et al.* (2001) showed that potential invertebrate hosts and actinospore stages isolated from oligochaetes in PKD enzootic waters were not related to the agent for PKD. Until a successful laboratory based complete lifecycle is achieved, the role of other hosts in transmission of *T. bryosalmonae* to salmonids or to bryozoans remains a possibility. PKD is considered to be a disease of salmonids, but the presence of PKX cells in pike (Morris *et al.*, 2000a) and PKX-like cells in carp (Voronin, 1993) brings into question the role of other fish hosts in the dissemination and transmission of the disease.

The cues that the parasite responds to during entry into the host remain unknown. When fish are exposed to either the fish or bryozoan stage of the parasite by gavage, transmission of the disease is unsuccessful (Kent and Hedrick, 1985a; Feist *et al.*, 2001). In addition, whilst intraperitoneal injection of kidney homogenate is successful (Clifton-Hadley *et al.*, 1984a), intraperitoneal injection of the bryozoan phase is not (Feist *et al.*, 2001). This provides strong evidence that the parasite is either responding to specific cues or needs to be at a specific state of development in order for successful transmission to occur. Elucidation of those factors may provide a possible means of control to effectively combat the disease by disruption of these cues. The involvement of sporoplasmosomes in entry of the parasite into the fish and avoidance of the host immune responses in the early phases of infection remain unknown. Detailed study of these enigmatic parasite organelles may provide some answers to these questions.

If fish are able to survive the disease, there is a strong, long lasting immune response, providing a real possibility for vaccine control. This immune response is used to good effect in the management of the disease by fish farmers who deliberately expose the fish to the parasite in the latter half of the summer. This controlled exposure allows the limited development of the disease as the temperatures decrease whilst inducing an immune response that provides protection to the fish the following summer. It is not known whether the bryozoan phase of *Buddenbrockia plumatellae* can induce an immune response with minimal clinical signs in salmonids. If this were the case, it may be possible to inoculate susceptible fish with *B. plumatellae* which could prove useful as a means to control or negate the effects of PKD in salmonid aquaculture.

Most studies of the parasite and the disease have been concerned with its pathological impact on the host. However, the physiological and behavioural aspects of the disease have been largely overlooked, especially in wild fish. The effect of the disease on aspects of the fish biology such as swimming speeds, feeding and foraging behaviour, food assimilation rates, predator avoidance, smoltification and general behaviour need to

be better studied in order to gain a greater understanding of the role, if any, of the parasite in regulating wild fish populations through direct mortality or through more subtle, sublethal effects. There is evidence from Switzerland that PKD, may in part be responsible for declines in wild salmonids (Burkhardt-Holm, 2002) and that water quality may exacerbate the progression and severity of the disease in wild salmonids (El-Matbouli *et al.*, 2002). A multidisciplinary approach, as proposed by Feist *et al.*, (2002) to examine the distribution and impact of PKD on wild fish seems appropriate.

It is clear that whilst we have gained a greater understanding of the disease in the ten years since Hedrick *et al.* (1993) produced a review of the disease, there still remains a number of gaps in knowledge, including immunological responses and completion of the lifecycle that need to be addressed in order to successfully negate the effects of this economically important disease. The production of vaccines remains a long term goal.

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CHAPTER THREE

DESCRIPTIONS, SEASONALITY AND DISTRIBUTION OF ACTINOSPORES (MYXOZOA) RELEASED FROM FRESHWATER OLIGOCHAETES IN SOUTHERN ENGLAND.

To be submitted to *Folia Parasitologica* as Longshaw, M. and Feist, S.W. Descriptions, seasonality and distribution of actinospores (Myxozoa) released from freshwater oligochaetes in southern England.

Abstract

The actinospore fauna of oligochaetes associated with mud and root systems of aquatic plants were studied from six river sites in southern England and a river site in north Wales. Three of the sites were associated with salmonid fish farms. A total of 17,482 Tubificidae, 1,110 Nadidae and 240 *Lumbriculus variegatus* were examined between summer 1996 and autumn 2002. A total of 138 tubificids were infected with actinospores from seven collective groups. Of these, there were three Aurantiactinomyxon types, seven Echinactinomyxon types, one Guyenotia type, one Neoactinomyxum type, one Synactinomyxon type, two Raabeia types and six Triactinomyxon types. Twelve of the actinospores found during the study appear to be new to science. The majority of actinospores were released in spring and summer, with echinactinomyxons and triactinomyxons being found throughout the year. Prevalence data for the actinospores ranged from 0.11% for Echinactinomyxon type 1 and *Triactinomyxon ignotum* sp. inq. up to 5.83% for the triactinomyxon stage of *M. pseudodispar* during the course of the study. Differences in the spatio-temporal distribution of actinospores are discussed in relation to abiotic and biotic factors.

Introduction

Actinospores have been recognised as parasites of oligochaetes for over 100 years and until the pioneering work of Wolf and Markiw (1984), they were considered parasites exclusively of these hosts. Wolf and Markiw (1984) demonstrated that the agent for whirling disease, *Myxobolus cerebralis*, underwent a two-host lifecycle, alternating between an oligochaete and a fish host. Subsequently, several further studies confirmed those results and links between actinospores and myxospores have now been established for a number of myxozoans (Kent et al. 2001). Whilst there have been suggestions that all myxozoans will ultimately possess an actinospore stage, only 33 myxozoan lifecycles have been elucidated. Of these, one has been shown to utilise a polychaete (Bartholomew et al. 1997), one utilises a bryozoan (Feist et al. 2001) and four transmit directly without the need for an invertebrate host (Diamant 1997, Redondo et al. 2002, Yasuda et al. 2002). However, to date transmission of actinospores to oligochaetes have been unsuccessful (Marques 1984) therefore it is likely that whilst some myxospores will not possess an actinospore stage, most, if not all actinospores will posses a myxospore stage.

Despite the economic and ecological importance of myxozoans, there have been limited studies on the actinospore fauna of oligochaetes (Özer et al. 2002a), and most studies have been concerned with the actinospore fauna of oligochaetes associated with fish farming (Hamilton and Canning 1987, McGeorge et al. 1997, El-Mansy et al. 1998a, Özer et al. 2002a, Székely et al. 2002, Oumouna et al. 2003). Few studies have considered the actinospore fauna of oligochaetes in wild environments (El-Mansy et al. 1998b, MacKinnon and Adam 1924, Xiao and Desser 1998a, b, c, Negredo and Mulcahy 2001, Székely et al. 2003, Rácz and Timm 2003). Many of the studies have been concerned with the taxonomy of the actinospores and fewer have considered the ecology and seasonality of oligochaetes. In more recent years there has been a change in emphasis towards examining temporal trends in infection patterns as well as a greater understanding of host-parasite interactions. Perhaps the best studied actinospore is the triactinomyxon stage of the agent

for salmonid whirling disease, *Myxobolus cerebralis*. In addition to examining host specificity of the parasite in the oligochaete stage, studies have examined the physico-chemical factors that impact on the actinospore and the spatio-temporal trends exhibited by the parasite in oligochaete hosts. A clearer understanding of those major factors that affect actinospore release and production will prove useful in control of myxozoans in both farmed and wild fish.

The purpose of the current study was to investigate the types, geographical distribution, host specificity and seasonality of actinospore release in selected river systems predominately in southern England. By examining river systems with different abiotic and biotic characteristics it was expected that a wider range of actinospore types would be encountered. Sites associated with fish farms were initially selected in the hope of finding an actinospore stage for the agent for salmonid proliferative kidney disease (PKD). Subsequent research has demonstrated that the agent for PKD utilises a bryozoan in at least part of its lifecycle (Longshaw et al. 1999, Feist et al. 2001). Additionally, the study aimed to provide data on the seasonality of release in order to better understand the ecology of actinospores in wild riverine environments in the United Kingdom.

MATERIALS AND METHODS

Oligochaetes were collected at sites in Dorset and Wiltshire selected for their different biotic and abiotic characteristics between May 1996 and October 2002. Due to discovery of the role of *Tetracapsuloides bryosalmonae* in salmonid PKD efforts were diverted towards PKD research during 1999 and so limited numbers of oligochaete samples were taken during the summer and autumn of 1999. In addition, due to the outbreak of foot and mouth disease (FMD) in the UK during the summer of 2001, no samples of oligochaetes were taken during that period. The sampling locations selected were sites on the River Cerne (Nether Cerne, OSGB SY669990) and River Avon (Standlynch, OSGB SU181236) associated with fish farms, a site on the River Frome (East

Stoke, OSGB SY873865) and three sites on the River Stour (Wimborne, OSGB SZ030989; Canford School, OSGB SZ003998 and Blandford OSGB ST884061). Grid references were taken on-site using a hand held GPS system (Garmin GPS12, software ver. 4.02 and 4.53) and confirmed using Ordinance Survey maps of Great Britain. In addition, a single sample of oligochaetes were collected from a rainbow trout farm in North Wales. Oligochaete samples were collected either by mud cores, sediment scrapes or sweeps through weed beds. The collected material was transported to the laboratory in 10 litre buckets overlaid with water. On return to the laboratory, the water was aerated and oligochaetes removed within 24 hours. Oligochaetes were separated from the mud by passing the sediment with tap water through sieves of the following mesh sizes: 1.40 mm, 710µm, 425µm, 250µm and 125µm. The invertebrate fauna trapped on each mesh was examined and all oligochaetes were removed using a plastic pipette. The water/mud was sifted twice to ensure that most oligochaetes had been removed from the substrate. Oligochaetes were isolated using the cell-well method described by Yokoyama et al. (1991). Five worms were placed in each well of 48-well trays with approx. 2ml of distilled water. These were then maintained for a minimum of four weeks.

A drop of water was removed bi-weekly from each well and examined by phase-contrast microscopy for the presence of actinospores. Actinospores were identified according to published descriptions, measured using the criteria of Lom et al. (1997) on a Nikon Eclipse E800 with LUCIA Screen Measurement System and images stored on a Lucia imaging system or photographed conventionally. All measurements are in µm and expressed as range (mean). Measurements are based on a minimum of twenty individual spores and terminology follows that of Lom et al. (1997). To identify the worm releasing the spores, the five worms were isolated in individual wells and the water containing them tested on a daily basis. Oligochaetes were identified utilising Brinkhurst (1971) and Timm and Veldhuijzen van Zanten (2003). Due to the large numbers of oligochaetes collected, only those oligochaetes releasing actinospores were identified to species. As described in

Feist et al. (2001) and chapter 8, all isolated actinospores were examined by polymerase chain reaction (PCR) to determine the relatedness of actinospore stages to *T. bryosalmonae,* the agent for PKD.

The prevalence of infection of actinospores was defined as the percentage of oligochaetes releasing mature actinospores into the water (Xiao and Desser 1998a, Margolis et al. 1982). For examination of seasonal release, samples were grouped into the four seasons spring (March to May), summer (June to August), autumn (September to November) and winter (December to February).

RESULTS

Between May 1997 and September 2002, 21 actinospore types belonging to seven collective groups were identified in oligochaetes collected at the five sites. A total of 17,482 tubificid oligochaetes, 240 *Lumbriculus variegatus* and 1110 Nadidae were examined. Of these, 138 tubificid worms were infected (see tables 3.4 – 3.11 for details). No actinospores were detected in the Nadidae or *L. variegatus*. The actinospores released belonged to the collective groups Aurantiactinomyxon (three types), Echinactinomyxon (seven types), Guyenotia (one type), Neoactinomyxum (one type), Raabeia (two types), Synactinomyxon (one type) and Triactinomyxon (six types). Actinospores were released from *Limnodrilus hoffmeisteri* Claparède, 1862, *Psammoryctides barbatus* (Grube, 1861), *Psammoryctides* sp., *Spirosperma ferox* (Eisen, 1879), *Tubifex ignotus* (Štolc, 1886) and *Tubifex* cf. *tubifex* (Müller). None of the actinospores released during the current study were related to the agent for PKD when tested using *T. bryosalmonae* specific primers.

COLLECTIVE GROUP AURANTIACTINOMYXON

Style-less, rounded spore body, three equal sized, leaf shaped, short caudal processes curved downwards with pointed, and in some cases rounded, ends. Base of caudal process

almost completely embraces spore body. Spores always found singly. Propeller shaped in apical view.

Aurantiactinomyxon sp. of McGeorge et al. (1997) Fig. 3.1

Tubificid host unidentified. One oligochaete infected. Spore body spherical, 9-15 (12) in diameter with three apical polar capsules, 2.1-2.7 (2.5) in diameter. Secondary cells, 64. Three caudal processes of equal length tapering to a sharp point, 16-29 (22) long and 7.5-13 (11) wide at base. Locality: Nether Cerne.

***Aurantiactinomyxon raabeiiunioris* sp. inq.** Figs. 3.2 and 3.3

Spores released from *Tubifex ignotus* and *Limnodrilus hoffmeisteri.* Six oligochaetes infected. Spore body spherical, 16-21 (18) diameter with three apical polar capsules, 2.7-3.3 (3.1) diameter. Secondary cells, 16. Three caudal processes of equal length tapering to a sharp point, 20-31 (26) long × 13-18 (15) wide at widest point. Caudal processes marginally wider along length. Locality: Canford School.

Aurantiactinomyxon type 1 Figs. 3.4 and 3.5

Spores released from immature tubificid. Three oligochaetes infected. Spore body spherical, 17.5-18 (18) diameter with three apical polar capsules, 2.3-2.9 (2.6) in diameter. Secondary cells, 32. Three caudal processes of equal length with rounded ends, 21-25 (25) long × 9.5-10 (10) width at base. Locality: East Stoke.

Figure 3.1 Line drawing of Aurantiactinomyxon sp. of McGeorge *et al.* (1997)

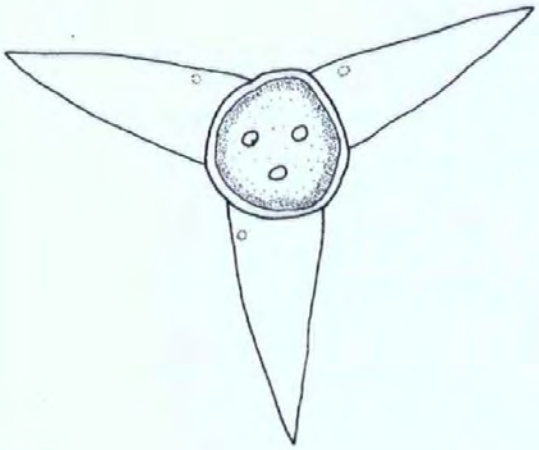
Figure 3.2 Line drawing of *Aurantiactinomyxon raabeiiunioris* sp. inq. of Janiszewska (1952)

Figure 3.3 Photomicrograph of *Aurantiactinomyxon raabeiiunioris* sp. inq. of Janiszewska (1952). Scale bar 10µm, DIC

Figure 3.4 Line drawing of Aurantiactinomyxon type 1.

Figure 3.5 Photomicrograph of Aurantiactinomyxon type 1. Scale bar 10µm, phase contrast microscopy.

3.1



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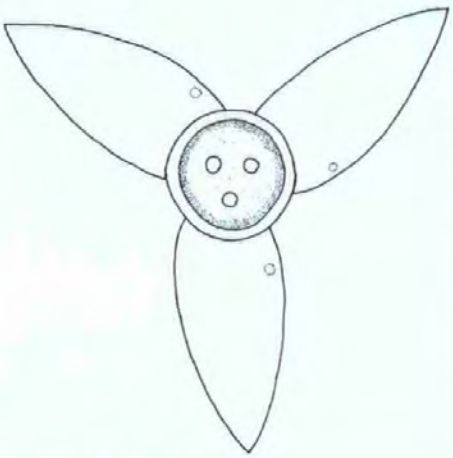

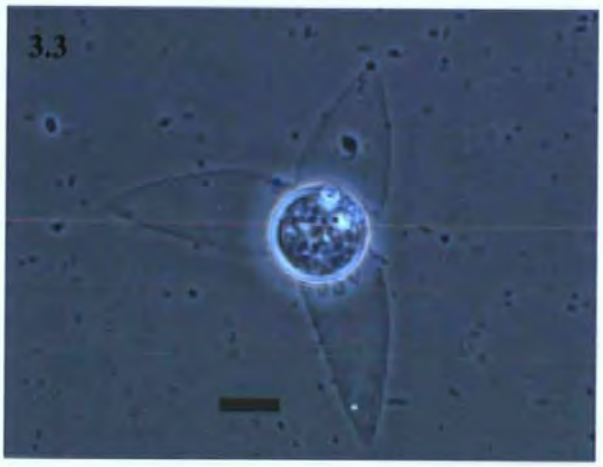

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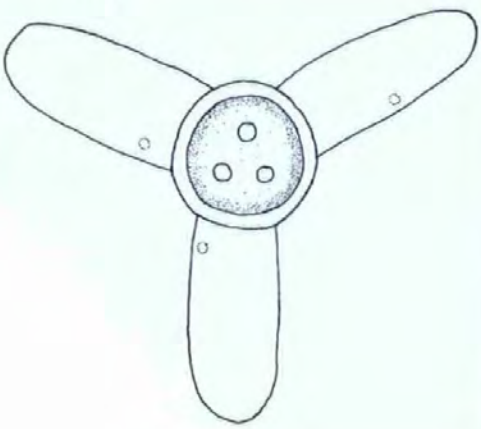
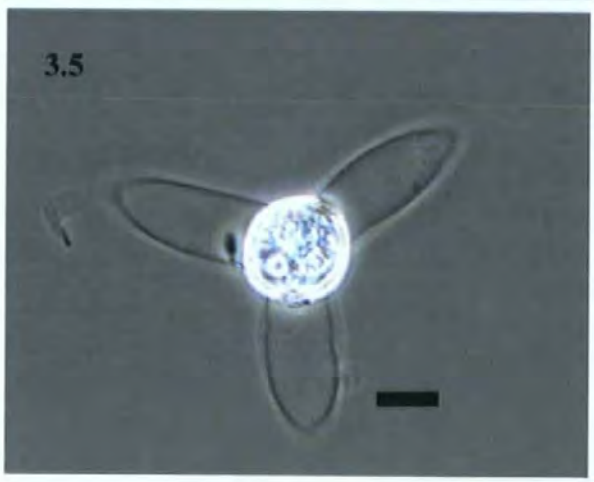




Remarks

Two of the three Aurantiactinomyxon types described here have previously been reported by other authors. McGeorge et al. (1997) described an Aurantiactinomyxon from an Atlantic salmon hatchery with caudal processes that tapered to a point. The measurements for that type were: caudal process 25.6 µm x 12 µm and spore body diameter 13.7 µm. Whilst the Aurantiactinomyxon reported here resembles types 6 and 7 of El-Mansy et al. (1998a), the current type differs from both of those in possessing a much smaller spore body (12µm versus approx. 19µm). In addition, whilst similar in overall dimensions to the actinospore stage of *Hoferellus carassii* described by El-Matbouli et al. (1992) and Troullier et al. (1996), it can be discriminated from this type as it possesses rounded tips to the caudal processes. Based on overall dimensions and geographical locality, we consider the form described here to be conspecific with Aurantiactinomyxon sp. of McGeorge et al. (1997). Özer et al. (2002a) reported that their Aurantiactinomyxon type 1 was conspecific with Aurantiactinomyxon sp. of McGeorge et al. (1997) based on overall dimensions and seasonality of release and described a new type (Aurantiactinomyxon type 4 of Özer et al. 2002a). However, comparison of the measurements and images provided by both McGeorge et al. (1997) and Özer et al. (2002a) would strongly suggest that Aurantiactinomyxon type 1 of Özer et al. (2002a) is not Aurantiactinomyxon sp. of McGeorge et al. (1997), rather that Aurantiactinomyxon type 4 of Özer et al. (2002a) is Aurantiactinomyxon sp. of McGeorge et al. (1997).

The overall appearance of the spore and the measurements provided by Janiszewska (1957) for *A. raabeiiunioris* sp. inq. match the current type and we thus consider the current type described here to be conspecific with *A. raabeiiunioris* sp. inq.

Aurantiactinomyxon type 1 is characterised by possessing rounded tips to the caudal processes and is morphologically similar to type 2 of Negredo and Mulcahy (2001), type 3 of El-Mansy et al. (1998b), type 12 of El-Mansy et al. (1998a), Aurantiactinomyxon

of Burtle et al. (1991), the Aurantiactinomyxon stage of *Hoferellus carassii* (Troullier et al. 1996) and *A. trifolium* sp. inq. of Marques (1984). However, A. type 2 of Negredo and Mulcahy (2001) has a smaller spore body and longer caudal processes; A. type 3 of El-Mansy et al. (1998b) has both a smaller spore body and caudal processes; A. type 12 of El-Mansy et al. (1998a) has narrower caudal processes, and smaller spore body; Aurantiactinomyxon type of Burtle et al. (1991) has larger spore body and longer caudal processes; Aurantiactinomyxon stage of *Hoferellus carassii* has longer and wider caudal processes and larger spore body (Troullier et al. 1996) and *A. trifolium* sp. inq. of Marques (1984) is larger in its overall dimensions, especially in the size of the caudal processes. As the current Aurantiactinomyxon type 1 can be discriminated from all other members of the collective group, we consider that it is a previously undescribed form of Aurantiactinomyxon.

Table 3.1 Measurements of main taxonomic features of Aurantiactinomyxon types isolated during the current study and comparison with known forms where previous descriptions apply.

Aurantiactinomyxon types	Caudal process length × width	Spore body (diameter)	Number of secondary cells	Polar capsule (W)	Reference
Aurantiactinomyxon sp. of McGeorge et al (1997)	16-29 (22) × 7.5-13 (11)	9-15 (12)	64	2.1-2.7 (2.5)	This study
Aurantiactinomyxon sp. of McGeorge et al (1997)	19-31 (25.6) × 10-14 (12)	12-15 (13.7)	ND	2-3 (2.7)	McGeorge et al (1997)
Aurantiactinomyxon raabeiiunioris	20-31 (26) × 13-18 (15)	16-21 (18)	16	2.7-3.3 (3.1)	This study
Aurantiactinomyxon raabeiiunioris	25-35 long	17	16	ND	Janiszewska (1957)
Aurantiactinomyxon type 1	21-25 (25) × 9.5-10 (10)	17.5-18 (18)	32	2.3-2.9 (2.6)	This study

Style-less spore with three equal, spiny, straight, rigid and pointed processes. Spore body less than half the length of the caudal processes, sporoplasm completely filling the spore body. Three polar capsules.

Echinactinomyxon radiatum sp. inq. of Janiszewska (1957) Figs. 3.6 and 3.7

Spores released from *Psammoryctides barbatus*. Three oligochaetes infected. Spore body style-less, 23-30 (27) long × 13-16 (15) at widest point with three apical polar capsules, 7.1-8.3 (7.5) long × 4.2–4.4 (4.5) wide. Secondary cells, 28. Three straight caudal processes of equal length tapering to a point, 85-105 (97) long × 8.7-10.2 (10) wide at base of caudal process. Locality: Standlynch and Canford School.

Echinactinomyxon type 1 Figs 3.8 and 3.9

Spores released from *Psammoryctides barbatus*. Six oligochaetes infected. Spore body style-less, 17.7-22.1 (20) long × 10.2-13.5 (12) at widest point with three apical polar capsules, 5.8-6.0 (5.9) long × 3.9-4.4 (4.1) wide. Secondary cells, >20. Three straight caudal processes of equal length tapering to a point, 47-82 (66) long × 4.2-6.3 (6) wide at base of caudal process. Locality: Canford School, Wimborne, East Stoke.

Echinactinomyxon type 2 Figs 3.10 and 3.11

Spores released from *Spirosperma ferox* and *Psammoryctides barbatus*. Twelve oligochaetes infected. Spore body style-less, 18.4-21.2 (19.7) long × 14.5-16.3 (15.3) at widest point with three apical polar capsules, 6.2-7.5 (6.7) long × 3.2-4.3 (4) wide.

Figure 3.6 Line drawing of *Echinactinomyxon radiatum* sp. inq. of Janiszewska (1957)

Figure 3.7 Photomicrograph of *Echinactinomyxon radiatum* sp. inq. of Janiszewska (1957). Scale bar 10µm, phase contrast microscopy.

Figure 3.8 Line drawing of Echinactinomyxon type 1.

Figure 3.9 Photomicrograph of Echinactinomyxon type 1. Scale bar 10µm, DIC

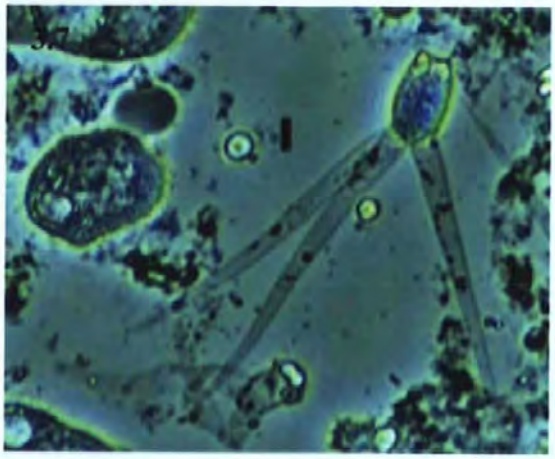
Figure 3.10 Line drawing of Echinactinomyxon type 2

Figure 3.11 Photomicrograph of Echinactinomyxon type 2. Scale bar 10µm, phase contrast microscopy

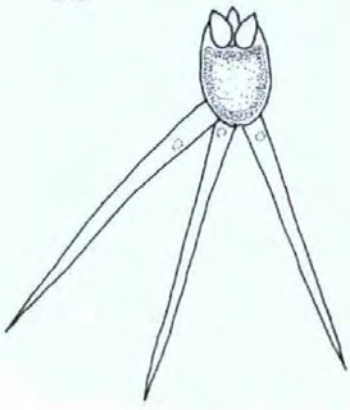
Figure 3.12 Line drawing of Echinactinomyxon type 3

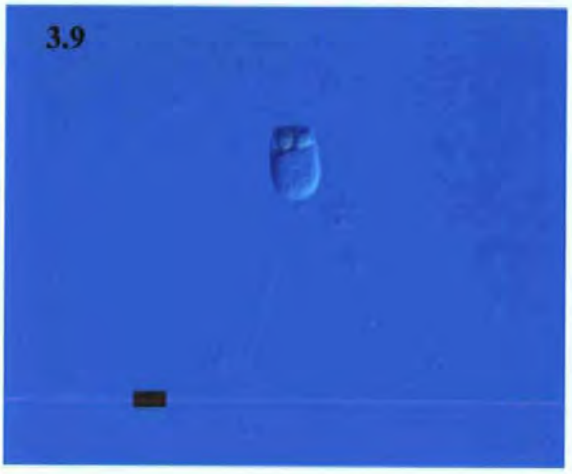
Figure 3.13 Photomicrograph of spore body of Echinactinomyxon type 3 showing details of secondary cells and polar capsules. Scale bar 100µm, phase contrast microscopy

3.6

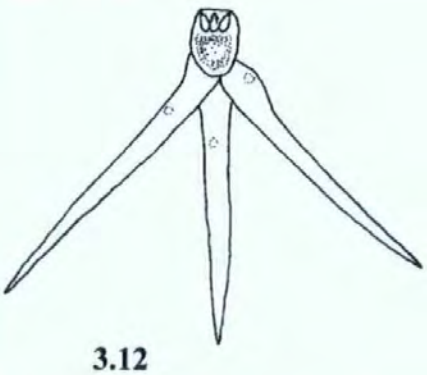


3.8





3.10



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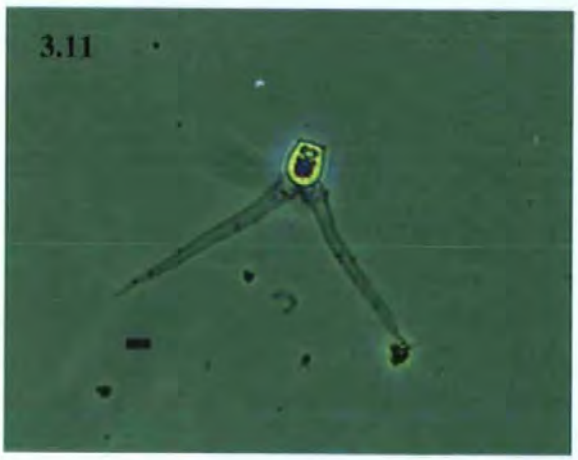
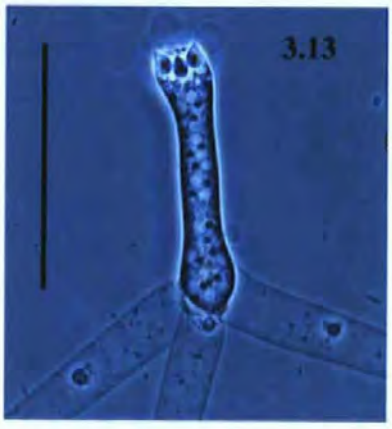




Secondary cells, >20. Three straight caudal processes of equal length tapering to a point, 84-104 (89.9) long × 9-11.3 (10.4) wide. Base of caudal processes marginally inflated to give the appearance of shoulders. Locality: Standlynch, East Stoke.

Echinactinomyxon type 3 Figs 3.12 and 3.13

Spores released from *Tubifex* cf. *tubifex*. Two oligochaetes infected. Spore body style-less, 33-51 (44) long × 6.9-8.4 (8) at widest point with three apical polar capsules, 4.3-4.7 (4.5) long × 2.5-3.3 (2.9) wide. Secondary cells, 32. Three straight caudal processes of equal length tapering to a point, long 100-181 (135) × 9.8-10.8 (10) wide at base of caudal process. Locality: Standlynch.

Echinactinomyxon type 4 Figs 3.14, 3.15 and 3.16

Spores released from *Tubifex* cf. *tubifex*. Four oligochaetes infected. Spore body style-less, 30.2-32.1 (31) long × 20-21.5 (21) at widest point with three apical polar capsules, 6.4-7.1 (6.8) long × 4.7-5.4 (5.2) wide. Secondary cells, 16. Three straight caudal processes of equal length tapering to a point, 99-126 (115) long × 9-11 (10) wide at base of caudal process. Caudal processes with small bifurcations near tips (Fig 3.18). Locality: Wimborne, East Stoke, Canford School.

Echinactinomyxon type 5 Figs 3.17 and 3.18

Spores released from unknown tubificid. Two oligochaetes infected. Spore body style-less, 24.3-27.9 (26) long × 17.1-19.2 (17.6) at widest point with three apical polar capsules, 7.2-7.5 (7.3) long × 3.7-4.1 (4) wide. Secondary cells, 16. Three straight caudal processes

Figure 3.14 Line drawing of Echinactinomyxon type 4

Figure 3.15 Photomicrograph of Echinactinomyxon type 4. Scale bar 100µm, phase contrast microscopy.

Figure 3.16 Photomicrograph of detail of small projections on caudal process of Echinactinomyxon type 4. Scale bar 10µm, phase contrast microscopy.

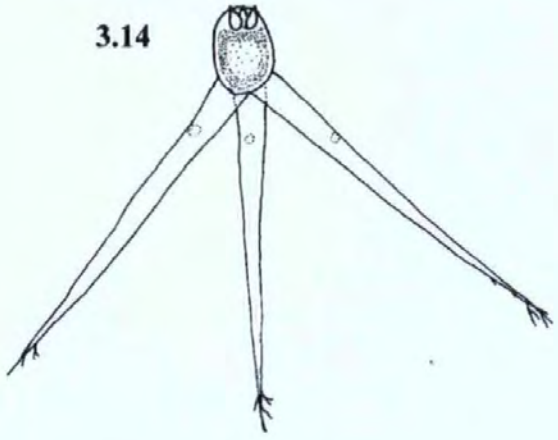
Figure 3.17 Line drawing of Echinactinomyxon type 5.

Figure 3.18 Photomicrograph of Echinactinomyxon type 5. Scale bar 10µm, phase contrast microscopy

Figure 3.19 Line drawing of Echinactinomyxon type 6.

Figure 3.20 Photomicrograph of Echinactinomyxon type 6. Scale bar 100µm, DIC

3.14



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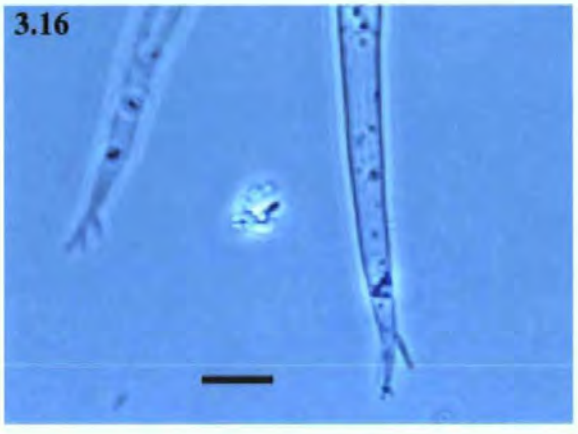

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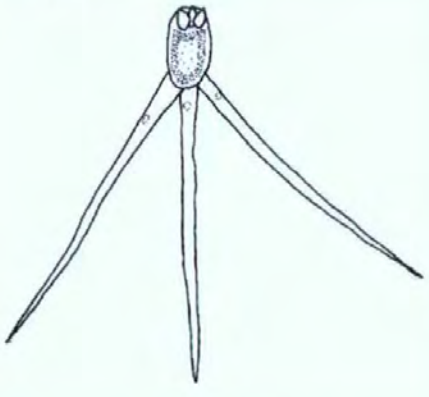
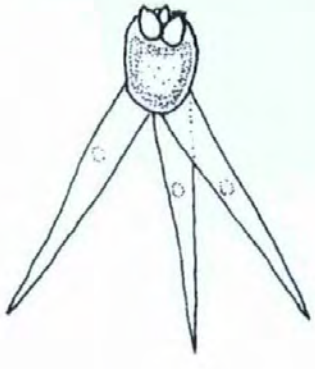
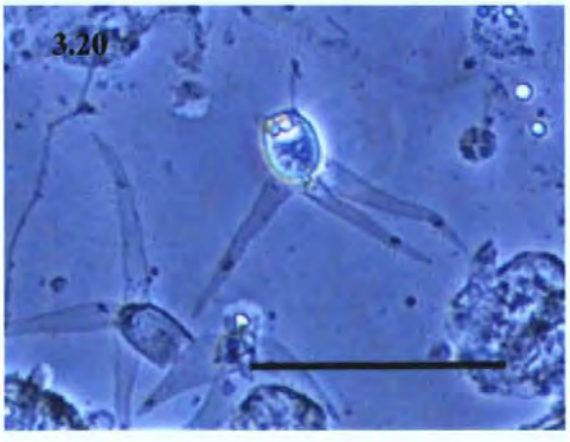
3.17



3.18

3.19





3.20

of equal length tapering to a point, 79-115 (100) long × 5-8.5 (7.5) wide at base of caudal process. Locality: Canford School, Wales.

Echinactinomyxon type 6 Figs 3.19 and 3.20

Spores released from unknown tubificid. One oligochaete infected. Spore body style-less, 23.8-27.2 (25.1) long × 19-20.2 (19.8) at widest point with three apical polar capsules, 5.9-6.4 (6.3) long × 4.2-5 (4.8) wide. Secondary cells, 32. Three straight caudal processes of equal length tapering to a point, 59-99.7 (71.8) long × 7.2-11.3 (9.2) wide at base of caudal process. Locality: East Stoke.

Remarks

According to Janiszewska (1957), *E. radiatum* sp. inq. have caudal processes measuring 100-125µm, and a spore body length of 25-30µm containing 32 secondary cells. Whilst the original description is lacking in detail, the overall dimensions of the current type match with those provided by Janiszewska (1957) and is therefore considered conspecific with that type.

E. type 1 possesses long, narrow caudal processes and a rounded spore body and is most similar to E. type 2 of Özer et al. (2002a) in overall appearance. However, it can be discriminated form this type as the caudal processes are shorter, narrower and outside the range reported by Özer et al. (2002a).

E. type 2 is characterised as possessing caudal processes that are inflated at the proximal end to give the appearance of "shoulders" and is similar to E. types D and E of Xiao and Desser (1998b) and E. type 3 of Özer et al. (2002a) in overall dimensions. Özer et al. (2002a) reported that E type 3 possessed caudal processes that were much widened at the proximal end. The spore body length of E. type 3 of Özer et al. (2002a) is longer than

the current type 2 and whilst the caudal processes lengths are similar, the caudal process width of the current type is wider than reported by Özer et al. (2002a). E. type 1 of Negredo and Mulcahy (2001) also possesses a caudal processes that are widened at the proximal end but has a wider spore body and longer and wider caudal processes than E. type 2 of the current study. The measurements of E. type 3 of Negredo and Mulcahy (2001) are similar to the current type 2. However, E. type 2 from the current study can be discriminated from E. type 3 of Negredo and Mulcahy (2001) in the size and shape of the polar capsules and by the greatly enlarged caudal processes at the proximal end. In view of the absence of similar type in the literature, we consider the current form to be a previously unrecorded type of Echinactinomyxon.

E. type 3 has been included within the collective group Echinactinomyxon as it possesses straight caudal processes and a spore body that is completely filled by a sporoplasm. Only one other type of with a similar morphology to the current E. type 3 has been reported previously. Xiao and Desser (1998a) considered that Triactinomyxon F could belong either in the collective group Raabeia as it possessed curved caudal processes or Triactinomyxon, as the spore body was elongate. Whilst Janiszewska (1964) originally described *E. astilum* sp. inq. as belonging to the collective group Triactinomyxon due to the elongate nature of the spore body, it was transferred to the collective group Echinactinomyxon as it does not possess a style. The current E. type 3 can be discriminated from *E. astilum* sp. inq. as it is much larger in all dimensions. The Triactinomyxon type F of Xiao and Desser (1998a) possesses curved caudal processes which are both longer and wider than E. type 3 in the current study, although the spore body has similar dimensions in length and width. Since the current type does not conform to the description of Triactinomyxon F of Xiao and Desser (1998a) and there are no other echinactinomyxons reported with a similar morphology, we consider the current type 3 as a previously unrecorded type.

The most striking feature of E. type 4 is the presence of small extensions to the distal portion of the caudal processes. Unlike those reported for example in E. type of Székely et al. (2002) or R. types 1 and 2 of Özer et al. (2002), the extensions are small and the caudal processes taper to a sharp point. Özer et al. (2002a) report the presence of small branches to the tips of the caudal process in their Raabeia types 4, 5 and 6. E. type 4 is similar to Raabeia type 5 of Özer et al. (2002a) in its overall dimensions and the size, shape and position of the caudal process bifurcations but is smaller in all dimensions. E types 1 and 4 of Özer et al. (2002a) have caudal processes of a similar size, but smaller spore bodies and lack the small bifurcations of the caudal process. As there are no other Raabeia or Echinactinomyxon types described with small bifurcations similar to those reported here, we consider the current form to be a previously undescribed form of Echinactinomyxon.

E. type 5 is similar to E. type 4 of Özer et al. (2002a) in general morphology which they considered to be conspecific with *E. radiatum* sp. inq. However, the current type 5 can be discriminated from E. type 4 of Özer et al. (2002a) as it has a narrower spore body and shorter, wider caudal processes.

E. type 6 has a relatively large, almost rounded spore body and short, thin caudal processes. Only *E. astilum* sp. inq. of Janiszewska (1964) and E type 1 of Negredo and Mulcahy (2001) possess spore bodies of similar size. However, the current type 6 can be discriminated form both these types on the basis of the caudal process length. *E. astilum* sp. inq. has much shorter caudal processes (72µm vs. 45-60) and E. type 1 of Negredo and Mulcahy (2001) has longer caudal processes (72µm vs. 126µm)

Table 3.2 Measurements of main taxonomic features of Echinactinomyxon types isolated during the current study and comparison with known forms where previous descriptions apply.

Echinactinomyxon types	Caudal process length × width	Spore body length × width	Number of secondary cells	Polar capsule length × width	Reference
Echinactinomyxon radiatum sp. inq. of Janiszewska (1957)	85-105 (97) × 9-10 (10)	23-30 (27) × 13-16 (15)	28	7.1-8.3 (7.5) × 4.2–4.4 (4.5)	This study
Echinactinomyxon radiatum sp. inq. of Janiszewska (1957)	100-125 × ?	27-30 ×?	32?	6 ×?	Janiszewska (1957)
Echinactinomyxon type 1	47-82 (66) × 4-6 (6)	18-22 (20) × 10-13.5 (12)	>20	5.8-6.0 (5.9) × 3.9-4.4 (4.1)	This study
Echinactinomyxon type 2	84-104 (90) × 9-11 (10)	18-21 (20) × 14.5-16 (15)	>20	6.2-7.5 (6.7) × 3.2-4.3 (4)	This study
Echinactinomyxon type 3	100-181 (135)	33-51 (44) x 7-8 (8)	32	4.3-4.7 (4.5) × 2.5-3.3 (2.9)	This study
Echinactinomyxon type 4	99-126 (115) × 9-11 (10)	30-32 (31) × 20-21.5 (21)	16	6.4-7.1 (6.8) × 4.7-5.4 (5.2)	This study
Echinactinomyxon type 5	79-115 (100) × 5-8.5 (7.5)	24-28 (26) ×17-19 (18)	16	7.2-7.5 (7.3) × 3.7-4.1 (4)	This study
Echinactinomyxon type 6	59-100 (72) × 7-11 (9)	24-27 (25) × 19-20 (20)	32	5.9-6.4 (6.3) × 4.2-5 (4.8)	This study

Triradiate spores possessing three finger shaped, long caudal processes arising below the spore body, pointed downwards. Tips of caudal processes rounded.

***Guyenotia sphaerulosa* sp. inq. of Naville (1930)** Figs 3.21 and 3.22

Spores released from *Tubifex* cf. *tubifex*. One oligochaete infected. Spore body sub-circular, 15.9-17.2 (17) long × 14.8-15.1 (15) at widest point with three apical, sub-spherical polar capsules protruding from spore body, 5.9-6.0 (6.0) long × 4.8-5.1 (5.0) wide. Secondary cells, 32. Three caudal processes of equal length, straight with slightly rounded tips, (41-46) 45 long and 8.5-9.6 (9) wide at widest point. Locality: Canford School.

Remarks

The dimensions of *Guyenotia sphaerulosa* sp. inq. by Naville (1930) are as follows: Caudal process length 40 µm; Spore body diameter 15µm; polar capsule length × width 6 µm × 5 µm. The measurements of the current Guyenotia type match those of Naville (1930) and whilst the tips of the current type are not as rounded as reported and drawn diagrammatically by Naville (1930), we consider this form to be conspecific with *Guyenotia sphaerulosa* sp. inq.

COLLECTIVE GROUP NEOACTINOMYXUM

Triradiate spherical valves with semicircular or triangular lobes greater than half the total length of spore, swellings increase in size when parasite is released into the water. Caudal processes joined together at edges. Three polar capsules project from spore.

Neoactinomyxum type of Özer et al. (2002a) Figs 3.23 and 3.24

Spores released from *Tubifex* cf. *tubifex*. One oligochaete infected. Spore body spherical, 14-18 (16.5) in diameter with three apical polar capsules, 2.3-2.9 (2.7) in diameter. Secondary cells, more than 20. Three caudal processes of equal length, rounded, 23-29 (27.2) long and 15-18 (17.3) wide. Locality: Canford School.

Remarks

Two general forms of Neoactinomyxum have been reported, those with triangular caudal processes, and those with rounded, semicircular caudal processes. Morphologically, the current type fits with those types with rounded caudal processes. The Neoactinomyxum form of Xiao and Desser (1998b) has larger caudal processes and a smaller spore body than the current type. *N. globosum* sp. inq. of Granata (1922b) has a smaller spore body and fewer secondary cells, *N. minutum* sp. inq. of Marques (1984) has smaller caudal processes and *N. eiseniellae* sp. inq. of Ormières and Frezil (1969) is smaller overall compared with the current type. Although the current type is marginally smaller than that described by Özer et al. (2002a), we consider it to be conspecific with that type.

COLLECTIVE GROUP RAABEIA

Spore possessing three long, pointed and curved processes arising from spore body. Does not possess a style. Spore body length less than half the length of the caudal processes. Sporoplasm is rounded or barrel shaped, contains numerous germ cells and completely fills spore body. Three polar capsules.

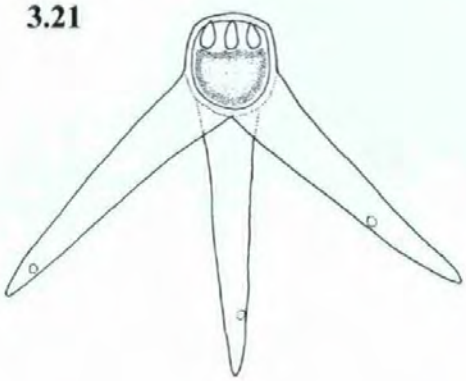
Figure 3.21 Line drawing of *Guyenotia sphaerulosa* sp. inq. of Naville (1930)

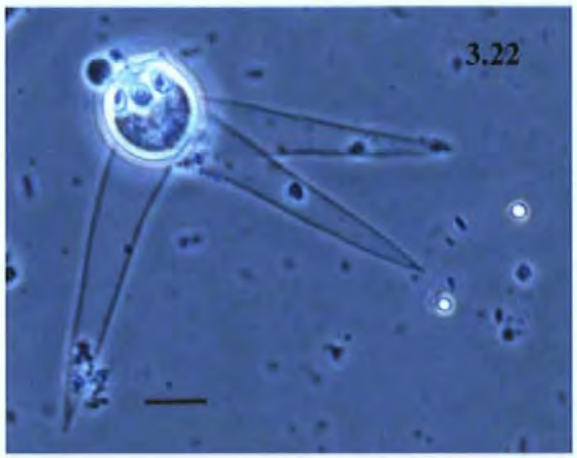
Figure 3.22 Photomicrograph of *Guyenotia sphaerulosa* sp. inq. of Naville (1930). Scale bar 10µm, phase contrast microscopy

Figure 3.23 Line drawing of Neoactinomyxum type 1 of Özer *et al.* (2002)

Figure 3.24 Photomicrograph of Neoactinomyxum type 1 of Özer *et al.* (2002) following release of spore body. Scale bar 10µm, phase contrast microscopy

3.21





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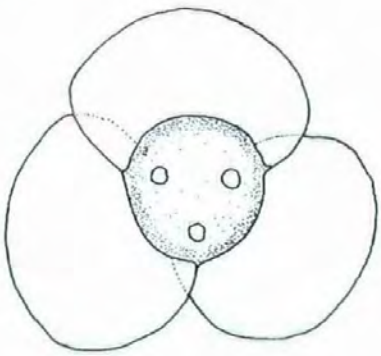
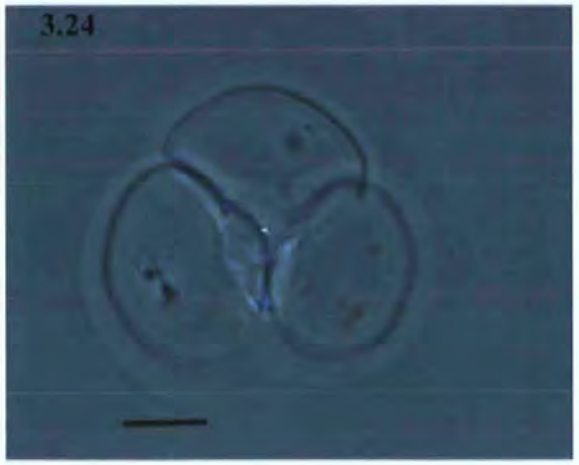




Raabeia type 1 **Figs. 3.25 and 3.26**

Tubificid host unidentified. One oligochaete infected. Spore body style-less, slightly barrel shaped, 25-32 (27) long × 8-12 (10) at widest point with three apical polar capsules, 4.5 long × 2.7 wide. Secondary cells, 32. Three curved caudal processes of equal length tapering to a point, 168-218 (191) long × 8.5-9.2 (9) wide. Locality: Nether Cerne.

Raabeia type 2 Figs 3.27 to 3.30

Spores released from *Limnodrilus hoffmeisteri* and *Tubifex* cf. *tubifex*. Five oligochaetes infected. Spore body style-less, barrel shaped, 9-10 (10) long × 5-6 (5) wide, with three apical, cylindrical polar capsules, 4.0-4.7 (4.4) long × 1.3-1.9 (1.6) wide. Secondary cells, 8. Three short, curved caudal processes of equal length tapering to a point, 30-45 (35) long × 2.9-3.4 (3) wide. Locality: Wimborne and East Stoke.

Remarks

Raabeia type 1, is characterised as possessing a relatively short spore body and long, curved caudal processes. A number of Raabeia forms bear a morphological resemblance to the current Raabeia type 1. Özer et al. (2002a) consider that their type 3 is conspecific with Raabeia sp. of McGeorge et al. (1997). The current type, whilst morphologically similar to that type, can be discriminated from Raabeia type 3 of Özer et al. (2002a) as the spore body is smaller and more barrel shaped, and possesses smaller caudal processes. The caudal processes of *R. gorlicensis* sp. inq. of Janiszewska (1955) are of a similar length to type 1 but the spore body is rounded, unlike type 1 in the current study which is elongate and barrel shaped. Of the four Raabeia types reported by El-Mansy et al. (1998a), three are morphologically similar to the current Raabeia type 1.

Figure 3.25 Line drawing of Raabeia type 1

Figure 3.26 Photomicrograph of spore body of Raabeia type 1. Scale bar 10μm, phase contrast microscopy.

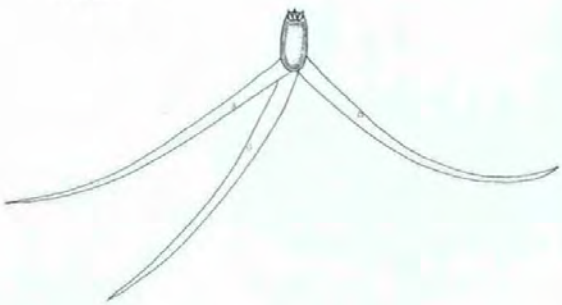
Figure 3.27 Line drawing of "curved" form of Raabeia type 2

Figure 3.28 Photomicrograph of "curved" form of Raabeia type 2. Scale bar 10μm, phase contrast microscopy.

Figure 3.29 Line drawing "straight" form of Raabeia type 2.

Figure 3.30 Photomicrograph of "straight" form of Raabeia type 2. Scale bar 10μm, phase contrast microscopy.

3.25



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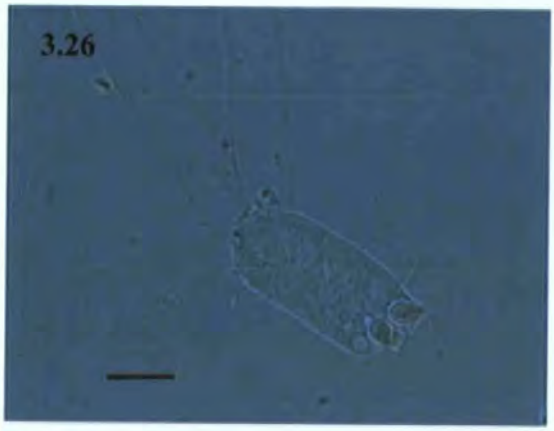

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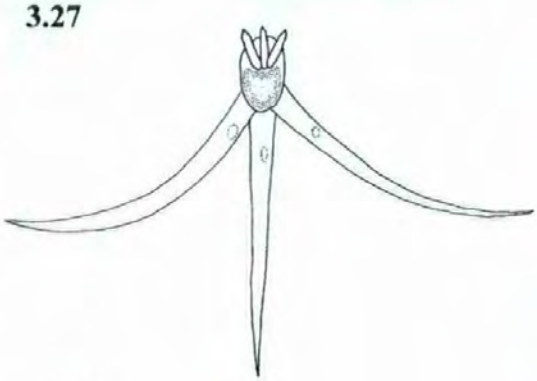
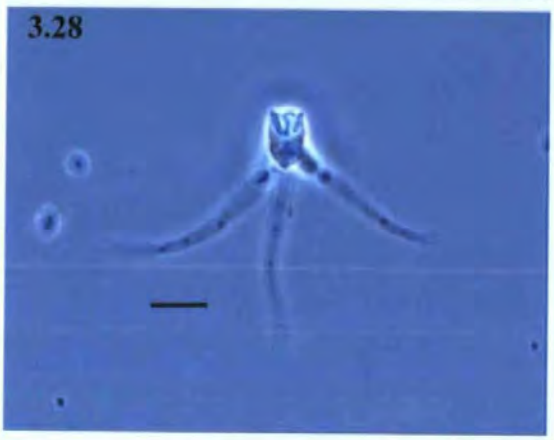
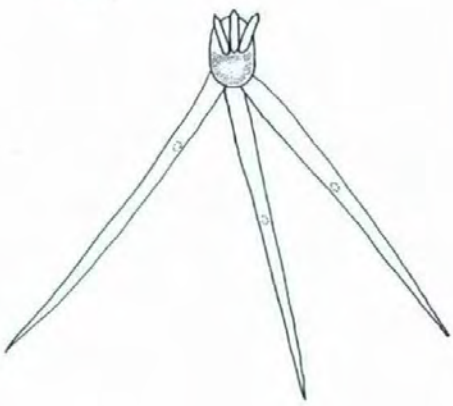


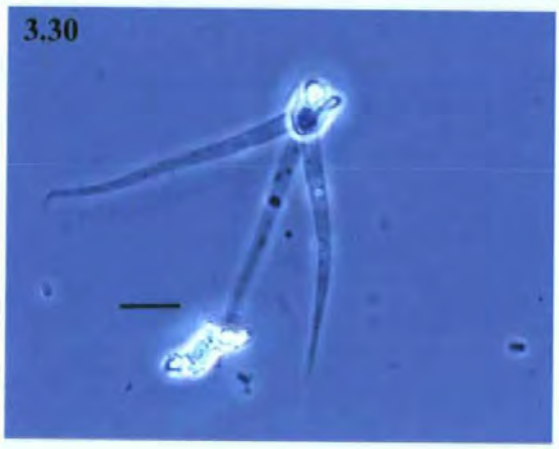
3.28



3.29



3.30



Raabeia type 1 of El-Mansy et al. (1998a) possess a longer caudal process, Raabeia type 3 of El-Mansy et al. (1998a) has a wider spore body and wider caudal process and Raabeia type 4 of El-Mansy et al. (1998a) has a narrower spore body and markedly narrower caudal process.

The lengths of all of the caudal processes of the Raabeia types described by Xiao and Desser (1998a) are outside the ranges of the current type. Raabeia type 1 of Oumouna et al. (2003) is similar in morphology but larger in all dimensions than the current type. We therefore consider the current Raabeia type 1 to be a previously undescribed type.

The classification of Raabeia type 2 is problematic. Whilst the majority of the time the caudal processes were curved, on occasions they were straight, like echinactinomyxons (Figures 3.31 and 3.32), and unlike most Raabeia types, were extremely short (average 35µm length). Additionally, the polar capsules were unlike any previously reported for actinospores (or myxospores). They were elongate, rectangular, and protruded markedly from the apical surface of the spore body. Whether in the "straight" or "curved" form, the spores occasionally aggregated together, forming loose attachments to each other via the tips of the caudal processes to form "webs" with up to 50 or more spores. Despite these differences, we do not consider them sufficient to erect a new collective group. The only Raabeia types that have caudal process lengths close to the current type are Raabeia types 1 and 2 of Özer et al. (2002a). These have mean caudal process lengths of 94.5µm and 85.6µm respectively. Both of these however, have bifurcations at the tip of the caudal process and have larger spore bodies. Because of the unique features demonstrated by the current Raabeia type 2, and due to the fact that most of the time the caudal processes were curved, we consider this to be a previously undescribed form of Raabeia.

Style-less spores possessing either one short caudal process and two longer caudal processes or three equal sized caudal processes. Joined together in groups of eight by either the short caudal process or the tip of one caudal process to form a circle.

***Synactinomyxon longicauda* sp. inq. of Marques and Ormières (1982)** Figs. 3.31 and 3.32

Eight spores joined together by one tip of caudal process, arranged in a circular formation. Occasionally, free, individual spores present in water. Mature spores with characteristics of the Echinactinomyxon group with three straight, equal sized caudal processes that taper to a pointed end with pointed ends, style-less spore body. Spores released from *Tubifex* cf. *tubifex*. Sixteen oligochaetes infected. Spore body 16-19 (18) long, 12-15 (14) at its widest point; goblet shaped when viewed laterally, circular when viewed apically. Secondary cells, 16. Polar capsules with six coils, sub-spherical measuring 4.5-5.8 (5.4) × 2.6-3.8 (3.6). Caudal process equal in size, 65-83 (75) × 8-9.4 (8.5). Locality: East Stoke, Wimborne and Wales.

Remarks

The Synactinomyxon form isolated most closely resembles *Synactinomyxon longicauda* sp. inq. Marques & Ormières, 1982 in general morphology. It was also reported by McGeorge et al. (1997) and Özer et al. (2002a) associated with fish farms in Scotland. Marques and Ormières (1982) described *S. longicauda* as having a truncated ellipsoidal spore body measuring 22-25µm, caudal processes 80 µm × 8 µm and polar capsules 7 µm long. McGeorge et al. (1997) tentatively identified their Synactinomyxon

'B' as *S. longicauda* because the measurements broadly reflect those recorded for *S. longicauda* (Caudal process 64.4 µm (55-80 µm) × 8 µm (6.5-9 µm); Spore body 21.3 µm (20-23 µm) × 18.3 (16-20); polar capsule 6 µm × 4µm). The dimensions given by Özer et al. (2002a) broadly reflect the measurements from the current study, except that the measurements given by them for the spore body are larger and contain only 16 secondary cells. In the present study, the parasite is marginally smaller than the measurements given by Marques and Ormières (1982) in all dimensions but not sufficiently to consider it a new type. We therefore consider that this parasite is morphologically identical to *S. longicauda* sp. inq.

COLLECTIVE GROUP TRIACTINOMYXON

Triradiate spores possessing a style and three long, pointed or straight caudal processes. Style length greater than or equal to half the length of the caudal processes. Sporoplasm does not completely fill spore body. Three polar capsules.

***Triactinomyxon ignotum* sp. inq. of Štolc (1899)** Figs. 3.33 and 3.34

Spores released from *Tubifex* cf. *tubifex, Limnodrilus hoffmeisteri* and *Psammoryctides* sp. Twenty six oligochaetes infected. Sporoplasm, 41-60 (48) long × 11-15 (13) wide within a style containing 8 secondary cells. Polar capsules pyriform, 4.1-5.0 (4.8) × 2.7-3.3 (3.1). Style 165-190 (174) long × 21-26 (23) wide. Caudal processes curved upwards, 192-240 (220) long × 19-22 (20) wide at base. Locality: Nether Cerne, East Stoke, Wimborne, Canford School and Blandford.

Figure 3.31 Line drawing of *Synactinomyxon longicauda* sp. inq. of Marques and Ormieres (1982).

Figure 3.32 Photomicrograph of four individual spores of *Synactinomyxon longicauda* sp. inq. Scale bar 10µm, bright field microscopy.

Figure 3.33 Line drawing of *Triactinomyxon ignotum* sp. inq. of Štolc (1899).

Figure 3.34 Photomicrograph showing detail of sporoplasm and eight secondary cells in the style of *Triactinomyxon ignotum* sp. inq. Scale bar 10µm, DIC.

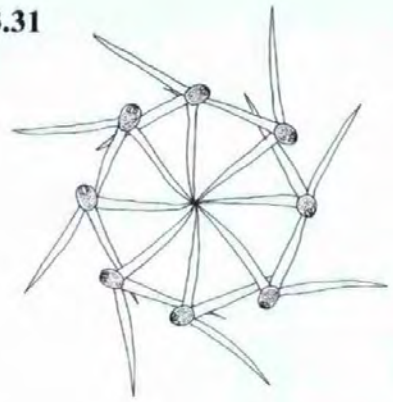
Figure 3.35 Line drawing of the triactinomyxon stage of *Myxobolus pseudodispar.*

Figure 3.36 Photomicrograph of the triactinomyxon stage of *Myxobolus pseudodispar.* Note the presence of one short caudal process and one long caudal process in the field of view. Scale bar 100µm, phase contrast microscopy

Figure 3.37 Line drawing of the triactinomyxon stage of *Myxobolus macrocapsularis.*

Figure 3.38 Photomicrograph of the triactinomyxon stage of *Myxobolus macrocapsularis.* Scale bar 100µm, phase contrast microscopy

3.31



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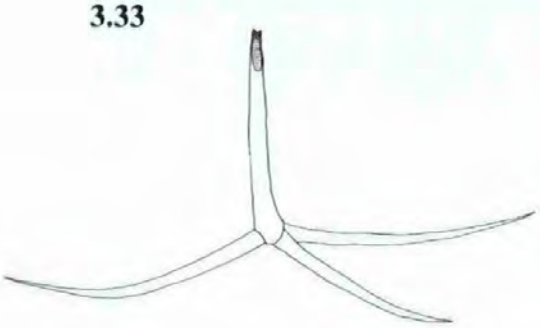

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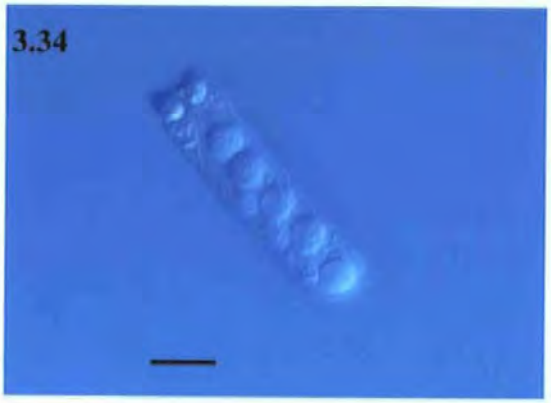

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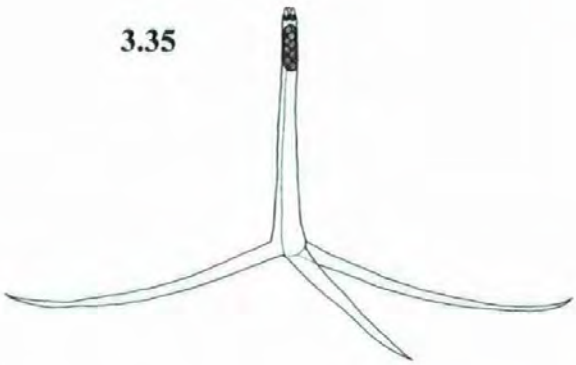

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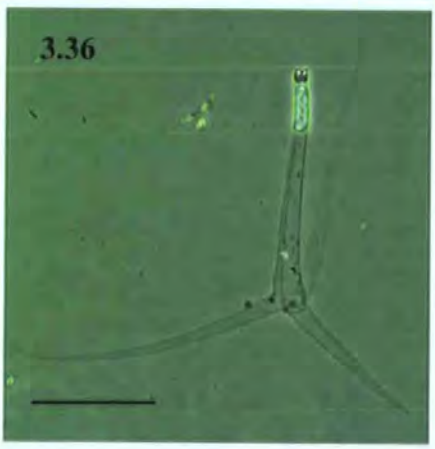

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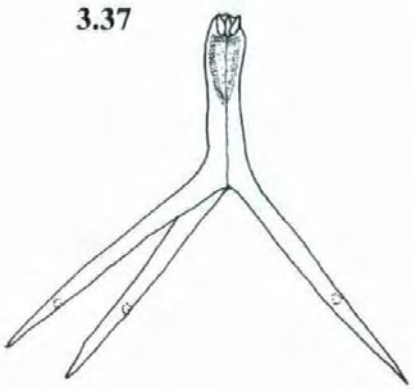


3.38

Triactinomyxon form of *Myxobolus pseudodispar* Figs 3.35 and 3.36

Spores released from *Tubifex* cf. *tubifex* and *Limnodrilus hoffmeisteri*. Thirty seven oligochaetes infected. Sporoplasm, 35.1-39 (37) long × 11.5-13.9 (12) wide within a style containing 8 secondary cells. Polar capsules pyriform, 4.9-5.7 (5.3) × 3.7-4.4 (4.1). Style 142-165 (150) long × 14.2-15.6 (15) wide. Two long caudal processes curved upwards, 189-207 (200) long × 13.2-15.7 (14) wide at base and one short caudal process 109-134 (120) long × 12.9-15.2 (15) wide at base. Locality: East Stoke, Standlynch, Canford School and Blandford.

Triactinomyxon form of *Myxobolus macrocapsularis* Figs 3.37 and 3.38

Spores released from *Tubifex* cf. *tubifex*. Two oligochaetes infected. Sporoplasm narrows to a point, 15-17.5 (17) long × 12.4-13.9 (13) wide within a style containing more than 20 secondary cells. Polar capsules pyriform, 4.4 × 2.5. Style 59-67(65) long × 9-11.5 (10) wide at midpoint of style. Caudal processes straight and pointed downwards, 89-100 (97) long × 9-10 (9.4) wide at base. Locality: East Stoke.

Triactinomyxon type 1 Figs 3.39 and 3.40

Spores released from *Psammoryctides barbatus*. Two oligochaetes infected. Sporoplasm at apex of style, wider than style, 27-32 × (29) long × 12-17 (15) wide within a style containing 32 secondary cells. Polar capsules pyriform, 7.4-7.6 (7.5) long × 4.3-4.7 (4.6) wide. Style 186-209 (197) long × 14-15 (14.5) wide. Caudal processes curved upwards, 170-257 (231) long × 13-16 (14.8) wide at base. Locality: Wimborne.

Figure 3.39 Line drawing of Triactinomyxon type 1

Figure 3.40 Photomicrograph of the sporoplasm of Triactinomyxon type 1. Scale bar 10μm, phase contrast microscopy.

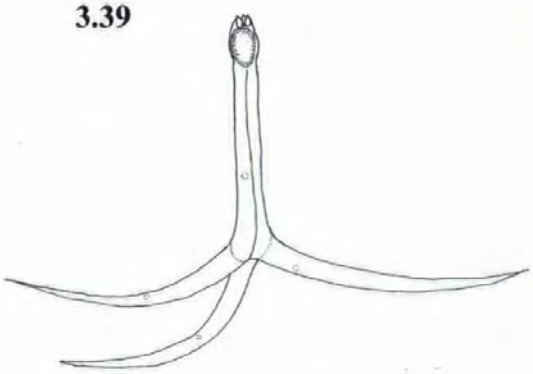
Figure 3.41 Line drawing of Triactinomyxon type 2.

Figure 3.42 Photomicrograph of Triactinomyxon type 2. Scale bar 100μm, phase contrast microscopy.

Figure 3.43 Line drawing of Triactinomyxon type 3.

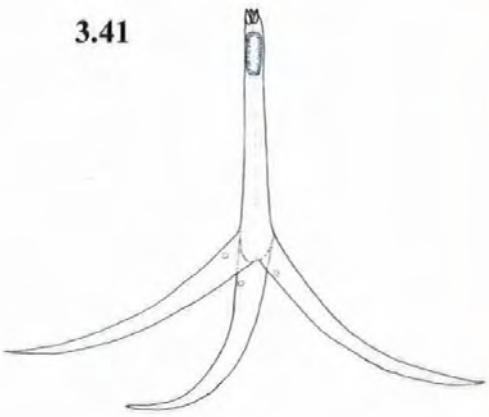
Figure 3.44 Photomicrograph of Triactinomyxon type 3. Note that the sporoplasm has been displaced away from the apical end of the style. Scale bar 100μm, phase contrast microscopy.

3.39



3.40

3.41



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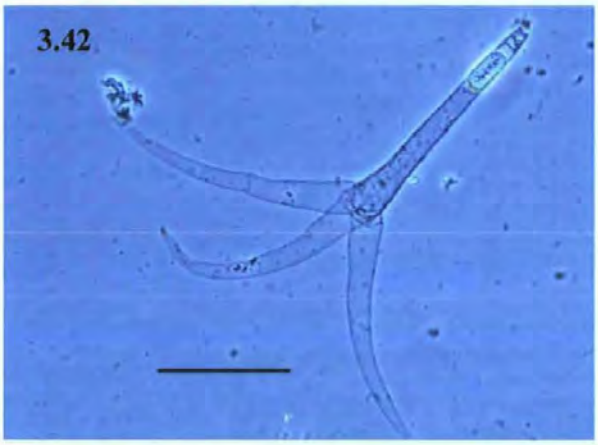

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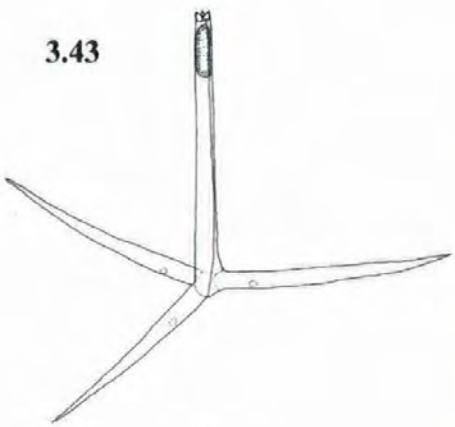

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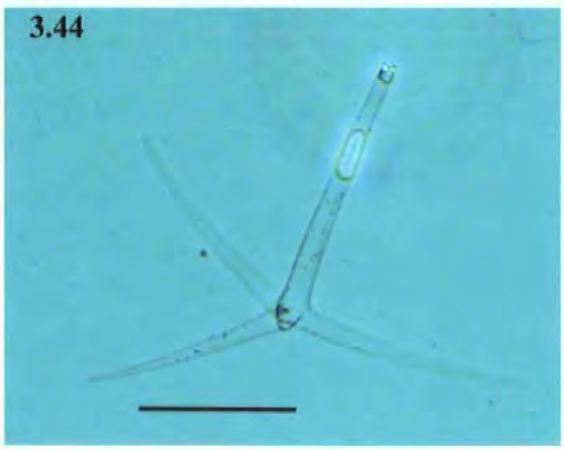

Triactinomyxon type 2 Figs 3.41 and 3.42

Tubificid host unidentified. Three oligochaetes infected. Sporoplasm 26.9-37.2 (34.5) long × 13.2-16.7 (15) wide within a style containing 32 secondary cells. Polar capsules pyriform, 5.2-5.9 (5.7) long × 3.1-3.4 (3.2) wide. Style 171-197 (189) long × 14-18.7 (18) wide at midpoint of style. Style gradually widens. Caudal processes curved upwards, 160-186 (171) long × 21-24 (22.5) wide at base. Locality: East Stoke.

Triactinomyxon type 3 Figs. 3.43 and 3.44

Tubificid host unidentified. Three oligochaetes infected. Sporoplasm 19.5-42.6 (32.2) long × 10.8-13 (11.5) wide within a style containing 32 secondary cells. Polar capsules pyriform, 5.3-5.6 (5.4) long × 3.2-4 (3.8) wide. Style 168.5-185.3 (179.5) long × 12.6-14.3 (13.5) wide at midpoint of style. Caudal processes straight, pointed slightly upwards, 120-156 (132.9) long × 11.4-14.9 (13.3) wide at base. Locality: Canford School.

Remarks

The collective group contains the most number of described types of all the actinospores and many have been implicated in myxozoan lifecycles, particularly within the myxospore genus *Myxobolus*. The measurements and general spore morphology of the *Triactinomyxon ignotum* sp. inq. described during the current study matches with descriptions provided by Štolc (1899), Marques (1984) and Xiao and Desser (1998a) and we therefore consider the form in the current study to be conspecific with *T. ignotum* sp. inq.

The Triactinomyxon stage of *M. pseudodispar* is easily distinguished from other Triactinomyxon types by the presence of two long caudal processes and one shorter one.

The measurements obtained during the current study for the triactinomyxon stages of *M. pseudodispar* and *M. macrocapsularis* agree with those reported by Székely et al. (1999) and Székely et al. (2002) respectively.

T. type 1 has an enlarged anterior portion of the style to encompass a sporoplasm wider than the remainder of the style. T. type B of Xiao and Desser (1998a) has an enlarged anterior portion of the style, but T. type 1 in the current study is larger in all dimensions. T. type C of Xiao and Desser (1998a), whilst similar in general morphology and dimensions is more robust than the current type 1, possessing a wider style and caudal processes and longer caudal processes. T. type D of Xiao and Desser (1998a) has an enlarged anterior portion of the style but overall is smaller than the current type 1. T. type 1 is also similar to the actinospore stage of *M. hungaricus* but possesses a smaller sporoplasm, does not possess the small conical structure at the posterior of the sporoplasm as reported by El-Mansy and Molnár (1997) and has a greater number of sporoplasm cells. Whilst the caudal processes of T. type 1 of El-Mansy et al. (1998b) are similar to those reported here for T. type 1, the style length of T. type 1 of El-Mansy et al. (1998b) is shorter and the sporoplasm is longer. T. type 3 of El-Mansy and T. type 5 of El-Mansy et al. (1998b) have caudal processes within the length range reported for T. type 1 but both possess a much shorter style. Types 2 and 3 of Rácz and Timm (2002) are smaller than the current type in all dimensions. Based on the current literature, we are unable to assign the current form to a previously reported Triactinomyxon and therefore consider T. type 1 from the current study to be a previously unreported type.

Both T. type 3 and 4 of Oumouna et al. (2003) possess styles that widen at the base. T. type 2 from the current study can be discriminated from both these forms as it possesses a much longer style than either of the forms reported by Oumouna et al. (2003) and slightly larger polar capsules. T. type 2 of Lowers and Bartholomew (2003) has a shorter style and similar sized caudal processes. No other Triactinomyxon types

previously reported have dimensions similar to the current type 2 and we therefore consider it to be a previously unrecorded type.

T type 3 is characterised by the presence of a style that is longer than the caudal processes and caudal processes that are more or less straight rather than curved. Only *T. mrazeki* sp. inq. of MacKinnon and Adam (1924), *T. ohridensis* sp. inq. of Georgevitch (1940), T. type 3 of El-Mansy et al. (1998a) and T. type 1 of Oumouna et al. (2003) have style lengths greater than the caudal length. The style of *T. mrazeki* sp. inq. of MacKinnon and Adam (1924) increases in size towards its base, which does not occur in the current type 4. The style of *T. mrazeki* sp. inq. is shorter than the current type (150µm vs. 180µm) although the caudal processes in both types are similar. T. type 3 of the current study is clearly different from *T. ohridensis* sp. inq. which is defined as possessing three very short caudal processes. T. type 3 of El-Mansy et al. (1998a) has straight caudal processes but the current type 3 has longer caudal processes, a longer style and a smaller sporoplasm. T. type 3 of the current study can be discriminated from T. type 1 of Oumouna et al. (2003) as it possess much shorter caudal processes. As the current T. type 3 does not conform to any previously described types of Triactinomyxon, we consider it a new form.

Spatial distribution

The site at East Stoke contained the most number of actinospore types with 12 representatives comprising A. type 1, E. types 1, 2, 4 and 6, Neoactinomyxum type of Özer et al. (2002a), R. type 2, *S. longicauda* sp. inq. of Marques and Ormières (1982), *T. ignotum* sp. inq. of Štolc (1899), Triactinomyxon stages of *M. pseudodispar* and *M. macrocapsularis* and T. type 2. The Canford School site contained 9 actinospore types comprising *A. raabeiiunioris* sp. inq., *E. radiatum* sp. inq., E. types 2, 4 and 5, *G. sphaerulosa* sp. inq., *T. ignotum* sp. inq., Triactinomyxon stage of *M. pseudodispar* and

Table 3.3 Measurements of main taxonomic features of Triactinomyxon types isolated during the current study and comparison with known forms where previous descriptions apply.

Triactinomyxon type	Style length × width	Caudal process length × width	Sporoplasm length ×width	No. of secondary cells	Polar capsule length × width	Reference
Triactinomyxon ignotum sp. inq. of Štolc (1899)	165-190 (174) × 21-26 (23)	192-240 (220) × 19-22(20)	41-60 (48) × 11-15 (13)	8	4.1-5.0 (4.8) × 2.7-3.3 (3.1)	Current study
Triactinomyxon ignotum sp. inq. of Štolc (1899)	140-170 × ?	175-220 × ?	30-50 × ?	8	-	Štolc (1899)
Triactinomyxon form of *Myxobolus pseudodispar*	142-165 (150) × 14-16 (15)	Longer 189-207 (200) × 13-16 (14) Shorter 109-134 (120) × 13-15 (15)	35-39 (37) × 11.5-14 (12)	8	4.9-5.7 (5.3) × 3.7-4.4 (4.1)	Current study
Triactinomyxon form of *Myxobolus pseudodispar*	145-173 (157) × 13-18 (16)	Longer 190-204 (197) × 12-16 (14) Shorter 104-144 (127)	42-48 (45) × 13-18 (16)	8	4-6 (5) × 3-3 (3)	Székely et al (1999)
Triactinomyxon form of *M. macrocapsularis*	59-67 (65) × 9-11.5 (10)	89-100 (97) × 9-10 (9)	15-17.5 (17) × 12-14 (13)	>20	3.7-5.2 (5.1) × 3.6-7.1 (4.3)	Current study
Triactinomyxon form of *M. macrocapsularis*	53-69 (60) × 9-10 (9.5)	94-141 (117) × 8-10 (8.5)	16-24 (21) × 10-14 (12)	32	4-6 (5) × 3-4 (4)	Székely et al (2002)
Triactinomyxon type 1	186-209 (197) × 14-15 (14.5)	170-257 (231) × 13-16 (15)	27-32 (29) × 12-17 (15)	32	7.4-7.6 (7.5) × 4.3-4.7 (4.6)	Current study
Triactinomyxon type 2	171-197 (189) × 14-19 (18)	160-186 (171) × 21-24 (22.5)	27-37 (34.5) × 14-19 (18)	32	5.2-5.9 (5.7) × 3.1-3.4 (3.2)	Current study
Triactinomyxon type 3	168.5-185 (179.5) × 13-14 (13.5)	120-156 (133) × 11-15 (13)	19.5-43 (32) × 11-13 (11.5)	32	5.3-5.6 (5.4) × 3.2-4 (3.8)	Current study

T. type 3. The Wimborne site contained 6 actinospore types comprising E. types 1 and 4, R. type 1, *S. longicauda* sp. inq., *T. ignotum* sp. inq. and T. type 1. At Standlynch, 4 actinospore types were recorded comprising *E. radiatum* sp. inq., E. types 2 and 3 and the Triactinomyxon stage of *M. pseudodispar*. At Nether Cerne, only three actinospore types were recorded comprising A. type of McGeorge et al. (1997), R. type 1 and the Triactinomyxon stage of *M. pseudodispar*. At the site in Wales, two actinospore types were identified, E. type 5 and *S. longicauda* sp. inq. At Blandford, two types were also identified namely *T. ignotum* sp. inq. and the triactinomyxon stage of *M. pseudodispar*.

At the level of the collective group, spatial distribution differences were noted where aurantiactinomyxons were only recorded from Canford School, Nether Cerne and East Stoke; echinactinomyxons from Canford School, Wimborne, Standlynch and East Stoke; Guyenotia from Canford School; Neoactinomyxum from East Stoke; raabeia types from Wimborne, Nether Cerne and East Stoke; Synactinomyxon from Wimborne, Wales and East Stoke and triactinomyxons from all sites, excluding the Wales site.

Temporal changes in the distribution of actinospores at all sites combined

There are marked seasonal patterns in the release of actinospores from their oligochaete hosts; both at the collective group level and at the type level (Tables 3.4 and 3.5). Despite clear spatial differences in the distribution of actinospores, there is some temporal stability in the release of actinospores at the collective and type level (Tables 3.4 and 3.5). The majority of actinospores were released during the summer (85 oligochaetes infected), followed by spring (39 oligochaetes infected), then autumn (10 oligochaetes infected) and winter when only 4 oligochaetes were infected. Representatives of the collective group Aurantiactinomyxon and Raabeia were released both in spring and summer. Guyenotia and Neoactinomyxum were released only during the summer. The peak of release for Synactinomyxon was in summer, with some release during autumn and

winter. In contrast, echinactinomyxons and triactinomyxons were released throughout the year, with the peak of release for echinactinomyxons being in spring, summer and autumn and for triactinomyxons, during spring and summer.

Aurantiactinomyxons showed a single pulse of infection, with *A. raabeiiunioris* sp. inq. and A. type 1 released in the summer at prevalences of between 0.01 and 0.06% for all sites combined. On the other hand, A. type of McGeorge et al. (1997) was released only once, during the spring. Echinactinomyxons had a broad temporal distribution pattern. *E. radiatum* sp. inq. was released in spring, summer and autumn, whereas E. type 1 was only released during summer and autumn. E. type 2 was released in all seasons except the summer when E. type 3 was released. E. types 4 and 5 were released both in spring and summer, whilst E. type 6 was only recorded during the spring. The peak of release for *T. ignotum* sp. inq. was during summer, with some release in both spring and winter, whilst the peak release of the triactinomyxon stage of *M. pseudodispar* was in spring and summer, with a single infected worm being detected in autumn. The triactinomyxon stage of *M. macrocapsularis* and T. type 2 were only recorded in spring, T. type 1 was only recorded in the summer and T. type 3 was recorded in both spring and summer.

Seasonal release of actinospores at the site level.

The Blandford site was visited for four seasons during the course of the study. Only two triactinomyxons were recorded with a peak of release in spring 1997, at a prevalence of 5.83% for the triactinomyxon stage of *M. pseudodispar* (Table 3.6). No actinospores were recorded in autumn 1996, and *T. ignotum* sp. inq. was recorded in one oligochaete in the winter of 1996.

The site at Standlynch was visited in 1997 and 1998, during which actinospores were only recorded during the 1997 sampling season (Table 3.7). Whilst the triactinomyxon stage of *M. pseudodispar* was recorded in two separate sampling seasons (spring and autumn 1997) at prevalences of between 0.23 and 1.11%, the three

Table 3.4 Summary data of seasonal release of actinospore collective groups isolated from all sites. Where % = percentage of oligochaetes infected in sample and n = number of oligochaetes infected.

Collective group	Spring		Summer		Autumn		Winter		Overall	
	%	n	%	n	%	n	%	n	%	N
Aurantiactinomyxon	0.03	1	0.1	10	-	-	-	-	0.063	11
Echinactinomyxon	0.3	11	0.1	10	0.37	8	0.05	1	0.173	30
Guyenotia	-	-	0.01	1	-	-	-	-	0.006	1
Neoactinomyxum	-	-	0.01	1	-	-	-	-	0.006	1
Raabeia	0.06	2	0.04	4	-	-	-	-	0.035	6
Synactinomyxon	-	-	0.14	13	0.05	1	0.1	2	0.09	16
Triactinomyxon	0.66	25	0.35	46	0.05	1	0.05	1	0.33	73
All actinospores	1.05	39	0.89	85	0.46	10	0.2	4	0.71	138
Number of oligochaetes examined	3618		9549		2170		2145		17482	

Table 3.5 Seasonal infection data for actinospores released from oligochaetes at all sites during the study period. Where % = percentage of oligochaetes infected and n = number of oligochaetes infected.

Actinospore type	Spring		Summer		Autumn		Winter		Total	
	%	n	%	n	%	n	%	n	%	n
Aurantiactinomyxon raabeiiunioris sp. inq.	-	-	0.06	6	-	-	-	-	0.035	6
Aurantiactinomyxon type of McGeorge et al (1997)	0.03	1	-	-	-	-	-	-	0.006	1
Aurantiactinomyxon type 1	-	-	0.04	4	-	-	-	-	0.02	4
Echinactinomyxon radiatum sp. inq.	0.03	1	0.01	1	0.05	1	-	-	0.017	3
Echinactinomyxon type 1	-	-	0.03	3	0.15	3	-	-	0.035	6
Echinactinomyxon type 2	0.19	7	-	-	0.2	4	0.05	1	0.07	12
Echinactinomyxon type 3	-	-	0.02	2	-	-	-	-	0.01	2
Echinactinomyxon type 4	0.03	1	0.03	3	-	-	-	-	0.02	4
Echinactinomyxon type 5	0.03	1	0.01	1	-	-	-	-	0.01	2
Echinactinomyxon type 6	0.03	1	-	-	-	-	-	-	0.006	1
Guyenotia sphaerulosa sp. inq.	-	-	0.01	1	-	-	-	-	0.006	1
Neoactinomyxum type of Özer et al (2002)	-	-	0.01	1	-	-	-	-	0.006	1
Raabeia type 1	0.03	1	-	-	-	-	-	-	0.006	1
Raabeia type 2	0.03	1	0.04	4	-	-	-	-	0.029	5
Synactinomyxon longicauda sp. inq.	-	-	0.14	13	0.05	1	0.1	2	0.09	16
Triactinomyxon ignotum sp. inq.	0.08	3	0.23	22	-	-	0.05	1	0.15	26
Triactinomyxon stage of *M. pseudodispar*	0.44	16	0.21	20	0.05	1	-	-	0.21	37
Triactinomyxon stage of *M. macrocapsularis*	0.06	2	-	-	-	-	-	-	0.01	2
Triactinomyxon type 1	-	-	0.02	2	-	-	-	-	0.01	2
Triactinomyxon type 2	0.08	3	-	-	-	-	-	-	0.017	3
Triactinomyxon type 3	0.03	1	0.02	2	-	-	-	-	0.017	3
Number of oligochaetes examined	3618		9549		2170		2145		17482	

echinactinomyxons were each only recorded once. E. type 3 was released in the summer of 1997 at a prevalence of 0.28%, *E. radiatum* sp. inq. was recorded in autumn 1997 at a slightly lower prevalence and E. type 2 was released from one oligochaete during the winter of 1997-1998. Actinospores were only recorded once at the Nether Cerne site, during spring 1997 (Table 3.8). Subsequent sampling in 1998, 1999 and 2000 failed to demonstrate the presence of further actinospore types. All three actinospore types, A. type of McGeorge et al (1997), R. type 1 and *T. ignotum* sp. inq. were recorded at a prevalence of 0.83%.

Actinospores were only detected at the Wimborne site in summer 1996, 1997 and 1998 and during winter 1997-1998 (Table 3.9). Prevalence of infection ranged from 0.11% for E. type 1 and *T. ignotum* sp. inq. in summer 1997 to 0.68% for *T. ignotum* sp. inq. and T. type 1 in summer 1998. Whilst E. type 1 was released twice during the study, in summer 1997 and 1998, E. type 4 was only released in summer 1996. R. type 2 was recorded once in summer 1998 and *S. longicauda* sp. inq. was released from one oligochaete in winter 1997-1998.

Actinospores were recorded at the East Stoke site during every sampling period with the exception of winter 2001-2002, when no actinospores were recorded (Table 3.10). Aurantiactinomyxons were only released during summer 1997, 1998 and 2000 at prevalences ranging from 0.15 to 0.29%. The most common actinospore types were echinactinomyxons, these being recorded in the spring, summer and autumn at prevalences ranging from 0.29 to 1.17%. E. type 1 was released from 3 oligochaetes in autumn 1997 and E. type 2 was recorded twice, at a prevalence of 0.91% in autumn 1997 and 1.17% in spring 1998. E. type 4 was recorded once in summer 1998 at a prevalence of 0.29% and E. type 6 was released in summer 2002 at a prevalence of 0.71%. N. type of Özer et al. (2002) released from one oligochaete in summer 2000, whilst R. type 2 was released in spring and summer 1998 at a prevalence of between 0.167 and 0.88%. *S. longicauda* sp. inq. was released from three oligochaetes, one each in autumn and winter 1997-1998 and

Table 3.6 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the Blandford site between summer 1996 and spring 1997. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Summer 1996		Autumn 1996		Winter 1996-1997		Spring 1997		Total	
	%	n	%	n	%	n	%	n	%	n
Triactinomyxon ignotum sp. inq.	1.44	12	-	-	2.5	1	-	-	1.17	13
Triactinomyxon stage of *M. pseudodispar*	2.28	19	-	-	-	-	5.83	7	2.24	26
Number of oligochaetes examined	834		120		40		120		1114	

Table 3.7 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the Standlynch site between spring 1997 and autumn 1998. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Spring 1997		Summer 1997		Autumn 1997		Winter 1997-1998		Spring 1998		Summer 1998		Autumn 1998		Total	
	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
E. radiatum sp. inq.	-	-	-	-	0.23	1	-	-	-	-	-	-	-	-	0.04	1
Echinactinomyxon type 2	-	-	-	-	-	-	0.29	1	-	-	-	-	-	-	0.04	1
Echinactinomyxon type 3	-	-	0.28	2	-	-	-	-	-	-	-	-	-	-	0.08	2
Triactinomyxon stage of *M. pseudodispar*	1.11	4	-	-	0.23	1	-	-	-	-	-	-	-	-	0.21	5
Number of oligochaetes examined	360		720		440		350		140		210		120		2340	

Table 3.8 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the Nether Cerne site between spring 1997 and summer 2000. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Spring 1997		Summer 1997		Autumn 1997		Winter 1998		Spring 1998		Summer 1998		Spring 1999		Summer 2000		Total
	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Aurantiactinomyxon type of McGeorge et al (1997)	0.83	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02
Raabeia type 1	0.83	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02
T. ignotum sp. inq.	0.83	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02
Number of oligochaetes examined	120		1320		680		320		620		740		160		435		4395

Table 3.9 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the Wimborne site between summer 1996 and summer 1998. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Summer 1996		Summer 1997		Autumn 1997		Winter 1997-1998		Spring 1998		Summer 1998		Total	
	%	n	%	n	%	n	%	n	%	n	%	n	%	n
Echinactinomyxon type 1	-	-	0.11	1	-	-	-	-	-	-	0.34	1	0.1	2
Echinactinomyxon type 4	0.42	1	-	-	-	-	-	-	-	-	-	-	0.05	1
Raabeia type 2	-	-	-	-	-	-	-	-	-	-	0.34	1	0.05	1
Synactinomyxon longicauda sp. inq.	-	-	-	-	-	-	0.37	1	-	-	-	-	0.05	1
Triactinomyxon ignotum sp. inq.	-	-	0.11	1	-	-	-	-	-	-	0.68	2	0.16	3
Triactinomyxon type 1	-	-	-	-	-	-	-	-	-	-	0.68	2	0.1	2
Number of oligochaetes examined	240		840		120		270		140		295		1905	

summer 1998. The triactinomyxon stage of *M. pseudodispar* was the most common triactinomyxon at East Stoke and was recorded in spring 1998 and 1999 as well as spring 2000. T. type 2 and the triactinomyxon stage of *M. macrocapsularis* were only recorded during the spring of 1998 at prevalences of 0.5 and 0.33% respectively. *T. ignotum* sp. inq. was only recorded during spring and summer 2002.

The site at Canford School is characterised by actinospores that were released only during the spring and summer, with no actinospores being detected in any of the autumn and winter samplings (Table 3.11). *A. raabeiiunioris* sp. inq. was recorded twice during the survey, in summer 1996 at a prevalence of 0.42% and again in summer 1997 at a prevalence of 1.22%. Echinactinomyxons were only recorded in summer 1997, spring 1998, summer 2000 and spring 2002 at prevalences ranging from 0.35 to 0.63%. *G. sphaerulosa* sp. inq. was only recorded in summer 1998 in one oligochaete at a prevalence of 0.26%. *T. ignotum* sp. inq. was the most common triactinomyxon at Canford School, being found four times during the study, mainly in the summer at prevalences of between 0.42 and 0.83%. T. type 3 occurred in a two year cycle, being found only in 1998, 2000 and 2002. The triactinomyxon stage of *M. pseudodispar* was only found once, in the summer of 1997.

Table 3.10 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the East Stoke site between summer 1997 and summer 2002. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Summer 1997		Autumn 1997		Winter 1997-1998		Spring 1998		Summer 1998		Spring 1999		Spring 2000		Summer 2000		Winter 2001-2002		Spring 2002		Summer 2002		Total	
Aurantiactinomyxon type 1	0.28	2	-	-	-	-	-	-	0.29	1	-	-	-	-	0.15	1	-	-	-	-	-	-	0.09	4
Echinactinomyxon type 1	-	-	0.68	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.07	3
Echinactinomyxon type 2	-	-	0.91	4	-	-	1.17	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.26	11
Echinactinomyxon type 4	-	-	-	-	-	-	-	-	0.29	1	-	-	-	-	-	-	-	-	-	-	-	-	0.02	1
Echinactinomyxon type 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.71	1	-	-	0.02	1
Neoactinomyxum type of Özer et al (2002)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.15	1	-	-	-	-	-	-	0.02	1
Raabeia type 2	-	-	-	-	-	-	0.167	1	0.88	3	-	-	-	-	-	-	-	-	-	-	-	-	0.09	4
S. longicauda sp. inq.	-	-	0.23	1	0.25	1	-	-	0.29	1	-	-	-	-	-	-	-	-	-	-	-	-	0.07	3
T. ignotum sp. inq.	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	0.71	-	0.26	1	0.05	2
Triactinomyxon stage of *M. pseudodispar*	-	-	-	-	-	-	0.5	3	-	-	0.83	1	0.45	1	-	-	-	-	-	-	-	-	0.12	5
Triactinomyxon stage of *M. macrocapsularis*	-	-	-	-	-	-	0.33	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	2
Triactinomyxon type 2	-	-	-	-	-	-	0.5	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.07	3
Number of oligochaetes examined	720		440		400		600		340		120		220		690		165		141		385		4221	

Table 3.11 Table showing seasonality of release of actinospore types and numbers of oligochaetes examined at the Canford School site between summer 1996 and autumn 2002. Where % = percentage of oligochaetes releasing actinospores of a particular type in the sampling period and n = number of oligochaetes infected with a particular actinospore.

Actinospore type	Summer 1996		Autumn 1996		Winter 1996-1997		Spring 1997		Summer 1997		Autumn 1997		Winter 1997-1998		Spring 1998		Summer 1998		Spring 1999		Spring 2000		Summer 2000		Winter 2001-2002		Spring 2002		Summer 2002		Autumn 2002		Total	
	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
A. raabeiiunioris sp. inq.	0.42	1	-	-	-	-	-	-	1.22	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	6
E. radiatum sp. inq.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.63	1	-	-	-	-	-	-	0.45	1	-	-	-	-	-	-	-	-	0.06	2
Echinactinomyxon type 1	-	-	-	-	-	-	-	-	0.49	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	2
Echinactinomyxon type 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.35	1	-	-	-	-	0.03	1
Echinactinomyxon type 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.35	1	-	-	-	-	0.03	1
G. sphaerulosa sp. inq.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.26	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03	1
T. ignotum sp. inq.	0.42	1	-	-	-	-	0.83	1	0.73	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.54	1	-	-	0.2	6
Triactinomyxon stage of *M. pseudodispar*	-	-	-	-	-	-	-	-	0.24	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03	1
Triactinomyxon type 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.26	1	-	-	-	-	0.45	1	-	-	0.35	1	-	-	-	-	0.1	3
Number of oligochaetes examined	240		120		240		120		410		90		150		160		385		100		210		220		120		285		185		40		3075	

DISCUSSION

The current study has identified a total of 21 actinospore types, of which 12 appear to be new to science. It is likely, given the current state of knowledge, that each of these will possess a myxospore counterpart in a vertebrate host. Indeed, two triactinomyxons identified in the current study have been shown experimentally to be the alternate stage of *Myxobolus* spp. and is the first record of these two actinospore types in the UK (Székely et al., 1999, 2002). It is known that both these myxozoans occur in the UK (Kennedy, 1974) and that both *M. pseudodispar* and *M. macrocapsularis* occur in fish hosts at the sites where the actinospores have been recorded (Longshaw, unpublished observations). This study has demonstrated for the first time the types and spatio-temporal trends of the actinospore fauna of oligochaetes from natural riverine habitats as well as providing further information on actinospores associated with sites of salmonid aquaculture in the UK. The numbers of actinospore types found at each site was less than reported for actinospores released from a farm site in Scotland (Özer et al. 2002a, b) but may reflect the smaller number of oligochaetes collected and the limited number of sampling visits made to certain sites, rather than a real difference in the numbers of actinospores present in each system. It is expected that the types of actinospores present at each site would be greater than was actually recorded in the current study as not all oligochaete types were examined.

Many of the actinospores identified during the current study were easily placed within their respective collective groups as they clearly conformed to the generic descriptions of the type. However, some were less easy to place including both Raabeia types and Echinactinomyxon type 3. Both these forms exemplify the problems in the classification of certain actinospore types that has led to some authors either reclassifying actinospores or in subjectively classifying actinospores as belonging to a particular collective group. The morphological differences between Raabeia and Echinactinomyxon types, whilst originally reasonably clear, have become blurred with the addition of a

number of new types to each collective group, to the point where there are a number of actinospores that appear to be intermediate between the two collective groups. Both Raabeia types 1 and 2 in the current study were characterised by possessing curved caudal processes which is one of the definitive characters of the collective group. However, Oumouna et al. (2003) described an Echinactinomyxon type which is similar in general morphology to the current Raabeia type 1. Whilst we have classified the current Raabeia type 1 as a Raabeia, it could also justifiably be placed within the echinactinomyxons in order to conform to the description provided by Oumouna et al. (2003). Raabeia type 2 in the current study was placed within the collective group Raabeia as most spores contained curved caudal processes. However, the presence of cylindrical polar capsules and the relative shortness of the caudal process would suggest that the current Raabeia type 2 should be classified within a new collective group. However, as this is currently the only one of its type reported in the literature, we are reluctant to erect a new collective group until further examples are described. Echinactinomyxon type 3 in the current study is another example where there is difficulty in placing certain actinospores within a defined collective group. Xiao and Desser (1998a) described a similar actinospore in oligochaetes collected in Canada that they placed within the collective group Triactinomyxon. Whilst E. type 3 in the current study has an elongated spore body, the fact that the sporoplasm completely fills the spore body has led us to classify the actinospore as an Echinactinomyxon. The morphological criteria used to define each collective group will need to be addressed in the near future There is a need, when describing either Raabeia or Echinactinomyxon types, to compare the new forms to representatives of both groups.

Xiao and Desser (1998b) considered that *A. trifolium* sp. inq. Marques 1984 and the Aurantiactinomyxon stage of *Hoferellus carassii* reported by Troullier et al. (1996) should be transferred to the collective group Guyenotia as the caudal processes are finger-like and possess rounded tips. Whilst this is a valid point since the original genus description stated that Aurantiactinomyxon types possess pointed tips, we disagree with this proposal for the

following reasons. *Guyenotia sphaerulosa* sp. inq. of Naville (1930) was originally reported as possessing rounded tips and discriminated from other genera of actinospores on the basis that the caudal processes pointed sharply downwards away from the spore body. However, the schematic diagram of *Guyenotia sphaerulosa* sp. inq. clearly shows that the tips are not as rounded as those reported for some aurantiactinomyxons, which tend to be blunt rounded, rather than pointed and rounded as in Guyenotia. The number of aurantiactinomyxons reported subsequently to Naville's report and the erection of the genus Aurantiactinomyxon by Janiszewska (1957) has risen sharply. Of those aurantiactinomyxons reported with rounded tips to the caudal processes, the main feature distinguishing them from Guyenotia is that the caudal processes only point slightly downwards and when viewed apically have a "propeller" type appearance. Most, if not all of the aurantiactinomyxons reported with rounded ends have been described from the apical view.

Whilst less problematic, the collective group Triactinomyxon contains some anomalies. For example Triactinomyxon type 7 of Lowers and Bartholomew (2003) should be classified as Pseudotriactinomyxon type 1 of Lowers and Bartholomew (2003) following the erection of the new collective group Pseudotriactinomyxon by Hallett et al. (2003). Granata (1922a) described *Triactinomyxon magnum* sp. inq. but failed to provide any drawings. It was stated that *T. magnum* sp. inq. had "a short median axis" which was "often only a little longer than the germinal mass, which measures 25 to 30µm long by 10 to 12µm wide". It is apparent that based on the description, *T. magnum* sp. inq. does not possess a clearly defined style as expected in the collective group Triactinomyxon and that the size of the spore body is closer to that expected of Raabeia and Echinactinomyxon types than Triactinomyxon types. However, since Granata (1922a) failed to describe the caudal processes (other than to state that they were as long as 500µm), we are unable to assign the actinospore to either of the collective groups Raabeia or Echinactinomyxon. Similarly, both Triactinomyxon major and T. minor of Styer et al. (1992) were reported as

not possessing a style. Again, as no drawings were provided by those authors, both forms cannot be reclassified into either the collective groups Raabeia or Echinactinomyxon where they clearly belong. Dresscher and Gispen-van der Weg (1958) reported the presence of a number of actinospores in water samples collected in the Amsterdam area. They considered that one of the forms found should be classified as *T. dubium* sp. inq. since it contained "32 amoeboid cells" within the style. However, the form reported by them should be transferred to the collective group Siedleckiella as individual spores are joined together in groups of eight by the tips of the caudal processes and are morphologically identical with other representatives of the collective group.

As well as experimental demonstration of the role of actinospores in myxozoan lifecycles, previous studies of actinospores have examined the actinospore fauna of oligochaetes associated with natural and farmed environments. In those studies of actinospores in fish farming situations, most authors have reported that the actinospores show strong host specificity to the oligochaete host and invariably, *Tubifex tubifex*, *Lumbriculus variegatus* and *Limnodrilus hoffmeisteri* have been implicated as the host of choice in these environments (McGeorge et al. 1997, El-Mansy et al. 1998a, Özer and Wootten 2000, Özer et al. 2002a, b, Oumouna et al. 2003). In contrast, studies of actinospores in oligochaetes collected in "wild" environments can show weaker host specificity and a wider variety of oligochaete types involved as hosts for actinospores (Székely et al. 2003, Xiao and Desser 1998a, Koprivnikar and Desser 2002). During the current study *Tubifex* cf. *tubifex* was implicated as hosts for 9 of the actinospores, of which 6 appeared to be specific to *T.* cf. *tubifex*. However, 5 actinospores, namely *A. raabeiiunioris* sp. inq., E. type 2, R. type 2, *T. ignotum* sp. inq. and the triactinomyxon stage of *M. pseudodispar* were isolated from at least two species of oligochaete. It is possible that the reason for the apparent lack of host specificity for these five actinospore types may reflect the greater availability of different potential oligochaete hosts in "wild" situations compared with fish farms which are characterised as possessing fewer

oligochaete species. In relatively pristine sites such as those visited during the current study, a greater diversity of oligochaete hosts would be anticipated.

The low prevalences agree with those found by other workers including Yokoyama (1993a, b), McGeorge et al. (1997), El-Mansy et al. (1998b), Xiao and Desser (1998c), Negredo and Mulcahy (2001), Székely et al. (2002), Özer et al. (2002b), Oumouna et al. (2003) and Székely et al. (2003). This is in part due to the method of examination where only those oligochaetes which released actinospore stages were considered to be positive. Additionally the oligochaetes collected at each site were only maintained for a maximum of two months. Oligochaetes were usually only sampled from the lateral margins of the river, thus excluding hosts from the central portion of the river. It is possible that oligochaetes in these areas may have different prevalences of infections. The low prevalences contrast markedly with those presented by El-Mansy et al. (1998a), who recorded prevalences in excess of 90% for Raabeia types in *Branchiura sowerbyi* collected from a fish farm in Hungary. The high prevalences recorded during that study may well be due to the relatively limited flushing of water and therefore myxospores from the still water system. In the fast flowing rivers sampled during the current study, the probability of oligochaetes coming into contact with myxospores will be reduced, thus reducing the overall prevalences. Indeed, when El-Mansy et al. (1998b) examined oligochaetes from Lake Balaton, levels of infection were much lower, the maximum levels being only around 45% for Triactinomyxon species, suggesting that whilst relatively still waters were beneficial for myxospore transfer, the greater volume of water reduced the risk of oligochaetes coming into contact with myxospores when compared with the pond culture system.

Differences in prevalence levels of the various actinospores collected at different sites may also be due to differences in the biotic and abiotic characteristics of the sites examined. Since each site was selected for these different factors, and that these factors will influence biological processes such as oligochaete and fish host distributions it follows

that the types of actinospores found and levels of infection will vary. Ultimately, actinospore diversity at the different sites reflects both the habitat characteristics and fish host diversity. It would be expected that sites with slow moving water, suitable substrate for oligochaete survival and high fish and myxospore diversity would contain greater diversity of actinospores. The East Stoke site, where actinospore diversity was highest, is a slow, meandering river with organically rich sediment composed of a mixture of river sand and fine, silty mud and during the summer, large amounts of *Ranunculus*. The river also supports a wide range of fish hosts including salmonids, perch, sticklebacks, bullheads, lampreys and cyprinids such as dace, chub, roach and minnow. The combination of *Ranunculus* and the reduced water flow are ideal conditions for the deposition of detritus and sediment which provide a suitable habitat for oligochaetes. Coupled with the presence of many fish hosts to provide myxospores, the large number of actinospore types would be expected. In contrast, the Nether Cerne site is shallow, being only about four miles below the source of the river, contains limited numbers of macrophytes and a reduced fish fauna comprising salmonids, sticklebacks, eels and some cyprinids. As a result, it would be expected that fewer actinospores would be isolated from this ecologically depauperate site. The three sites on the River Stour, Blandford, Wimborne and Canford School, provide an interesting insight into different factors determining actinospore diversity. The section of river sampled at Blandford was a small side stream off the main river with an input of municipal runoff from a nearby car park. The stream bed was composed mainly of thick, somewhat anoxic mud and contained no macrophytes. Whilst the site was only visited in four sampling seasons, it is apparent that the numbers of actinospores was greatly reduced compared with all other sites. Indeed, the only oligochaetes isolated with actinospore infections at that site were *T.* cf. *tubifex* and *L. hoffmeisteri*, both of which tend to be pollution tolerant. The impact of municipal runoff has been to reduce the diversity of fish species and possibly oligochaetes in this small section of river with a concomitant decrease in the types and diversity of actinospores

isolated. Ten miles downstream of Blandford is the Wimborne site which is far less impacted by anthropogenic inputs. This site is fast flowing, shallow with a river bed composed mainly of river sand overlaid with a layer of shale. In the central portion of the river, large beds of *Ranunculus* are present, whilst in the lateral margins there are reed beds and silt. The maximum depth of the river during the summer months is around 1 metre. Whilst only six actinospore types were isolated, they belonged to four collective groups. The site has a similar fish fauna to the East Stoke site to include cyprinids and pike and with the exception of T. type 1 has an identical actinospore fauna. The similarity in actinospore types would suggest that similar fish hosts are found at both sites even though they are on two different rivers. The Canford School site is three miles downstream of the Wimborne site. The section of river sampled has been canalised and as a result is much slower flowing. The mean depth in the middle of the river is approximately 4 metres. There is a noticeable absence of macrophytes in the river and the sediment is composed of a combination of leaf litter, mud and river sand. The different environmental conditions at Canford School and Wimborne are reflected in the actinospore types at both sites. These sites only share three actinospores in common, namely E. type 1, E. type 4 and *T. ignotum* sp. inq. Once again, these differences reflect the different biotypes at each site. In common with all other sites, Standlynch contained the triactinomyxon stage of *M. pseudodispar* which would be expected since cyprinids at this site are infected with *M. pseudodispar* (Longshaw, unpublished observations). The site is approximately 1 mile upstream of a salmonid fish farm, is at least 2m deep, contains limited macrophytes and is fast flowing. The only other actinospore types to be found at this site were echinactinomyxons, including E. type 3 which was not recorded at any other site. Although the site was infrequently sampled, it nevertheless demonstrates that the actinospore fauna can reflect both the environment and the fish hosts within a site.

Whilst the actinospore faunas at the different sites may reflect the myxospore component of the ecosystem, the physical characteristics of each river appear to determine

the types of actinospores found. For example, aurantiactinomyxons with short caudal processes are only found in the slower moving rivers such as Nether Cerne, Canford School and East Stoke and are absent from Wimborne, Standlynch and Blandford which are much faster flowing. Whilst this may be an artefactual effect due to the presence or absence of the relevant fish hosts possessing the myxospore equivalent, it is interesting to speculate that actinospore morphology may also be determined by the water flow characteristics in which myxozoans and hosts have evolved. Given the plethora of short caudal process forms such as aurantiactinomyxons and neoactinomyxums reported by El-Mansy et al. (1998a, b) in oligochaetes from a fish farm pond and from Lake Balaton, and the number of myxozoans from fish traditionally associated with still or slow moving water that possess an aurantiactinomyxon or neoactinomyxum alternate stage such as *Henneguya ictaluri*, *Hoferellus carassii*, *Hoferellus cyprini*, *Sphaerospora renicola*, *Thelohanellus hovorkai* and *T. nikolskii*, there is some evidence that these forms are associated with slower moving water. However, it is recognised that long caudal process forms, such as raabeias, triactinomyxons and echinactinomyxons are also found in slower moving water. However, if these short form actinospores have a predilection for slow water, it would explain why there appears to be limited conformity between myxospore genera and their actinospore counterparts as has been demonstrated in several myxozoan lifecycles (Kent et al., 2001). The general morphology of actinospores may be a function of the fish host in the myxospore stage. Actinospores with small or short caudal processes would be expected to remain in close proximity to the point of release and may therefore infect fish in close association to the substrate or those found in areas of slower moving water such as the lateral margins. Conversely, those actinospores with longer caudal processes would be expected to float higher in the water column or be dispersed more widely in the environment.

It is now established that seasonality occurs in myxozoan infections in both oligochaete and fish hosts (Kovacs-Gayer & Molnar 1983, Narasimhamurti and Kalavati

1984, El-Mansy et al. 1998a, b, Özer et al. 2002b, Oumouna et al. 2003). In agreement with other authors, during the current study most actinospores were released during spring and summer, at a time when water temperatures are elevated. At the collective group level, clear patterns of release of actinospores emerge. Aurantiactinomyxons, Guyenotia, Neoactinomyxum and Raabeia types were only released in spring and/or summer, whilst echinactinomyxons and triactinomyxons were released throughout the year, albeit at a much reduced prevalence during autumn and winter. The timing of release of actinospores is most likely to be intricately linked with the biology of both oligochaete and fish host as well as a direct effect of temperature on actinospore development. The release of actinospores in spring would tie in with the presence of large spawning shoals of adult fish thus maximising the probability of contact with a suitable fish host. Recently hatched, naïve fish hosts tend to congregate in large shoals in the lateral margins of rivers during late spring and summer in UK rivers (Pinder 2001, Cowx 2001). During this critical period of development they are susceptible to infections by a number of parasites, including myxozoans (Longshaw, unpublished). The release of actinospores during the summer months, when these large shoals are present would once again maximise the probability of infection by actinospore stages.

The current study has provided baseline data on the types, distribution and seasonality of release of actinospores in river systems in southern England in order to better understand the complex relationships between different myxozoan hosts and the environment. It is expected that most, if not all actinospores identified in the current study will possess a myxospore counterpart in vertebrate hosts. The data collected will assist in the targeting of candidate actinospores for lifecycle studies based on ecology and seasonality of myxozoans in both hosts. Future work should incorporate DNA sequence analysis in order to match stages and in the discrimination of actinospore types.

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CHAPTER FOUR

DESCRIPTIONS, DEVELOPMENT AND PATHOGENICITY OF MYXOZOAN (MYXOZOA: MYXOSPOREA) PARASITES OF JUVENILE CYPRINIDS (PISCES: CYPRINIDAE).

To be submitted to *Journal of Fish Diseases* as Longshaw, M., Frear, P. and Feist, S.W. "Descriptions, development and pathogenicity of myxozoan (Myxozoa: Myxosporea) parasites of juvenile cyprinids (Pisces: Cyprinidae)".

Abstract

A total of 4946 juvenile cyprinids, comprising 1001 roach, 1258 chub, 501 dace, 1825 minnow, 28 bleak, 51 bream and 228 gudgeon caught in the north of England were examined histologically for the presence of myxozoan infections. In addition, a further 250 freshly killed hosts were examined for the presence of myxozoans to allow, where possible, identification of the myxozoans found. Thirteen myxozoan species were identified in the genera *Myxobolus*, *Myxidium* and *Sphaerospora* in the skeletal musculature, gills, kidney, gall bladder and stomach epithelium. In addition, two types of extrasporogonic stages and one plasmodial stage were identified in these hosts and several *Myxobolus* spp. were noted but not identified to species due to the very low prevalences. The most common myxozoans were *M. pseudodispar* and *M. pfeifferi* infecting the muscles of roach, chub and dace. Roach contained the most number of myxozoan species.

The pathological responses of the hosts to parasite infections were variable, with chub appearing to respond most vigorously to sporogonic parasite stages. Dace on the other hand had fewer myxozoan parasites and were characterised by having a limited host response to myxospore sporogonic stages. Data is provided on the histopathological responses of each host to myxozoans, the host and geographical range of each type identified and representative images, line drawings and measurements are provided for all myxozoans identified.

Introduction

Over 1350 myxozoans have been described, mainly infecting teleost fishes (Kent, Andree, Bartholomew, El-Matbouli, Desser, Devlin, Feist, Hedrick, Hoffman, Khattra, Hallett, Lester, Longshaw, Palenzuela, Siddall, and Xiao 2001). Additionally there have been a few reports of myxozoans in reptiles (Jayasri and Hoffman 1982), amphibians (Mutschmann, 1999), elasmobranchs (Heupel and Bennett 1996; Stoffregen and Anderson 1990), cephalopods (Yokoyama and Masuda 2001), Digenea (Overstreet 1976), crustaceans (Korczynski 1988), birds (Lowenstine *et al.* 2002) and mammals (Friedrich, Ingolic, Freitag, Kastberger, Hohmann, Skofitsch, Neumeister, and Kepka 2000). Whilst most myxozoans are considered relatively innocuous to their fish host, some species are recognised as disease agents in both wild and cultured fish (Bucke and Andrews 1985; Chen, Kou, Wu, Wang, and Su 2001; Hedrick, El-Matbouli, Adkison, and MacConnell 1998; Moran, Whitaker, and Kent 1999). Whirling disease, caused by *Myxobolus cerebralis,* has been implicated in declines of wild salmonids in the USA (Hedrick *et al.*, 1998) and PKD in both wild and farmed salmonids is a contributory factor to mortality (Feist, Peeler, Gardiner, Smith, and Longshaw 2002). Parasites may act synergistically in a multiplicative or additive manner leading to a number of sublethal and lethal effects in their hosts. These effects can include alterations in blood parameters, physiological compromise, reduced growth, reduced swimming speeds, reduced assimilation rates, increased predation risks and behavioural alterations (Clifton-Hadley, Richards, and Bucke 1987; Barber, Hoare and Krause 2000; Hedrick, McDowell, Mukkatira, Georgiadis, and MacConnell 2001; Moles and Heifetz 1998). Additionally, parasites can cause pathological changes and directly lead to mortality.

Over 60 species of myxozoans parasitizing cyprinids have been described, including members of the genera *Myxobolus*, *Myxidium*, *Myxobilatus*, *Hoferellus* and *Sphaerospora*. These reports have often consisted of descriptions of the parasites and less frequently, the pathological effects of the parasite on the fish host. Few studies have

examined the distribution, development and pathogenicity of myxospores in juvenile and/or wild fish. Approximately 100 species of myxozoans have been described from cyprinids worldwide, although it is likely that many of these will turn out to be synonyms as more myxozoan sequences become available and experimental lifecycle studies are completed.

Myxozoan infections have been noted in general parasite surveys in freshwater fish in the United Kingdom (Andrews 1979; Campbell 1974; Chubb 1963; Lee 1977; Mishra and Chubb 1965). However, there have been few reports specifically concerning myxozoans in British fish (Athanassopoulou and Sommerville, 1993b; Bucke and Andrews, 1985; Bucke, Hudson, and McGregor 1982; Copeland 1982; Crawshaw and Sweeting 1986; Davies 1968; Elson 1969; Lom, Pike & Dyková, 1991, McGeorge et al., 1994, 1996; Nicholas and Jones 1959; Özer and Wootten, 2000; Poynton and Bennett, 1985; Quadri 1962; Walliker 1966). Additionally, there are numerous reports of PKD caused by the malacosporean *Tetracapsuloides bryosalmonae* (Feist *et al.*, 2002) in salmonid hosts. Other than the report by Williams (1964) on a mass mortality of roach caused by the myxozoan *Thelohanellus* sp., there have been few records of mortality directly associated with myxozoans in UK waters. However, a note of caution should be added to the report of Williams (1964) since it was not clearly established that *Thelohanellus* was responsible for the observed mortality as other pathogenic parasites such as *Trichodina* sp., *Ichthyobodo* sp. and *Gyrodactylus* spp. were also present at high levels in these fish.

In order to better understand the pathogenicity and pathogenic potential of any disease agent, perhaps the most useful point to examine the hosts is within the first few months of life. It is during this period that immunologically naive fish are exposed to disease agents and is potentially the life stage during which most mortalities occur in a population (Cowx 2001). Examination of adult fish will demonstrate the parasite fauna of

fish that have survived and thus may underestimate the pathogenicity, overall distribution and types of myxozoan infections in any given population.

As part of a large scale project examining factors affecting recruitment in juvenile cyprinids, histopathological studies have been conducted on myxozoan parasites in 0+ fish in selected rivers in Yorkshire, Humberside and Cleveland in northeast England. This paper describes the myxozoans found, their development in the fish hosts and the pathology that they cause and considers the impact that myxozoans have on the survival of individual fish.

Materials & Methods

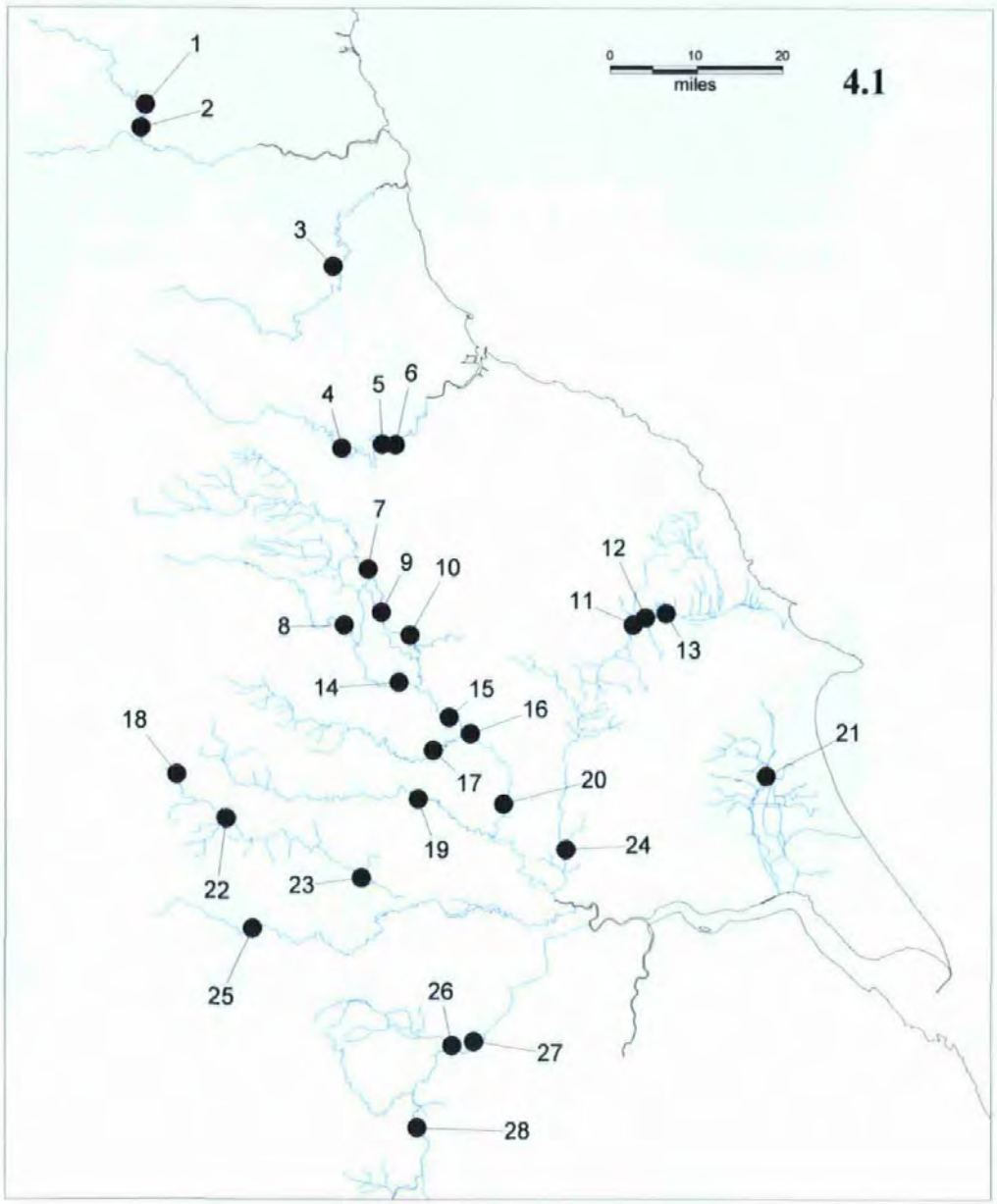
Samples

Young of the year (0+) cyprinid fish, comprising roach (*Rutilus rutilus*), chub (*Leuciscus cephalus*), dace (*Leuciscus leuciscus*), minnow (*Phoxinus phoxinus*), bream (*Abramis brama*), bleak (*Alburnus alburnus*), barbel (*Barbus barbus*) and gudgeon (*Gobio gobio*) were collected semi-quantitatively at selected sites in Yorkshire, Humberside and Cleveland between 1993 and 1999 (see Figure 4.1 for sites). Fish were caught using three sweeps with a 20m × 2m micromesh seine net set along the river margin at each site or caught by hand netting. All fish caught were killed by over anaesthetising in MS222 or Benzocaine and immediately transferred to 10% neutral buffered formalin (NBF).

Light microscopy

On return to the laboratory, fish were identified to species, fork length was taken and the fish stored in NBF by site and species. For histological studies a sub-sample of fry of each species from each site for each year were taken. A maximum of 30 fish of each species per site were examined. Tissues were softened in 10% formic acid for up to four days, transferred to 70% IMS prior and processed to wax blocks on an automatic vacuum

Figure 4.1. Map of sites sampled for fish examined during the current study between 1993 and 1999 where 1 = Tyne, Haughton. 2 = Tyne, Station House. 3 = Wear, Durham. 4 = Tees, Croft. 5 = Tees, Low Moor. 6 = Tees, Low Worsall. 7 = Swale, Maunby Demesne. 8 = Ure, Bellflask, 9 = Swale, Skipton, 10 = Swale, Thornton Bridge, 11 = Derwent, Fish Haven Low Marishes, 12 = Derwent, Yedingham Fish Haven, 13 = Derwent, Yedingham, 14 = Ure, Boroughbridge, 15 = Ouse, upstream of Linton Weir, 16 = Ouse, Beningbrough, 17 = Nidd, Kirk Hammerton, 18 = Aire, Carleton Bridge, 19 = Wharfe, Boston Spa, 20 = Ouse, Acaster, 21 = Hull, Hempholme, 22 = Aire, Keighley, 23 = Aire, Knostrop, 24 = Derwent, Bubwith, 25 = Calder, Elland, 26 = Dearne, Pastures Bridge, 27 = Don, Sprotborough, 28 = Rother, Woodhouse.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infiltration tissue processor. Longitudinal sections were cut at 3-5 μm and stained routinely with haematoxylin & eosin (H&E) or Giemsa. The numbers of cysts and/or location within an infected organ along with any pathological changes were noted. Myxozoan infections in the gill were characterised according to the criteria proposed by Molnár (2002).

Fresh spores were isolated from samples of fish collected in Yorkshire during June 2002 and measurements, photographs and drawings were made from these samples. Representative images of spores and pathologies were captured, and spores were measured using a Nikon Eclipse E800 microscope with LUCIA™ screen measurement system. Myxozoans were described and measured according to the criteria of Lom and Arthur (1989). Representative spores were air-dried on slides, acetic-methanol fixed, and stained with May-Grünwald-Giemsa. Stained spores and sectioned material for myxospores from each fish host were deposited in the Natural History Museum, London and in the Registry of Aquatic Pathology (RAP), CEFAS Weymouth.

Scanning Electron Microscopy

Spores isolated from dissected fish were fixed in 2.5% gluteraldehyde in sodium cacodylate buffer, pH 7.4, for a minimum of 24 hours then filtered through a 0.1μm ceramic filter; post fixed in 1% osmium tetroxide in sodium cacodylate buffer, pH 7.4, for 1 hour and dehydrated through an acetone series. Filters were critical point dried and sputter coated with 4nm of gold and examined in a JEOL 5200 SEM at 25kV.

Results

A total of 4946 fish were examined histologically comprising 1001 roach (*Rutilus rutilus*), 1258 chub (*Leuciscus cephalus*), 501 dace (*Leuciscus leuciscus*), 1825 minnow (*Phoxinus phoxinus*), 28 bleak (*Alburnus alburnus*), 51 bream (*Abramis brama*) and 228 gudgeon (*Gobio gobio*). In addition, 250 freshly killed fish were examined for the

presence of myxozoan infection to assist, where possible in the identification and descriptions of myxozoans observed in histological sections. Drawings of myxozoans and representative images of pathologies are shown in Figures 4.2-4.57.

A total of 13 identifiable myxozoan species belonging to three genera were found in the fish. This included *Myxobolus pseudodispar, M. musculi* and *Myxobolus pfeifferi* in the skeletal musculature, *Myxobolus oviformis, Myxobolus macrocapsularis, Myxobolus bramae* and *Myxobolus muelleri* in the gills, *Myxobolus* sp. in the head cartilage, *Sphaerospora ousei* n. sp. and *Sphaerospora leuciscusi* n. sp. in kidney tubules, *Myxidium rhodei* and *Myxidium barbatulae* in the kidneys, *Myxidium pfeifferi* in the gall bladder and *Myxobolus* cf. *cycloides* in the stomach epithelium and liver. Additionally, two types of extrasporogonic stages were found in the kidney tubule epithelium and in the gall bladder and plasmodial stages in the kidney tubule lumens. Some *Myxobolus* spp. infections that were detected in the gills were not identified to species. The most common parasites were *Myxobolus pseudodispar* and *Myxobolus pfeifferi* infecting the skeletal musculature of roach, chub and dace.

Roach contained the most myxozoan species, being parasitised by *Myxobolus pseudodispar* and *Myxobolus pfeifferi* in the muscle, *Myxobolus* sp. in gill cartilage, *Myxobolus* sp. in head cartilage, *Sphaerospora ousei*, *Myxidium rhodei*, *Myxidium pfeifferi*, *Myxobolus* cf. *cycloides* and the three different myxozoan developmental stages. Bleak were parasitised by *Myxobolus pseudodispar, Myxobolus* cf. *cycloides*, *Myxobolus* sp. in the gills, extrasporogonic type 2 and plasmodia type 1. Bream were parasitised by *Myxobolus pseudodispar, Sphaerospora* sp., *Myxidium rhodei*, extrasporogonic type 1 and plasmodia type 1. Chub were infected by *Myxobolus pseudodispar* and *Myxobolus pfeifferi* in the muscle, *Myxobolus* spp. in the gills, *M. macrocapsularis*, *Myxidium rhodei*, extrasporogonic type 1 and plasmodia type 1. Dace were parasitised by *Myxobolus pseudodispar, Myxobolus pfeifferi* in muscle, *Myxobolus* spp., *M. macrocapsularis, M. oviformis* and *M. muelleri* in the gills and *Myxobolus* sp. in the head cartilage,

Sphaerospora leuciscusi n. sp., *Myxidium rhodei*, *Myxidium pfeifferi* and all three myxozoan developmental stages. Gudgeon were infected with *Myxobolus musculi* in the muscle, *Myxobolus oviformis* in the gills and plasmodia type 1. Minnow were parasitised by *Myxobolus musculi*, *Myxobolus bramae* and *Myxobolus* spp. in the gills, extrasporogonic type 1 and plasmodia type 1.

All measurements are in µm unless otherwise stated. Values for numbers of cysts in muscle are based on the numbers of cysts seen in one histological section.

Myxobolus pseudodispar Gorbunova, 1936 Figures 4.2-4.10

Site of infection: Skeletal musculature

Infection data and affected sites:

Abramis brama - 13 infected, range of 1-10 cysts per section (mean 2.23). Dearne (Pastures Bridge); Hull (Hempholme); Ure (Boroughbridge).

Alburnus alburnus- 4 infected, range of 1-4 cysts per section (mean 2).

Ouse (Acaster Malbis, Beningbrough).

Leuciscus cephalus - 786 infected, range of 1-195 cysts per section (mean 9.1).

Aire (Keighley, Carleton Bridge, Knostrop); Dearne (Pastures Bridge); Derwent (Bubwith, Yedingham Fish Havens); Don (Sprotborough); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/s Linton Weir); Rother (Woodhouse); Swale (Maunby Demesne, Skipton, Thornton Bridge); Tees (Croft, Low Moor, Low Worsall); Ure (Bellflask, Boroughbridge); Wear (Durham); Wharfe (Boston Spa).

Leuciscus leuciscus - 60 infected, range of 1-26 cysts per section (mean 3.15).

Aire (Knostrop); Derwent (Bubwith, Yedingham, Yedingham Fish Havens); Ouse (Acaster Malbis, Beningbrough, U/s Linton Weir); Swale (Maunby Demesne, Thornton Bridge); Tees (Croft, Low Moor); Ure (Boroughbridge); Wharfe (Boston Spa).

Rutilus rutilus – 292 infected, range of 1-23 cysts per section (mean 2.68).

Figure 4.2. Fresh *Myxobolus pseudodispar* spores isolated from the musculature of *Leuciscus cephalus*. Scale bar = 10µm.

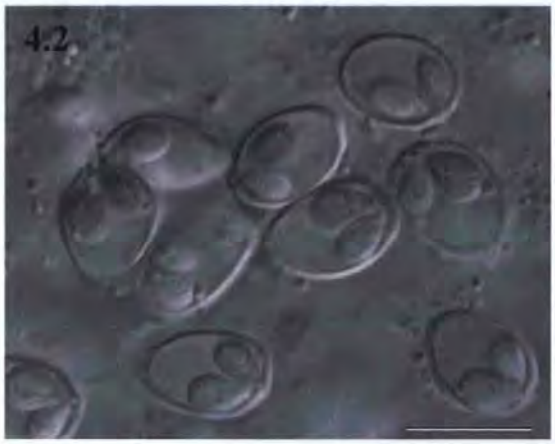
Figure 4.3. Histological section through *Myxobolus pseudodispar* cyst in the skeletal musculature of *Leuciscus cephalus*. Note lack of host response to developing plasmodia. Giemsa, scale bar = 50µm

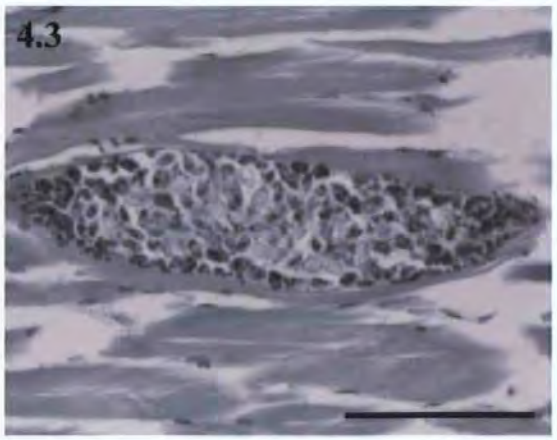
Figure 4.4. Line drawings of *Myxobolus pseudodispar* spores from *Leuciscus cephalus* showing wide morphological variation. Scale bar = 10µm

Figure 4.5. Fresh *Myxobolus pseudodispar* spores isolated from the musculature of *Leuciscus leuciscus*. Scale bar = 10µm.

Figure 4.6. Histological section through *Myxobolus pseudodispar* plasmodia in the musculature of *Leuciscus leuciscus*. More mature spores are noted towards the centre of the cyst compared with the periphery of the plasmodia. H&E. Scale bar = 50µm

Figure 4.7. Line drawings of *Myxobolus pseudodispar* spores from *Leuciscus leuciscus*. Note the reduced amount of morphological variation typified by spores from *Leuciscus cephalus*. Scale bar = 10µm





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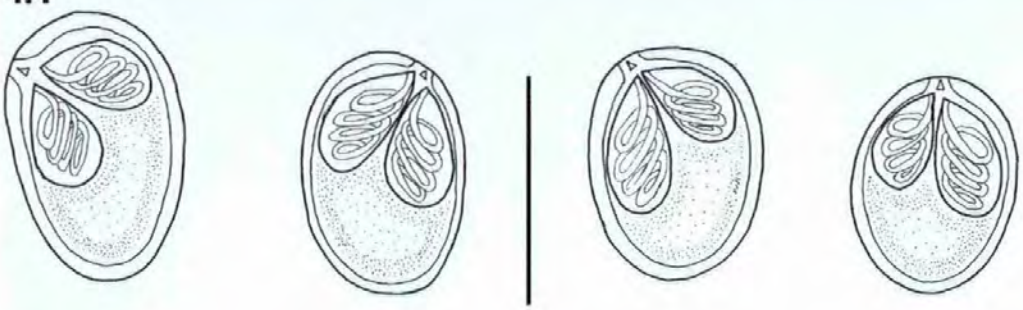

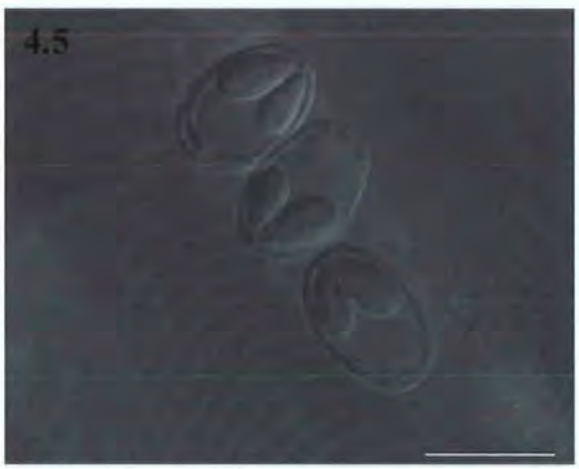

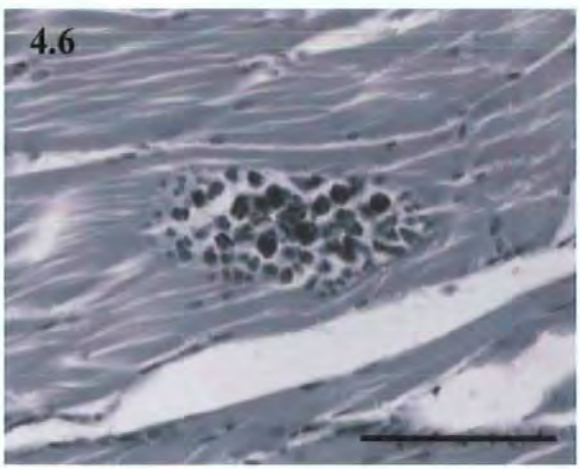

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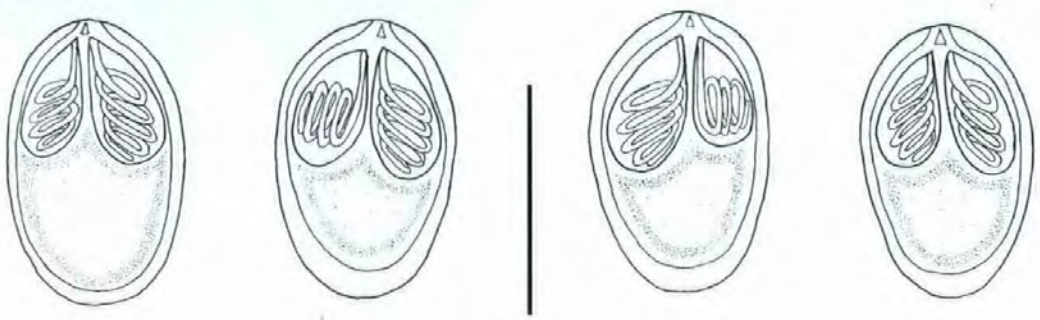


Aire (Keighley); Calder (Elland); Dearne (Pastures Bridge); Derwent (Bubwith, Fish Haven Low Marishes, Yedingham, Yedingham Fish Havens); Don (Sprotborough); Hull (Hempholme); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/s Linton Weir); Rother (Woodhouse); Swale (Thornton Bridge); Ure (Boroughbridge); Wharfe (Boston Spa).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa and May-Grünwald-Giemsa stained smears of isolated spores from roach, chub and dace have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Rutilus rutilus* RAP 2-F30; *Leuciscus leuciscus* RAP 2P-1; *Leuciscus cephalus* RAP 2-K3).

Description: Spores typical of the genus *Myxobolus.* Plasmodium polysporous. Mature spores both within and between cysts highly pleomorphic. Mature spores ellipsoid to elongate ellipsoid, wider at anterior end. Polar capsules apical, sub-apical or lateral, pyriform, most of unequal size, though some of equal size. Loosely coiled polar filament with 4-5 turns. No intercapsular process. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 specimens, as length × width range with means ± SD in parentheses: Spores from *Leuciscus cephalus* 8.38 - 11.48 (9.61 ± 0.83) × 6.99 - 8.37 (7.65 ± 0.34); Polar capsules of unequal size, large polar capsule 4.33 - 5.94 (5.01 ± 0.43) × 2.57 - 3.47 (2.95 ± 0.24), small polar capsule 2.91 - 4.84 (4.02 ± 0.46) × 1.77 - 2.93 (2.47 ± 0.28). Spores from *Rutilus rutilus* 9.54 - 12.13 (11.08 ± 0.61) × 7.02 - 8.59 (7.93 ± 0.38); Polar capsules of unequal size, large polar capsule 4.26 - 5.74 (5.10 ± 0.32) × 2.49 - 3.59 (3.03 ± 0.26); small polar capsule 3.43 - 5.14 (4.41 ± 0.39) × 2.20 - 3.08 (2.63 ± 0.19). Spores from *Leuciscus leuciscus* 11.17 – 12.73 (11.85 ± 0.45) × 6.91 – 8.09 (7.53 ± 0.35); polar capsules of unequal size, large polar capsule 5.25 - 7.09 (5.97 ± 0.4) × 2.37 – 3.59 (2.97 ± 0.38); small polar capsule 4 – 5.49 (4.78 ± 0.42) × 1.34 – 3.11 (2.43 ± 0.27).

Myxobolus musculi Keysselitz, 1908 Figures 4.11-4.16

Site of infection: Skeletal musculature

Infection data and affected sites:

Phoxinus phoxinus – 636 infected, range of 1-71 cysts per section (mean 4.79).

Aire (Keighley, Carleton Bridge, Knostrop); Calder (Elland); Dearne (Pastures Bridge); Derwent (Bubwith, Fish Haven Low Marishes, Yedingham, Yedingham Fish Havens); Don (Sprotborough); Nidd (Kirk Hammerton); Ouse (Beningbrough, U/S Linton Weir); Swale (Maunby Demesne, Skipton, Thornton Bridge); Tees (Croft, Low Moor, Low Worsall); Tyne (Haughton, Station House); Ure (Bellflask, Boroughbridge); Wear (Durham); Wharfe (Boston Spa).

Gobio gobio - 42 infected, range of 1-13 cysts per section (mean 2.21).

Aire (Carleton Bridge); Derwent (Bubwith, Fish Haven Low Marishes, Yedingham Fish Havens); Hull (Hempholme); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/S Linton Weir); Rother (Wilfholme); Tees (Croft); Ure (Boroughbridge).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa and May-Grünwald-Giemsa stained smears of isolated spores have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Phoxinus phoxinus* RAP 2-N2; *Gobio gobio* RAP 2-L5).

Description: Spores typical of the genus *Myxobolus*. Plasmodium polysporous. Mature spores both within and between cysts pleomorphic. Mature spores ellipsoid or oval. Polar capsules apical and pyriform, mostly of unequal size, occasionally equal in size. Loosely coiled polar filament with 4-5 turns. Intercapsular process absent. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 specimens, as length × width range with means ± SD in parentheses: Spores from *Phoxinus phoxinus* 8.67 - 11.86 (10.21 ± 0.89) × 6.82 - 9.16 (7.94 ± 0.53); Polar capsules of unequal size,

Figure 4.8. Isolated spores of *Myxobolus pseudodispar* from the musculature of *Rutilus rutilus*. Scale bar = 10µm.

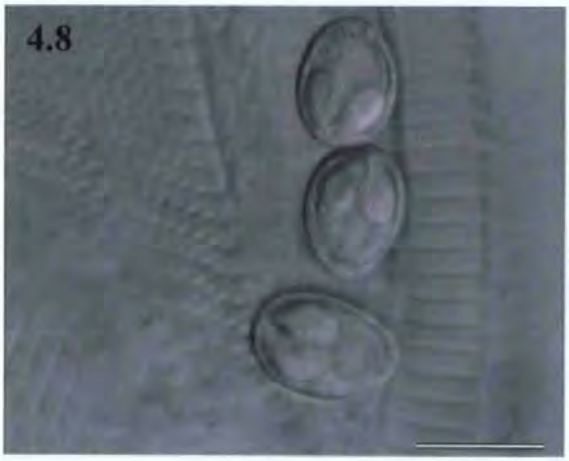
Figure 4.9. Histological section through *Myxobolus pseudodispar* plasmodia in the musculature of *Rutilus rutilus*. Giemsa, Scale bar = 50µm

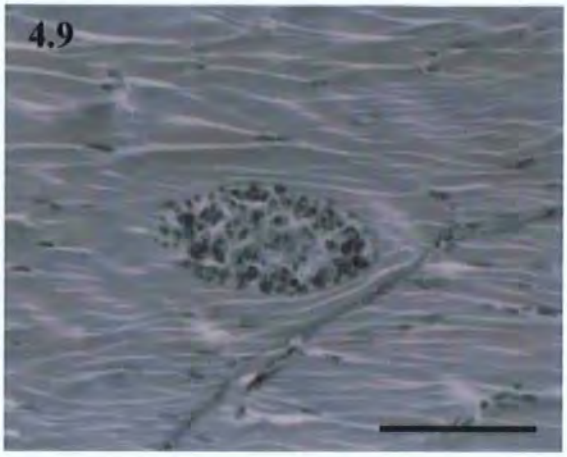
Figure 4.10. Line drawings of *Myxobolus pseudodispar* spores from *Rutilus rutilus*. Scale bar = 10µm

Figure 4.11. *Myxobolus musculi* spores from the musculature of *Phoxinus phoxinus*. Scale bar = 10µm

Figure 4.12. Histological section through musculature of *Phoxinus phoxinus* showing plasmodia of *Myxobolus musculi* containing sporogonic forms. H&E, scale bar = 50µm

Figure 4.13. Line drawings of *Myxobolus musculi* spores from *Phoxinus phoxinus*. Scale bar = 10µm





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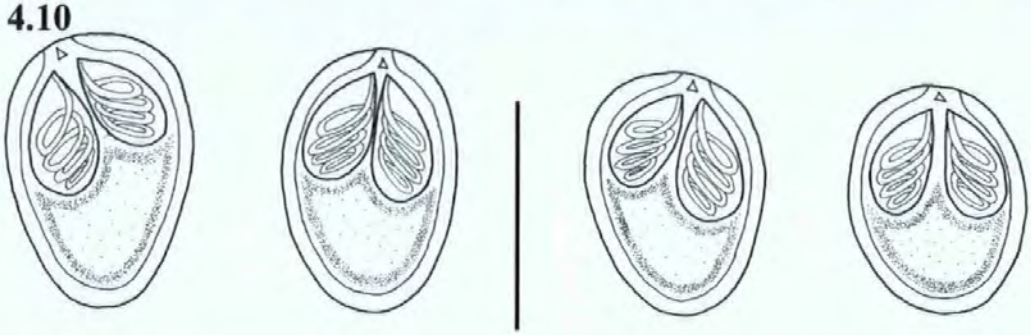

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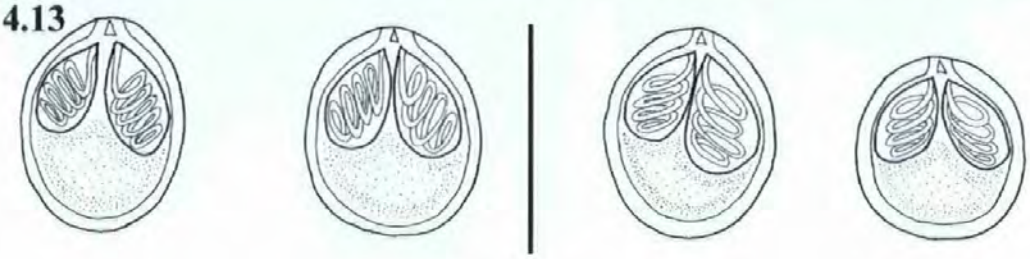


large polar capsule 4.18 - 6.60 (5.29 ± 0.66) × 2.17 - 3.68 (2.86 ± 0.39); small polar capsule 3.32 - 5.42 (4.27 ± 0.47) × 1.83 - 3.12 (2.41 ± 0.33). Spores from *Gobio gobio* 9.46 - 11.73 (10.56 ± 0.51) × 7.50 - 9.45 (8.62 ± 0.49); Polar capsules of unequal size, large polar capsule 4.94 - 6.71 (5.64 ± 0.42) × 2.58 - 3.79 (3.18 ± 0.33); small polar capsule 3.70 - 5.91 (4.60 ± 0.53) × 2.18 - 3.22 (2.79 ± 0.25).

Myxobolus pfeifferi Thélohan, 1895 Figures 4.17-4.19

Site of infection: Skeletal musculature

Infection data and affected sites:

Abramis brama - 3 infected, range of 1-3 cysts per section (mean 1.67).

Hull (Hempholme)

Leuciscus cephalus - 457 infected, range of 1-58 cysts per section (mean 4.83).

Aire (Keighley, Carleton Bridge, Knostrop); Dearne (Pastures Bridge); Derwent (Bubwith, Yedingham Fish Havens); Don (Sprotborough); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/s Linton Weir); Rother (Woodhouse); Swale (Maunby Demesne, Skipton, Thornton Bridge); Tees (Croft, Low Moor, Low Worsall); Ure (Bellflask, Boroughbridge); Wear (Durham); Wharfe (Boston Spa).

Leuciscus leuciscus - 23 infected, range of 1-3 cysts per section (mean 1.57).

Derwent (Bubwith, Yedingham, Yedingham Fish Havens); Ouse (Acaster Malbis, U/s Linton Weir); Swale (Skipton, Thornton Bridge); Tees (Low Moor); Ure (Boroughbridge).

Rutilus rutilus – 110 infected, range of 1-10 cysts per section (mean 2.1).

Calder (Elland); Dearne (Pastures Bridge); Derwent (Bubwith, Fish Haven Low Marishes, Yedingham, Yedingham Fish Havens); Don (Sprotborough); Hull (Hempholme); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/s Linton Weir); Swale (Thornton Bridge); Ure (Boroughbridge); Wharfe (Boston Spa).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa and May-Grünwald-Giemsa stained smears of

isolated spores deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Leuciscus cephalus* RAP 2-K4; *Leuciscus leuciscus* RAP 2-P2; *Rutilus rutilus* RAP 2-F31).

Description: Spores typical of the genus *Myxobolus*. Plasmodium polysporous. Mature spores almost rounded. Polar capsules apical, pyriform, of equal size. Polar filament with 8-9 coils, perpendicular to the longitudinal axis of the polar capsule. Small intercapsular process. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) from *Leuciscus cephalus* based on 30 specimens, as length × width range with means ± SD in parentheses: 10.24-11.94 (11.03 ± 0.45) × 8.32-10.11 (9.19 ± 0.41); Polar capsules of equal size, 4.6-6.43 (5.59 ± 0.44) × 2.65-3.53 (3.18 ± 0.21).

Development & Pathology of Myxobolus spp. *in muscle:*

Myxospores develop intracellularly within individual skeletal myofibrils (Figure 4.3), sporogony asynchronous, generative cells around outside of cyst with mature spores within centre of cysts (Figure 4.3). Sarcoplasm eventually replaced by parasite cyst. Growth of cysts results in marked enlargement of infected muscle fibres inducing pressure atrophy of adjacent myofibrils. Individual cysts do not extend beyond myoseptal boundaries (Figure 4.12). No host response generally noted towards developing plasmodial stages (Figure 4.15). However, some mature plasmodia rupture and spores are liberated into connective tissues between muscle fibres (Figure 4.20). A vigorous granulomatous response involving epitheloid-type cells and connective tissue cells is noted in the vicinity of the ruptured cyst (Figures 4.21 & 4.22). Infected myofibrils are destroyed and in some cases, remnants of myofibrils are sometimes visible within the inflammatory tissue (Figure 4.20). Within the musculature, spores traverse myoseptal boundaries (Figure 4.23) and localised blockage and necrosis of the surrounding tissue is apparent. Spores that manage to evade the host response are dispersed around the body via the blood and lymphatic systems and are subsequently located in most tissues including thymus, mucous

Figure 4.14. Fresh spores of *Myxobolus musculi* from *Gobio gobio*. Scale bar = 10µm

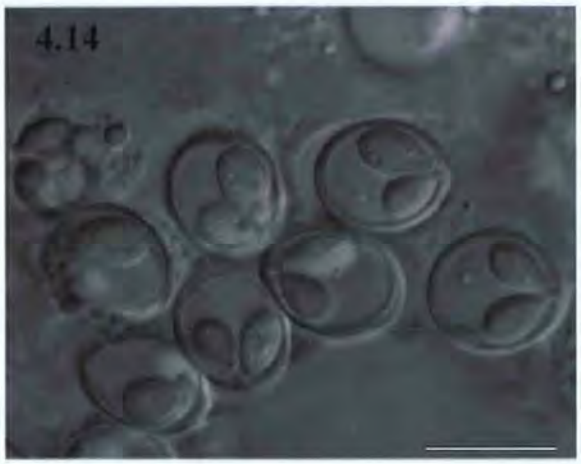
Figure 4.15. Histological section through skeletal musculature of *Gobio gobio* showing developing *Myxobolus musculi* plasmodia. H&E, scale bar = 50µm.

Figure 4.16. Line drawings of *Myxobolus musculi* spores from *Gobio gobio*. Scale bar = 10µm.

Figure 4.17. Fresh spores of *Myxobolus pfeifferi* isolated from the muscle of *Leuciscus leuciscus*. Scale bar = 10 µm

Figure 4.18. Plasmodia of *Myxobolus pfeifferi* in the musculature of *Rutilus rutilus*. H&E. Scale bar = 50µm

Figure 4.19. Line drawings of *Myxobolus pfeifferi* spores from the musculature of *Leuciscus leuciscus*. Scale bar = 10µm



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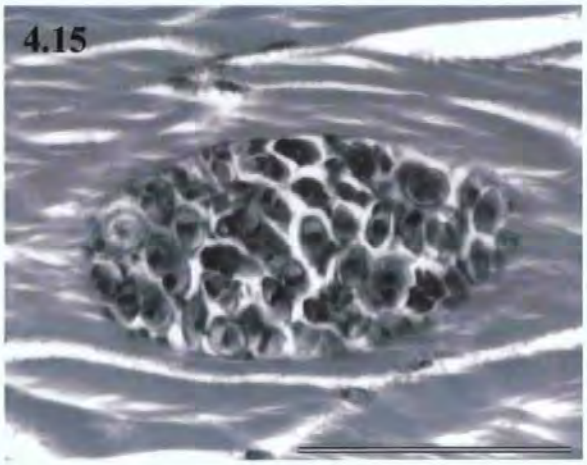

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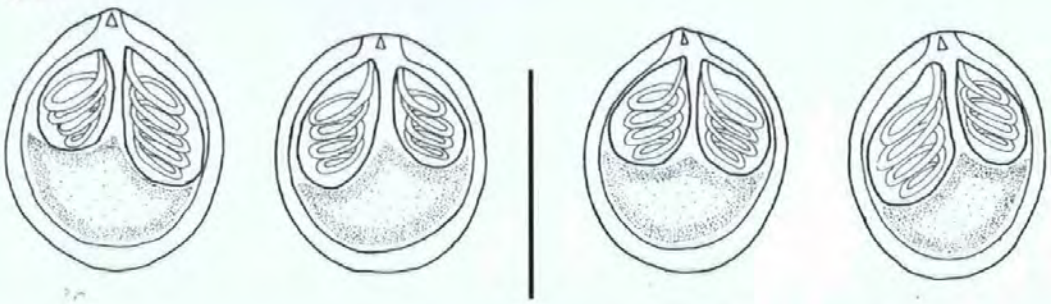

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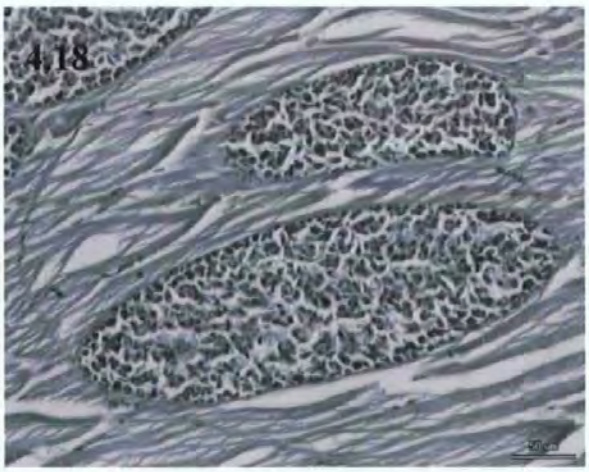

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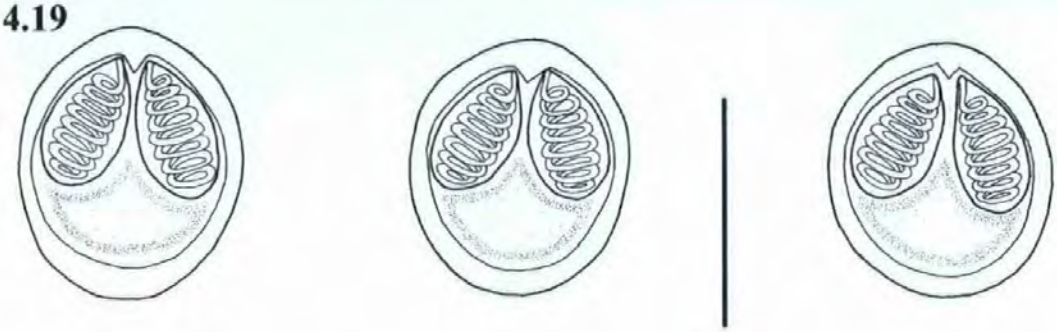


cells of intestinal epithelium, within the skin epithelium, occasionally in gills, liver, spleen and kidney, often in macrophage aggregates. Chub were the most heavily parasitised, individual fish containing up to 195 cysts in one histological section, with roach containing up to 28 cysts, dace containing up to 29 cysts and minnow containing up to 71 cysts. Bleak, bream and gudgeon were the least parasitised containing around 2-3 cysts per histological section on average, up to a maximum of 13 cysts.

Myxobolus macrocapsularis Reuss, 1906 Figures 4.24-4.28

Site of infection: Intralamellar within the gills and in pseudobranch

Infection data and affected sites:

Leuciscus cephalus – 46 fish infected. Aire (Keighley, Carleton Bridge); Dearne (Pastures Bridge); Derwent (Bubwith); Ouse (Acaster Malbis, Beningbrough, U/S Linton Weir); Swale (Maunby Demesne, Skipton, Thornton Bridge); Tees (Croft, Low Moor); Ure (Bellflask, Boroughbridge); Wharfe (Boston Spa).

Leuciscus leuciscus – 3 fish infected. Ouse (U/S Linton Weir).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Leuciscus leuciscus* RAP 2-P3; *Leuciscus cephalus* RAP 2-K5).

Description: Spores typical of the genus *Myxobolus.* Plasmodium polysporous, multilobed, developing intralamellarly. Mature spores pyriform with narrow and acuminate anterior pole. Mucous envelope apparent in posterior region of spore. Polar capsules pyriform, apical, of equal size. Polar filament with 7-9 coils, perpendicular to the longitudinal axis of the polar capsule. Sporoplasm binucleate, filling entire extracapsular

Figure 4.20. Disrupted *Myxobolus pseudodispar* plasmodia subjected to a host response. Note the remnants of host muscle within the destroyed cyst (arrowed). H&E, scale bar = 50µm.

Figure 4.21. Host response to *Myxobolus pseudodispar* cyst in the musculature of *Leuciscus cephalus*. Giemsa, scale bar = 50µm.

Figure 4.22. Destroyed *Myxobolus pseudodispar* spores trapped within a granulomatous host response. H&E, scale bar = 50µm

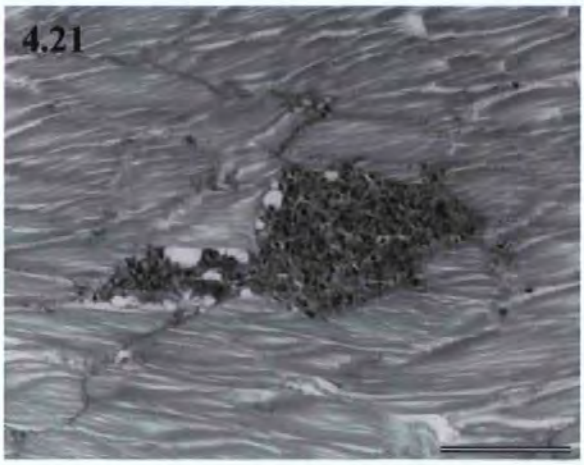
Figure 4.23. Spores of *Myxobolus pseudodispar* released from a plasmodia and moved across the myoseptal boundary by the host response. Giemsa, scale bar = 50µm

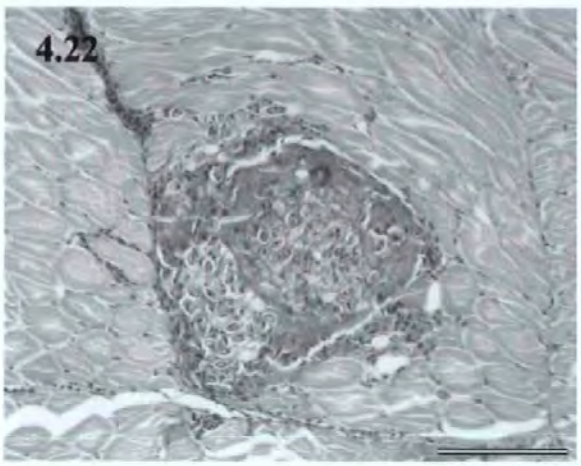
Figure 4.24. Fresh spores of *Myxobolus macrocapsularis* from the gills of *Leuciscus cephalus*. Scale bar = 10µm

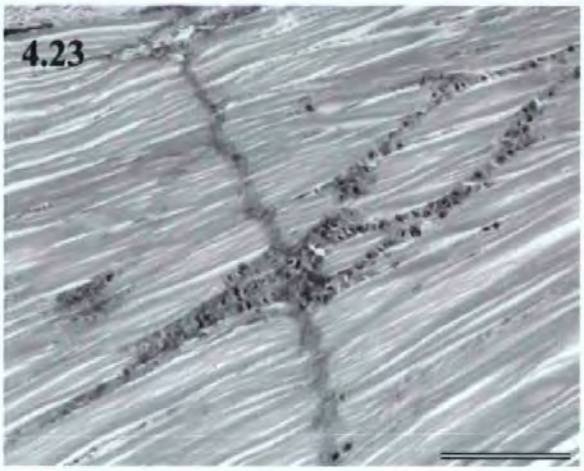
Figure 4.25. Line drawings of *Myxobolus macrocapsularis* spores from *Leuciscus cephalus*. Scale bar = 10µm

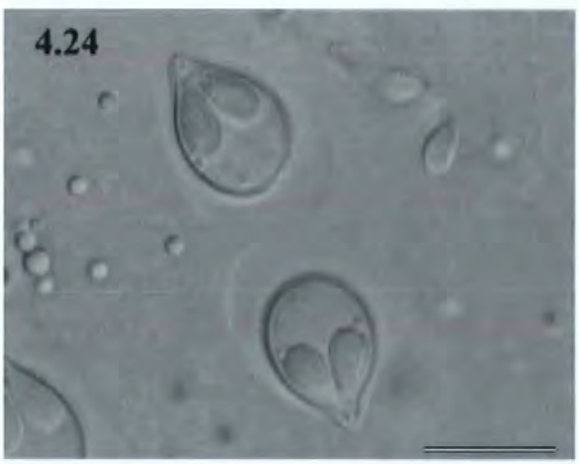
Figure 4.26. Fresh spores of *Myxobolus macrocapsularis* from the gills of *Leuciscus leuciscus*. Scale bar = 10µm

Figure 4.27. Line drawings of *Myxobolus macrocapsularis* spores from *Leuciscus leuciscus*. Scale bar = 10µm









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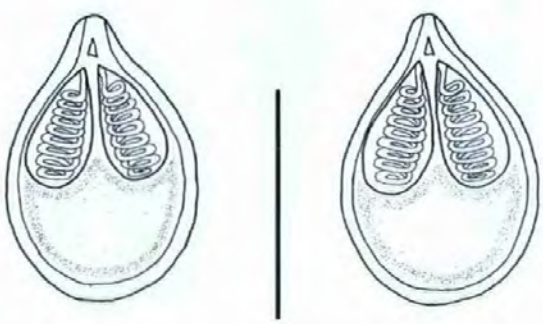

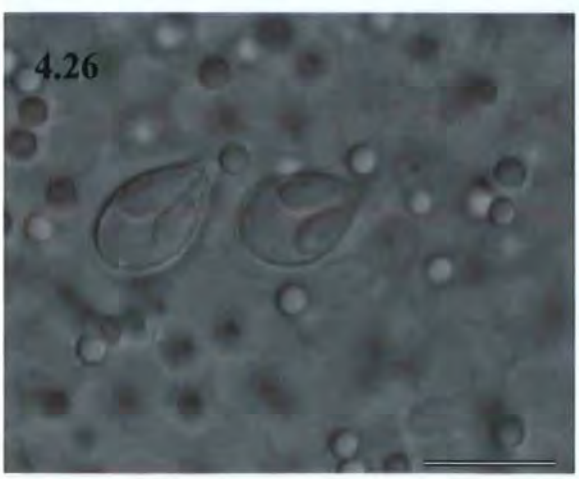

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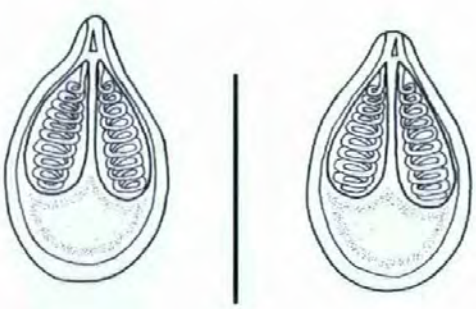


spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores from *Leuciscus cephalus* 10.23-13.21 (11.51 ± 0.76) × 6.31-8.37 (7.49 ± 0.43). Polar capsules of equal size extend to mid-length of spore and measure 3.97-6.02 (5.26 ± 0.58) × 2.13-3.26 (2.64 ± 0.28). Spores from *Leuciscus leuciscus* 9.12-12.35 (11.14 ± 0.67) × 5.46-7.32 (6.43 ± 0.44). Polar capsules of equal size extend to mid-length of spore and measure 4.74-7.08 (5.64 ± 0.47) × 1.85-3.43 (2.54 ± 0.36).

Pathology:

The developing plasmodia invade the intralamellar spaces in a series of finger-like projections. Various parts of individual plasmodia can be found throughout single gill lamellae. In low level or early stages of development, the host response is limited. However, in the later stages of plasmodium development and in heavy infections, the developing plasmodia can completely fill the intralamellar space (Figure 4.29). Host response however is limited until sporogony is complete after which there is a vigorous inflammatory response. Heavy infection completely filling the lamellae possibly leading to disruption of the respiratory function.

Myxobolus oviformis Thélohan, 1892 Figures 4.30-4.33

Site of infection: Intralamellar in gills

Infection data and affected sites:

Gobio gobio – 2 fish infected. Ure (Boroughbridge).

Leuciscus leuciscus – 4 fish infected. Derwent (Yedingham Fish Havens); Swale (Maunby Demesne, Skipton, Thornton Bridge).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Leuciscus leuciscus* RAP 2-P4).

Figure 4.28. Scanning electron micrograph of mucous coat on posterior pole of *Myxobolus macrocapsularis* spore from the gills of *Leuciscus cephalus*. Inset, scanning electron micrograph of *M. macrocapsularis* spore.

Figure 4.29. Histological section through the gill of *Leuciscus cephalus* infected with *Myxobolus macrocapsularis*. Giemsa, scale bar = 50µm

Figure 4.30. Fresh spores of *Myxobolus oviformis* from the gills of *Gobio gobio*. Scale bar = 10µm

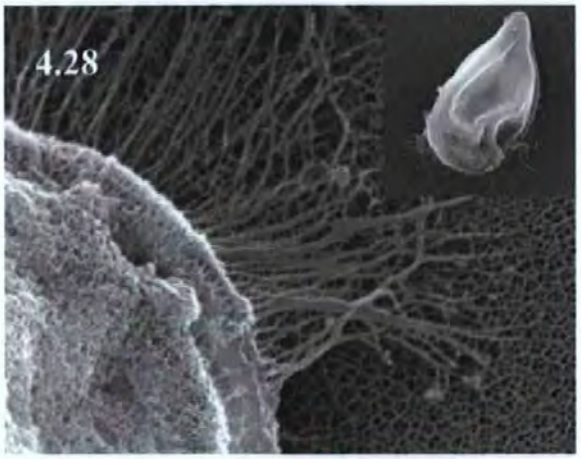
Figure 4.31. Line drawings of *Myxobolus oviformis* spores from the gills of *Gobio gobio*. Scale bar = 10µm

Figure 4.32. Fresh spores of *Myxobolus oviformis* from the gills of *Leuciscus leuciscus*. Scale bar = 10µm

Figure 4.33. Line drawings of *Myxobolus oviformis* spores from the gills of *Leuciscus leuciscus*. Scale bar = 10µm

Figure 4.34. Fresh spores of *Myxobolus bramae* isolated from the gills of *Phoxinus phoxinus*. Scale bar = 10µm

Figure 4.35. Line drawings of *Myxobolus bramae* spores from the gills of *Phoxinus phoxinus*. Scale bar = 10µm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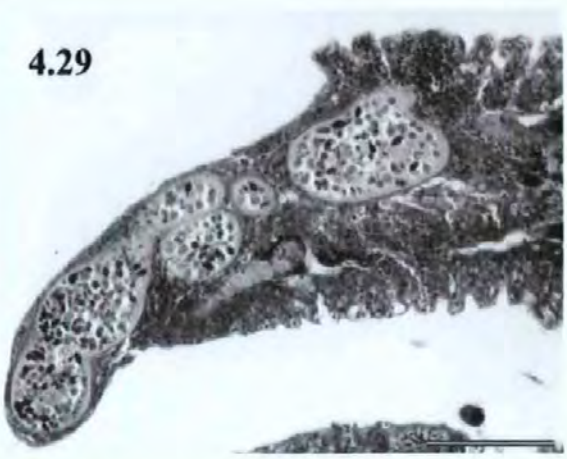
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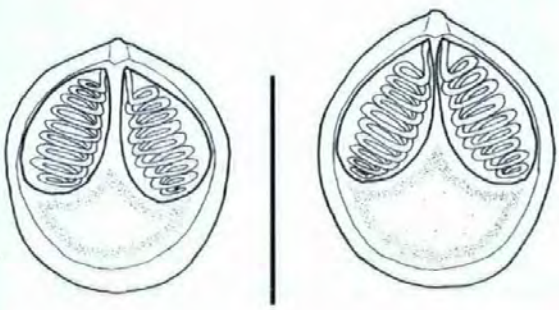
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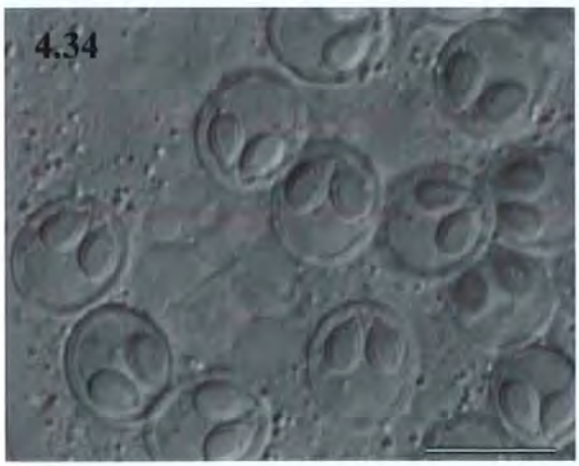
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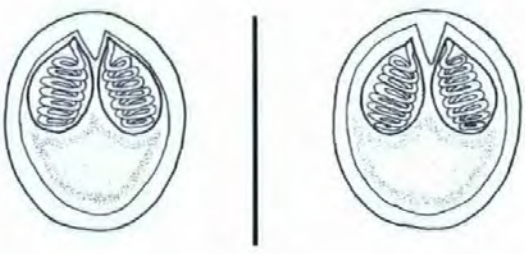
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Description: Spores typical of the genus *Myxobolus*. Plasmodium polysporous. Mature spores smooth, oval in shape, anterior pole tapered and slightly acuminate. Slight nipple-like protrusion at anterior pole. Polar capsules pyriform, terminal of equal size. Polar filament with 8-10 coils, coiled perpendicular to longitudinal axis of polar capsule. Small intercapsular process. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores from *Leuciscus leuciscus* 9.83-12.32 (11.08 ± 0.67) × 7.94-9.76 (8.65 ± 0.53). Polar capsules of equal size extend to mid-length of spore and measure 4.24-5.95 (5.34 ± 0.42) × 2.69-3.95 (3.22 ± 0.30). Spores from *Gobio gobio* 9.92-12.29 (11.05 ± 0.55) × 8.32-10.14 (9.29 ± 0.43). Polar capsules of equal size extend to mid-length of spore and measure 4.85-6.69 (6.03 ± 0.40) × 2.85-3.88 (3.38 ± 0.28).

Pathology:

Developing plasmodia completely fill individual lamellae with a concomitant loss of respiratory function of infected lamellae. However, infections in cyprinids were low grade with no more than two cysts per histological section of an individual fish.

Myxobolus bramae Reuss, 1906 Figures 4.34-4.35

Site of infection: Intralamellar with gills.

Infection data and affected sites:

Phoxinus phoxinus – 4 fish infected. Derwent (Yedingham) Tees (Croft).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (RAP 2-N3).

Description: Spores typical of the genus *Myxobolus*. Plasmodium polysporous. Mature spores round to ellipsoid. No sutural edge markings. Polar capsules pyriform, apical. Polar filament with 7-8 coils, coiled perpendicular to longitudinal axis of polar capsule.

Large intercapsular process. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores 8.23-10.14 (9.13 ± 0.51) × 7.16-8.48 (7.76 ± 0.34). Polar capsules of equal size measuring 3.28-4.94 (4.09 ± 0.46) × 1.61-2.90 (2.51 ± 0.26).

Pathology:

Plasmodial development as per *M. oviformis* with limited host response to the parasites (Figure 4.38). Low intensity infection and minimal host response appear unlikely to significantly impair host respiratory function.

Myxobolus muelleri Buetschli, 1882 sensu lato Figures 4.36-4.37

Site of infection: Intralamellar within gills.

Infection data and sites affected:

Leuciscus leuciscus – 2 fish infected. Derwent (Yedingham Fish Haven); Swale, Skipton.

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (RAP 2-P5).

Description: Spores typical of the genus *Myxobolus*. Plasmodium polysporous. Mature spores smooth with several sutural edge markings (normally 4-6), slightly variable in shape. Polar capsules pyriform, terminal. Polar filament with 9-10 coils. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores 7.86-9.99 (9.12 ± 0.56) × 6.49-8.62 (7.84 ± 0.59); large intercapsular appendix. Polar capsules of equal size do not extend beyond the mid-length of the spore and measure 3.63-5.36 (4.51 ± 0.40) × 2.25-3.30 (2.67 ± 0.28).

Pathology:

The small plasmodia within the gills coupled with a low number of cysts per fish limit the pathological effect caused by this parasite. No host response to sporogonic forms of the parasite were observed (Figure 4.39).

Sphaerospora ousei n. sp. Figures 4.40-4.41

Site of infection: Lumens of kidney tubules

Infection data and affected sites:

Rutilus rutilus – 3 fish infected. Ouse (Beningbrough).

Etymology: The parasite is named for the type locality river form which it was caught.

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic Pathology, CEFAS Weymouth (RAP 2-F31).

Description: Pseudoplasmodia mono- and di-sporous, elongate, measuring 18-21 × 10-11. Cytoplasm containing numerous refringent granules. Mature spores almost spherical with smooth valve shells and sutural line protruding slightly at anterior end, less so at posterior end. Polar capsules spherical to sub-spherical, equal in size. Polar filament with 3-4 turns, coiled perpendicular to the longitudinal axis of the polar capsule. No mucous envelope. Two uninucleate sporoplasms fill entire extracapsular space. Spore dimensions (μm) based on 30 specimens, as range with means ± SD in parentheses: Spores (length × thickness) 7.56 - 9.12 (8.46 ± 0.52) × 7.54 - 8.89 (8.21 ± 0.44) with slightly protruding sutural line especially at the anterior end; Polar capsules of equal size, spherical to sub-spherical 2.60 - 3.89 (3.22 ± 0.34) with 3-4 turns of the polar filament.

Differential diagnosis:

Three described *Sphaerospora* sp. have been reported from *Rutilus rutilus*, namely *S. minima* Kaschkovsky, 1974, *S. carassii* Kudo, 1919 and *S. poljanskii* Kulemina,

Figure 4.36. Fresh spores of *Myxobolus muelleri* from the gills of *Leuciscus leuciscus.* Scale bar = 10µm

Figure 4.37. Line drawings of *Myxobolus muelleri* spores from the gills of *Leuciscus leuciscus*. Scale bar = 10µm

Figure 4.38. Histological section through a *Myxobolus bramae* plasmodia in the gill of *Phoxinus phoxinus*. H&E, Scale bar = 100µm

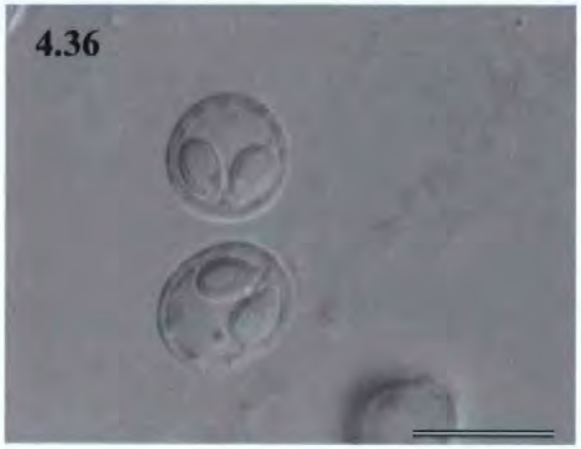
Figure 4.39. Histological section through a *Myxobolus muelleri* plasmodia in the gill of *Leuciscus leuciscus*. H&E, Scale bar = 100µm

Figure 4.40. Spores of *Sphaerospora ousei* in a disporous pseudoplasmodia in the kidney of *Rutilus rutilus*. Scale bar = 10µm

Figure 4.41. Line drawings of *Sphaerospora ousei* spores from the kidney of *Rutilus rutilus*. Scale bar = 10µm.

Figure 4.42. Fresh preparation of *Sphaerospora leuciscusi* spores in a monosporous pseudoplasmodia from the kidney of *Leuciscus leuciscus.* Scale bar = 10µm

Figure 4.43. Line drawings of *Sphaerospora leuciscusi* spores from the kidney of *Leuciscus leuciscus.* Scale bar = 10µm.



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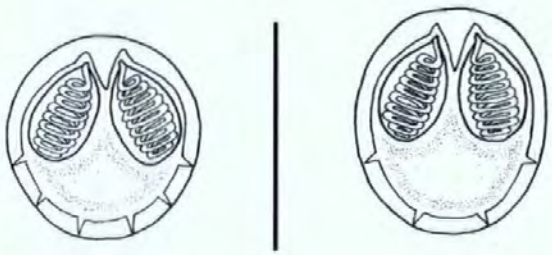

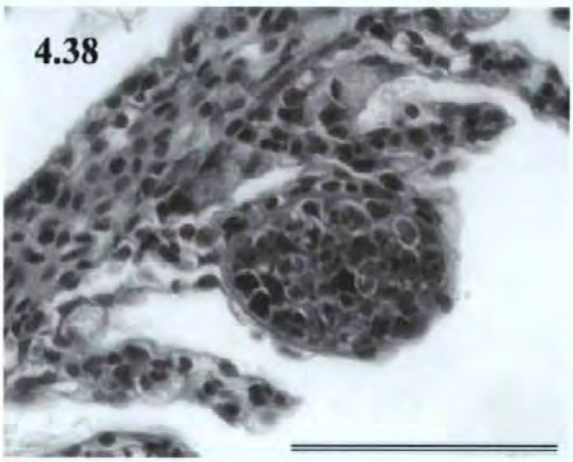

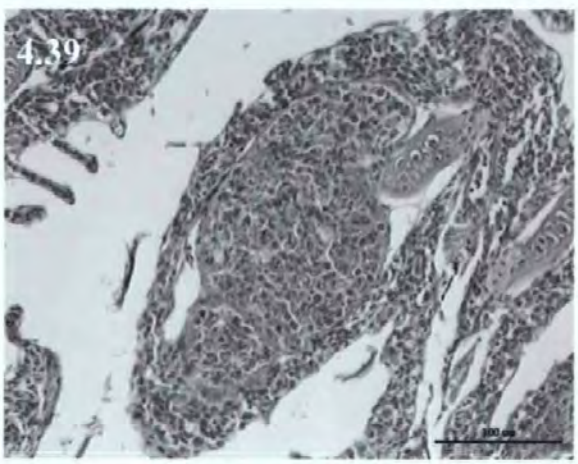

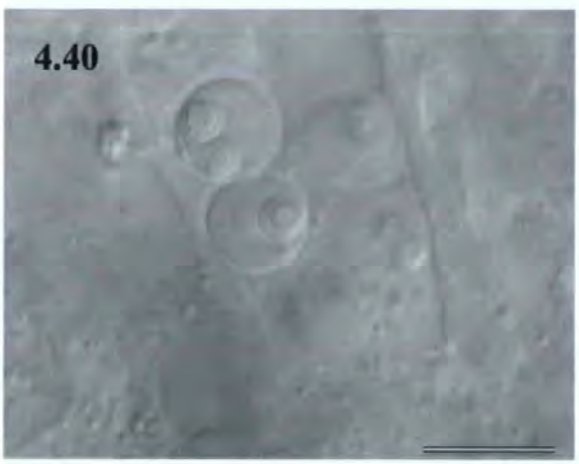

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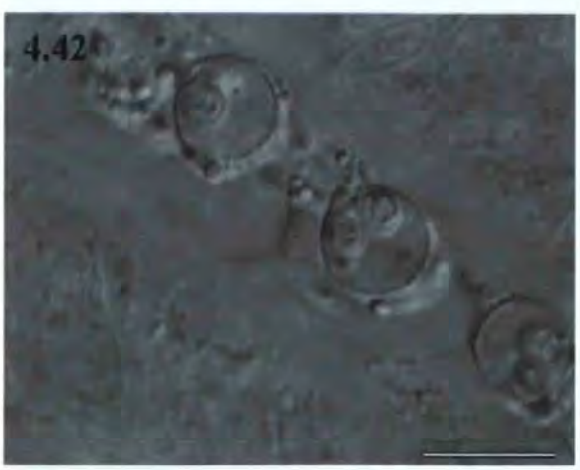


4.43

1969. In addition, at least a further two have been reported but remain undescribed. Lom et al., (1985) reported the presence of two *Sphaerospora* spp. in roach. The first (*Sphaerospora* sp. 1) was spherical (mean dimensions 8.5 × 8.2μm), with equal-sized, subspherical polar capsules measuring 3.5 × 2.9μm with 3 turns to the polar filament. Spores were described as smooth with a slightly protruding sutural line. The pseudoplasmodia were monosporous. The second form (*Sphaerospora* sp. 2) was smaller (7 × 7μm), smooth, subspherical with a slightly pointed anterior end, with equal-sized, rounded-pyriform polar capsules measuring 3.3 × 2.9 with 5 turns of the polar filament. Kepr (1991) provided a drawing of an undescribed *Sphaerospora* sp. in the renal canaliculi of roach from Czechoslovakia. Sitja-Bobadilla and Alvarez-Pellitero (1994) consider this parasite to be *S. minima*. Of the two undescribed forms, *S. ousei* appears to be conspecific with *Sphaerospora* sp. 1 of Lom et al., (1985) as it matches the morphological characteristics and the host details reported. However, in images provided by Lom et al., (1985), the pseudoplasmodia of *S.* sp. 1 were monosporous. In the current study, both monosporous and disporous pseudoplasmodia were recorded.

In sutural view, *S. ousei* most resembles *S. gobionis* Lom, Pavlásková and Dyková, 1985, *S. molnari* Lom, Dyková, Pavlásková and Grupcheva, 1983, *S. renicola* Dyková and Lom, 1982, *S. sapae* Donets in Shulman, 1966, *S. tincae* Plehn, 1925 in Lom, Körting and Dyková, 1985 and *S. minima* Kaschkovsky, 1974 in Kepr, 1991. *S. gobionis* has been reported from the kidneys of gudgeon. *S. ousei* can be differentiated from *S. gobionis* by having completely smooth shell valves and by being larger both in thickness and length compared with *S. gobionis*. *S. molnari* pseudoplasmodia are monosporous and the spores are subspherical, measuring 10.3μm in length × 10.5μm thickness. *S. molnari* spores and polar capsules are larger than *S. ousei*. *S. renicola* spores are smaller than *S. ousei* and the anterior edge of the sutural line does not extend as much as *S. ousei*. *S. sapae* spores occur in *Abramis sapa*. The polar capsules of *S. sapae* are pyriform, occupy more than half of the spore cavity and are larger than *S. ousei*. However, *S. ousei* spores are larger than *S.*

sapae. The sutural edge of *S. tincae* protrudes in both the anterior and posterior poles of the spore, unlike *S. ousei* in which it only protrudes in the anterior pole. Spores of *S. tincae* are also surrounded by a mucous envelope which is absent from *S. ousei*.

The three sphaerospores reported from *R. rutilus* can be differentiated from *S. ousei* by the following characters. *S. carassii* has been reported from the gills of *Carassius* spp., *Cyprinus* spp. and *R. rutilus*, unlike *S. ousei* which occurs within the kidney. Like *S. ousei*, *S. carassii* pseudoplasmodia are disporous. However, the polar capsules in *S. carassii* are pyriform, the sutural line protrudes in both the anterior and posterior apices of the spore and in overall dimensions, *S. ousei* is smaller. *S. minima* from the kidneys of roach can be differentiated from *S. ousei* by the presence of an ornate posterior end and in being smaller in overall dimensions than *S. ousei*. *S. poljanskii* spores are larger than *S. ousei* both in thickness and length. In addition, spores of *S. poljanskii* possess two small projections on the posterior end, which are absent from *S. ousei*.

Overall, *S. ousei* can be discriminated from all known *Sphaerospora* spp. by a combination of its measurements, a smooth spherical spore with a slightly protruding sutural line at the anterior end and is unlike any of the *Sphaerospora* spp. occurring in *R. rutilus*.

Sphaerospora leuciscusi n. sp. Figures 4.42-4.43

Site of infection: Lumens of kidney tubules

Type locality: Upstream of Linton Weir, River Ouse.

Infection data and affected sites:

Leuciscus leuciscus – 25 fish infected. Derwent (Yedingham Fish Havens); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, U/S Linton Weir); Swale (Thornton Bridge); Tees (Croft, Low Worsall); Ure (Boroughbridge); Wharfe (Boston Spa).

Etymology: Named for the generic name of the type host.

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (RAP 2-P6).

Description: Pseudoplasmodia monosporous, rounded, measuring 14μm in diameter. Cytoplasm with numerous refringent granules. Mature spores spherical with smooth shell valves. Sutural line protruding slightly at anterior pole. Polar capsules sub-spherical, of equal size. Polar filament with 3-4 turns, coiled perpendicular to the longitudinal axis of the polar capsule. No mucous envelope. Two uninucleate sporoplasms fill entire extracapsular space. Spore dimensions (μm) based on 30 specimens, as range with means ± SD in parentheses: Spores (length × thickness) 5.11 - 6.01 (5.56 ± 0.27) × 5.41 - 6.72 (5.87 ± 0.13); Polar capsules of equal size, spherical 1.97 - 3.13 (2.35 ± 0.33) with 3-4 turns of the polar filament.

Differential diagnosis:

To the best of our knowledge, no sphaerospores have been reported or described previously from *Leuciscus leuciscus.* However, *S. rota* has been reported in *L. l. baicalensis*, a subspecies of dace in Lake Baikal. *S. rota* spores have a highly protruding sutural edge, three lateral protuberances and a prominent ridge on the posterior pole. In addition, *S. rota* spores are larger than *S. leuciscusi* spores and possess much larger polar capsules. In sutural view, *S. leuciscusi* most resembles *S. amurensis, S. carassii, S. gobionis, S. molnari,* and *S. renicola. S. amurensis, S. molnari* and *S. carassii* are all larger than *S. leuciscusi* in all dimensions. *S. gobionis* spores have a slightly uneven surface and are also larger than *S. leuciscusi. S. renicola* spores are similar in size to *S. leuciscusi* but possess ovoid polar capsules, unlike *S. leuciscusi* which has subspherical polar capsules. In addition, *S. molnari* occurs in the gills of its hosts. On the combination of its small size, equal sized, subspherical polar capsules and smooth shell valves we propose to establish the species as *Sphaerospora leuciscusi.*

Development & Pathology of kidney sphaerospores:

Mature and maturing spores were present within the renal tubule lumens. The possibility that plasmodial stages type 1 and 2 described later represent different developmental stages of *Sphaerospora* spp. in roach and dace is discussed further later.

Infected tubules become completely occluded by the parasite and the tubules themselves become significantly dilated (Figure 4.44) with a concomitant reduction in epithelial cell height and a loss of the brush border.

Myxidium rhodei Léger, 1905 sensu lato Figure 4.48

Site of infection: kidney

Infection data and affected rivers:

Abramis brama – 2 fish infected, range of 16-16 plasmodia in infected fish, average 16 plasmodia per infected fish. Hull (Hempholme).

Leuciscus cephalus – 1 fish infected, 1 plasmodia seen. Ouse (Beningbrough).

Leuciscus leuciscus - 2 fish infected, range of 1-3 plasmodia in infected fish, average 2 plasmodia per infected fish. Derwent (Bubwith); Ouse (U/s Linton Weir).

Phoxinus phoxinus - 2 fish infected, range of 2-5 plasmodia in infected fish, average 3.5 plasmodia per infected fish. Tyne (Haughton).

Rutilus rutilus – 30 fish infected, range of 1-43 plasmodia in infected fish, average 6.13 plasmodia per infected fish. Hull (Hempholme); Derwent (Bubwith); Ouse (Acaster Malbis, Beningbrough); Swale (Thornton Bridge).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Abramis brama* RAP 2-H8; *Leuciscus cephalus* RAP 2-K6; *Leuciscus leuciscus* RAP 2-P7; *Rutilus rutilus* RAP 2-F32).

Figure 4.44. Histological section through a kidney tubule of *Leuciscus leuciscus* showing the presence of a large number of *Sphaerospora leuciscusi* spores in the lumen. Note the widely dilated tubule and the reduction in epithelial height of the host kidney. H&E, scale bar = 50μm

Figure 4.45. Histological section through the kidney of *Rutilus rutilus*. Note presence of *Myxidium rhodei* plasmodia in the kidney interstitium subjected to a granulomatous response. H&E, scale bar = 50μm

Figure 4.46. Vigorous host response in kidney interstitium of *Abramis brama* towards *Myxidium rhodei* plasmodia. H&E, scale bar = 50μm

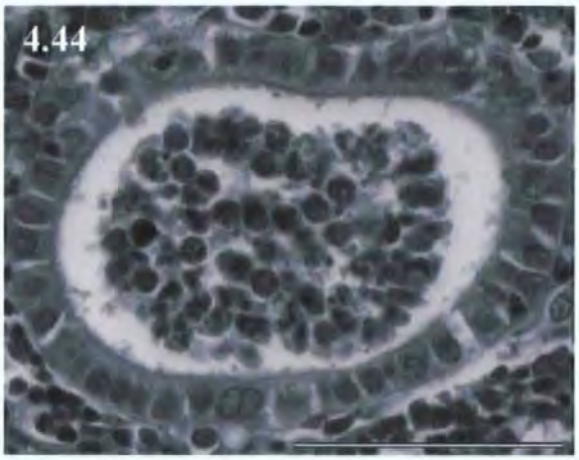
Figure 4.47. *Myxidium pfeifferi* plasmodia in a the bile duct of *Rutilus rutilus*. H&E, scale bar = 50μm

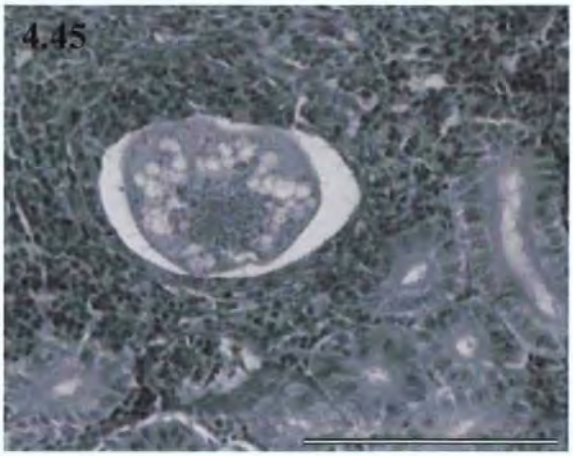
Figure 4.48. Line drawings of *Myxidium rhodei* spores from the kidney of *Rutilus rutilus*. Scale bar = 10μm.

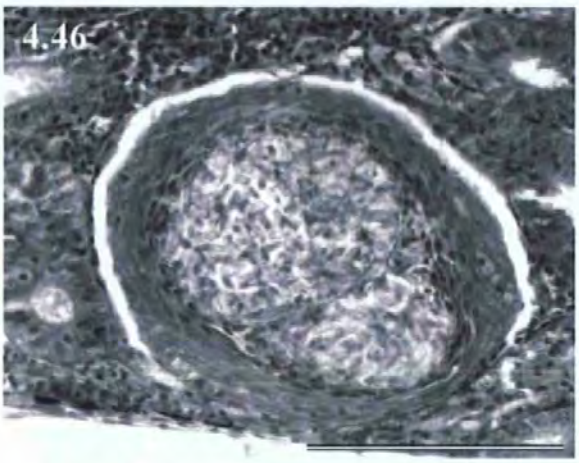
Figure 4.49. Line drawings of *Myxidium barbatulae* spores from the kidney of *Barbatula barbatula*. Scale bar = 10μm.

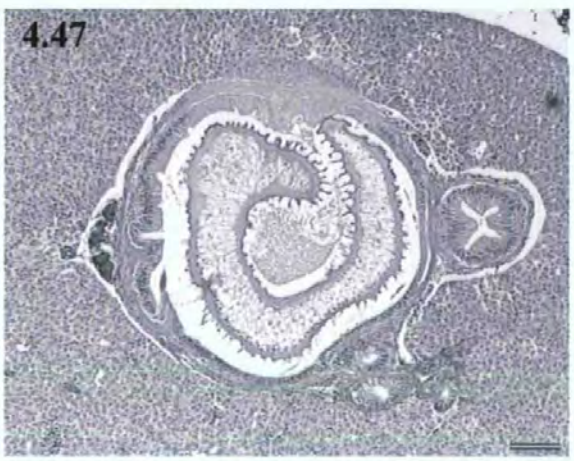
Figure 4.50. Histological section through kidney of *Leuciscus leuciscus* infected with plasmodial type 1 and extrasporogonic type 1. Extrasporogonic type 1 occur within the kidney epithelial cells, plasmodia type 1 are coelozoic. Note the greatly enlarged kidney tubule compared with surrounding, uninfected tubules. H&E, scale bar = 50μm

Figure 4.51. Type 1 plasmodial stages coelozoic in the kidney tubule of *Gobio gobio*. Unlike forms in Figure 4.50, the forms in *G. gobio* do not appear to attach to the kidney tubule epithelium. H&E, scale bar = 50μm









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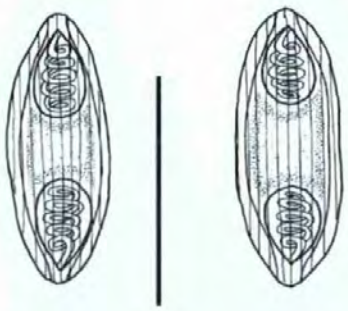

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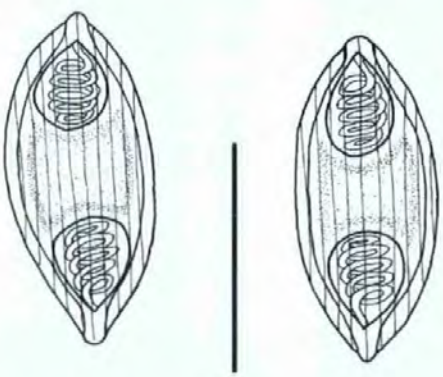
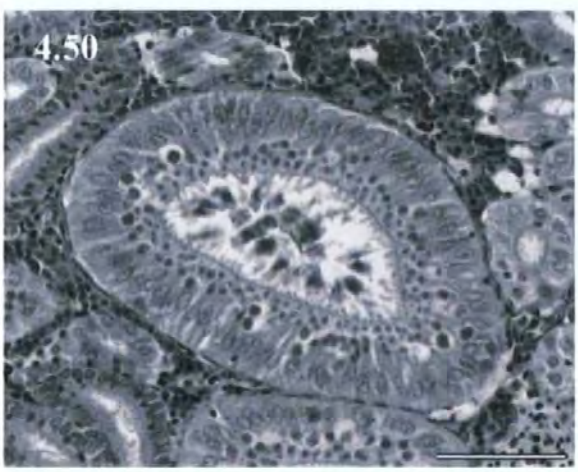




Description: Plasmodium polysporous. Spore elongate, ellipsoidal, with 20-30 fine ridges on valves. Polar capsules pyriform, at opposite ends of spore along longitudinal axis of spore. Polar filament with 4-5 coils. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores from *Rutilus rutilus*, 10.94 - 12.82 (11.82 ± 0.48) × 3.69 - 4.68 (4.25 ± 0.30). Polar capsules of equal size, 2.80 - 3.83 (3.36 ± 0.29) × 1.79 - 3.07 (2.37 ± 0.27).

Myxidium barbatulae Cépède, 1906 Figure 4.49

Site of infection: kidney

Infection data and affected sites:

Barbatula barbatula – 2 fish infected. Swale (Maunby Demesne)

Description: Plasmodium polysporous. Pansporoblasts are disporous. Spore elongate, ellipsoidal, with 20-30 fine ridges on valves. Polar capsules pyriform, at opposite ends of spore along longitudinal axis of spore. Polar filament with 4-5 coils. Sporoplasm binucleate, filling entire extracapsular spore cavity. Spore dimensions (μm) based on 30 fresh specimens, as range with means ± SD in parentheses: Spores 12.36 - 14.36 (13.49 ± 0.662) × 4.69 - 6.18 (5.53 ± 0.45). Polar capsules of equal size, 2.97 - 4.49 (3.42 ± 0.31) × 2.47 - 3.66 (2.85 ± 0.26).

Development & Pathology of renal Myxidium *spp.*:

No very early developmental stages were detected. Plasmodia were noted in the renal interstitium and glomeruli of the kidney (Figure 4.45). Mature spores were not detected free within the interstitium. Compression and atrophy of the tissues immediately surrounding plasmodia was apparent. Plasmodia were subjected to an inflammatory granulomatous response (Figure 4.46) and nearby tubules were atrophied as a result of pressure from the granuloma.

Myxidium pfeifferi Auerbach, 1908

Site of infection: Bile ducts

Infection data and affected sites:

Rutilus rutilus – 3 fish infected. Don (Sprotborough)

Leuciscus leuciscus – 1 fish infected. Ouse (U/S Linton Weir).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Rutilus rutilus* RAP 2-F33; *Leuciscus leuciscus* RAP 2-P8).

Description: Due to the low prevalence of this parasite, no description of the spores was possible from fresh material. This parasite has previously been reported infecting the bile ducts of roach and dace.

Pathology:

Large plasmodia were found within the bile ducts of both roach and dace (Figure 4.47). They were characterised by possessing finger-like projections on their surface and almost completely filled the bile duct lumen leading to occlusion of the duct. The parasite elicited a hyperplastic response of the epithelial cells of the bile duct with degeneration of the epithelial cells. Hepatocytes immediately surrounding the bile duct were necrotic.

Extrasporogonic type 1 Figure 4.50

Site of infection: Intracellularly within the epithelial cells of the mid and posterior kidney tubules and of the urinary bladder.

Infection data and sites affected:

Abramis brama – 1 fish infected. Hull (Hempholme).

Leuciscus cephalus – 9 fish infected. Ouse (Acaster Malbis, U/S Linton Weir); Swale (Skipton); Tees (Croft); Wharfe (Boston Spa).

Leuciscus leuciscus – 41 fish infected. Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough, U/S Linton Weir); Swale (Maunby Demesne, Skipton, Thornton Bridge); Tees (Croft, Low Moor, Low Worsall); Ure (Boroughbridge); Wharfe (Boston Spa).

Phoxinus phoxinus - 2 fish infected. Derwent (Yedingham Fish Havens); Swale (Thornton Bridge).

Rutilus rutilus – 30 fish infected. Derwent (Bubwith); Hull (Hempholme); Nidd (Kirk Hammerton); Ouse (Acaster Malbis, Beningbrough); Swale (Thornton Bridge); Ure (Boroughbridge); Wharfe (Boston Spa).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa from roach, chub, dace, bream and minnows have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Rutilus rutilus* RAP 2-F44; *Leuciscus leuciscus* RAP 2-P9; *Leuciscus cephalus* RAP 2-K7; *Phoxinus phoxinus* RAP 2-N4).

Description: Intracellular stages were found predominately in the mid and posterior kidney and in the urinary bladder wall. Almost all epithelial cells of infected tubules were filled with extrasporogonic parasite stages. The earliest identifiable stage contained a primary and secondary stage, located towards the lumen of the tubule. Later trophozoite stages comprising a primary cell with secondary and up to 6 tertiary cells and were generally found towards the base of the epithelial cell.

Development & Pathology:

The host cell nucleus in infected epithelial cells were found towards the base of the cell. The cell borders between successive cells were still distinct. No syncitium was formed. Infected tubules were greatly enlarged compared to uninfected tubules, being up

to 8 times larger than uninfected tubules. The brush border lining the tubule epithelium was clearly visible.

Plasmodial type 1 Figures 4.51-4.54

Site of infection: Within lumens of kidney tubules and of urinary bladder

Infection data and affected sites:

Abramis brama – 1 fish infected. Hull (Hempholme).

Alburnus alburnus - 1 fish infected. Ouse (Acaster Malbis).

Barbatula barbatula – 1 fish infected. Wharfe (Boston Spa).

Gobio gobio - 42 fish infected. Derwent (Bubwith); Nidd (Kirk Hammerton); Ouse (Beningbrough, U/S Linton Weir); Swale (Thornton Bridge); Ure (Boroughbridge).

Leuciscus cephalus – 4 fish infected. Aire (Knostrop); Ouse (U/S Linton Weir); Tees (Croft, Low Moor).

Leuciscus leuciscus – 15 fish infected. Derwent (Bubwith); Ouse (Acaster Malbis, Beningbrough); Swale (Skipton, Thornton Bridge); Tees (Low Moor); Ure (Boroughbridge); Wharfe (Boston Spa).

Phoxinus phoxinus – 9 fish infected. Aire (Keighley); Derwent (Bubwith); Nidd (Kirk Hammerton); Swale (Maunby Demesne, Thornton Bridge); Ure (Boroughbridge); Wharfe (Boston Spa).

Rutilus rutilus – 41 fish infected. Dearne (Pastures Bridge); Derwent (Bubwith, Yedingham Fish Havens); Don (Sprotborough); Hull (Hempholme); Ouse (Acaster Malbis, Beningbrough, U/S Linton Weir); Swale (Thornton Bridge); Ure (Boroughbridge).

Accession numbers: Syntype slides of histological sections of whole fish stained with Haematoxylin & Eosin and with Giemsa from roach, chub, dace and minnows have been deposited in the Registry of Aquatic pathology, CEFAS Weymouth (*Rutilus rutilus* RAP 2-F45; *Leuciscus leuciscus* RAP 2-P10; *Leuciscus cephalus* RAP 2-K8; *Phoxinus phoxinus* RAP 2-N5; *Abramis brama* RAP 2-H9).

Description: Coelozoic in lumens of tubules and urinary bladder of fish. In dace co-infected with the digenean *Phyllodistomum* sp., plasmodial stages found attached to the digenean (Figures 4.53 and 4.54). Plasmodia loose within the lumen of the kidney tubule (Figure 4.51) or, most often, attached to epithelia by finger-like projections and containing numerous refringent granules (Figure 4.52). In a single roach specimen, sporogonic stages within plasmodia were noted in the kidney tubules. Polar capsules were elongate and pyriform.

Pathology:

No pathology associated with the parasite was apparent.

Other infections

Occasionally, other myxozoan infections were encountered during the survey. However, due to the extremely low prevalences, more detailed descriptions were not possible. These included infections by *M.* cf. *cycloides* (Figure 4.55) in 2 bleak from the river Ouse and 3 roach from the river Swale. Small, spherical extrasporogonic stages (type 2) were observed in the gall bladders and bile ducts of one roach, one dace and one bleak. An unidentified *Sphaerospora* sp. was noted in the kidney tubule lumens of 1 bream from the river Hull at Hempholme and an unidentified *Myxobolus* sp. eliciting a host response in the kidney of minnow was also noted (Figure 4.56). Numerous infections by unidentified *Myxobolus* spp. were recorded in various elements of the gill to include the following types (following Molnár, 2002): interlamellar-type (3 chub and 4 minnow), intralamellar-type (19 chub), filamental, vascular-type (7 chub, 1 minnow, 2 dace), filamental, epithelial-type (1 bleak, 3 minnow, 1 chub), basifilamental-type (1 chub, 2 minnow) and cartilaginous tissue of the gill arch (2 roach). An unidentified *Myxobolus* sp. in the head cartilage (Figure 4.57) was recorded in 7 roach from the rivers Hull, Ure and Derwent and in 8 dace from the rivers Tees, Nidd, Ure and Ouse.

Figure 4.52. Attachment of type 1 plasmodial stages in the ureter of *Rutilus rutilus*. Note also the presence of early stages of extrasporogonic type 1 stages in the kidney tubule epithelium and the apparent lack of host response. H&E, scale bar = 10µm

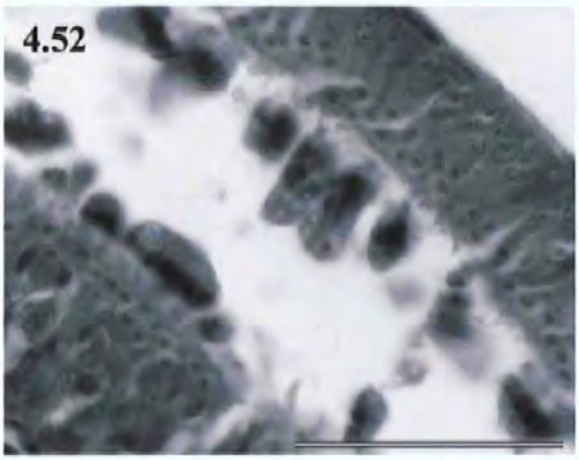
Figure 4.53. Scanning electron micrograph of type 1 plasmodial stages attached to the cuticle of *Phyllodistomum* sp. from the urinary bladder of *Leuciscus leuciscus*.

Figure 4.54. Scanning electron micrograph showing details of type 1 plasmodial stages attached to cuticle of *Phyllodistomum* sp. isolated from the urinary bladder of *Leuciscus leuciscus*.

Figure 4.55. Plasmodia of *Myxobolus* cf. *cycloides* in the basal epithelium of the intestine of *Rutilus rutilus*. H&E, scale bar = 50µm

Figure 4.56. Vigorous host response to a *Myxobolus* sp. in the kidney of *Phoxinus phoxinus*. Only one minnow examined had a myxozoan infection in the kidney. H&E, scale bar = 10µm

Figure 4.57. Histological section through the head cartilage of *Rutilus rutilus* showing the presence of numerous *Myxobolus* sp. spores. Giemsa, scale bar = 10µm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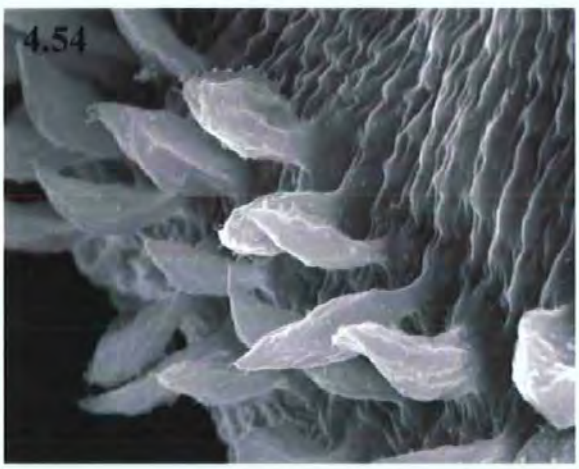
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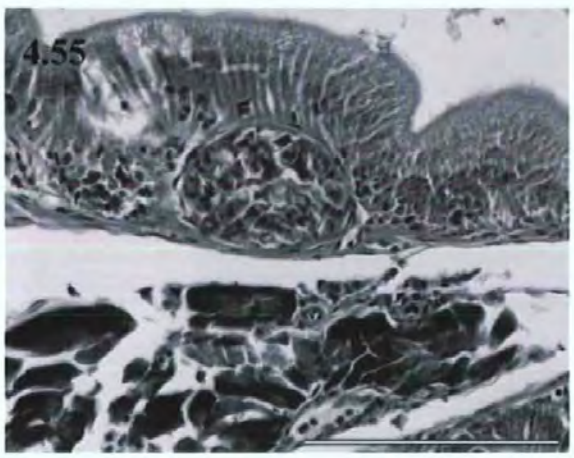
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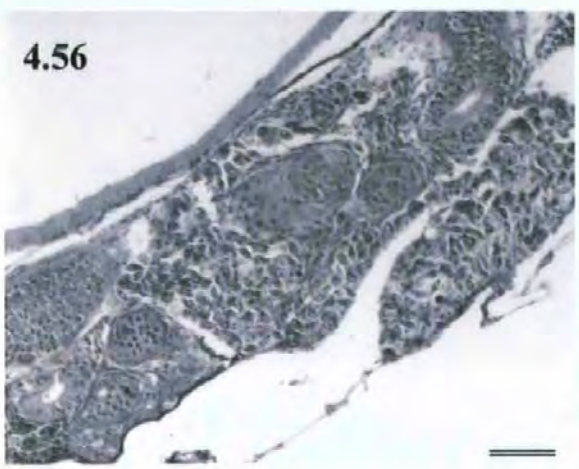
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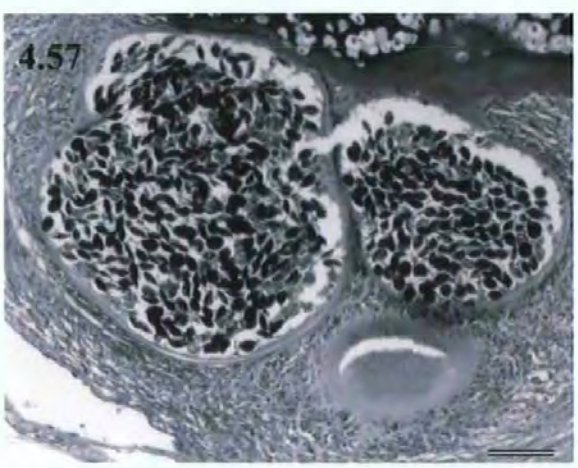
4.57

Although not reported as part of this study, it was recognised that all hosts examined were variously infected by other parasites including protistans, monogeneans, digeneans, nematodes and occasionally cestodes and acanthocephalans.

Discussion

This large-scale study of the myxozoan parasites of 0+ cyprinids in the UK advances the work of Athanassopoulou & Sommerville (1993b) who provided information on the pathogenicity of myxozoan infections in adult roach. Whilst the most of the parasite fauna of the fish species examined in the current study have been studied by a variety of workers, few studies have examined parasitism in juveniles or in fish from wide geographical locations. The method employed in the current study of examining a single sagittal section of fish carries with it the risk of underestimating the types, prevalence and abundance of myxozoan infections in the hosts. For example, only 4 fish were recorded as being infected by *Myxidium pfeifferi* in the bile ducts. Since bile ducts and/or gall bladders were not always present in the section, it is highly likely that the numbers of fish infected and the geographical distribution of the parasite has been been underestimated. In addition, it is recognised that the numbers of *Myxobolus* spp. cysts in the musculature has been underestimated as a result of only taking one sagittal section per fish. Whilst histological examination of fish allows the exact localisation and pathology to be determined, it can lead to misidentifications or provide insufficient material to allow speciation to be determined. The identities of *Myxobolus* spp. in the gills are examples where the use of histology has proved problematic. Many were recorded as *Myxobolus* sp. due to their low prevalence in the current study and the inability to identify parasites to species level using histological techniques.

From the current study, although there are a number of different myxozoan species parasitizing juvenile cyprinids, most appear to be innocuous or elicit only a limited host response. Since the fry examined were approximately between 3 and 5 months old and

myxozoan development is known to be at least that long, it is unlikely that these fish represent survivors of pathological myxozoans and thus, the parasites found can be considered as representative of the range of myxospores of cyprinid fry in the study area.

The measurements and general features of the parasites identified in this study agree well with the published data of known species. However, based on morphological data only, it appears that there are at least two species of *Myxidium* in the kidneys of cyprinids - *Myxidium* cf. *rhodei* in roach, chub, dace, bream and minnows, and *Myxidium barbatulae* in stone loach. Cépède (1906) described *M. barbatulae* from the kidneys of *Barbatula barbatula*, which was relegated to a junior synonym by a number of authors (Shul'man, 1966), due to the wide variation in the measurements reported for *M. rhodei* and the wide host and geographical range for the parasite. Jayasri and Hoffman (1982), however, included *M. barbatulae* in their review of the genus with no comments on the synonomy of the parasite. It has also been suggested that the closely related *Myxidium pfeifferi* in the bile ducts of cyprinids may also be a junior synonym of *M. rhodei* and there have been numerous calls for re-examination of the differences between these two species (Alvarez-Pellitero 1989; Athanassopoulou and Sommerville 1993a; Dyková, Lom, and Grupcheva 1987). Whilst it is feasible for a parasite to be found in a number of different hosts, organs and geographical areas, as exemplified by *M. pseudodispar,* it seems odd that whilst we consider the almost morphologically indistinguishable *Myxidium pfeifferi* and *M. rhodei* as two distinct species, the *M. barbatulae* from stone loach has previously been relegated as a junior synonym of *M. rhodei.* This is in spite of clear morphological evidence in the current paper that *M. rhodei* and *M. barbatulae,* isolated from fish caught in the same river can be clearly distinguished using established morphological criteria (Lom and Arthur, 1989). We thus consider *M. barbatulae* a valid species. Recent advances in molecular biology techniques and transmission trials will prove invaluable in confirming the speciation of *Myxidium* spp. in the kidneys of coarse fishes. It is possible that the

Myxidium sp. in the kidneys of roach, chub, dace and minnows represent different species, even though the site of infection and pathology appear to be identical.

The morphology of *M. pseudodispar* and to a lesser extent *M. musculi* spores in the current study were found to be highly variable both within individual fish and between species of fish. Indeed wide variability was apparent even within the same cyst of individual fish. *M. pseudodispar* from chub appears to be the most variable, those from dace least variable. *M. musculi* shows less variability, especially in plasmodia isolated from gudgeon. It is highly unlikely given the known development of myxospores that any given cyst represents a co-infection of two or more sympatric *Myxobolus* species and thus the variability seen can be considered as normal. Baska (1987) reported that *M. pseudodispar* from roach were of uniform size and shape. However, the current study clearly demonstrates that even spores from roach are variable and thus lack of variability cannot be considered as a taxonomic discriminator.

In carp, the majority of infections by *M. cyprini* are considered to cause only sub-clinical damage to the muscle and local necroses in other organs (Molnar & Kovacs-Gayer, 1985). The potential for disruption of the anatomical and functional unity of the muscle fibres as a result of the host response was reported. In one heavily infected population, clinical and histopathological signs similar to malignant anaemia characterised by hydropic degeneration and dropsy were noted. Similar pathologies have not been reported for *M. pseudodispar* in roach where "no mortalities were noted in intensively infected fish" (Baska, 1987). Additionally, Athanassopoulou & Sommerville (1993a) did not record any changes in the melanomacrophage centres of roach infected by *M. pseudodispar*, a feature which has also been noted for carp infected with *M. cyprini* (Molnar & Kovacs-Gayer, 1985). A probable explanation for this was promulgated by Holzer & Schachner (2001) who suggested that *M. pseudodispar* in chub was a parasite of the haematopoietic tissues, rather than of the musculature. Data from the current study clearly demonstrates that the parasite develops in the musculature, and following sporogenesis, the parasite is moved to

various organs via the reticulo-endothelial system. The most likely explanation for the presence of the parasite in these other tissues is to assist in the destruction or release of the spores into the environment, rather than to complete development. Holzer & Schachner (2001) suggested that chub had lower prevalences of *M. pseudodispar* compared to other cyprinid species due to the enhanced defence capacity of their haematopoietic organs. This view was not supported by the findings in the present study as juvenile chub were the most parasitised both in terms of prevalence and abundance compared with the other hosts examined and reacted most vigorously to the presence of sporogonic forms of both *M. pseudodispar* and *Myxobolus pfeifferi*. Infections by *Myxobolus pfeifferi* were considered to be more detrimental to host survival than *M. pseudodispar* as *M. pfeifferi* plasmodia were much larger and contained more spores than *M. pseudodispar*. In cross section, some individual *M. pfeifferi* plasmodia filled over 20% of a myotome block. *M. pfeifferi* has previously been implicated in the lethal "boil disease" of barbel in Europe and in the current study, large numbers of fish from a wide geographical range were infected by the parasite. Fish in which there has been a response to the sporogonic forms of the *Myxobolus* spp. in the musculature clearly show muscle destruction, not only of the infected muscle fibre but also in the surrounding tissues. It is expected that there is a concomitant impairment of the swimming ability of the fish, such that natural behaviours like predator avoidance and foraging abilities would be reduced.

Regarding the extrasporogonic / plasmodial stages in the kidneys found during the current study, Lom & Dyková (1985) considered the intracellular extrasporogonic stages (extrasporogonic stage type 1) as blocked development stages of *Sphaerospora* or of a *Hoferellus* sp. Molnár (1988) on the other hand considered both the intracellular (extrasporogonic stage type 1) and coelozoic (plasmodial type 1) stages in the kidneys of cyprinids as being different stages in the development of *Myxobilatus legeri* sensu lato based on seasonal differences between the two stages. Molnár (1988) proposed that the parasite first had an intracellular stage, followed by the coelozoic stages in which

sporogenesis eventually took place. Induction of a *Sphaerospora renicola* infection in common carp by injection of swimbladder stages of the parasite into naïve fishes failed to produce the intracellular plasmodial stages and thus it has been suggested that the intracellular stages could not be related to a *Sphaerospora* spp. (Molnár and Kovács-Gayer, 1986). Furthermore, Troullier *et al.* (1996) completed the lifecycle of *Hoferellus carassii* by experimental infections. In their study, they did not find intracellular stages of *H. carassii* despite examination of the fish up to 1½ years post-exposure. The presence of both coelozoic and intracellular extrasporogonic parasite stages in 3-4 month old fish as demonstrated in the current study strongly suggests that development can occur both rapidly and concurrently in these fish. It is possible that this rapid, early phase of the infection was not observed by Troullier et al (1996) since they did not examine their fish until 90 days post-exposure. During the current study, polar capsules were visible in the coelozoic stages of two fish that were pyriform and apical, suggestive of either a *Myxobilatus* spp. or a *Hoferellus* spp. The most likely explanation for the two developmental forms described during the current study are that they are *M. legeri*, based on the shape of the polar capsules and the variety of hosts infected. However, if the view is taken that the intracellular stage leads to the coelozoic stage, it does not explain why, in some fish, only the coelozoic stages were found (see Figure 4.51). Even if the parasite had completed its intracellular development and moved onto the coelozoic stage, it could be expected that pathological or cellular changes would still be visible within the epithelial cells. Although in some fish, both stages occurred simultaneously, most fish examined contained single infections of either the coelozoic or intracellular stage and it is clear from Figure 4.51 that no damage is apparent in the epithelial cells and therefore it is suggested that the coelozoic stages cannot be related to the intracellular stages. Subtle differences occurred in the appearance of the coelozoic stages in different hosts, with those from gudgeon both free within the lumens and attached to the luminal epithelium, those from roach and dace appearing to only be attached to epithelial surfaces. The forms in dace

were also often found intimately associated with the co-occurring digenean *Phyllodistomum* sp. There remains therefore the possibility that both stages represent two different myxozoan development cycles and indeed that they represent different species or even genera of myxozoans in the different hosts. We are therefore unable to assign species names these to developmental stages. The use of molecular techniques such as PCR and sequencing and the use of *in-situ* hybridisation are needed to verify their identity.

In addition to extrasporogonic or plasmodial stages, fish in the current study also contained mature and maturing spores. It would suggest that development of the myxospores in some cases is either rapid or occurs very early on in the life history of the host. It is known that some myxospores can develop to a sporogonic state under experimental conditions 40-80 days after initial infection (Bartholomew *et al.*, 1997; Benajiba and Marques 1993; Székely, Molnár, and Rácz 2001). The presence of mature and maturing myxospores in juvenile wild caught fish confirms that even under fluctuating temperature regimes, present under natural conditions, development can be comparatively rapid. Although the route of entry and exit by the parasite was not explicitly studied in the current study, a number of observations were made. The histozoic *M. pseudodispar*, *Myxobolus pfeifferi* and *M. musculi* were subjected to a vigorous host response once sporogony was complete. The movement of spores from the initial site of sporogony to other tissues and organs may facilitate the release of spores into the environment, prior to death of the host. It has been shown previously that spores of the myotropic *M. artus* infecting carp are released during the lifetime of the host, with up to 3×10^5 spores per fish per day being released (Yokoyama *et al.*, 1996). Although those authors did not state which organs the spores were released from, it is presumed that this occurred via the gills and digestive tract. Nehring *et al.*, (2002) demonstrated that viable *M. cerebralis* myxospores were expelled from live brown trout, contrary to the long standing view that myxospores trapped within tissues could only be released on death and decay of the host. The presence of spores in the mucous cells of the intestine and in the upper layers of the

skin epithelium found in the current study strongly suggest that spores of the myotropic *Myxobolus* spp. in cyprinid fry can be released over the lifetime of the host.

Cyprinid fry are, in some river systems, heavily parasitised. In combination with variability in the quality of habitats, predation and competition this may have a detrimental effect on the physiological tolerance of individuals and populations. The levels of parasitism and pathological responses in some of these hosts were such that it is unlikely that heavily infected fish would survive to adulthood. Consequently, attempts to quantify temporal changes in population structure should consider the role of disease in regulating populations. Failure to consider disease and parasitism as a fundamental component of the ecology, survivability and behaviour of the host may reduce the accuracy of predictions of populations or recruitment based solely on factors such as length, abundance or temperature.

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CHAPTER FIVE

STUDIES ON *Myxobolus buckei* IN THE SPINAL COLUMN OF CHUB, ROACH AND BREAM.

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Abstract

Myxobolus buckei sp. n. is described from the spinal column of *Leuciscus cephalus* (L.), *Rutilus rutilus* (L.) and *Abramis brama* (L.) from freshwater rivers in the North of England. The plasmodia develop within the remnants of the embryonic notochord in the intervertebral spaces. The spores are large, measuring (in μm) 14.0 ± 0.7 × 11.5 ± 0.6 (mean ± SD), smooth, round to ellipsoid in valvular view with several sutural edge markings. The polar capsules are pyriform and of equal size, measuring 7.5 ± 0.5 × 4.2 ± 0.2 (mean ± SD), with 11-12 turns of the polar filament arranged perpendicularly to the longitudinal axis of the polar capsule. The parasite has a large intercapsular appendix and large iodinophorous vacuole. The parasite can be differentiated from all known species of *Myxobolus* by a combination of the morphological characters defined. Infected fish show marked longitudinal compression of the body compared to uninfected individuals of the same year class, a feature which is pathognomonic for the disease. Histologically, host responses ranged from mild hypertrophy of the zygapophyseal process and expansion of the intervertebral membrane to complete hypertrophy and fusion of the vertebrae. Prominent notochord is present in the intervertebral spaces of infected fish and sporogony of the parasite leads to a vigorous focal inflammatory response involving proliferating fibroblast and osteogenic cells. The parasite causes a radial expansion of the centra and extensive dorsal and ventral outgrowths of the vertebrae leading to compression of the spinal cord and blood vessels running through the neural and haemal spines respectively. The parasite is considered highly pathogenic to juvenile cyprinids.

Introduction

Skeletal abnormalities have been reported for a number of fish species under natural and experimental conditions. Most skeletal deformities occur during embryogenesis or early in the life of the fish. A number of factors can lead to these abnormalities including exposure to pesticides including organochlorides and organophosphates (Couch et al. 1979, Middaugh et al. 1990), heavy metals (Roberts 1989), inappropriate temperature or movement during critical phases of embryogenesis (Brown and Núñez 1998) and disease. Arguably, one of the most studied disease causing skeletal deformities is whirling disease caused by the myxozoan *Myxobolus cerebralis* Hofer, 1903. The parasite has a predilection for cartilage and infects fish prior to ossification. Subsequent destruction of the cartilage leads to skeletal deformities in salmonids (Hedrick et al. 1998). A number of other reports of myxozoans causing skeletal deformities have been published. They either cause deformities by direct association with and destruction of the skeletal elements as exemplified by *M. cerebralis* or indirectly by infecting nervous tissues leading to spinal curvatures (see Table 5.1). The majority of spinal deformities associated with myxozoan infection appear to be related to infections in the nervous tissues. Lom et al. (1991) reported vertebral deformities in perch (*Perca fluviatilis* L.) which they attributed to *Myxobolus sandrae* Reuss, 1906 infections of the spinal cord leading to myopathy of the axial muscles and/or of asymmetrical changes in muscle tone as a result of damage to the nerves controlling muscle contraction. Lom et al. (1991) did not examine the brains of affected fish, although it has been suggested that myxozoan infection in brain tissues can lead to spinal deformities (Egusa 1985, Langdon 1987, Maeno et al. 1990, Rothwell and Langdon 1990) Myxozoan infections in brain tissues does not always produce spinal deformities but may produce abnormal or altered swimming behaviour and may thus be a function of the number, size and specific location of spores and/or plasmodia (Langdon 1990, Egusa 1985). Additionally, reductions in the swimming speeds of sockeye salmon

Table 5.1 Host, site of infection and spore dimensions of *Myxobolus* spp. reported from cartilage or implicated in spinal deformities.

Parasite	Host	Site of infection	Spore shape	Intercapsular appendix	Sutural edge markings	Spore length × width	Equal sized polar capsules?	Polar capsule length × width	Polar filament coils
M. aeglefini Auerbach, 1906	Numerous marine fish	Cartilage	Elliptical to subspherical	No	Yes	11.0 × 9.2	Yes	4.2 × ?	5
M. buri Egusa, 1985	*Seriola quinqueradiata*	Brain	Oval	Small	Yes	10.6 × 9.2	Yes	4.5 × 2.8	?
M. cerebralis (Hofer, 1903)	Salmonids	Cartilage	Oval to circular	Very small	No	7.4-9.7 × 7.0-10.0	Yes	4.2-6.0 × 3.0-3.5	5-6
M. cartilaginis (Hoffman, Putz & Dunbar, 1965)	*Lepomis macrochirus*	Cartilage	Subspherical	No	Yes	9.5-10.5 × 8.4-9.5	Yes	5.2-5.6 × 3.0-3.5	5-7
M. dentium Fantham, Porter & Richardson, 1939,	*Esox masquinongy*	Cartilage	Elongate-oval	No	No	11.8-14.5 × 5.5-7.3	Yes	4.5-7.3 × 1.3-3.2	?
M. divergens carassii Jukhimenko, 1986	*Carassius auratus gibelio*	Cartilage	Ellipsoid	No	No	11.0-12.8 ×7.3-7.8	Yes	4.2-5.2 × 2.3-2.6	?
M. encephalicus (Muslow, 1911),	*Cyprinus carpio*	Brain	Oval to subspherical	Small	No	7.0-12.0 × 6.3-10.5	Yes	3.9 × 3.0	5-6
M. eucalii (Guilford, 1965),	*Eucalia inconstans*	Cartilage	Pyriform	No	No	12.0-15.6 × 8.4-10.8	Yes	9.6-12.0 × 3.0-4.8	9-11
M. filamentosus (Haldar, Mukherjee & Kundu, 1981)	*Puntius filamentosus*	Cartilage	Ovoid	?	?	11.2-17.3 × 8.1-12.2	Yes	4.0-7.1 × 2.0-4.0	5-6
M. gangulli (Sarkar, Halder & Chakraborti, 1982),	*Sillago maculata*	Cartilage	Ovoid to ellipsoid	No	Yes?	8.0-10.0 × 4.8-6.5	No	L: 2.8-4.6 × 1.5-2.5 S: 2.2-3.0 × 1.3-2.0	5-6
M. hoffmani (Meglitsch, 1963)	*Pimephales promelas*	Cartilage	Round to oval	No	Yes	8.6-10.8 × 7.8-8.9	Yes	4.6-5.7 × 2.2-2.7	Up to 10

Table 5.1 (Continued)

Parasite	Host	Site of infection	Spore shape	Intercapsular appendix	Sutural edge markings	Spore length × width	Equal sized polar capsules?	Polar capsule length × width	Polar filament coils
M. hyborhynchi Fantham, Porter & Richardson, 1939	*Hyborhynchus notatus*	Cartilage	Oval to subspherical	No	No	9.1-10.9 × 7.3-8.6	Yes	4.1-5.9 × 2.3-2.5	?
M. indirae (Kundu, 1985)	*Cirrhina mrigala*	Cartilage	Oval to spherical	No	No	11.0-14.0 × 9.0-11.0	Yes	4.0-6.0 × 2.0-2.5	8-10
M. intrachondrealis Molnár, 2000	*Cyprinus carpio*	Cartilage	Elongate ellipsoid	Large	No	9.0-11.0 × 6.0-7.0	Yes	3.7-4.7 × 2.0-2.6	9-11
M. nuevoleonensis Segovia, Jiménez, Galaviz & Ramirez, 1991	*Poecilia* spp.	?	?	?	?	?	No	?	10-12
M. petruschewskii Zhukov, 1962	*Myoxocephalus axillaris*	Cartilage	Round	No	No	11.0-12.0 × 11.0-12.0	Yes	5.5-6.5 × 2.7-3.6	?
M. sandrae Reuss, 1906	*Perca fluviatilis*	Spinal cord	Oval	No	Yes	8.0-11.0 × 7.0-9.0	No	L: 3.5-5.7 × 2.0-2.3 S: 3.0-5.1 × 2.0-2.3	6-7
M. scleroperca (Guilford, 1963)	*Perca flavescens*	Cartilage	Pyriform	No	No	10.0-19.2 × 7.2-9.6	No	L: 7.2-13.0 × 2.4-3.6 S: 7.2-12.6 × 2.4-3.6	?
M. spinacurvatura Maeno, Sorimachi, Ogawa & Egusa, 1990	*Mugil cephalus*	Brain	Oval	No	Yes	10.5-12.5 × 9.0-11.0	Yes	3.5-5.0 × 2.5-3.5	?
Myxobolus sp. Rothwell & Langdon, 1990	*Platycephalus bassensis*	Brain	Ovoid	?	No	9.0-11.0 × 6.0-8.5	Yes	3.5-4.0 × ?	6

infected with *M. arcticus* Pugachev & Khokhlov, 1979 in the brain have been reported (Moles and Heifetz 1998).

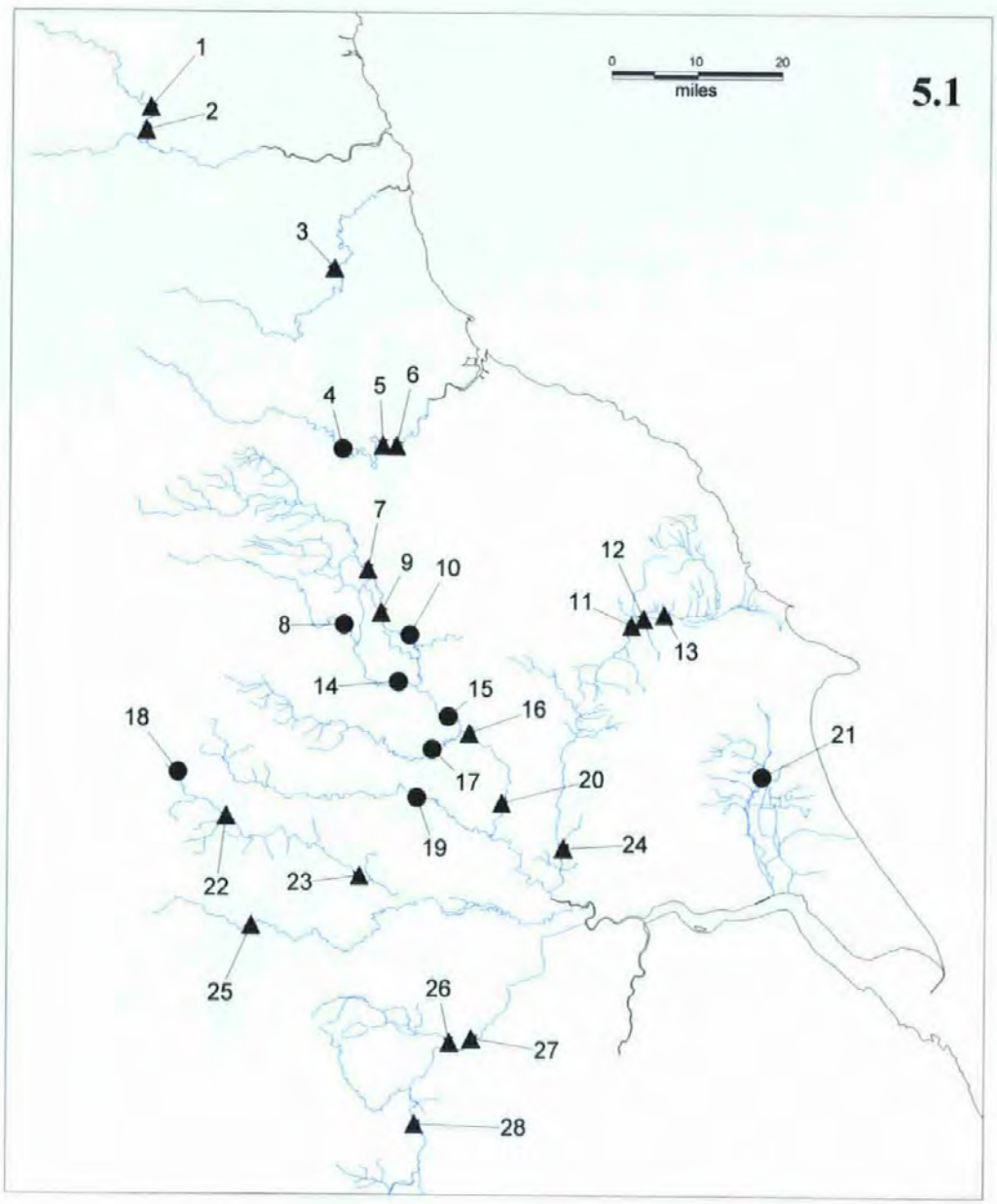
Roach, chub and bream are parasitised by at least 41 species of *Myxobolus*. If subspecies of *L. cephalus* (L.) and *R. rutilus* (L.) are included, the list becomes greater. The morphological measurements and site(s) of infection of *Myxobolus* spp. from these three hosts are listed in Table 5.2. (Bucke and Andrews 1985) reported vertebral deformities in juvenile chub and suggested, without any measurements or detailed morphological studies apparently being done, that the parasite may be *M. ellipsoides* Thélohan, 1892. This tentative assignment to species has become accepted and numerous publications have referred to *M. ellipsoides* as a pathogenic species with a predilection for cartilage (Lom and Dyková 1992, Feist and Longshaw 2000). Following a survey of the parasites of juvenile cyprinids in Yorkshire and Humberside in the north of England, including the "type-locality" where deformed chub were first reported from, we are able to describe the parasite as a previously unidentified species, *Myxobolus buckei*. In addition, this paper describes the pathology associated with the parasite, examines its spatial and temporal distribution and considers the potential impact of the parasite on host populations.

MATERIALS AND METHODS

Young-of-the-year cyprinid fish were collected semi-quantitatively at selected sites in Yorkshire, Humberside, Dorset and Wiltshire between 1993 and 1998 (see Figure 1). Fish were either caught using three sweeps with a 20m × 2m micromesh seine net set along the river margin at each site or caught by hand netting. All fish caught were killed by over anaesthetising in MS222 then fixed in 10% neutral buffered formalin (NBF).

On return to the laboratory, fish were identified to species, fork length was taken and the fish stored in NBF by site and species. For histological studies a sub-sample of fry of each species from each site for each year were taken. A maximum of 30 fish of each species per site were examined. Tissues were softened in 10% formic acid for up to four

Figure 5.1 Map of rivers and sites sampled for cyprinid fry. Where ● = *M. buckei* present and ▲= *M. buckei* absent. 1 = Tyne, Haughton. 2 = Tyne, Station House. 3 = Wear, Durham. 4 = Tees, Croft. 5 = Tees, Low Moor. 6 = Tees, Low Worsall. 7 = Swale, Maunby Demesne. 8 = Ure, Bellflask, 9 = Swale, Skipton, 10 = Swale, Thornton Bridge, 11 = Derwent, Fish Haven Low Marishes, 12 = Derwent, Yedingham Fish Haven, 13 = Derwent, Yedingham, 14 = Ure, Boroughbridge, 15 = Ouse, upstream of Linton Weir, 16 = Ouse, Beningbrough, 17 = Nidd, Kirk Hammerton, 18 = Aire, Carleton Bridge, 19 = Wharfe, Boston Spa, 20 = Ouse, Acaster, 21 = Hull, Hempholme, 22 = Aire, Keighley, 23 = Aire, Knostrop, 24 = Derwent, Bubwith, 25 = Calder, Elland, 26 = Dearne, Pastures Bridge, 27 = Don, Sprotborough, 28 = Rother, Woodhouse.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days, transferred to 70% IMS until being processed to wax blocks on an automatic vacuum infiltration tissue processor. Sections were cut at 3-5 μm and stained routinely with Haematoxylin & Eosin (H&E) or Giemsa stain.

Fresh spores were isolated from samples of fish collected in Yorkshire during June and September 2002 and measurements, photographs and drawings were taken from these samples. Representative images of spores and pathologies were captured, and spores were measured using a Nikon Eclipse E800 microscope with LUCIA™ screen measurement system. Myxozoans were described and measured according to the criteria of (Lom and Arthur 1989). Representative samples of spores were air-dried on glass slides, acetic-methanol fixed, and stained with May-Grünwald-Giemsa. Stained spores and sectioned material from each fish host were deposited in the Registry of Aquatic Pathology (RAP), CEFAS Weymouth and stained spores from *Leuciscus cephalus* and *Rutilus rutilus* were deposited in the collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice. Measurements and drawings of *M. buckei* spores were compared to published descriptions of *Myxobolus* spp. reported to occur in cartilage or implicated in spinal deformities of fish (Table 5.1) as well as those reported from roach, chub and bream (Table 5.2).

For scanning electron microscopy (SEM) studies of spores, two methods were used: (1) Spores isolated from dissected fish were fixed in 2.5% gluteraldehyde in sodium cacodylate buffer, pH 7.4, for a minimum of 24 hours. Spores were then filtered through a 0.1μm ceramic filter; post fixed in 1% osmium tetroxide in sodium cacodylate buffer, pH 7.4, for 1 hour and dehydrated through an acetone series. Filters were critical point dried and sputter coated with 4nm of gold and examined in a JEOL 5200 SEM at 25kV. (2) Histological blocks containing infected fish were dewaxed and post-fixed in xylene containing liquid osmium tetroxide (1% w/v). Samples were held at 37°C for up to 60 minutes until the wax had dissolved then rinsed in several changes of xylene at room temperature. Samples were critical point dried, sputter coated and examined as above. For

Table 5.2 Comparison of *Myxobolus buckei* sp. n. with all *Myxobolus* spp. reported from *Abramis brama, Leuciscus cephalus* and *Rutilus rutilus*. Where Ab = *Abramis brama*, Lc = *Leuciscus cephalus*, Rr = *Rutilus rutilus*, + = present, - = absent, +/- = sometimes, ND = No data.

Parasite	Presence in hosts			Site of infection	Spore shape	Intercapsular appendix	Sutural edge markings	Spore length × width	Equal sized polar capsules	Polar capsule length × width	Polar filament coils
	Ab	Lc	Rr								
***M. buckei* sp. n.**	+	+	+	**Spinal column**	**Round to ellipsoid**	**Large**	+	**12.6-15.4 × 10.2-12.4**	+	**6.0-8.6 × 3.3-4.6**	**11-12**
M. albovae	+	+	-	Gills	Oval	Large	-	10.5-13 × 8-9.8	+	4.8-5.5 × 2.7-3.3	ND
M. bramae	+	+	+	Gills and others	Widely oval	Large	+/-	9-15 × 8-12	+	4-7.2 × 2.5-3	6-7
M. branchialis	-	-	+	Gills	Close to round	Small	-	6.8-8.5 × 5.8-7.6	+	2.5-4.1 × 1.6-2	ND
M. carassii	+	-	+	Body cavity	Ovate, narrow anteriorly	Small	-	13-17 × 8-11	+	5.6-8.5 × 3.5-4	ND
M. chernovae	-	-	+	Gills	Pyriform	No	-	8.5-10.0 × 6.5-7.0?	+	5.5 × 3-4	ND
M. circulus	+	-	-	Gills and others	Close to round	No	-	8.5-12 × 7.5-12	+	3.5-6 × 3.4	5?
M. cycloides	+	+	+	Gills	Elongate ellipsoid	Small	+	12.6-15.0 × 9.0-11.0	+	5.4-7.2 × 3.0-4.5	5-6
M. dispar	+	+	+	Gills	Oval, narrow anterior end	Large	+/-	9-14 × 7.5-10	-	L: 5-8 × 3-4.5 S: 2.5-4.5 × 2-2.7	L: 6-8 S:3-6
M. diversicapsularis	-	-	+	Gills	Widely oval to round	Small	-	8.5-13 × 8.5-12.5	-	L: 3.8-5.5 × 2.2-3.7 S: 2.2-3.3 × 1.1-2.5	L:7-8 S:4
M. dogeli	+	-	+	Various	Round or oval	Large	-	9-16 × 8-15	+	4-6.5 × 3.5-4	ND
M. donecae	-	-	+	Muscles	Elongate-oval	Medium	-	10-13 × 7.4-8.7	-	L: 5 × 3 S: 3.7 × 2.5	6-8?
M. dujardini	+	-	+	Gills	Pyriform	No	-	11-13 × 5-8	+	5-7 × 1-2	ND
M. elegans	+	-	+	Gills	Pyriform	Small	-	13.5-17 × 7.4-10	+	6.8-8 × 2.5-3.5	ND
M. ellipsoides	+	+	+	Various organs	Ellipsoid, wider anteriorly	Small	-	13.0-16.0 × 9.0-11.0	+	4.5 × 2.8	3-6
M. exiguus	+	-	+	Various organs	Close to oval, narrow anterior end	Large	+/-	8-12 × 6-9.3	+	4-7 × 2.5-2.7	5-7
M. gigas	+	+	+	Gills, fins, skin	Round to ellipsoid?	Small	-	17-21.6 × 13-16.2	+	7.5-8 × ?	ND
M. hungaricus	+	-	-	Gills	Oval	No	-	7.3-9.3 × 4.8-6.8	+	4.3-4.4	ND
M. impressus	+	+	-	Gills	Ellipsoid	Small	+	10.5-13.7 × 9.2-11.0	+	5.5-6.8 × 2.8-4.0	6-7
M. infundibulatus	-	+	-	Kidneys, swim bladder	Oval, narrow posteriorly	No?	-	13.4-15.4 × 11-13	-	L: 6.9-7.9 × 4.5-4.8 S: 5.7-6 × 3.6-4.1	ND
M. intimus	-	-	+	Gills	Round to oval	No	-	11.2-12.6 × 9.8-11.2	+	5.6 × 4.2	ND
M. isakovi	-	+	-	Kidney	Ellipsoid	Small	-	13.0-14.0 × 8.4-8.8	+	6.6-7.7 × 3.3-4.2	4-5?

Table 5.2 continued

Parasite	Presence in hosts			Site of infection	Spore shape	Intercapsular appendix	Sutural edge markings	Spore length × width	Equal sized polar capsules	Polar capsule length × width	Polar filament coils
	Ab	Lc	Rr								
M. kubanicus	+	-	-	Intestinal wall, muscles, gills	Rectangular with rounded edges	Small	+/-	11-13.3 × 7-9.6	+	4-5.6 × 3-3.5	ND
M. lomi	-	+	+	Gills	Pyriform	No	-	9-13 × 7-9	+	4-7 × 2-2.7	ND
M. macrocapsularis	+	+	+	Gills	Pyriform	No	-	9-14.5 × 6-9.5	+	5-8.6 × 2.4-3.6	7-9
M. marginatus	-	-	+	Skull	Ellipsoid	Small	-	8.5-10.0 × 5.0-7.0	+	3.0-4.0 × 1.4-1.7	ND
M. minutus	+	+	-	Gills	Oval	Very small	-	6-7 × 4.2-7.5	+	3-4 × 2	ND
M. muelleri	+	+	+	Gills, fins	Oval to ellipsoid	Large	+	7.2-15.0 × 5.8-11.5	+	3.6-5.5 × ?	5-8
M. muelleriformis	-	-	+	Gills	Round	Small	+/-	7.9-12.0 × 7.2-10.8	+	4.5-6.3 × 3.0	5?
M. multiplicatus	-	+	+	Muscles, gills	Oval	No	+	12 × 9.5	+	4.0 × 2.3	ND
M. musculi	+	+	+	Muscles	Oval	Small	-	9.0-13.0 × 8.0-11.0	-	L: 4.5-7.0 × 3.0-4.2 S: 4.2-6.3 × 2.0-3.5	ND
M. nemachili	+	-	+	Connective tissue	Oval	No	+	9.0-12.0 × 8.0-9.0	+	4.0-5.0 × 2.0-3.0	ND
M. obesus	-	-	+	Gills	Ovate	Small	-	11.0-12.0 × 7.5-8.0	+	5.0 × ?	ND
M. oviformis	+	+	+	Gills	Pyriform	Small	-	10.0-13.0 × 8.0-11.0	+	5.0-6.7 × 2.8-4.0	ND
M. pfeifferi	-	-	+	Muscles, gills	Oval to round	Small	-	10.0-13.0 × 9.0-12.2	+	?	ND
M. pseudodispar	-	-	+	Muscles	Elongate oval	No	-	10.0-12.0 × 7.0-9.5	-	L: 4.4-5.6 × 3.0 S: 3.9-4.2 × 2.7	ND
M. rotundus	+	-	+	Gills	Round	Very small	-	9.5-10.0 × 9.3-10.0	+	3.8-5.0 × ?	ND
M. rutili	-	-	+	Gills, fins	Round	Medium	+/-	14.0-16.5 × 10.5-13.5	+/-	4.8-8.4 × 3-4.5	5-6
M. schulmani	+	-	+	Fins	Widely oval	Large	-	16.1-19.0 × 12.2-14.4	+	6.0-9.0 × 5.0-5.5	ND
M. squamaphilus	+	-	-	Scales	Ellipsoid	Large	+	17.0-19.5 × 13.0-14.0	+	6.5-7.0 × 4.0-4.5	7
M. subepithelialis	-	-	+	Skin	Oval, pointed anteriorly	Small	-	8.0-12.0 × 6.0-10.0	+	6.0 × 3.0	ND

examination of vertebral elements by SEM, spinal columns of freshly killed fish were removed intact from fish showing external symptoms of infection and placed in distilled water. The soft tissues were allowed to disintegrate for approximately 1 month at 15°C, after which individual vertebrae were rinsed in fresh distilled water to remove remnants of soft tissues, mounted and coated with 4nm of gold and examined as above.

RESULTS

A total of 4,866 fish were examined histologically, comprising 1,001 roach [*Rutilus rutilus* (L.)], 1,258 chub [*Leuciscus cephalus* (L.)], 501 dace [*Leuciscus leuciscus* (L.)], 1,798 minnow [*Phoxinus phoxinus* (L.)], 28 bleak [*Alburnus alburnus* (L.)], 52 bream [*Abramis brama* (L.)] and 228 gudgeon [*Gobio gobio* (L.)]. In addition, 30 freshly killed fish were examined to assist in the identification and descriptions of the parasite. Infected fish were only found at 9 sites on 8 rivers, out of 28 sites from 15 rivers examined (see Fig. 5.1). Roach, chub and bream were infected each exhibiting similar (see below) pathological responses. Prevalence in rivers positive for *M. buckei* ranged between 0 and 68.42% (see Table 5.3). In addition, one minnow from Boston Spa collected in 1993 and one minnow each from Kirk Hammerton collected in 1997 and 1998 harboured a *Myxobolus* infection in the spinal column. One dace each collected in 1996 and 1998 from Low Moor, River Tees and three dace collected from upstream of Linton Weir in 1998 also had a *Myxobolus* infection in the spinal column. In dace and minnow, this *Myxobolus* sp. may be conspecific with *M. buckei*; however the host response to the parasite was minimal. Prevalence of infection in infected rivers was between 3.33% and 68.42%. Infected bream were only found at Boston Spa in 1996 and at Hempholme in 1997. Infected chub were found at Bellflask in 1998, Boroughbridge in 1993, Boston Spa in 1993, 1994, 1995, 1996 and 1997, Carleton Bridge in 1993, Croft 1998, Kirk Hammerton in 1998, upstream of Linton Weir in 1998 and Thornton Bridge in 1996. Infected roach were found only found

Table 5.3 List of sites, grid references, numbers of fish examined (N) and prevalence (%) of *M. buckei* in *A. brama*, *L. cephalus* and *R. rutilus*

				1993		1994		1995		1996		1997		1998	
Site	NGR	Number on map	Species	N	%	N	%	N	%	N	%	N	%	N	%
Ure, Bellflask	SE295775	8	*A. brama*	-	-	15	0	-	-	-	-	-	-	-	-
			L. cephalus	-	-	-	-	-	-	-	-	-	-	30	16.67
			R. rutilus	-	-	-	-	-	-	-	-	-	-	-	-
Ure, Boroughbridge	SE397670	14	*A. brama*	-	-	-	-	-	-	7	0	-	-	-	-
			L. cephalus	14	21.43	23	0	9	0	20	0	31	0	-	-
			R. rutilus	10	0	-	-	8	0	20	0	20	0	-	-
Wharfe, Boston Spa	SE433457	19	*A. brama*	-	-	-	-	-	-	1	100	-	-	-	-
			L. cephalus	19	68.42	10	20	10	40	29	51.72	27	44.44	2	0
			R. rutilus	10	0	11	0	18	0	15	0	18	0	26	0
Aire, Carleton Bridge	SD985502	18	*A. brama*	-	-	-	-	-	-	-	-	-	-	-	-
			L. cephalus	5	20	6	0	2	0	24	0	12	0	1	0
			R. rutilus	-	-	-	-	-	-	-	-	-	-	-	-
Tees, Croft	NZ290098	4	*A. brama*	-	-	-	-	-	-	-	-	-	-	-	-
			L. cephalus	-	-	19	0	-	-	20	0	30	0	28	3.57
			R. rutilus	-	-	-	-	-	-	-	-	-	-	-	-
Hull, Hempholme	TA079498	21	*A. brama*	-	-	-	-	-	-	-	-	5	80	-	-
			L. cephalus	-	-	-	-	-	-	-	-	-	-	-	-
			R. rutilus	-	-	24	12.5	18	61.11	15	26.67	30	23.33	-	-
Nidd, Kirk Hammerton	SE461546	17	*A. brama*	-	-	-	-	9	0	-	-	-	-	-	-
			L. cephalus	-	-	4	0	10	0	-	-	30	0	27	18.52
			R. rutilus	5	0	23	0	-	-	27	0	7	0	3	0
Ouse, u/s Linton Weir	SE491606	15	*A. brama*	-	-	-	-	-	-	-	-	-	-	-	-
			L. cephalus	-	-	-	-	-	-	-	-	-		30	3.33
			R. rutilus	-	-	-	-	-	-	-	-	-	-	30	0
Swale, Thornton Bridge	SE432713	10	*A. brama*	-	-	-	-	-	-	-	-	-	-	-	-
			L. cephalus	10	0	22	0	20	0	22	4.55	5	0	4	0
			R. rutilus	10	0	18	0	10	0	24	0	37	0	8	0

at Hempholme in 1994, 1995, 1996 and 1997. All other species of fish examined were negative by histological assessment.

***Myxobolus buckei* sp. n.** Figs. 5.2-5.4

Vegetative stages. Small, white, round to ellipsoid plasmodia measuring 0.3-0.6 mm present in the intervertebral spaces. In latter stages of infection, plasmodia trapped within ossified elements of spine.

Spores. Spores typical of the genus *Myxobolus*. Mature spores smooth, round to ellipsoid with several sutural edge markings (normally 5-7), slightly variable in shape (Figures 2, 3 and 4). Polar capsules pyriform, apical of equal size. Polar filament with 11-12 coils, perpendicular to the longitudinal axis of the polar capsule. Large intercapsular appendix. Sporoplasm binucleate, filling entire extracapsular spore cavity. Large iodinophorous vacuole. Spore dimensions (μm) based on 30 fresh specimens from *Leuciscus cephalus*, as range with means ± SD in parentheses: Spores 12.6-15.4 (14.0 ± 0.7) × 10.2-12.4 (11.5 ± 0.6). Polar capsules of equal size extend beyond the mid-length of the spore and measure 6.0-8.6 (7.5 ± 0.5) × 3.3-4.6 (4.2 ± 0.23).

Type host: *Leuciscus cephalus* (L.) (Pisces: Cyprinidae).

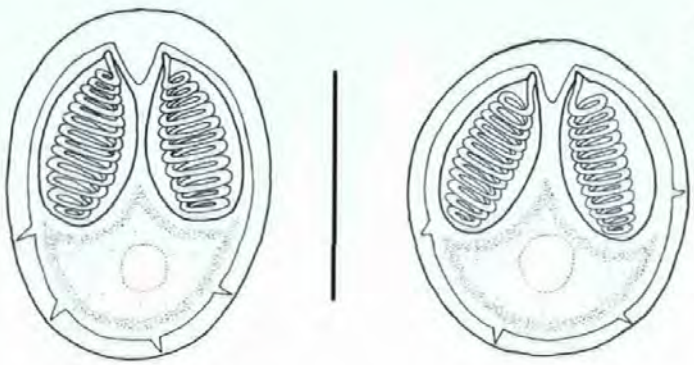
Other hosts: *Abramis brama* (L.), *Rutilus rutilus* (L.). Possibly *Phoxinus phoxinus* (L.) and *Leuciscus leuciscus* (L.).

Type locality: Boston Spa, River Wharfe, UK (NGR SE433457).

Other localities: Bellflask and Boroughbridge, River Ure; Carleton Bridge, River Aire; Croft, River Tees; Hempholme, River Hull; Kirk Hammerton, River Nidd; upstream of Linton Weir, River Ouse; Thornton Bridge, River Swale.

Site of infection: Plasmodia initially in the intervertebral spaces of the spinal column, replacing the notochord. Spores and plasmodia eventually found in cartilaginous and ossified elements of the spinal column.

Figure 5.2. *Myxobolus buckei* sp. n., frontal view of mature spore. Scale bar = 10μm



5.2

Prevalence: Up to 68%

Type material: Syntype slides of histological sections of whole fish stained with H&E and with Giemsa stain have been deposited in the Registry of Aquatic Pathology, CEFAS Weymouth [*Abramis brama* (RAP Accession No. 807); *Leuciscus cephalus* (RAP Accession No. 808); *Rutilus rutilus* (RAP Accession No. 809)]. May-Grünwald-Giemsa stained smears of isolated spores from roach and chub have also been deposited in the RAP with the same accession numbers and in the collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice Nos. DPF-007 and DPF008.

Etymology: This parasite is named for Mr David Bucke formerly of the CEFAS Weymouth Laboratory who first reported the infection in juvenile *Leuciscus cephalus* (Bucke and Andrews 1985).

Taxonomic affinities

The original report of *M. buckei* sp. n. in chub was tentatively referred to as *M. ellipsoides*. However, *M. ellipsoides* is elliptical, with either a small or non-existent intercapsular appendix and is smaller than *M. buckei* both in length and width and has smaller polar capsules.

Most of the 41 *Myxobolus* spp. reported from roach, chub and bream can be discriminated from *M. buckei* as they do not resemble it morphologically in valvular view with the exception of *M. bramae*, *M. branchialis*, *M. dogeli*, *M. muelleri*, *M. muelleriformis*, *M. multiplicatus*, *M. nemachili*, *M. pfeifferi*, *M. schulmani* and *M. squamaphilus*. *M. bramae* occurs on the gills and overall is smaller than *M. buckei* in all dimensions. The polar capsules of *M. branchialis* are widely spaced and in overall dimensions, the spores of *M. branchialis* are smaller. Spores of *M. dogeli* narrow slightly at the anterior end, have a much wider sutural line and do not possess sutural edge

Fig. 5.3. *Myxobolus buckei* sp. n. viewed under differential interference contrast microscopy. Scale bar = 10 µm.

Fig. 5.4. Scanning electron microscopy images of *Myxobolus buckei* spores. a-c - isolated spores, note presence of sutural edge markings. d – view of spores *in-situ* in material previously prepared for histological examination. Presence of sutural edge denoted by arrow. Scale bar = 5 µm.

Fig. 5.5. Normal (a) and infected (b) young of the year *Leuciscus cephalus*. Note very apparent longitudinal compression of infected fish compared with uninfected fish from the same year class. Scale bar = 10 mm.

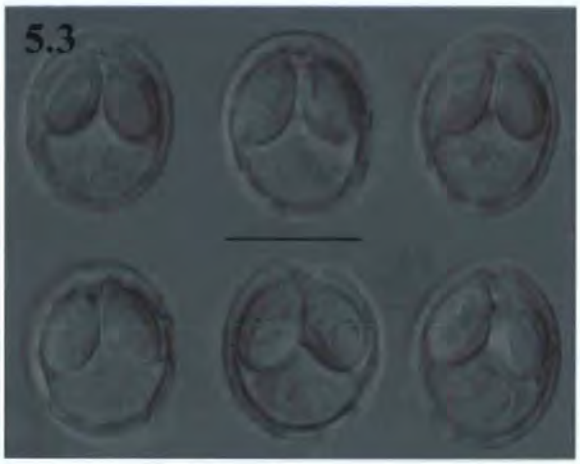
Fig. 5.6. Histological section through an uninfected chub vertebrae, where sc= spinal cord, d= digenean, ns= neural spine, n= notochord, ivm= intervertebral membrane. Giemsa stain. Scale bar = 50 µm.

Fig. 5.7. Scanning electron microscopy image showing anterior face of vertebrae isolated from chub parasitised by *M. buckei*. Note the distended and distorted anterior face and zygapophyseal elements. Scale bar = 500 µm.

Fig. 5.8. Scanning electron microscopy image of the posterior face of infected vertebrae. Scale bar = 500 µm.

Fig. 5.9. Histological section through vertebrae of chub showing mild symptoms of infection with *M. buckei*. Giemsa stain. Scale bar = 50 µm.

Fig. 5.10. Severe compression and fusion of vertebral elements of bream infected with *M. buckei*. Note inflammatory response in periphery of neural and haemal spines. H&E. Scale bar = 50 µm.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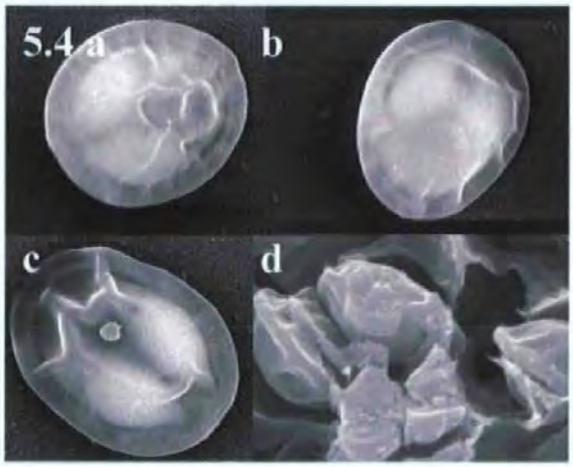
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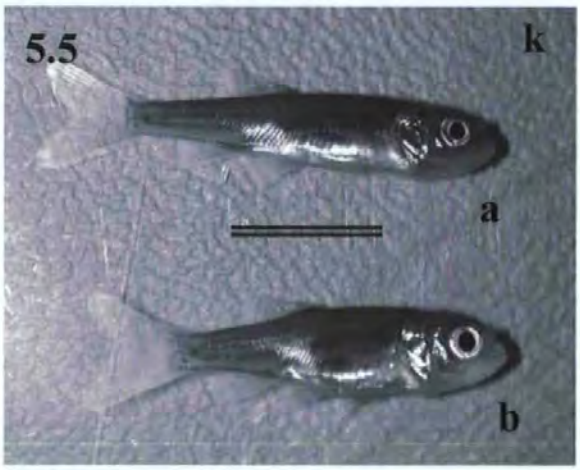
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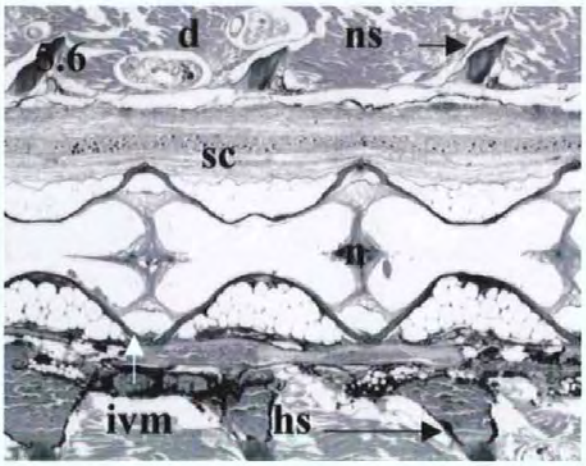
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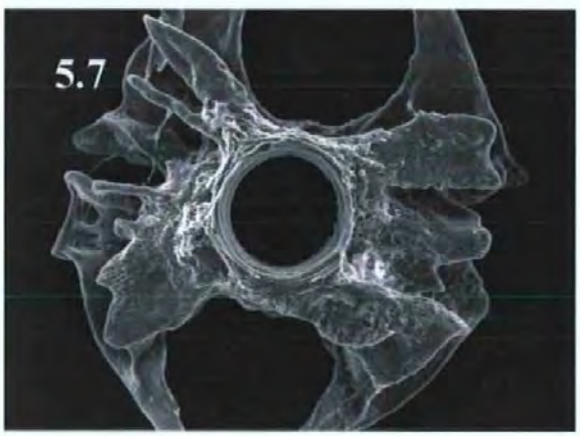
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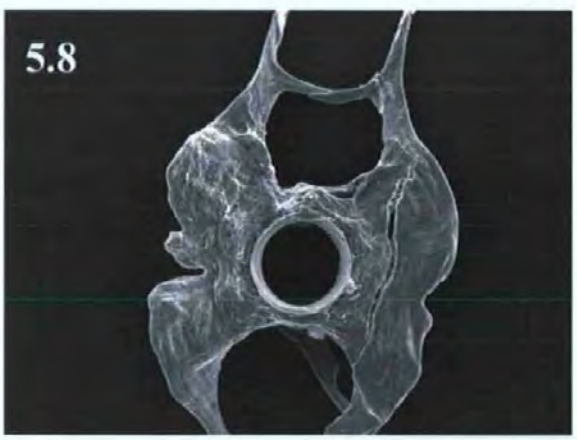
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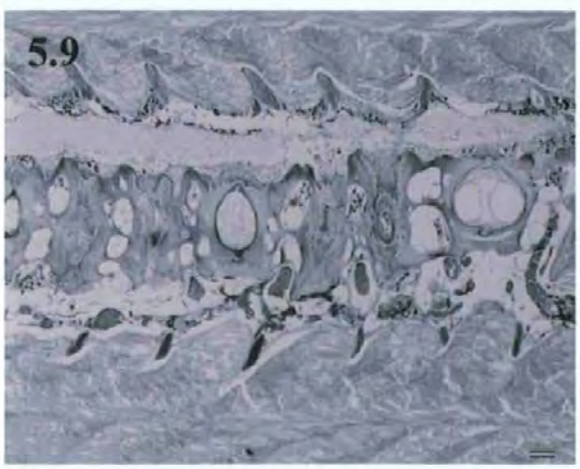
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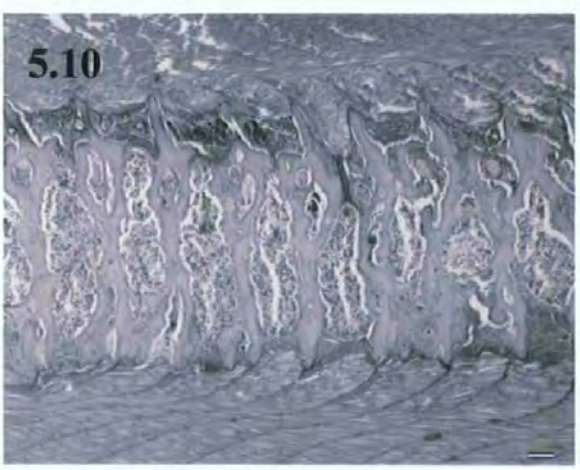
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markings. *M. muelleri* morphology is highly variable, however, it can be discriminated from *M. buckei* by a number of features. The intercapsular appendix is smaller than *M. buckei*, has more sutural edge markings and only 5 to 8 filament turns in the polar capsule. In addition, the polar capsules are smaller than in *M. buckei* and do not extend beyond the midline of the spore. *M. muelleriformis* is much rounder than *M. buckei*, is smaller and does not possess sutural edge markings. *M. multiplicatus* is more ellipsoid and has 12-14 sutural edge markings, the polar capsules do not extend beyond the midline of the spore, and are shorter than those in *M. buckei*. *M. nemachili* possess a greater number of sutural edge markings, smaller polar capsules and a smaller intercapsular appendix. The polar capsules of *M. pfeifferi* do not extend beyond the midline of the spore, and the intercapsular appendix is small. Whilst the length of *M. pfeifferi* spores falls within the range of *M. buckei* the width is smaller. *M. schulmani* is much larger than *M. buckei* in all dimensions and has a large intercapsular appendix. *M. squamaphilus* spores are larger than in both length and width and are more ellipsoid in shape and there are only 7 turns to the polar filament in *M. squamaphilus* compared with 11-12 in *M. buckei*.

Of the *Myxobolus* spp. implicated in spinal deformities or having a predilection for cartilaginous tissues, *M. buckei* can be differentiated from them by a number of morphological features. *M. aeglefini* is smaller than *M. buckei*, possesses a large sutural edge, and only 5-6 turns of the polar filament. *M. cerebralis* is smaller than *M. buckei*, possess no intercapsular appendix, no sutural edge markings and only has 5-6 turns to the polar filament. *M. cartilaginis* is smaller than *M. buckei*, has a greater number of sutural edge markings and 7 turns to the polar filament. In addition, the polar capsules do not extend beyond the midline of the spore. *M. dentium* spores are ellipsoid in valvular view. *M. encephalicus* is variable in morphology, but are generally subcircular in valvular view, smaller than *M. buckei* with only 5-6 turns of the polar filament. *M. eucalii* spores are pyriform with, occasionally, unequal polar capsules extending to the posterior end of the spore. *M. filamentosus* spores are narrower than *M. buckei* with smaller polar capsules and

5-6 turns of the polar filament. *M. gangulli* spores are ovoid to ellipsoid in valvular view, with a slightly pointed anterior end and possess unequal polar capsules. *M. hoffmani* is smaller; possess more sutural edge markings with fewer filament turns. *M. hyborhynchi* is also smaller and the polar capsules extend well beyond the midline of the spore. *M. indirae* are oval to spherical in valvular view with a slightly pointed anterior end. Polar capsules are smaller, do not extend beyond the midline of the spore and possess 8-10 turns of the polar filament. *M. intrachondrealis* spores are elongate ellipsoid in valvular view and smaller than *M. buckei*. *M. nuevoleonensis* spores possess markedly unequal polar capsules. *M. petruschewskii* is completely spherical in valvular view, and is smaller than *M. buckei* both in its overall size and in the size of the polar capsules. *M. sandrae* spores are elliptical, smaller and do not possess an intercapsular appendix. *M. scleroperca* is pyriform in valvular view and possess two unequal polar capsules. *M. spinacurvata* is rounder and smaller than *M. buckei*, does not possess an intercapsular appendix or sutural edge markings and its polar capsules do not extend beyond the midline of the spore. *M.* sp. spores reported from *Platycephalus bassensis* are ovoid and smaller than *M. buckei*, the polar capsules are smaller, do not extend beyond the midline of the spore and have 6 turns to the polar filament.

In summary, *M. buckei* does not conform to any of the descriptions of *Myxobolus* spp. previously reported from cyprinids in the UK or Europe and can be differentiated from all known species of *Myxobolus* by the presence of a large intercapsular appendix, its somewhat large size and overall dimensions, polar capsules extending below the mid-line, 11-12 coils in the polar capsule and its tissue tropism.

Development & Pathogenicity

Externally infected fish show marked longitudinal compression of the body compared to uninfected individuals of the same year class (Fig. 5.5). This compression of the fish is pathognomonic for the disease. Lordosis or scoliosis not seen. The normal

Fig. 5.11. Plasmodia of *Myxobolus buckei* (arrowed) in the intervertebral spaces. Both precaudal and caudal vertebrae are infected as denoted by the position of the hind kidney (k). Scale bar = 1 mm.

Fig. 5.12. Inflammatory response (*) surrounding centra of infected vertebrae. Parasite plasmodia (p) are apparent in between successive vertebrae. H&E. Scale bar = 50 μm.

Fig. 5.13. Expansion of the intervertebral membrane (ivm) leading to compression of the spinal cord (*). Note the difference in morphology of the anterior (asc) and posterior (psc) spinal cord. Scale bar = 50 μm.

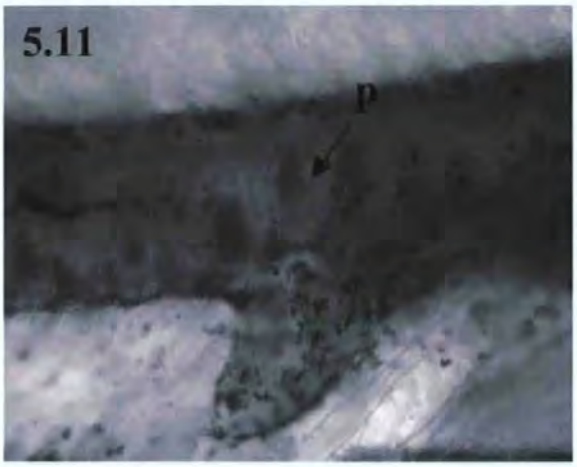
Fig. 5.14. Limited expansion of intervertebral membrane causing compression of the spinal cord. Scale bar = 50 μm.

Fig. 5.15. Development of spores within plasmodia (p) situated between successive vertebrae. Note the difference in development of spores between the two adjacent plasmodia. H&E. Scale bar = 50 μm.

Fig. 5.16. Presence of *M. buckei* spores in the spaces between the spinal cord, vertebral elements and nerve bundles. The parasite is absent from the nervous tissue. Giemsa stain. Scale bar = 50 μm.

Fig. 5.17. *M. buckei* spores (arrowed) trapped in a fibroblast matrix. Giemsa stain. Scale bar = 50 μm.

Fig. 5.18. *M. buckei* spores (arrowed) trapped within ossified cartilage of the vertebrae. Giemsa stain. Scale bar = 50 μm.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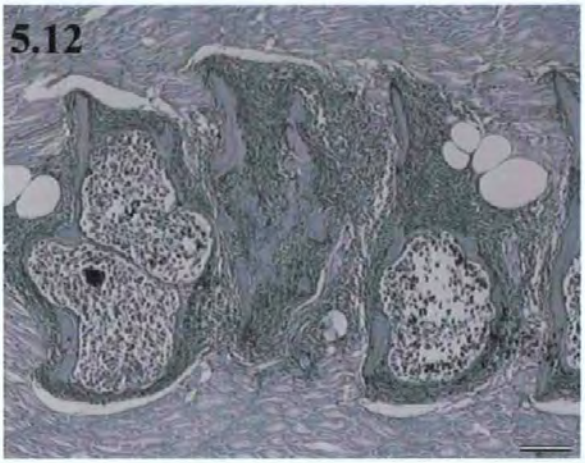
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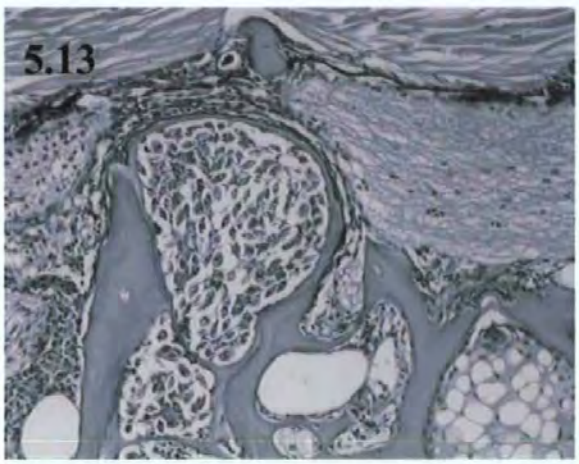
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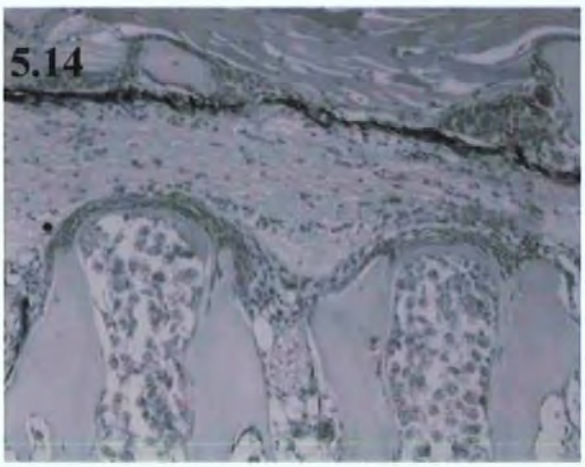
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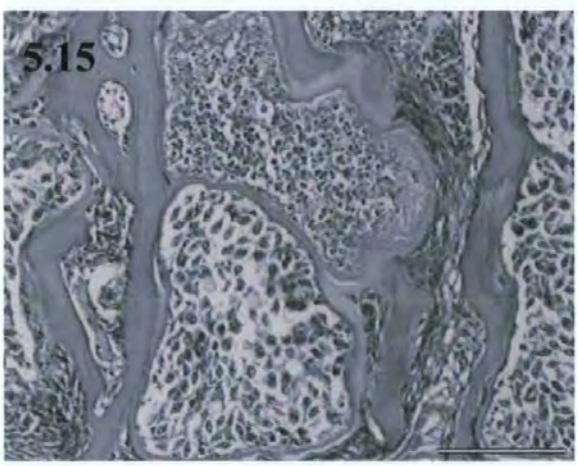
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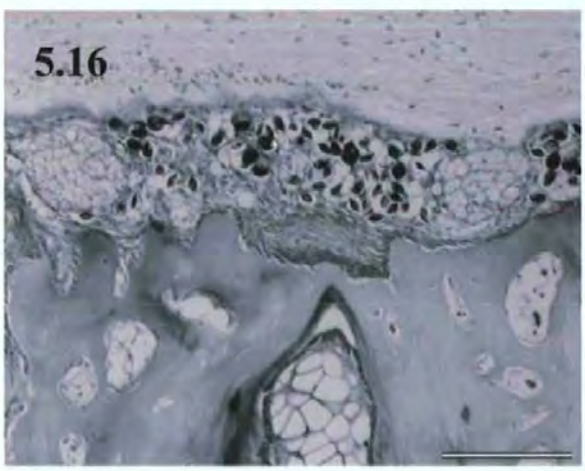
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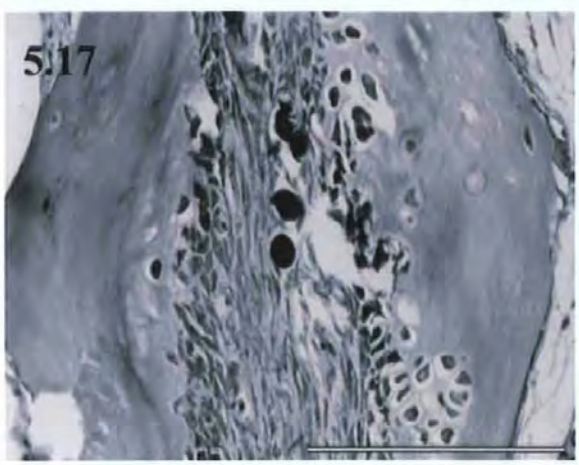
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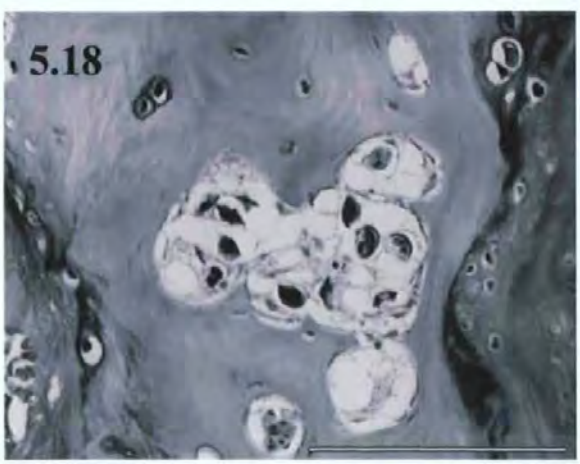
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architecture (Fig. 5.6) of the vertebrae is disrupted in the presence of the parasite plasmodia and spores (Figs. 7.5, 7.8). A variety of vertebral lesions were exhibited in fish parasitised by *M. buckei*, ranging from mild hypertrophy of the zygapophyseal process and expansion of the intervertebral membrane to complete hypertrophy and fusion of the vertebrae (Figs. 5.9, 5.10). The extent of pathological change appears to be dependent on the degree of ossification of the host at the time of exposure and presumably the parasite invades the host prior to ossification. The parasite has a predilection for cartilaginous tissue and the remnants of the embryonic notochord and plasmodia are found in the intervertebral spaces (Fig. 5.11) of both the caudal and precaudal vertebrae. The longitudinal compression noted externally is apparent in histological sections (Fig. 5.10) with a concomitant marked hypertrophy of the vertebral walls (Figs. 5.12-5.14). In heavy infections, all elements of the vertebrae are affected including hypertrophy of the zygapophyseal processes to accommodate the developing plasmodia. Hypertrophy of the vertebral centra wall and fusion of adjoining centra is common. In intervertebral spaces infected by the parasite, prominent notochord is present. There is a vigorous focal inflammatory response involving proliferating fibroblast (Fig. 5.12) and osteogenic cells to the presence of the parasite. The parasite causes a radial expansion of the centra and intervertebral membrane (Fig. 5.13) and extensive dorsal and ventral outgrowths of the vertebrae (Fig. 5.11) leading to compression of the spinal cord and blood vessels running through the neural and haemal spines respectively (Figs. 5.13, 5.14). Sporogony is asynchronous with different plasmodia developing at different rates (Fig 5.15). Loose parasites become trapped in a number of spaces associated with the vertebrae (Figs. 5.16, 5.17) and within the ossifying cartilage (Fig. 5.18) although no destruction of parasite stages is apparent.

DISCUSSION

Bucke and Andrews (1985) reported the presence of a *Myxobolus* sp. associated with wild *Leuciscus cephalus* which they tentatively assigned to *M. ellipsoides*. We have been able to collect samples of fish from the same site that Bucke and Andrews (1985) used to describe the pathology associated with that *Myxobolus* sp. In addition, a re-examination of the original samples collected by Bucke and Andrews (1985) has confirmed that the pathology described in the original report conforms to that contained herein. Consequently the parasite infection described by Bucke and Andrews (1985) is due to *M. buckei* and not *M. ellipsoides*. We propose that *M. ellipsoides* is not a pathogenic species, at least not as previously reported in chub with spinal deformities. The report by Bucke and Andrews (1985) was based on the histological assessment of 3 fish from one site on a Yorkshire river. We have been able to extend the work to include a larger number of rivers and collect temporal data on parasite prevalence. In many of the sites sampled, *M. buckei* occurs sporadically, appearing in some years and not in others. At Boston Spa, 0+ chub are consistently infected with prevalences ranging from 20% to 68%. In contrast, chub from Boroughbridge were infected at a prevalence of around 21% in 1993, and the parasite has not been recorded from this site since. Interestingly, infected roach were only recorded at Hempholme, despite being caught at other sites where chub were infected. The factors which specifically predispose roach to infections by *M. buckei* at that site and for the variations in host susceptibility and in spatial and temporal differences are unknown but may be related to a number of multifactorial biotic and abiotic factors. It is known that at each site and in different years other diseases are present or absent (Longshaw and Feist, unpublished data), including other myxozoans, digeneans such as *Bucephalus polymorphus* and coccidian infections which may interact synergistically with *M. buckei* to exacerbate the host susceptibility.

Landsberg and Lom (1991) listed 444 species of *Myxobolus* that has been described from fish and amphibians. That number has risen over the past decade due to further

descriptions (e.g. Chen and Ma 1998) and confirmation of the taxonomic status of some species (Molnár et al. 2002). A large number of *Myxobolus* spp. have been described from cyprinids around the world, and within the genera *Rutilus, Leuciscus* and *Abramis* at least 40 species have been reported. There was some controversy regarding the use of tissue tropism as a valid taxonomic criterion for myxozoans until the work of Andree et al. (1999) that demonstrated, using rDNA sequence information, that tissue specificity in *Myxobolus* spp. could be used as a diagnostic criterion. The extreme tissue specificity exhibited by *M. buckei* is further evidence of this phenomenon. It is recognised that some myxozoan infections can show low host specificity, especially amongst the cyprinid-infecting myxozoans, whilst maintaining high tissue specificity (Hedrick et al. 1993, Hedrick et al. 1998, Molnár et al. 2002). Since *M. buckei* appears to be a parasite of at least four genera and five cyprinid species it is not unusual in this respect.

The presence of the parasite in *P. phoxinus* and *L. leuciscus* is unusual in that the tissue responses to the parasite are markedly different to the responses in the other three hosts. In dace and minnow there is only a slight expansion of the intervertebral membrane. The reason for this is unclear but may be due to minnow and dace being accidental hosts or related to the biology of the fish. It is known that dace spawn earlier than roach, bream and chub in UK waters. By spawning earlier, dace are able to ossify sooner than other hosts in the river system and will presumably move out of the river margins once they reach a suitable size. If the ossification occurs prior to the release of presumptive actinospore stages, dace may be able to mitigate the effect of the parasite.

The severity of the disease caused by *M. buckei* in roach, chub and bream appears to be related to the degree of oesteological development. Data on the ossification of cartilage in fish is lacking, though Economou et al. (1991) suggested that ossification of the spinal column of *L. cephalus* occurred in fish over 8mm in length. Therefore it would be expected that the infection of fish by the parasite should occur when fish are smaller than 8mm. The presence of spore forms of the parasite in fish that are approximately 3-5

months old would support the idea that sporogony occurs rapidly in these hosts and that infections occur very early in the development of the fish. Markiw (1992) demonstrated that *M. cerebralis* developed in two-month-old rainbow trout that were ossifying when exposed to actinospore stages of the parasite. However, many did not display clinical signs of whirling disease. Whilst Markiw (1992) was able to infect older, ossified fish with actinospore stages of *M. cerebralis*, all of these fish were asymptomatic for clinical signs of whirling disease. Whether chub, roach and bream can be infected but not display the characteristic spinal compression and fusion after ossification occurs remains unknown. In *M. cerebralis* infections, fish show signs of the characteristic "whirling" motion and in some cases skeletal deformities (MacConnell and Vincent 2002). Rose et al. (2000) demonstrated that the whirling behaviour is a consequence of pressure on the spinal cord and lower brain stem. Whilst cyprinids infected with *M. buckei* do not exhibit signs of whirling suggestive of an effect on the nervous control mechanisms, the obvious compression of the spinal cord strongly implies that the developing parasite is detrimental to the nervous system. Infected fish show erratic swimming patterns characterised by a short, jerky lateral motion. Burst swimming is compromised with few infected fish able to sustain long periods of burst activity or typical escape behaviour (M. Longshaw, unpublished observations). The combination of spinal cord compression and fusion of the vertebrae appear to lead to poor swimming performance ultimately compromising host survival.

The pathology associated with the parasite is, especially during the early stages, reminiscent of fish exposed to pesticides as described by Couch et al. (1979) and Middaugh et al. (1990) respectively. Couch et al (1979) suggested a number of mechanisms by which herbicides may directly or indirectly influence vertebral dysplasia in fish including influencing or stimulating osteogenic tissues and cells or the hormonal control of calcium utilisation and compartmentalisation in the fish host. It is interesting to speculate on whether *M. buckei* has a similar mechanism in fish. The determination of the

exact mechanisms by which the fish responds to the parasite or the parasite influences the fishes response may assist in development of control methods for myxozoans such as *M. buckei* and *M. cerebralis* that lead to cartilage destruction.

There is strong circumstantial evidence from the current study that *M. buckei* may have a detrimental effect on host populations. Based on histological assessment, the prevalence can be as high as 68% in certain rivers. In the rivers Ure and Swale, no adults with external symptoms of the disease have been recorded and in the river Wharfe, the number of "stumpy" adults recovered varies between 5 and 20% (P Frear, unpublished observations). As it is unlikely that fish are able to recover from the disease, it can be assumed, based on the severe pathological response, that in some affected populations most of the fry do not survive to recruitment age. The parasite is extremely pathogenic leading ultimately to the death of the host, almost certainly during the first winter. The actual prevalence and distribution of the parasite across the UK and in other areas where the hosts are found are as yet unknown and it would be interesting to address the impact on populations using a multidisciplinary approach involving disease specialists and population biologists. The factors determining host susceptibility and the lifecycle of the parasite are currently unknown.

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CHAPTER SIX

MODELLING THE EFFECT OF DISEASE ON HOST POPULATIONS

To be submitted to *Canadian Journal of Fisheries and Aquatic Sciences* as Longshaw, M., Jones, S.E., Frear, P.A., Nunn, A.D., Cowx, I.G. and Feist, S.W. "Do diseases have any impact on fish populations? Evidence from freshwater cyprinid populations in England"

Abstract

Previous studies on the factors that determine recruitment success, fish length and relative abundance in fish populations have excluded the role that disease may have in determining these factors. To attempt to address this situation, a total of 1053 young of the year (0+) cyprinid fry, comprising 563 chub (*Leuciscus cephalus*) and 490 roach (*Rutilus rutilus*), were collected semi-quantitatively from Acaster, Beningbrough, and upstream of Linton Weir on the river Ouse, Thornton Bridge on the River Swale, and from Boston Spa on the river Wharfe in Yorkshire, northern England every September between 1993 and 2002. Histopathological studies were conducted on these fish to determine the types of pathogens present, to provide an assessment of the pathological responses in individuals and to quantify the levels of parasitism in the population using parasite prevalence, and where appropriate by abundance. Statistical regression models were developed, incorporating a number of abiotic and biotic factors along with parasite prevalence and abundance in order to determine the role of parasitism in roach and chub population success at the selected sites. These models are presented and indicate that parasitism may be a major contributory factor in host population success. It is recommended that future studies should incorporate a measure of disease in population modelling.

Introduction

Reports of parasite induced host mortality (PIHM) in teleost fishes and their impact of disease on host population dynamics are relatively rare. Many studies demonstrating an effect of PIHM have been inferred from data collected post mortality in wild fish. None have successfully linked these mortality events to an effect on year class strength or recruitment success. Indeed, most studies on recruitment and population success have failed to consider PIHM as a factor in host mortality. Numerous studies on fish population dynamics have investigated the role of a number of biotic and abiotic factors in determining population success. Few have considered the role of disease and indeed those that have, invariably did not consider disease to be a major driver. Additionally, any measures of mortality have been calculated post-mortality. No studies to date have considered the role of disease in regulating juvenile coarse fish populations, despite Cowx (2001) stating the importance of identifying "the critical factors which act upon or within coarse fish to regulate their population structure and dynamics".

Various methods have been developed to measure PIHM. Lester (1984) suggested six possible methods for estimating a mortality effect due to parasites in fish populations including (1) through autopsies, (2) by determining the frequency of infections known to be lethal, (3) by observing a decrease in the prevalence of long lived parasites with host age, (4) observation of a decrease in variance to mean ratio with host age, (5) comparison of observed frequencies of a combination of two independent events with the theoretical probability of their occurrence and (6) comparison of the observed frequency distribution with a projected frequency based on lightly infected fish. These methods, whilst useful, can generally only be used once mortality events have occurred in the population and are not predictive and cannot be used to measure the impact of parasitism on year class strength. During the current study, autopsies were undertaken to gain an understanding of the pathological impact of myxozoan diseases on individual host fitness (Chapter 4).

Previous studies examining the impact of parasitism on host population success has used complex mathematical modelling and, due to a lack of suitable data, have mainly been theoretical models (Anderson and Gordon, 1982; Grenfell and Dobson, 1995; Hudson *et al.*, 2001). The data utilised for these studies is usually derived from experimental studies. Unfortunately, these models rarely considered the role of multiple infections, possibly due to the complexities involved in dealing with large, complex data sets. Another approach that has been tried is the manipulation of wild populations through the use of anthelmintics to reduce parasite loads and measuring the subsequent effect on the host population. For example, Stein *et al.* (2002) treated individual reindeer in Spitsbergen, Norway then measured the effect of the reduction in parasite loads on body mass and fecundity. They were able to demonstrate that parasitism had a significant effect on host body condition and fecundity. Whilst these intervention studies provide useful data, they are impractical in wild fish populations. An alternative approach is to measure the effect on host success by the addition of pathogens to a system. The ethics of this approach however are questionable and unacceptable on ecological grounds. Alternatively, data collected from experimental studies may be included. However, as with mathematical models, these studies invariably utilise one pathogen in each trial and thus could not account for the interaction of multiple infections.

Parasitism can have a number of lethal and sub-lethal effects. In lethal infections in captive situations, these are relatively easy to quantify since dead fish will be visible. However, in wild fish, this may not be so readily determined since dead fish will rapidly be removed from the system by scavengers and water movements. Sub-lethal infections by parasites include alterations in host behaviour and increased susceptibility to predation by prey hosts (Barber *et al.*, 2000; Millán *et al.*, 2002). Parasitism may also lead directly to a reduction in swimming speeds (Sprengel and Lüchtenberg, 1991; Moles and Heifetz, 1998), thus increasing the host susceptibility to predation. Alterations in the swimming ability and behaviour of a host will in some cases lead ultimately to death of the host.

Munro *et al.* (1983) consider that disease may be an important factor in pre-recruit populations and may indeed be more severe at this stage than during the post-recruit phase due to pre-recruits being immunologically naïve and unable to combat infections. However, they suggest that disease-induced mortality *per se* may be less significant in these hosts than losses due an increase in predation rates as the avoidance responses of diseased individuals diminishes. Disease is the outcome of the interaction of many inter-related factors including parasites and the presence of other disease agents. Poor environmental conditions may exacerbate the effect of disease leading to host mortality (Kirk, 2003) and thus for diseases to cause death there may be a requirement for other factors to increase susceptibility to pathogens.

Mass mortalities have been reported in fish populations, without an assessment made on the ultimate impact on population success (Wales and Wolf, 1955; Johnsen and Jensen, 1991; Yamamoto *et al.*, 1984; Hedrick *et al.*, 1998; Modin, 1998; Murray *et al.*, 2003). Despite the plethora of literature on mass mortality events or on pathogenic parasite species reported, fisheries scientists still appear to be reticent about including disease as a factor in host mortality when developing fisheries and population models.

Previous fish population models have implicated predation, temperature, hydrological conditions and the position of the north wall of the Gulf Stream in determining year class strength and in recruitment success (Mooij *et al.*, 1996; Nilo *et al.*, 1997; Nash and Geffen, 2000; Nunn *et al.*, 2003; Tolonen *et al.*, 2003). For cyprinid populations, particular attention has been paid to the role of water temperature on fish growth and recruitment success and some authors consider that year class strength is determined by the growth of fish in the first year of life (Mills and Mann, 1985; Copp, 1990; Mann, 1997; Nunn *et al.*, 2003). Fewer studies have shown that disease may have a role to play in host population success (Jones and Taggart, 1998).

The current study aims to redress this imbalance by examining the role of parasitism on the population success of roach and chub in selected rivers in Yorkshire. A

previous study by Nunn *et al.* (2003) used regression models to demonstrate that the main factors driving cyprinid fry populations in the same rivers were temperature and flow rate.

Materials & Methods

Sample collection and preparation

A total of 1053 young of the year (0+) cyprinid fry, comprising 563 chub (*Leuciscus cephalus*) and 490 roach (*Rutilus rutilus*), were collected semi-quantitatively from Acaster, Beningbrough, and upstream of Linton Weir on the river Ouse, Thornton Bridge on the River Swale, and from Boston Spa on the river Wharfe in Yorkshire, northern England every September between 1993 and 2002. Fish were caught using three sweeps of a 20m × 2m micromesh seine net set along the river margin at each site, killed by over anaesthetising in MS222 and fixed in 10% neutral buffered formalin (NBF). On return to the laboratory, fish were identified to species, fork length was taken and the fish stored in NBF by site and species. For histological studies a sub-sample of fry of each species from each site for each year were taken. A maximum of 30 fish of each species per site were examined (see Table 6.1 for details of numbers of fish examined at each sampling point). Tissues were softened in 10% formic acid for up to four days, transferred to 70% IMS prior and processed to wax blocks on an automatic vacuum infiltration tissue processor. Longitudinal sections of fish to include, where possible, the majority of the internal organs, gills, musculature and spinal column, were cut at 3-5 μm and stained routinely with haematoxylin & eosin (H&E) or Giemsa. Numbers of myxozoan cysts and individual digeneans in a single sagittal section of the musculature were enumerated. The location of, and pathological changes associated with, all infections were noted.

Table 6.1 Numbers of 0+ roach and chub examined at each sampling site and in each river during each year between 1993 and 2002 in the rivers Ouse, Swale and Wharfe to provide data on prevelance and abundance of parasite infections in the respective hosts.

River	Site	Host	Year							
			1993	1994	1995	1996	1997	1998	1999	2002
Ouse	Acaster	Chub	-	20	12	-	30	-	-	-
Ouse	Acaster	Roach	5	6	-	-	15	-	-	-
Ouse	Beningbrough	Chub	10	20	15	30	-	-	-	10
Ouse	Beningbrough	Roach	10	10	-	24	30	30	-	10
Ouse	U/S Linton Weir	Chub	-	-	-	-	-	30	-	-
Ouse	U/S Linton Weir	Roach	-	-	-	-	-	30	-	-
Ouse		Chub	10	40	17	30	30	30	-	10
Ouse		Roach	15	16		24	45	60	-	10
Swale	Maunby Demesne	Chub	-	-	-	33	29	-	30	-
Swale	Skipton	Chub	-	-	-	29	-	-	-	-
Swale	Thornton Bridge	Chub	10	22	20	22	5	-	30	10
Swale	Thornton Bridge	Roach	10	20	10	24	37	8	30	-
Swale		Chub	10	22	20	84	34	-	60	10
Swale		Roach	10	20	10	24	37	8	30	-
Wharfe	Boston Spa	Chub	19	10	10	30	27	-	-	10
Wharfe	Boston Spa	Roach	10	11	18	15	18	26	-	-
Wharfe		Chub	19	10	10	30	27	-	-	10
Wharfe		Roach	10	11	18	15	18	26	-	-

Measures of parasite infection used were prevalence, defined as the number of fish infected by a particular parasite species divided by the number of fish within the sample, expressed as a percentage and abundance, defined as the total number of individual digeneans or myxozoan cysts in section of a particular parasite species in a sample of hosts divided by the total number of individuals of the host species in the sample. For myxozoan cysts in the musculature, variance to mean ratios (V:M) were calculated.

Year class strength (YCS) data was only available for roach and chub from the rivers Wharfe and Ouse and was calculated according to the methods of Nunn *et al.* (2003) with data provided by the Environment Agency. In brief, the instantaneous mortality rate Z was calculated from the linear relationship of $Z = (\ln N_t - \log N_0) \div t$, where t is age and N_t is the number of fish at age t. The number of fish at time zero (N_0) was calculated independently for each age group using $N_0 = N_t \exp Z_t$. The mean number of fish in each age group (N_{mean}) was determined as $\sum (t_{max} N \div t_{max})$ where t_{max} is the total number of age classes present in the sample. Finally YCS was determined at age t by $t = (N_0 \div N_{mean}) \times 100$.

Daily water temperature between 1993 and 1999 obtained from Yorkshire Water plc for the river Ouse was used to calculate the cumulative degree-days greater than 12°C between April and September inclusive for each year. Daily river discharge data for the river Ouse was obtained from the Environment Agency multipath ultrasonic flow gauging station at Skelton and used to calculate the annual cumulative number of discharge days above the basal discharge rate between April and September inclusive. Position of the north wall of the Gulf Stream (NWGS) between 1993 and 2000 was obtained from the Plymouth Marine Laboratory (http://www.pml.ac.uk/gulfstream/inetdat.htm).

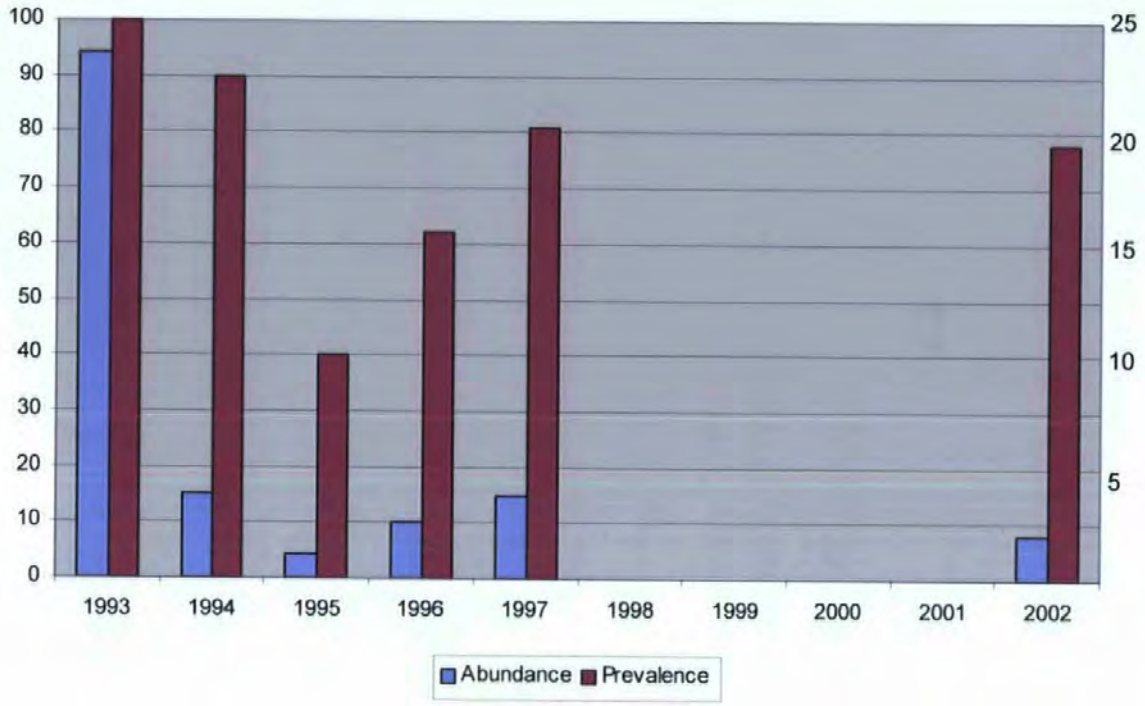
Following determination of correlations between variables and responses, linear and multiple regression models were produced using MINITAB (ver. 13.20) and confirmed using stepwise regressions.

Results

There was considerable spatio-temporal and interspecific variation in parasitism throughout the study. In general terms, chub were the most parasitized in the number of parasite species, prevalence and abundance and pathological responses were greatest in this species. Roach have fewer parasite species compared with chub, show a lesser pathological response and parasites are found at lower prevalence and abundance. Despite the wide variations noted, patterns of parasite prevalence and abundance were similar across all sites with fish collected in 1993, 1997 and 1998 having comparatively higher levels of parasitism compared with 1994, 1995 and 1996 (Figures 6.1 to 6.3). Parasite prevalence and abundance was lowest in the 1995 year class for all species examined. For myxozoans in the muscle, abundance of cysts followed a similar pattern to that seen in the prevalence data. Around forty different parasite species were identified in the study and with the exception of *Rickettsia*-like organisms (RLO's), no bacterial or viral induced pathology were noted in the fish examined. Typically, the parasites found had direct lifecycles or for those with a complex lifecycle, via water borne transmission stages, typified by the digenean *Bucephalus polymorphus* and by myxozoans (Figures 6.4 to 6.6). Mean to variance ratios for *Myxobolus* spp. in the muscle are attached as Table 6.2.

Models developed are attached as Table 6.3.

Figure 6.1 Histogram of prevalence and abundance of *Myxobolus* spp. from the musculature of 0+ chub caught in the river Ouse between 1993 and 2002. Where left hand Y axis is prevalence and right hand Y axis is abundance.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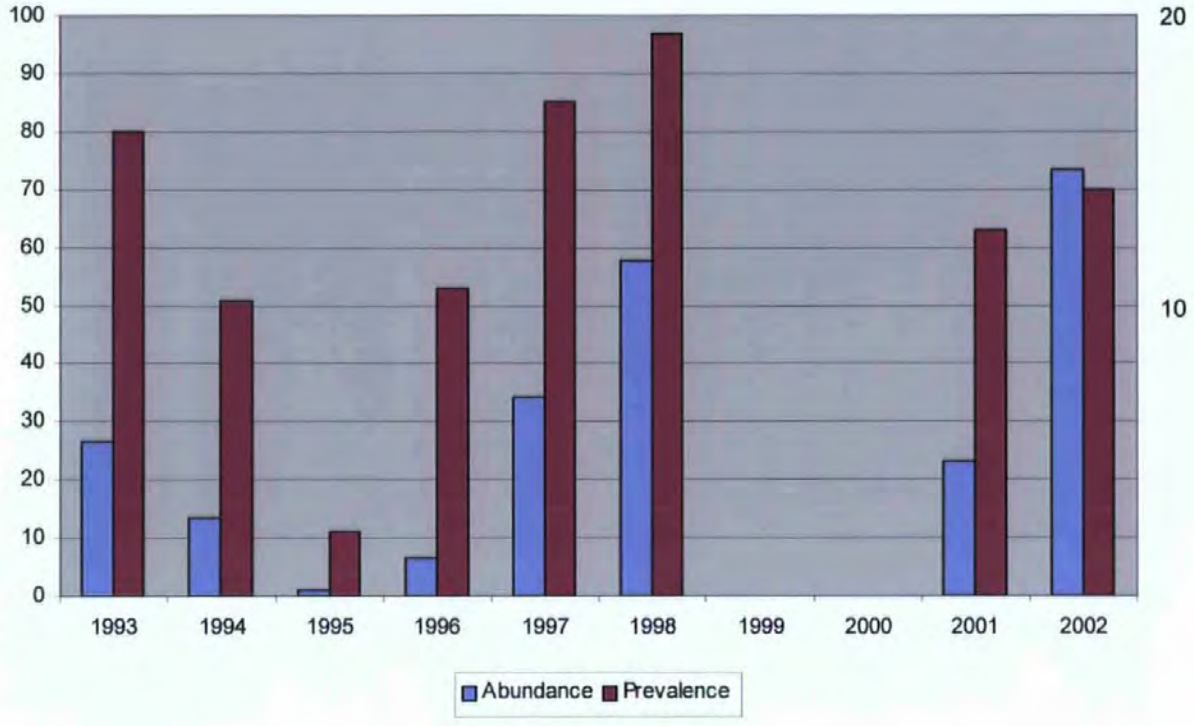
Abundance
Prevalence

Figure 6.2 Histogram of prevalence and abundance of *Myxobolus* spp. from the musculature of 0+ chub caught in the river Swale between 1993 and 2002. Where left hand Y axis is prevalence and right hand Y axis is abundance.

Figure 6.3 Histogram of prevalence and abundance of *Myxobolus* spp. from the musculature of 0+ chub caught in the river Wharfe between 1993 and 2002. Where left hand Y axis is prevalence and right hand Y axis is abundance.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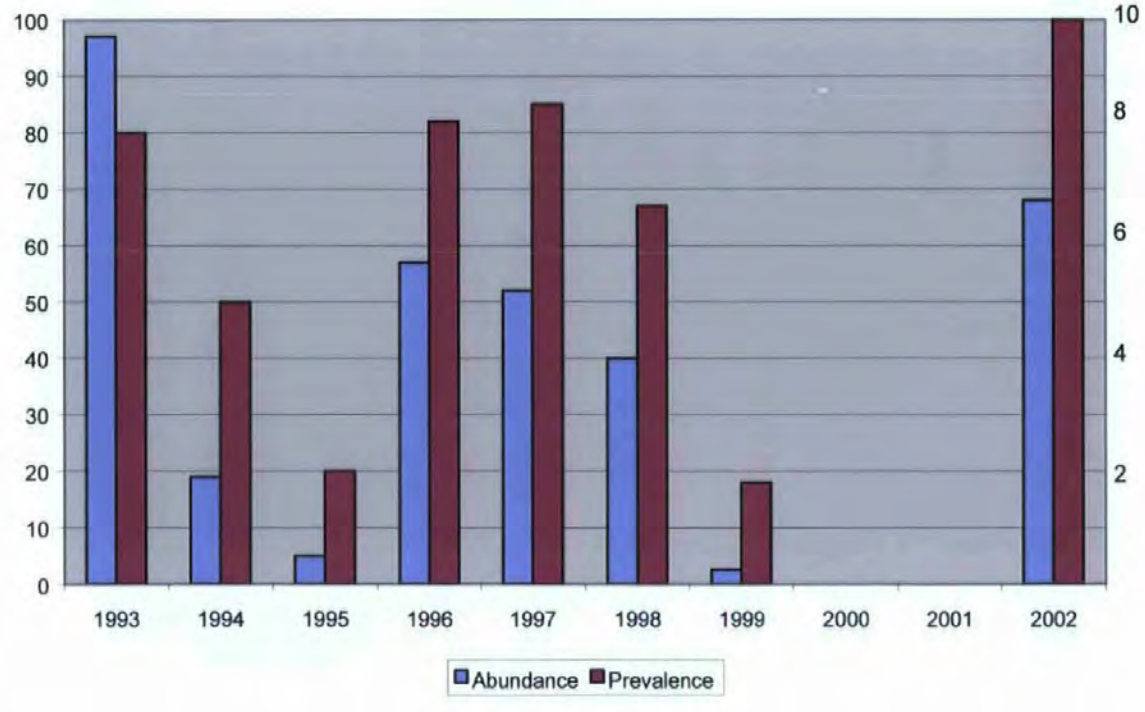
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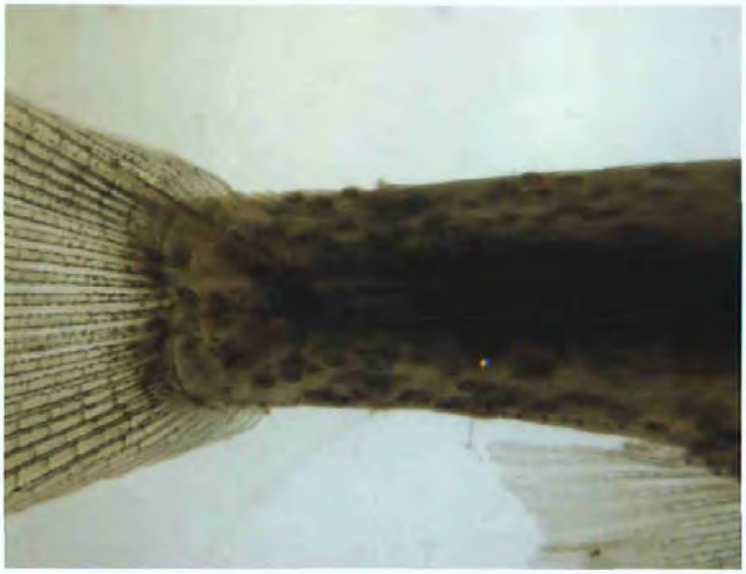
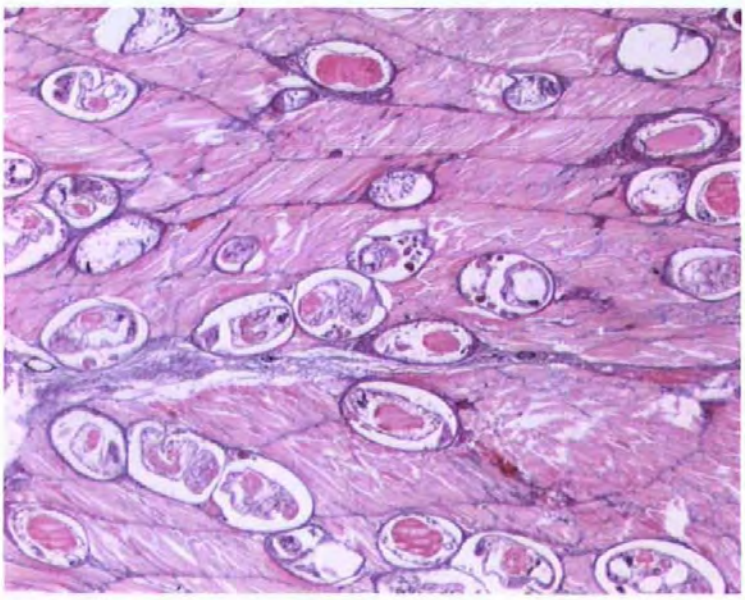
2002
Abundance
Prevalence

Figure 6.4 Photograph of the caudal region of a chub infected with *Bucephalus polymorphus*. Note the large numbers of parasites visible in the musculature.

Figure 6.5 Histological section of encysted *Bucephalus polymorphus* metacercaria in the musculature of chub. Note the disruption to the normal architecture of the somatic muscle. H&E

Figure 6.6 Destruction of stomach epithelial cells associated with coccidian infection of roach (*Rutilus rutilus*). Note presence of different developmental stages of coccidians. H&E





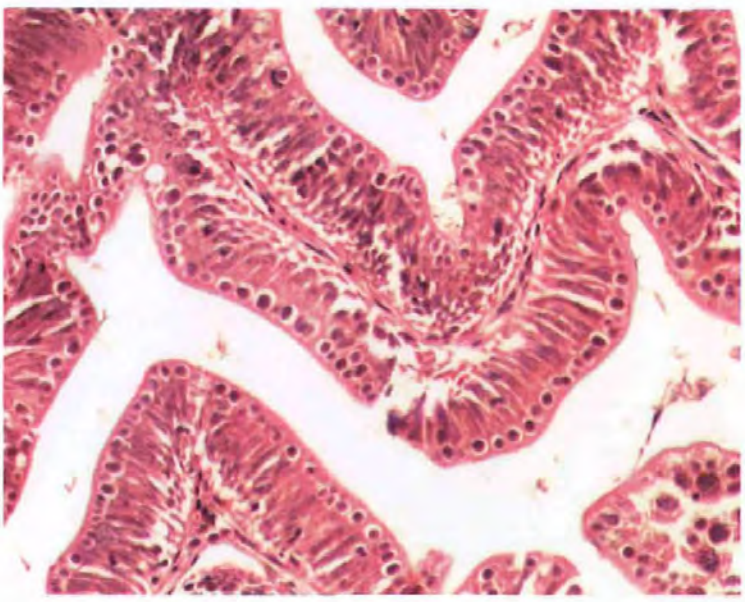


Table 6.2. Variance to mean ratios of *Myxobolus* spp. in the musculature of 0+ roach and chub from the rivers Swale, Ouse and Wharfe caught between 1993 and 2002

River	Site	Host	Year							
			1993	1994	1995	1996	1997	1998	1999	2002
Ouse	Acaster	Chub	-	22.000	1.945	-	13.047	-	-	-
Ouse	Acaster	Roach	2.000	1.000	-	-	3.591	-	-	-
Ouse	Beningbrough	Chub	3.482	7.458	-	2.077	-	-	-	44.264
Ouse	Beningbrough	Roach	0.778	-	-	2.082	6.302	4.429	-	1.556
Ouse	U/S Linton Weir	Chub	-	-	-	-	-	3.069	-	-
Ouse	U/S Linton Weir	Roach	-	-	-	-	-	2.395	-	-
Swale	Maunby Demesne	Chub	-	-	-	8.067	4.932	-	1.172	-
Swale	Skipton	Chub	-	-	-	4.328	-	-	-	-
Swale	Thornton Bridge	Chub	17.802	7.329	3.894	6.490	4.625	-	2.138	3.523
Swale	Thornton Bridge	Roach	1.909	1.000	2.333	-	0.984	4.000	0.965	-
Wharfe	Boston Spa	Chub	12.443	8.877	3.323	3.678	4.212	-	-	2.000
Wharfe	Boston Spa	Roach	1.000	-	4.004	-	1.857	3.752	-	-

Table 6.3. Linear and multiple regression models. Parasite data refers to prevalence unless otherwise stated and *Myxobolus* spp. data refers to those found in musculature unless otherwise stated. L = linear, M = multiple. [1] = Models developed including environmental data. Where *P* = probability, R^2 = r-squared values, N = the number of cohorts used in the analysis.

Site/River	Model type	Regression model	*P*	R^2 (%)	N
1. Boston Spa, Wharfe	L	Length $_{chub}$ = 23.8 + 1.45 (*Bucephalus polymorphus*)	0.032*	72.5	6
2. Beningbrough, Ouse	M	Length $_{chub}$ = 8.02 +1.10 (*Phyllodistomum* sp.) + 0.199 (*Bucephalus polymorphus*)	0.014*	94.2	6
3. Beningbrough, Ouse	L	Length $_{chub}$ = 36.2 – 0.147 (*Myxobolus* spp.)	0.063NS	62.1	6
4. Beningbrough, Ouse[1]	L	Length $_{chub}$ = 33.2 – 2.33 (*Myxobolus* spp. abundance)	0.029*	94.2	4
5. Beningbrough, Ouse[1]	M	Length $_{chub}$ = 34.9 + 0.0771 (*Myxobolus* spp.) – 1.38 (*Myxobolus* spp. abundance)	0.002*	100	4
6. Boston Spa, Wharfe[1]	M	Length $_{chub}$ = 28.1 +1.00 (*Bucephalus polymorphus*) – 0.268 (*Myxobolus* spp. abundance)	0.029*	97.1	5
7. Thornton Bridge, Swale[1]	M	Length $_{chub}$ = 23.4 + 0.926 (NWGS $_{January}$) + 0.766 (*Myxobolus buckei*)	0.049*	86.5	6
8. Beningbrough, Ouse	L	Relative abundance $_{chub}$ = 58.7 – 5.09 (*Phyllodistomum* sp.)	0.005**	95.0	5
9. Beningbrough, Ouse	M	Relative abundance $_{chub}$ = 64.6 – 0.088 (*Myxobolus* spp.) – 5.52 (*Phyllodistomum* sp.)	0.036*	96.4	5
10. Boston Spa, Wharfe	L	Relative abundance $_{chub}$ = 17.3 + 0.775 (*Myxobolus buckei*)	0.055NS	75.7	5
11. Thornton Bridge, Swale	L	Relative abundance $_{chub}$ = 22.2 + 4.02 (*Myxobolus macrocapsularis*)	0.075NS	58.8	6
12. Boston Spa, Wharfe[1]	M	Relative abundance $_{chub}$ = 28.5 – 6.15 (*Myxobolus* spp. abundance) + 10.1 (ciliates)	0.03*	97.0	6
13. Beningbrough, Ouse[1]	M	Relative abundance $_{chub}$ = 58.1 – 0.0371 (*Myxobolus* spp.) – 18.0 (NWGS $_{July}$)	0.069NS	99.5	4
14. River Wharfe	L	YCS $_{chub}$ = 31.0 + 17.3 (*Bucephalus polymorphus*)	0.001***	98.3	5
15. River Ouse	L	YCS $_{chub}$ = 217.0 – 2.18 (RLO's)	0.006NS	88.0	6
16. River Ouse	L	YCS $_{chub}$ = 245.0 – 12.9 (variance to mean ratio of *Myxobolus* spp.)	0.016*	88.9	5
17. River Ouse[1]	M	YCS = 256 + 26.2 (RLO's) - 1.00 (*Myxobolus* spp. in muscle) + 13.7 (*M. macrocapsularis*) - 2.53 (*Bucephalus polymorphus*)	0.001***	100	6
18. Boston Spa, Wharfe	L	Relative abundance $_{roach}$ = 12.9 + 9.32 (*Bucephalus polymorphus*)	0.011*	83.2	6
19. Beningbrough, Ouse	L	Relative abundance $_{roach}$ = 39.3 + 8.75 (myxozoan plasmodia in kidney)	0.038*	80.8	5
20. Thornton Bridge, Swale	M	Relative abundance $_{roach}$ = 9.86 + 0.368 (*Bucephalus polymorphus*) + 10.7 (RLO's)	0.027*	76.5	8
21. Beningbrough, Ouse	M	Relative abundance $_{roach}$ = 57.8 + 6.94 (myxozoan plasmodia in kidney) – 3.86 (Coccidia)	0.028*	97.2	5
22. River Ouse	L	YCS $_{roach}$ = 103 – 4.10 (*Trichodina* spp.)	0.003**	99.3	4
23. River Ouse	M	YCS $_{roach}$ = 127 – 4.64 (*Trichodina* spp.) – 0.031 (degree days)	0.011*	100	4
24. River Wharfe	L	YCS $_{roach}$ = 51.0 + 22.4 (*Bucephalus polymorphus*)	0.004**	80.2	5

Where * = P≤0.05; ** P≤0.01; *** P≤0.001; NS = not significant

Chub models – length

The best model for length of chub was from fish collected at Beningbrough (equation 5: $P = 0.002$, $R^2 = 100$) which showed that length was negatively correlated with the prevalence and abundance of *Myxobolus* spp. in the muscle, even with the inclusion of environmental data. Chub length was also positively correlated with the presence of the digeneans *B. polymorphus* and *Phyllodistomum* sp. and *Myxobolus buckei* in the spinal column at different sites with *P* values ranging between 0.014 and 0.063 and R^2 values of between 62.1 and 97.1%. Fitting environmental variables did not significantly increase the explained variation (R^2) with the exception of chub length at Thornton Bridge which in addition to *M. buckei*, included the position of the NWGS in January (equation 7).

Chub models – relative abundance

The best model to describe chub relative abundance was for chub caught in Beningbrough and included the prevalence of *Myxobolus* spp. in the musculature and position of the NWGS in July (equation 13: $P = 0.069$, $R^2 = 99.5\%$). At the different sites, chub relative abundance was also correlated with the digenean *Phyllodistomum* sp. (equation 8: $P = 0.005$, $R^2 = 95\%$; equation 9: $P = 0.036$, $R^2 = 96.4\%$), *Myxobolus* spp. in the musculature (equation 9: $P = 0.036$, $R^2 = 96.4\%$, equation 12: $P = 0.03$, $R^2 = 97\%$) *M. buckei* (equation 10: $P = 0.055$, $R^2 = 75.7\%$) and *M. macrocapsularis* (equation 11: $P = 0.075$, $R^2 = 58.8\%$).

Chub models – year class strength

A significant relationship between YCS and the prevalence of RLO's and between YCS and the variance to mean ratio of muscle myxozoans was found in chub from the

river Ouse (equations 15 and 16). However, a positive relationship was noted in YCS of chub from the river Wharfe with *B. polymorphus* (equation 14).

Roach models

Fewer models were developed to demonstrate a link between disease and roach compared with chub and were related to the presence of *B. polymorphus* in the musculature, *Trichodina* in the gills, myxozoan plasmodia in the kidney tubule epithelium and to a lesser extent, Coccidia in the intestinal epithelium and RLO's in the gills. Relative abundance of roach was positively correlated with disease (equations 18-21). As with chub, YCS was positively correlated with the presence of *B. polymorphus* (equation 24), but unlike chub, YCS was negatively correlated with the presence of *Trichodina* spp. in the gills (equations 22 and 23).

Discussion

The current study has, for the first time, demonstrated a correlation between disease levels in juvenile fish, year class strength and recruitment success. Whilst it has been suggested from previous studies that disease may impact on population success, these studies have tended to be conducted post-mortality and once population structure has been fixed by a series of abiotic and biotic factors, including disease. Previous studies by Nunn *et al.* (2003) on the same population of fish in the rivers Ouse, Wharfe and Swale suggested that the main drivers for population success were the position of the north wall of the Gulf Stream (NWGS), temperature and flow rate. It was considered by Nunn *et al.* (2003) that the position of the NWGS determined the summer temperature of the rivers, which in turn enhanced fish growth at higher temperatures. In addition, the position of the NWGS influences river discharge rates which Nunn *et al.* (2003) consider determines

"realised" YCS. However, those authors failed to consider the role that parasites and diseases may have on fish survivability.

There is evidence from the current study that these juvenile fish are infected during the fish few weeks of life as demonstrated by the presence of sporogonic myxozoan stages in these fish aged 3-5 months old. Since it is known that myxozoans take approximately 3-5 months to complete sporogony, it follows that the fish must have been infected shortly after hatching and that limited myxozoan infections take place in these hosts after those first initial weeks. Due to the nature of infection, the fish are unlikely to be able to avoid the infectious stages released by the invertebrate hosts and indeed, transmission of these infectious stages may be exacerbated in the shallow, warm river margins in which the juvenile fish reside in the first few months of life. It is during the period within the margins that these juvenile fish attempt to attain a maximum size prior to moving into the main body of the river. Newly hatched larvae are poor swimmers due to the limited muscle structure and small size (Cowx, 2001). As the fish increase in length, their ability to maintain station in high water flows increases due to an increase in muscle mass. Episodic flooding events either during the summer months, or later in autumn and winter may cause direct fish mortality by washing the smaller fish out of the riparian zones. Therefore, factors that alter the growth rate and length of the fish will impact on host survival. During the current study, models were developed that demonstrate a correlation between parasitism and fish length for chub.

A strong negative correlation between *Myxobolus* spp. abundance in the muscle and chub length was determined. There are multiple effects of muscle infections on the fish host. Myxozoan cysts in individual muscle fibres lead ultimately to the destruction of the infected muscle fibre and following the host response, adjacent muscle fibres may be destroyed. Loss of muscle function in these hosts will restrict the fishes ability to actively swim to catch prey food items, thus a reduction in energy intake would be expected. This would then be translated into reduced growth and a smaller overall size. Consequently, the

smaller sized would be expected to be less able to maintain a suitable position in the water column during episodic flooding events, and as suggested by Munro *et al.* (1983) more susceptible to predator induced mortality as their ability to swim away from predators would be reduced.

Conversely, a positive relationship was found between chub length and the prevalence of *Bucephalus polymorphus* in the musculature of chub. This may be explained by the fact that, unlike myxozoan infections which predominately occur in the first few weeks of life, infectious cercarial stages may be present over a protracted number of months in these rivers. The presence of metacercaria in different host organs and at different developmental stages would support this view (Longshaw and Feist, unpublished observations). Larger fish in the riparian zone would therefore be expected to have a higher abundance and prevalence of digeneans in the musculature compared with smaller fish, which may have hatched at a later date. Alternatively, the positive relationship between length and *B. polymorphus* in the muscle may be due to the spawning behaviour of adult chub which are known to possess the ability to spawn more than once in the summer (Nunn *et al.*, 2002). Larger juveniles in the population may represent an early batch or batches of fish that hatch out during the period when digenean cercaria are present and that the smaller, less infected fish are those that hatch later in the season, outwith the critical main period for infection. Due to the nature of sample collection and analysis, it was not possible during the current study to determine which process was operating in the chub populations and could be addressed by assessing the levels of parasitism in monthly samples of chub throughout the summer months post-hatching. It follows that the positive correlation between the presence of *B. polymorphus* and roach YCS may be due to the larger individuals which hatch earlier are able to survive flooding events thus contributing to a strong year class.

There was a strong negative correlation between chub YCS in the River Ouse and RLO's and *Myxobolus* spp. infections in the musculature. As described above, the impact

of myxozoan infections in the muscle are likely to disrupt the normal function and architecture of the skeletal muscle and impact on the host's ability to capture prey items, maintain position in high water flows and to avoid predators. Host losses associated with these factors would be expected to be reflected in the YCS, as demonstrated here. Chub YCS was also strongly correlated with the variance to mean ratio of *Myxobolus* spp. in the muscle. Variance to mean ratios can be a measure of the degree of overdispersion within a parasite population, with higher values suggestive of a greater degree of overdispersion. Intuitively, one would expect that overdispersion may be beneficial to host success since by its very nature, overdispersion suggests that most hosts have low levels of infection with a small proportion of the host population being heavily parasitized (Crofton, 1971). The more heavily infected individuals would be killed by the heavy parasite burdens thus allowing transmission of the parasite to the next host as appropriate and continuing the lifecycle. However, in host populations with highly overdispersed parasite populations, more fish will be infected at much higher levels and thus host mortality may increase (Hudson *et al.*, 2001). Direct evidence of the effect of higher values of variance to mean ratios on poor host survival was provided by Lester (1977) and Adjei *et al.* (1986) following a mortality event. These authors showed that by comparing the actual parasite distribution in a population with a theoretical negative binomial distribution derived from data obtained from a lightly infected population and the data collected after the supposed mortality event, there were fewer fish with higher parasite burdens than expected. They attributed this loss of parasites in the right tail of the distribution to parasite induced host mortality. Lester (1984) also suggested that a decrease in variance to mean ratio with increasing age may be due to PIHM. By implication, host populations with higher variance to mean ratios pre-mortality would be more at risk of suffering PIHM than those with lower variance to mean ratios. By calculating the exponent of Taylor's power law from variance and means of sea lice distributions in wild sea trout, Bakke and Harris (1998) suggested that higher variance to mean ratios were responsible for the higher

mortalities noted in sea trout from Scotland compared with Irish sea trout. The high degree of overdispersion present in the current study and its correlation with chub YCS would suggest that a similar process is in operation here. However, confirmation of a demonstrable effect on chub YCS would require calculations of variance to mean ratios in this population post-mortality. Any decrease in the ratios would be suggestive of a mortality effect. Anderson and Gordon (1982) suggested that decreases in the variance to mean ratio (decrease in aggregation) would occur as a result of parasite mortality, reductions in parasite fecundity and PIHM. Since myxozoans do not undergo reproduction in the host, other than through the production of extrasporogonic proliferative stages and that dead parasites in the host would still be apparent, these two factors could be excluded from any reductions in variance to mean ratios and allow a demonstration of PIHM.

Whilst high variance to mean ratios have been shown to negatively correlated with chub YCS in the River Ouse, regulation of the chub populations by myxozoans, in which lower levels of parasitism are found when population levels are low and are higher when host population numbers increase, appears not to be occurring since myxozoan prevalence and abundance was lowest in 1995 when YCS was high and large numbers of fish were present in the system. Indeed, Hudson *et al.* (2001) suggest that "for highly overdispersed distributions, regulation of the host population will be difficult to achieve because too many parasites will be lost from the system by parasite-induced host mortality".

The data presented during the current study has some limitations, not least the small data sets that were used in the development of the models. However, in spite of this, it is noteworthy that a clear statistical correlation exists in the data. Clearly this is an advance on previous studies that have dismissed disease in any population models and should be considered further. Additionally, the method in which the parasite data was collected (i.e. histologically) provides an underestimate of the number of cysts/parasites present in individual fish.

It is known that chub can undergo multiple spawnings over the summer (Nunn *et al.* 2002) and it has been suggested that highly fecund hosts may be able to negate the effect of disease by producing more progeny than can be removed though PIHM. However, since it is assumed that the probability that the infective stages contacts a host increases with increasing host density and that infection of juvenile cyprinids by the parasites under investigation takes place in the highly aggregated host populations along the river margins, the production of progeny by the fish hosts will merely serve to propagate the parasite. Since mortality of the juveniles is known to occur and that this is a source of infection back to the oligochaete host for myxozoans, this provides the source of infectious stages, thus completing the cycle and increasing parasite levels in the susceptible hosts. The effect of abiotic and biotic factors on parasite stages in the invertebrate hosts remain largely unknown and so studies should be instigated to determine why clear spatio-temporal differences exist in parasite prevalence and whether these are due to environmental impacts on the invertebrate host.

Under experimental or culture conditions, management of infectious agents can, to an extent be controlled or negated. However, for wild fish this may be almost impossible. Management intervention and accurate information on the drivers for population success may be one of the few tools available to fisheries managers to ensure healthy stocks of fish. In the current study it has been shown that parasites are able to exert an effect on host population success. Given this information, fisheries managers should be able to better manage a fishery by making accurate and correct predictions regarding the effect of any planned changes to the environment or indeed regarding decisions about if and when restocking should take place. Providing refuges in rivers for juvenile fish where water flows are reduced by either canalising stretches of rivers or by the addition of islands in the main part of the river is an example of current practice (Cowx, 2001). This has been based on the prior demonstration that fry survival is negatively correlated with discharge rates. However, the impact of such management intervention, by its very nature is that it provides

ideal conditions for transmission of parasite stages to naïve hosts in these areas due to the reduced flow rates, increased sediment and thus increased numbers of potential invertebrate hosts. Rather than making management decisions based on partial information as currently occurs, fisheries managers need to consider all risk factors associated with planned alterations to a river system if correct management decisions are to be made.

It is clear that parasites of cyprinid fry have an impact on fish length, relative abundance and have a role in determining year class strength. However, for each river system and indeed for each host species, the determining factors differ. Synergistic effects between parasite loads, host factors and environmental influences most likely determine the host survivability. There is a need to extend the study to determine the links between the different factors involved, and their influence on both parasite and host "success" and to determine the lethal and sub-lethal role of disease in wild fish populations. Future studies should consider the development of more robust, predictive population models for freshwater fisheries management that may ultimately be applied to marine fisheries management.

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CHAPTER SEVEN

TRANSMISSION OF MYXOSPORES TO OLIGOCHAETES

Abstract

Laboratory based experimental trials were initiated to attempt to transmit myxospore stages from cyprinid fish hosts to naïve oligochaetes. Samples of 0+ and 1+ fish were collected from selected sites in Yorkshire and Humberside to provide myxospore stages. Oligochaetes were exposed to the following myxospores: *Myxobolus pseudodispar* from the musculature of chub, *Myxobolus musculi* from the musculature of minnow and gudgeon, *Myxobolus buckei* from the vertebral column of chub, *Myxobolus* sp. from a plasmodium in the mouth of perch, *Myxobolus* sp. from a plasmodium in the fins of gudgeon, *Myxobolus oviformis* from plasmodia in the gills of gudgeon and dace, *Myxidium barbatulae* from the kidney of stone loach, *Myxidium rhodei* from the kidney of roach, *Sphaerospora leuciscusi* from the kidney of dace and *Sphaerospora ousei* from the kidney of roach. Samples of water were examined approximately every four weeks for up to one year for the presence of actinospore stages. No parasite development occurred in this experimental trials. The results are discussed in relation to abiotic and biotic factors affecting parasite success and host susceptibility.

Introduction

Since the first discovery of myxozoan parasites there has been a lack of understanding regarding the life cycle of these parasites. Many workers considered that myxospores from fish required a period of maturation in mud, after which they were directly infective to fish. For actinospores, within the same phylum, the mode of transmission was less clear, although this was also believed to be direct. Marques (1984) failed to transmit actinospores to naïve oligochaetes, which given the current knowledge that myxozoans alternate between oligochaetes and the fish host is unsurprising. Wolf and Markiw (1984) demonstrated that the agent for salmonid whirling disease, *Myxobolus cerebralis*, underwent a two-host life cycle, alternating between an oligochaete and a fish host. Whilst initially viewed with some scepticism (Hamilton and Canning, 1987; Lom, 1986, 1987), further studies on *M. cerebralis* and on other myxozoans clearly showed that at least some myxozoans underwent a two-host life cycle (Kent *et al.*, 2001). Whilst there have been suggestions that all myxozoans will ultimately possess an actinospore stage, to date only around 33 myxozoan lifecycles have been elucidated. Of these, one has been shown to utilise a polychaete (Bartholomew *et al.*, 1997), one utilises a bryozoan (Feist *et al.*, 2001) and four transmit directly without the need for an invertebrate host (Diamant, 1997; Redondo *et al.*, 2002; Yasuda *et al.*, 2002). With the exception of those myxozoans with a direct life cycle, only freshwater myxozoan life cycles have thus far been elucidated. Additionally, it is known that for some myxozoans the intraperitoneal injection of extrasporogonic stages into naïve fish can transmit the parasite between fish hosts. This however has only successfully demonstrated for *T. bryosalmonae* (Kent and Hedrick, 1985), *C. shasta* (Ibarra *et al.*, 1991), *S. renicola* (Molnár and Kovacs-Gayer, 1986), *S. truttae* (McGeorge *et al.*, 1996b) and *K. thyrsites* (Moran *et al.*, 1999a). It is clear therefore that there is a wide range of different transmission strategies available to the group.

Many of the earlier works in the late 1980's and early 1990's on myxozoan life cycles, were generally based on complete lifecycles in which myxozoans were transmitted between both hosts (Troullier *et al.*, 1996; Yokoyama *et al.*, 1993b; Benajiba and Marques, 1993). However, these studies may need to be revisited as many failed to provide adequate descriptions of the actinospore stages, making comparative studies difficult.

More recently, there has been a propensity to carry out small scale transmission studies by attempting to expose oligochaetes to myxospores isolated from fish (Molnár *et al.*, 1999a, b; Özer and Wootten, 2000; Székely *et al.*, 1998, 1999, 2002). Furthermore, molecular biology via DNA sequence matches has been used to complement these biological studies (Andree *et al.*, 1997; Feist *et al.*, 2001; Bartholomew *et al.*, 1997; Székely *et al.*, 1998) or as the sole method employed (Lin *et al.*, 1999).

Perhaps the most studied myxozoan lifecycle has been that of *M. cerebralis.* It is clear from these studies that not only is the parasite extremely specific to the invertebrate host, *Tubifex tubifex* (Granath and Gilbert, 2002), it may be specific to distinct genetic lineages of *T. tubifex* (Beauchamp *et al.*, 2001). With the exception of *M. cerebralis*, to date no other studies using oligochaetes have considered the genetic lineages of the oligochaetes used.

Following the understanding of the role of myxozoans in regulating cyprinid fry populations and their pathogenicity to individuals (chapter 6), it was necessary to determine whether oligochaetes had a role in transmitting myxozoan infections to these cyprinids. The current study therefore aimed to transmit myxospores isolated from a variety of freshwater cyprinid fishes to naïve, laboratory reared oligochaetes.

Materials and Methods

Samples of 0+ and 1+ fish were collected from the River Ouse in Yorkshire using a micromesh seine net and transported live in buckets containing river water to the CEFAS Weymouth laboratory. Fish were held overnight in flow through tanks at ambient

temperature. Fish were killed by over-anaesthetizing in MS222 followed by a cut to the vertebral column just posterior to the cranium. Following identification using standard keys, fish were dissected and examined for the presence of myxozoan parasites. All organs in the fish were examined with particular emphasis on gills, muscle, gall bladder and kidney. As far as possible, myxozoan cysts were isolated from surrounding host tissue and spores were resuspended in approximately 40ml of deionised water prior to infection trials. The myxospores used in the trials were *Myxobolus pseudodispar* from the musculature of chub, *Myxobolus musculi* from the musculature of minnow and gudgeon, *Myxobolus buckei* from the vertebral column of chub, *Myxobolus* sp. from a plasmodium in the mouth of perch, *Myxobolus* sp. from a plasmodium in the fins of gudgeon, *Myxobolus oviformis* from plasmodia in the gills of gudgeon and dace, *Myxidium barbatulae* from the kidney of stone loach, *Myxidium rhodei* from the kidney of roach, *Sphaerospora leuciscusi* from the kidney of dace and *Sphaerospora ousei* from the kidney of roach. Myxospore viability was determined by the Trypan blue method.

Parasite free, laboratory reared oligochaetes, comprising approximately 90% *Tubifex tubifex* and 10% *Limnodrilus hoffmeisteri*, were obtained from the laboratory of Dr Mansour El-Matbouli (Munich, Germany). It is known that the batch of oligochaetes used were susceptible to infections with *M. cerebralis* (Mansour El-Matbouli, pers. comm.). Naïve worms were maintained in small aquaria containing sterilised river mud and de-ionised water prior to infection trials.

Following isolation of myxospores from fish, 100 randomly selected oligochaetes were used for each infection trial. Worms were placed in petri dish containing each isolate of myxospores and left for a minimum of 30 minutes. The contents of each petri dish, including oligochaetes and myxospores were transferred to small, plastic containers containing approximately 1.5L of deionised water and sterilised river mud/sand. Each container was labelled, aerated continuously and maintained at approximately 14°C for the length of the study. Negative controls comprising 100 oligochaetes each in two plastic

containers under the same conditions as experimental trials and a stock tank held at room temperature were maintained. Oligochaetes were fed on a diet of commercially available macerated fish food containing *Spirulina* and liquid fish fry food on a weekly basis.

Approximately every four weeks, 150ml of water was removed from each tank and filtered through a 50µm sieve. The contents trapped on the sieve were resuspended in approximately 10ml of deionised water, and examined using a stereomicroscope for the presence of presumptive actinospore stages. In addition, a drop of the suspension was examined under a coverslip on a slide using phase contrast microscopy.

Oligochaetes were maintained and examined for one year and on termination of the experiment, all the water from each tank was examined for the presence of actinospores as described above.

Results

Myxospore viability was determined as being between 90 and 100%. Attempts to transmit myxospores to naïve oligochaetes were unsuccessful. No actinospores were recorded in any of the experimental challenges or negative controls throughout the study period. During the initial exposures in the petri dish, it was noted that oligochaetes quickly became surrounded with myxospores.

Discussion

Over 1300 species of myxozoans within approximately 50 genera and 15 families have been reported in vertebrate hosts. In contrast, only an estimated 200-300 actinospore types have been described within 16 collective groups. This paucity of data on actinospore forms complements the lack of knowledge on myxozoan life cycles, for which only 33 have been completed, either partially or in full. This may reflect a reluctance or inability by researchers to carry out such experimental trials due to time and funding availability or

an inherent biological problem with physically being able to transmit the parasite between hosts. Indeed, when the life cycle of *M. cerebralis* was first proposed it was met with some scepticism (Lom, 1986, 1987) and other researchers were unable to replicate the studies of Wolf and Markiw (1984) (Hamilton and Canning, 1987). Other researchers have found that different actinospore forms were produced in different laboratories when apparently working with the same myxospore form (Yokoyama *et al.*, 1993b; Troullier *et al.*, 1996) and that morphologically different actinospores can possess the same genotype (Hallett *et al.*, 2002). Although a relatively small number of myxozoan lifecycles have been completed, three have been consistently replicated under laboratory conditions (El-Matbouli *et al.*, 1999b; Székely *et al.*, 2001, Longshaw *et al.*, 2002).

During the current study a number of potential reasons that transmission of myxospores to oligochaetes was unsuccessful relating to environmental conditions, experimental design, the parasite and the host(s) are considered below.

Temperature

Temperature affects growth and development rates of all living creatures, including parasites and hosts. Oligochaetes were reared in the laboratory at a constant 12°C (Mansour El-Matbouli, pers. comm.) prior to being used in the current study. Although the temperatures were raised slightly to 14°C this differences is within the physiological tolerance range of both oligochaetes and the myxozoans used, therefore it is unlikely to have had a detrimental influence on actinospore development. Additionally, this temperature is within the ranges reported for successful development of actinospores. Whilst the temperatures were maintained at a constant 14°C, this again is unlikely to have affected development since it is known that *M. cerebralis* actinospore develops optimally at 15°C, and developmental stages degenerate at temperatures above 25°C (El-Matbouli *et al.*, 1999a). Under both natural, fluctuating temperature and controlled laboratory conditions actinospores develop within approximately 3 months. The maintenance and infection of oligochaetes under constant temperature regimes has been used successfully

for a number experimental trials and is therefore unlikely to have contributed to a lack of transmission.

Photoperiod

Experimental challenges were carried out in a constant temperature room with a variable light-dark regime, with oligochaetes being maintained in the dark for most of the time. It is possible that a lack of consistency in the photoperiod may have contributed to unsuccessful transmission. However, the oligochaetes used were laboratory reared and acclimated to laboratory conditions, including variable photoperiod, so are unlikely to have been affected in the same manner as wild oligochaetes. Previous studies have shown that actinospore release follows a circadian pattern in oligochaetes. The majority of spores are released in the late evening and early morning (Yokoyama *et al.*, 1993a; Özer and Wootten, 2001), although spores can be released throughout the day. There is a lack of data on the impact of alteration of photoperiod on actinospore development, although it is unlikely to have impacted on actinospore development during the present trials.

Seasonality

Seasonality of release is a recognised phenomenon for myxozoans in both fish and oligochaetes, which appears to be driven more by ambient temperature, rather than photoperiod since parasites can be found within the invertebrate host throughout the year, irrespective of photoperiod (Gay *et al.*, 2001). The main factor determining development appears to be ambient temperature rather than season, since parasites will develop in the laboratory if invertebrates are moved to higher temperatures following a period of cold weather in the wild (Gay *et al.*, 2001). Whilst the definitive hosts (oligochaetes) can release actinospore stages throughout the year, most are released in spring and summer in temperate regions, indicative of a temperature effect (El-Mansy *et al.*, 1998a, b; Oumouna *et al.*, 2003; Özer *et al.*, 2002; Xiao and Desser, 1998b). The transmission trials were commenced in May, at a time of year when it would be expected that myxospores would

be available to actively feeding oligochaetes in the wild. Therefore seaonality is unliley to have influenced the outcome of the present trial.

Sediment type

It is recognised that sediment type affects the distribution of oligochaete species (Xiao and Desser, 1998b). Blazer *et al.* (2003) showed that the number and duration of release of *M. cerebralis* actinospores was greatest in oligochaetes maintained in mud and sand, and least in a leaf litter substrate. During the current study oligochaetes were maintained in a mud/sand mix collected from river systems in which it was known that oligochaetes were able to survive successfully. Given that oligochaetes were reared and exposed in sediments conducive for maximum actinospore production, this seems to have been an unlikely reason for failure.

Length of exposure

Since oligochaetes were exposed initially to myxospores in a small volume of water for a short period and then transferred to a 1.5L plastic tank along with myxospores, there is a high probability that actively feeding oligochaetes would have come into contact with myxospores at some point, either in the initial phases of the exposure or during the trial. The exposure of oligochaetes was therefore maximised through these two main routes. It is known that previous trials have utilised this approach successfully and is thus the methodology employed is unlikely to have been detrimental to the success of the trial (El-Mansy and Molnár, 1997; Molnár *et al.*, 1999a, b).

Myxospore dose

As above, since exposures were carried out in small volumes of water, there was a high probability of oligochaetes becoming exposed to myxospores. Indeed, it was noted that individual oligochaetes quickly became surrounded by myxospores when exposed within the petri dish. Given also the relatively small container in which the oligochaetes

were maintained (along with myxospores), they are likely to have been exposed to myxospores over the period of the trial. Although no accurate measurement was taken of the number of myxospores used in each trial, it was estimated that a minimum of 500 spores were used for each exposure. Even if only a small percentage of the oligochaetes in each trial ingested a small proportion of the myxospores available, it would be expected that a much greater number of actinospores would be released compared with the number of myxospores introduced into the culture. This is due to the proliferative stages that are known to occur in the actinospore phase of the lifecycle to increase the numbers of actinospores released. Özer and Wooten (2001) suggested that up to 80,000 actinospores per infected oligochaete may be released daily, which could only have derived from a proliferative stage in the oligochaete host. Although Stevens *et al.* (2001) found that the initial myxospore dose affected the number of actinospores produced and that the parasite reduced the biomass, abundance and individual weights of oligochaetes, oligochaete mortality appeared to be minimal and it was noted that eggs were produced in some of the tanks. However, there are some uncertainties with the current experiments since any potential effects on oligochaetes were not measured, absolute amount of myxospores were not measured and ingestion of myxospores were not observed.

Myxospore viability

Prior to commencement of the trials, myxospores were examined under phase contrast for two reasons – one to ensure the parasites were correctly identified and two, to visualise the state of myxospores. Additionally, myxospore viability was measured using Trypan blue dye. Viability was assessed as being between 90 and 100% for each myxospore type. It was noted that in most of the myxospores, a clear sporoplasm was present. Since the sporoplasm is the infective stage to oligochaetes, and since it was present, it was assumed that at least some of the myxospores would have been infective to the oligochaete. The trial was started in May, several months after the young of the year

fish host would have become infected and at least a year after the older fish would have become infected. Sporogonic forms of the parasite were present in both 0+ and 1+ fish. Myxospore sporogony is known to occur relatively rapidly in the fish host (less than three months in many cases), so the parasite should have been viable. 1+ fish were used in the trial so even if those myxospores collected from 0+ fish were immature, those from the 1+ fish should have been mature and viable. This was the case with the trials using *M. musculi* from minnows, which were at least one year old. Therefore, it would have been expected that in at least some of the trials, successful transmission should have occurred, so this seems an unlikely reason for the failure to transmit myxospores to oligochaetes.

Development time

Two possibilities for the apparent lack of transmission were that, either transmission did occur and was missed between successive sampling points or that development would have taken longer than a year under the current experimental regime. It seems unlikely that the infection would have been missed since the current author is skilled in isolating and identifying actinospore infections in water samples. Additionally, although the actinospore may have been released between sampling periods, it is known that infections in oligochaetes can persist for at least a year under laboratory conditions and possibly for the lifetime of the oligochaete under natural conditions (El-Matbouli *et al.*, 1999 a, b; Granath and Gilbert, 2002) and that actinospores can survive for between 11 and 25 days following release from the host (Yokoyama *et al.*, 1993a; Xiao and Desser, 2000). Therefore, even if all actinospores were released during a single day, it is highly likely that some evidence would have been seen to suggest successful transmission.

Most studies on myxozoan lifecycles have shown that development in the oligochaete takes approximately 90-120 days (Grossheider and Körting, 1992; Benajiba and Marques, 1993; Kent *et al.*, 1993; Estzerbauer *et al.*, 2000; El-Matbouli and Hoffman, 1993; Székely *et al.*, 1998; 1999; Molnár *et al.*, 1999b), including that of *M. pseudodispar*

from the musculature of roach. During the current study, oligochaetes were exposed to *M. pseudodispar* spores from chub and therefore, even if all other myxozoans used had a longer development time, one would expect that at least this trial would have been completed within 90-120 days. This was not the case and since all oligochaetes were maintained for a year without successful transmission, it seems that the length of time given to allow development was sufficient, and therefore is not the reason why transmission was unsuccessful.

Susceptibility related to biology of the host

The biology of the host, including host age and within- and between-species susceptibility may have impacted on the success of the trial. The stock used was viable and acclimated to laboratory conditions, including being fed on an artificial diet. The stock has been successfully used to conduct transmission trials with *M. cerebralis* (El-Matbouli *et al.* 1998) and was considered susceptible to myxospore infections and acclimated to laboratory maintenance since the worms were actively breeding. Therefore, the lack of transmission is unlikely to have been due to the conditions in which the hosts were maintained. Whilst some immature tubificids would have been used in at least some of the trials, this should not have impacted on the transmission potential, since it is known that immature oligochaetes can also be infected with actinospore stages (Xiao and Desser, 1998b; Özer *et al.*, 2002; Granath and Gilbert, 2002; Oumouna *et al.*, 2003; Longshaw and Feist, unpublished). It is known that oligochaetes can develop a resistance to re-infection following successful elimination of the parasite, although infections can persist for the lifetime of the host (Granath and Gilbert, 2002). In the current study it is known that naïve, previously unexposed oligochaetes were used (M. El-Matbouli, pers. comm.) and so this is also unlikely to have determined the final outcome.

The most likely reason for the lack of transmission is due to the species of oligochaete used. The fish from which the myxospores were isolated came from a relatively pristine river system where few *T. tubifex* are found (P. Frear, Environment

Agency, York, pers. comm.). Whilst numerous previous studies of myxozoan lifecycles have implicated *T. tubifex* as the alternate host, it is possible that the oligochaetes used in those trials represent a collection of more than one species or subspecies. Coupled with the understanding that actinospores occur in a wider range of tubificids (Székely *et al.*, 2003; Xiao and Desser 1998a, b; Koprivnikar and Desser, 2002 and see chapter 3), it seems likely that myxozoans used in the current study are adapted to utilising specific tubificids other than *T. tubifex*. Additionally, from studies of the actinospore fauna of oligochaetes collected in river systems in Yorkshire and Humberside it is known that several do not alternate through *T. tubifex* (Longshaw, unpublished data).

Furthermore, the oligochaetes used in the trials were derived from a laboratory stock, originally sourced from a *M. cerebralis* enzootic area in the USA. They were selected and bred for their susceptibility to infections with *M. cerebralis*. It has been shown that susceptibility to infections with *M. cerebralis* in the oligochaete stage has a genetic basis and appear to be specific to genetic lineages of *T. tubifex* (Beauchamp *et al.*, 2001, 2002). S. Hallett (pers. comm.) failed to transmit *Sphaerospora elegans* and *Myxobilatus gasterostei* from sticklebacks using the same stock of oligochaetes. It therefore seems highly likely that the main reasons for lack of transmission during the current study is that either the myxospores used are specific to tubificids other than *T. tubifex* or, more likely, that the genetic strain of *T. tubifex* used is only susceptible to infections with *M. cerebralis*.

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CHAPTER EIGHT

INVERTEBRATES AS HOSTS FOR *TETRACAPSULOIDES BRYOSALMONAE*

Abstract

Prior to the recognition of bryozoans as hosts for the agent for proliferative kidney disease (PKD), it was anticipated that an actinospore stage would be involved in the lifecycle. Therefore, studies were initiated to examine, using PKX specific primers, if any of the actinospores found during the current study were implicated in the lifecycle of the PKD organism. All actinospores examined by the PCR reaction were negative. In addition, over sixty invertebrate species were examined using PKX specific PCR primers. Agaian, all were negative. Data is provided on these studies, along with the first report using molecular information on the presence of the PKD agent in UK bryozoans. Finally, given the current state of knowledge regarding the malacospore lifecycle, suggestions are made on possible reservoir hosts for *Tetracapsuloides bryosalmonae.*

Introduction

Proliferative kidney disease (PKD) is a serious pathogen of wild and farmed salmonid fish in Europe and the USA (Hedrick *et al.*, 1993). Whilst the disease has been recognised since 1924 (Plehn, 1924), the exact aetiological agent remained a mystery until the mid 1980's and was thus named PKX, where X stood for disease agent unknown. Following ultrastructural studies of the parasite, it was classified within the Myxozoa (Kent and Hedrick, 1985a, b; Clifton-Hadley and Feist, 1989). However, the lack of spores in the fish host precluded the possibility of further classification. Following the understanding of the role of oligochaetes in the lifecycle of *Myxobolus cerebralis*, and given that PKX is a myxozoan, it was anticipated that it too would utilise an oligochaete. Studies were therefore directed towards the examination of suitable oligochaetes (Hedrick *et al.*, 1992). Filtration experiments on water and sediments from a recirculating aquaculture facility in France demonstrated that PKD was induced in naïve fish exposed to filtrates trapped on a 500µm and in water passed through a 50µm mesh. Based on this data, Hedrick *et al.* (1992) suggested that the parasite most likely utilised the oligochaete *Stylaria lacustris* in its lifecycle although no actinospore stage was observed.

Subsequently, Canning *et al.* (1996) described a new order, genus and species of myxozoan in bryozoans, namely *Tetracapsula bryozoides* (now *Buddenbrockia plumatellae*) in the order Saccosporidae. Feist (1997) and Kent *et al.* (1998) suggested that bryozoans may be a possible host for the PKD agent. Following the report by Anderson *et al.* (1999) that the DNA sequence of a related malacospore in bryozoans was the agent for PKD, studies were directed towards confirming its presence in PKD enzootic waters in the UK (Longshaw *et al.*, 1999) and towards completing the lifecycle under laboratory conditions (Feist *et al.*, 2001; Longshaw *et al.*, 2002). The parasite was named as *Tetracapsuloides* (=*Tetracapsula*) *bryosalmonae* based on ultrastructural studies and DNA sequence data by Canning *et al.* (2000, 2002).

Whilst the partial lifecycle has been completed (Feist *et al.*, 2001; Longshaw *et al.*, 2002), researchers have so far been unable to complete Koch's postulate and transmit the parasite from salmonids to bryozoans or from bryozoans to bryozoans (Sylvie Tops, University of Reading, personal communication; Longshaw and Tops, unpublished data). Although extrasporogonic *Tetracapsuloides*-like organisms have been reported infecting the gills of carp (Voronin, 1993; Voronin and Chernysheva, 1993), and stages in the kidney of pike reported by Bucke *et al.* (1991) were confirmed as *T. bryosalmonae* (Morris *et al.*, 2000), no other fish hosts have been reported as containing these extrasporogonic stages of unique appearance. Since *T. bryosalmonae*-infected bryozoans have been reported from sites lacking salmonids, pike and carp hosts, it appears likely that fish may be a facultative host (Okamura *et al.*, 2001). However, the lack of transmission from fish to bryozoans and from bryozoan to bryozoan could indicate the necessity of another, as yet undiscovered invertebrate host since fish appear not to be an obligate host. Additionally, the possibility that the parasite could cycle between different species of bryozoans appears not to be possible since there are sites where only one species of bryozoan occurs (Okamura and Wood, 2002). The current study considers the role of oligochaetes and other invertebrates in the lifecycle of *T. bryosalmonae* through the screening of actinospores and potential invertebrate hosts by the use of *T. bryosalmonae*-specific primers and histological examination of a wide range of invertebrates in a PKD-enzootic river. Consideration is given to other potential hosts and suggestions are provided for future research needs in the elucidation of malacosporean lifecycles.

Materials and methods

Sampling

Oligochaetes were collected from five sites around Dorset and Wiltshire, including two farms enzootic for PKD and isolated using the methods described previously (chapter

3, this thesis) between summer 1996 and Autumn 2002. Released actinospores were collected and frozen to –20°C for DNA sequence analysis. Invertebrates were collected from the same sites in Dorset and Wiltshire in February, March, June, July and August 1998. To ensure as broad a range of types of species, four sampling methods were employed - kick samples, weed scrapes, aufwuchs (rock and log washes) and mud scrapes. Samples were returned to the laboratory and isolated from the abiotic component by using three sieves 1.40mm, 710µl and 425µm. Invertebrates were either treated for histology as described below or frozen or stored in 100% ethanol for DNA sequence analysis. Bryozoans were collected from two sites enzootic for proliferative kidney disease (PKD), namely Pound Fish Farm on the River Cerne and Barford Fish Farm on the River Avon in June and July 1999. Any collected bryozoans were transported back to the CEFAS Weymouth Laboratory in small containers or in resealable bags. As soon as possible, colonies were examined using light microscopy for the presence of myxozoan sacs as reported by Longshaw *et al.* (1999).

Histology

Invertebrates were maintained in distilled water at 4°C for 24-48 hours to reduce gut contents then fixed in Davidson's fixative for 24-48 hours with constant agitation. Samples were decalcified either in rapid decalcifying compound (RDC) for 1 hour and stored in 70% alcohol or in 5.5% EDTA solution for up to 4 days, washed in 70% alcohol, and stored in 70% alcohol prior to processing for histology. Samples were processed according to standard histological methods and embedded in wax. Sections were cut at between 3-5µm thickness, stained with Haematoxylin and Eosin, and examined using a Nikon E800 light microscope for the presence of protistan parasites.

Molecular biology

Samples maintained at –20°C were defrosted prior to use, resuspended in 450 µl of lysis buffer (44µl SDS, 5 µl proteinase K, 400 µl TE buffer) and incubated at 60° C for 1 hour. For samples maintained in 100% ethanol were washed twice in TE Buffer and then soaked in TE buffer for one hour. DNA was extracted using a standard SDS, proteinase K extraction followed by a phenol/chloroform/isoamyl alcohol and ethanol precipitation. The resultant pellet was resuspended in 20 µl of RNAase/DNAase free water. 400 µl of phenol/chloroform/IAA were added to the digested sample and vortexed for 20-30 seconds then centrifuged at 13000 rpm for 10-15 minutes. The upper (aqueous) phase was removed and transferred to a centrifuge tube. The process was repeated with the inclusion of 40µl of 3M sodium acetate. 900 µl of absolute ethanol was added, vortexed and centrifuged at 13000 rpm for 10 minutes. The ethanol was removed as soon as possible by vacuum suction and then air-dried. The pellet was resuspended in 20 µl of RNAase / DNAase free water and stored at -20°C until required. 18S rDNA fragments were amplified using a single round PCR in standard 100µl reactions containing 10µl reaction buffer IV (10×), 10µl 25mM Magnesium Chloride, 0.5 µl of each primer (1µg/µl), 1µl Red Hot DNA polymerase (5U/µl), 1µl 25mM dNTP's, 67 µl of DNAase / RNAase water and 10 µl of extracted DNA. The *T. bryosalmonae*-specific primers were PKX5f (5'-CCTATTCAATTGAGTAGGAGA-3') and PKX6r (5'-GGACCTTACTCGTTTCCGACC-3') of Kent *et al.* (1998).

T. bryosalmonae positive material was expected to produce a fragment of approximately 435 base-pairs. PCR reactions were temperature cycled in a Stratagene Robocycler 40 for thirty five cycles of 1 minute at 94°C, followed by 1 minute at 55°C, followed by a 1 minute at 72°C were used in the amplification. This was ended by a 5-minute extension at 72°C. Positive and negative controls were included in all reactions in the form of PKD affected kidney homogenate and water respectively. PCR products were resolved on a 2% agarose gel with ethidium bromide by loading 20µl of the 100µl reaction

volume with 6× loading buffer into each well. Fragment size was determined by running 5-10µl of a 100bp DNA ladder (Promega) in one of the lanes. The DNA was visualised with UV light. The DNA fragments of interest were purified using the Geneclean®Spin Spin system (Bio101 Inc.) following manufacturers protocol. PCR products were ligated and cloned using the pGEM-T Easy Vector II system (Promega). The pGEM-T Vector was briefly centrifuged and standard 10µl ligation reactions were used containing 1µl Ligase 10× buffer, 1µl pGEM-T vector (50ng), Xµl PCR product, 1µl T4 DNA Ligase (3 Weiss units/µl) and made up to 20µl using nuclease-free water. Positive controls were also used in 10µl reactions containing 1µl Ligase 10× buffer, 1µl pGEM-T vector (50ng), 2µl of control insert DNA , 1µl T4 DNA Ligase (3 Weiss units/µl), 5µl Nuclease-Free water. Samples were incubated overnight at 4°C or at room temperature for approx. 6 hours. Following ligation, PCR products were transformed in JM109 competent cells. One LB/ampicillin plates for each ligation reaction were prepared. Plates were equilibrated to room temperature prior to plating. Tubes containing ligation products were centrifuged and 5µl of each ligation reaction was added to a sterile 1.5ml microcentrifuge tube on ice. High efficiency JM109 Competent Cells were just defrosted from –70°C and gently mixed by flicking. 50 µl of cells was added to each tube containing ligation products. Tubes mixed by flicking and placed on ice for 20-40 minutes. Cells were then heat shocked for 2 minutes at exactly 42°C in a heated water bath and returned to ice for 2 minutes. Tubes were then incubated at 37°C for 1.5 hours. 10µl of X-Gal (50mg/ml) and 10µl IPTG (500mM) was added to each tube and each transformation culture was plated onto duplicate LB/ampicillin plates and incubated overnight at 37°C. Blue colonies were unligated and therefore not used. Two white colonies contained inserts and were selected for sequencing. Colonies were picked out using sterile toothpicks and added to 10ml LB broth containing 100µl of 10mg/ml ampicillin. Samples were incubated overnight at 37°C for 16-24 hours with shaking. DNA from clones was extracted using alkaline lysis as follows: cells were 1.5ml of cells was suspended for 20 seconds at 13,000 rpm.

Supernatant was removed and cells were re-suspended in 100µl GTE solution (solution I) (50mM glucose, 10mM EDTA, 25mM Tris pH 8.0). Cells left at room temperature for 5 minutes. 200µl of NaOH/SDS (solution II) (0.2M NaOH, 1% SDS) was added, solution was mixed and placed on ice for 5 minutes. Finally, 150µl of 3M potassium acetate solution (solution III) (60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml DNAase free water). Samples were placed on ice for 5 minutes and then spun at 13,000 rpm for 3 minutes. Supernatant was added to a new tube to which was added 400µl phenol/chloroform/IAA. Samples were centrifuged for 10 minutes at 13,000 rpm after which the aqueous layer was transferred to a new tube. 2½× volume (900µl) of ethanol was added to the tube. Samples were vortexed briefly and centrifuged at 13,000 rpm for 10 minutes. Ethanol was removed by vacuum suction. Pellets were air-dried, and 40 µl of RNAase A (10µg/ml) added. To confirm presence of an insert, 5µl of each sample was added to 2µl buffer II, 0.5µl EcoRI and 12.5µl water. Samples were incubated at 37°C for up to 1 hour. Samples were run on a 2% agarose gel at 140v and DNA was visualised using ethidium bromide.

Generally, 10µl of the eluted DNA were used in sequencing the DNA. 0.2ml thin walled microcentrifuge tubes were used containing the following in each: 10µl of eluted DNA, 8µl of Terminator Ready Reaction mix and 2µl of a 10pmol/µl solution of primer (forward or backward). This was overlaid with 20µl of mineral oil and prepared for sequencing in a GeneAmp 2400 PCR system. Twenty-five cycles of 10 seconds at 95°C, followed by 5 seconds at 50°C, followed by 4 minutes at 60°C were used in the amplification. After this, the 20µl of DNA was added to a new tube along with 70µl DNAase/RNAase free water, 10µl of 3M sodium acetate, 250 µl ethanol, briefly vortexed and centrifuged at 13000rpm for 15 minutes. The supernatant was removed under vacuum suction and the pellet then rinsed in 500µl of 70% ethanol. This was then removed under vacuum suction and the pellet dried under vacuum or in the air for 2-5 minutes. The pellet was re-suspended in 30µl of Template Suppression Reagent (ABI) and transferred to a

0.5ml Genetic Analyser Sample Tube with septa. This was then denatured at 95°C for 2 minutes, centrifuged briefly if necessary to remove bubbles and placed on ice or in the fridge until ready for use. DNA was sequenced on an ABI 310 Genetic Analyser. Sequences were aligned and analysed using DNAsis ver. 2.1.

To confirm that invertebrate host DNA did not interfere with the PCR reaction and provide erroneous results, a sub-sample of *Gammarus pulex*, leeches and ostracods were spiked with approximately $2cm^3$ of PKD-infected kidney from a rainbow trout and a standard extraction and PCR reaction completed as described above. Additionally, dilutions of "standard" PKD infected kidneys were performed and PCR reactions carried out to determine the limit of the PCR reaction to detect the parasite. Dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 of infected kidneys were performed and subsequently amplified using the methods described above.

During the PCR reactions against potential snail hosts, excess mucus interfered with the reactions, making interpretation of the subsequent DNA visualisation on agarose gels almost impossible. Since it was anticipated that oligochaetes may be involved in the lifecycle of the PKD organism, several hundred symbiotic oligochaetes (*Chaetogaster limnaei*) were removed from pulmonate snail hosts and as above, examined using standard PCR reactions with *T. bryosalmonae*-specific primers.

Results

Histology

Invertebrates examined for parasite infections using histology included leeches; the gastropods *Theodoxus fluviatilis*, *Bithynia* sp., *Planorbis* sp., *Valvata* cf. *piscinalis*, *Lymnea peregra* and *Potamopyrgus jenkinsi;* the bivalves *Pisidium* sp. and *Anadonta* sp.; mites of the suborder Hydracarina; ostracods; the amphipod *Gammarus pulex*; the isopod *Asellus aquaticus*; the hemipteran *Aphelocheirus aestivalis*; the caddis fly larvae

Brachycentrus subnubilis; the oligochaete *Sparganophilus tamesis* and various dipteran fly larvae (true flies) including members of the family Tipulidae and *Chironimus* spp. Although protistan organisms were present in some of the animals examined, none were thought to be myxozoan in origin. In addition, many larger helminth parasites such as digeneans were seen in histological sections. It was noted that many invertebrates, including oligochaetes, were infected with peritrichous ciliates.

PCR studies on invertebrate hosts and on actinospores

A total of 2295 invertebrates from at least 60 species were tested using *T. bryosalmonae*-specific primers (see Table 8.1). All PCR reactions on invertebrate material and on actinospores were negative. PCR reactions were able to amplify dilutions of 1:1,000 of a standard PKD infected kidney and were able to detect the presence of *T. bryosalmonae* in invertebrate samples "spiked" with PKD-infected kidneys.

Bryozoan studies

Plumatella sp. (possibly *P. emarginata*) was present at both PKD-enzootic sites, whereas *Fredericella sultana* was only found at Pound Fish Farm (Figure 8.1). Bryozoans were found predominately associated with willow roots at Pound Fish Farm and within water inlet boxes at Barford Fish Farm. Colonies of both species were widespread and numerous in the rivers feeding the fish farms. Myxozoan sacs were present in approximately 50% of the *Fredericella* zooids examined and in approximately 20% of the *Plumatella* zooids. In *Plumatella* sp., the majority of infected zooids contained a single sac, but occasionally there were several. *F. sultana* commonly harboured up to five sacs per zooid. No appreciable difference was noted in the behaviour or appearance of infected colonies.

Table 8.1 List of invertebrate species examined by the PCR reaction with *T. bryosalmonae* specific primers and numbers examined. Where CS = Canford School; BFF = Barford Fish Farm; PFF = Pound Fish Farm; IFE = IFE, East Stoke; JB = Julians Bridge.

Invertebrate species examined	Site	Numbers examined	Result with *T. bryosalmonae* Specific primers
Acari	CS	10	-
Acroloxus lacustris	CS	20	-
Ancylus fluviatilis	CS	20	-
Aphleocheirus aestivalis	BFF	50	-
Asellus aquaticus	PFF, BFF, CS	100	-
Bithynia spp.	IFE, BFF, PFF, JB, CS	50	-
Brachycentrus subrilis	BFF, JB, CS, IFE	20	-
Calopteryx sp.	PFF	10	-
Caddis flies assorted	BFF	50	-
Chaetogaster limnaei	PFF	150	-
Cyclops spp.	PFF	20	-
Daphnia spp.	IFE, PFF	60	-
Damson fly larvae	BFF	10	-
Dendrocoelum lacetum	CS	30	-
Elmis aenea	PFF	10	-
Ephemera sp.	CS, JB, PFF	10	-
Gammarus pulex	CS, BFF, IFE, PFF, JB	150	-
Glossiphonia complanata	PFF	50	-
Heptagenia sp.	CS, PFF	45	-
Ithytrichia	PFF	20	-
Ilyocoris cimicoides	PFF	15	-
Leeches	IFE, PFF, BFF, JB	70	-
Lymnea sp.	BFF	20	-
Lymnea peregra	CS, PFF	50	-
Lymnea palustris	CS, PFF	50	-
Mayfliy larvae (burrowing)	PFF	25	-
Mites	PFF	50	-
Naucoridae	BFF, JB, CS, IFE	10	-
Notonectidae	JB, CS, PFF	10	-
Ostracods	CS, PFF	100	-
Physa spp.	PFF, BFF, JB, CS, IFE	65	-
Piscicola geometra	PFF, CS	15	-
Pisidium spp.	IFE, BFF, PFF, CS, JB	80	-
Planorbis spp.	CS, IFE, PFF, BFF, JB	20	-
Plecoptera	CS	15	-
Polycelis spp.	IFE, PFF, CS	15	-
Potamopyrgus jenkinsi	PFF, JB, IFE, CS	100	-
Sericostoma personatum	CS	35	-
Sialis sp.	CS, IFE	20	-
Simulidae	CS	100	-
Snails	PFF	150	-
Stone flies	PFF	30	-
Theodoxus fluviatuis	IFE, BFF, PFF, JB, CS	100	-
Tipulidae	PFF	120	-
Triclads	PFF, BFF, IFE	50	-
Tricoptera	CS	20	-
Valvata spp.	CS	50	-
Zygoptera	CS	10	-

Myxozoan sacs were morphologically similar to those of *T. bryozoides* (Okamura, 1996) at the light microscope level but were generally smaller measuring up to approximately 300μm in diameter. In examples from *Plumatella* numerous spores and earlier stages were observed (Figs. 8.2 and 8.3). Sacs from the *Fredericella* zooids examined contained only immature stages. Four polar capsules were observed in spores from *Plumatella* sp., indicating that the species belongs to the genus *Tetracapsuloides*.

Following successful PCR on two isolates of myxozoans from *Plumatella* collected from Barford Fish Farm and on myxozoans from *Fredericella* and *Plumatella* collected from Pound Fish Farm, bands of approximately 435bp were resolved on 2% agarose gels (as expected with the primers used). In order to confirm its homology with *T. bryosalmonae*, the bands were excised, cloned and sequenced. Sequence data indicated a greater than 98.5% homology between the four isolates and that of *T. bryosalmonae* from salmonid fish (GenBank U70623) (Saulnier and de Kinkelin, 1997). The two isolates of *T. bryosalmonae* from *F. sultana* and *Plumatella* sp. collected at Pound Fish Farm were 100% identical in their sequences between position 463-897 based on the SSU-DNA sequence determined by Saulnier and de Kinkelin, (1997).

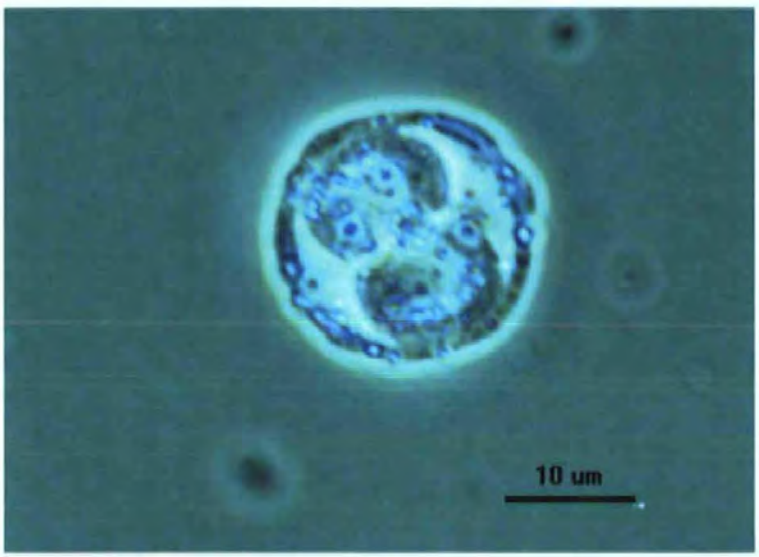
Discussion

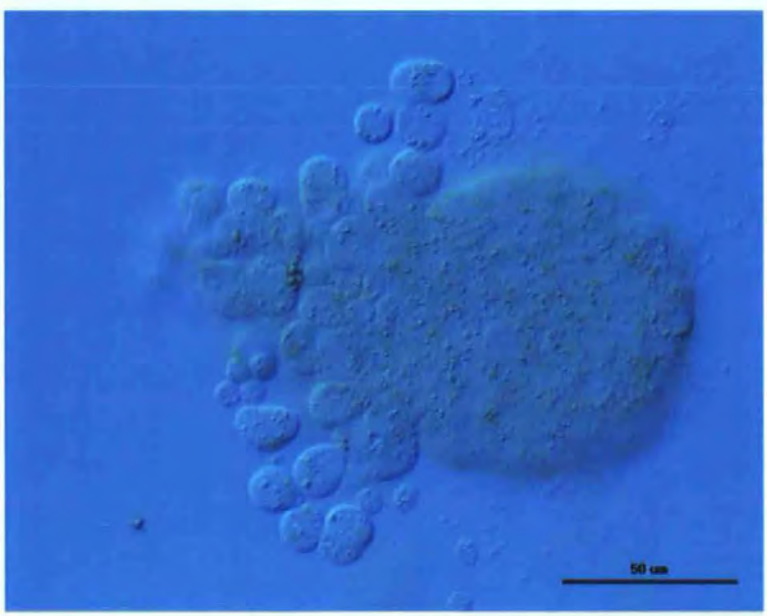
All of the work on screening of invertebrates as potential hosts for the PKD agent was done prior to the recognition that bryozoans are a host for the parasite. Many of the invertebrates sampled were done so due to their relative abundance at each site or seasonal availability. Since a wide range of different hosts were examined, it was hoped that at least one of them would be a host for *T. bryosalmonae*. Whilst it has been demonstrated using molecular, ultrastructural and experimental transmission trials that bryozoans are hosts for

Figure 8.1. Individual zooids of *Fredericella sultana* from a site enzootic for PKD

Figure 8.2. *T. bryosalmonae* spore isolated from *Plumatella* sp. (Phase Contrast)

Figure 8.3. Ruptured myxozoan sac released from *Fredericella sultana* showing immature stages of the *T. bryosalmonae* parasite. (Differential Interference Contrast)


10 um


50 um

the parasite (Canning *et al.*, 2000, 2002; Feist *et al.*, 2001; Longshaw *et al.*, 2002; Longshaw and Feist, unpublished), researchers have failed to transmit the parasite from the salmonid host back to bryozoans or between bryozoan species. The current study demonstrated for the first time using molecular biology studies, the presence of *T. bryosalmonae* sacs and spores in bryozoans associated with PKD-enzootic fish farms in the UK (Longshaw *et al.*, 1999).

Since transmission between bryozoans and from fish has thus far failed, there is a strong possibility that another, as yet unidentified host may be implicated in the lifecycle of the parasite. Fish-to-fish transmission has been demonstrated for four marine myxozoans (Diamant, 1997; Redondo *et al.*, 2002; Yasuda *et al.*, 2002) but natural fish-to-fish transmission of *T. bryosalmonae* is known not to occur (Ferguson and Ball, 1979; D'Silva *et al.*, 1984) and can thus be excluded as a route of transmission. The presence of malacospores in rainbow trout kidney suggests the possibility that transmission from the fish host to another invertebrate may be possible (Morris *et al.*, 2002a; Hedrick *et al.*, 2004). Consideration has therefore been given to the possibility that an actinospore form released from oligochaetes was involved in part of the lifecycle, as proposed by Hedrick *et al.* (1992). PCR reactions using *T. bryosalmonae*-specific primers during the current study and by Morris *et al.* (1999) and Longshaw and Feist (2000) against at least 30 different actinospore types released from oligochaetes collected at sites enzootic for PKD failed to demonstrate a link between *T. bryosalmonae* and these actinospores. DNA from many of the actinospores examined was amplified using myxozoan-specific primers (Longshaw and Feist, unpublished data), and since positive and negative controls were utilised in PCR reactions, it is unlikely that the negative results obtained using the *T. bryosalmonae*-primers is due to errors in the methodology used. In addition, attempts to transmit bryozoan derived *T. bryosalmonae* spores to oligochaetes failed to produce an actinospore equivalent (Longshaw, unpublished results).

It is known that both actinospores and malacospores undergo sexual reproduction in the invertebrate host (El-Matbouli and Hoffman, 1998; Canning *et al.*, 2000) but two sexual reproductive stages occurring in two separate hosts in the same lifecycle would be highly unusual for any parasite species and suggests that either an actinospore stage is present but doesn't undergo a sexual phase in the lifecycle or that an actinospore counterpart is not produced. Seasonal samples of actinospores were collected throughout the study period, and thus the methodology employed should have provided sufficient numbers and types of actinospores to be able to identify, using PCR primers, any potential actinospore stage of *T. bryosalmonae.* The wide range of spatio-temporal actinospore samples utilised minimised the possibility that an actinospore stage was missed due to seasonal effects of release. In addition, since no *T. bryosalmonae* DNA was detected in the snail symbiotic oligochaete *C. limnaei,* it seems highly unlikely that oligochaetes are involved in the lifecycle of *T. bryosalmonae.*

Alternatively, *T. bryosalmonae* may utilise another invertebrate host in its lifecycle. During the current investigation, several hundred individuals of common, mainly benthic invertebrates were examined using the *T. bryosalmonae*-specific primers and by histology. None were found to contain the DNA of *T. bryosalmonae* and, with the exception of some protistan-like organisms in snails, recognisable myxozoan stages were not detected by histology in any of the invertebrates examined. However, since only a small number of each invertebrate species was examined these species cannot entirely be precluded as being involved in the lifecycle of *T. bryosalmonae.* However, samples of invertebrates were collected immediately prior to, during and after the PKD season. If one of the invertebrates examined was a potential host, it would be expected that *T. bryosalmonae* DNA would be present either before spore formation in bryozoans, thus allowing transmission to the bryozoan host or after release of spores from the bryozoans and into an invertebrate host. This was not the case. Gay *et al.* (2001) reported that the examination of similar invertebrates in a recirculating system in France had failed to elucidate a

potential invertebrate host for *T. bryosalmonae*. Given this data, it seems unlikely that any of the invertebrates mentioned here are an alternative host for the parasite.

Recently, Audemard *et al.* (2002) showed that the free-living copepod *Paracatia grani* was involved in the lifecycle of the oyster pathogen *Marteilia refringens*. As with *T. bryosalmonae*, only part of this lifecycle has been elucidated. Similarities in the mode of development, such as the production of pluricellular "extrasporogonic" stages and apparent lack of spores in the oyster (Longshaw *et al.*, 2001) has led to some authors suggesting a taxonomic link between *M. refringens* and *T. bryosalmonae* (Seagrave *et al.*, 1980a, b). It is possible that similar divergent lifecycles in these two serious pathogens may be occurring. Bryozoans are known to filter feed and motile free-living copepods occur at many freshwater sites, including those enzootic for PKD. Given that *P. grani* has been shown to be capable of transmission of the distantly related *M. refringens* to oysters, it is possible that motile copepods in freshwater systems are a source of *T. bryosalmonae* infections to either bryozoan or fish hosts. The ability of these free living copepods to maintain their position in a water column, their feeding behaviour and their relatively common abundance at most sites make them a suitable candidate for consideration as a host for the PKD organism.

The role of fish hosts in the dissemination of *T. bryosalmonae,* either to other fish, bryozoans or invertebrates also seems possible, although unlikely. Morris *et al.* (2002b) demonstrated by PCR that salmonids release *T. bryosalmonae* into the environment. However, they were unable to conclude whether this PCR positive material contained viable *T. bryosalmonae* spores or developmental stages which were capable of transmission back to any possible host. Subsequently, Hedrick *et al.* (2004) demonstrated the presence of *T. bryosalmonae* spores in urine of rainbow trout recovering from PKD. These spores were bilaterally symmetrical, and unlike those described by Kent and Hedrick (1985b), were sub-spherical and fully formed. These spores were clearly related to *T. bryosalmonae* based on molecular, antibody and morphological data obtained but

since they degenerated within a few minutes after release from the fish host, were unlikely to remain viable long enough to transmit to bryozoans unless they were released in immediate proximity to these invertebrate hosts. There is a lack of diversity in the mtDNA of *T. bryosalmonae* (Beth Okamura, University of Reading, personal communication) from farmed rainbow trout and since there has been widespread movement of this host through aquaculture practises, it follows that transmission and dissemination of the parasite through the movement of fish has not occurred. Additionally, the relative rarity of these spores (Feist *et al.* 2002; Feist and Bucke, 1993) in farmed and wild-caught salmonid hosts, coupled with the apparent short longevity of the spores and absence of a fish species common to all sites in which *T. bryosalmonae* has been reported would strongly suggest that fish are not an essential component of the lifecycle of the parasite.

Gay *et al.* (2001) reported that stages of *T. bryosalmonae* infective to naïve rainbow trout were present in bryozoans collected from a PKD enzootic river throughout the year. They therefore suggested the possibility that the parasite was able to remain in a dormant state within *Fredericella sultana* colonies, which are known to over-winter as live colonies (Okamura *et al.*, 2001; Longshaw *et al.*, 2002). These so-called "cryptic stages" were reported as occurring in the body wall of bryozoans by Canning *et al.* (2002) and Tops and Okamura (2003). It was suggested that proliferation of these stages then produced sacs within the *Fredericella* colony once permissive temperatures were reached. Whilst this may provide an explanation for the source of infection within these overwintering colonies, it does not explain how the parasite is subsequently able to infect other bryozoan colonies, including those that over-winter as statoblasts. Okamura and Wood (2002) suggested that dispersal of the parasite in bryozoans with a statoblast stage could result from the incorporation of cryptic stages into the statoblast. However, to date, this route of transmission has not been demonstrated. It is possible, given the wide variety in bryozoans known to be infected with *T. bryosalmonae* that several routes of transmission are possible, including proliferation from cryptic stages in the body wall and

statoblast, as well as through another invertebrate host. It is clear that much remains to be elucidated concerning the lifecycle of the parasite, both in terms of completing the cycle and in clarifying the factors that determine the mode and choice of development.

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CHAPTER NINE

STUDIES ON THE LIFECYCLE OF *TETRACAPSULOIDES BRYOSALMONAE* IN SALMONID HOSTS

Published as two papers:

1. Feist, S.W., Longshaw, M., Canning, E.U and Okamura, B. (2001) Induction of proliferative kidney disease (PKD) in rainbow trout *Oncorhynchus mykiss* via the bryozoan *Fredericella sultana* infected with *Tetracapsula bryosalmonae*. *Diseases of Aquatic Organisms* **45:** 61-68
2. Longshaw, M., Le Deuff, R.M., Harris, A.F. and Feist, S.W. (2002) Development of proliferative kidney disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following short-term exposure to *Tetracapsula bryosalmonae* infected bryozoans. *Journal of Fish Diseases* **25:** 443-449

Introduction

It is now established that bryozoans are involved in at least part of the life cycle of *Tetracapsuloides bryosalmonae*. Initially, this was demonstrated through the use of DNA sequence matching (Anderson *et al.*, 1999a, b; Longshaw *et al.*, 1999) and confirmed using experimental transmission trials. The following two papers were written as a result of these experimental trials. The initial work by Feist *et al.* (2001) was an attempt to show the effect of route of exposure on the development of the disease. Once it was established that bryozoans were indeed implicated in the lifecycle, the route of entry by the parasite and minimum length of exposure required to induce the disease were determined (Longshaw *et al.*, 2002). The main findings of this research are that *T. bryosalmonae* stages from bryozoans are able to infect naïve rainbow trout through bath challenge but not through gavage, that the minimum length of exposure required to induce PKD in fish is 1 minute and the route of entry by the parasite into the fish host is via mucous cells in the skin epithelium.

INDUCTION OF PROLIFERATIVE KIDNEY DISEASE (PKD) IN RAINBOW TROUT *Oncorhynchus mykiss* (WALBAUM, 1792) VIA THE BRYOZOAN *Fredericella sultana* (BLUMENBACH, 1779), INFECTED WITH *Tetracapsula bryosalmonae* CANNING, CURRY, FEIST, LONGSHAW & OKAMURA, 1999.

Abstract

Proliferative kidney disease (PKD) is a serious pathogen of wild and farmed salmonids, affecting mainly the kidney and spleen but becoming systemic in most susceptible fish hosts. This report deals with the transmission of *Tetracapsula bryosalmonae* from naturally infected bryozoans (*Fredericella sultana*) to naïve rainbow trout, thereby confirming the recent conclusion, based on partial 18S rDNA sequence data, that bryozoans are hosts of the myxozoan parasite *T. bryosalmonae* (formerly PKX organism) that causes the disease. Parasite transmission using *T. bryosalmonae* spores was successful by short-term exposure to disrupted bryozoans known to contain *T. bryosalmonae* spores and *T. bryosalmonae* sacs liberated from the bryozoans and by long-term cohabitation with infected bryozoan colonies. Infection was confirmed by examination of kidney imprints, detection of the parasite in stained tissue sections, Polymerase Chain Reaction (PCR) using *T. bryosalmonae* specific primers, and comparison of amplified 18S rDNA sequences from the bryozoans and experimentally infected fish. Transmission was not apparent, nor was PKD induced, in fish challenged by intraperitoneal injection of spores isolated from *F. sultana*.

Introduction

Research into aspects of proliferative kidney disease (PKD) in salmonids has been successful in providing information related to the pathogenesis of the disease in the fish host and in the development of the parasite within the host tissues (Kent & Hedrick 1986, Clifton-Hadley et al. 1987a,b, Feist & Bucke 1987, Hedrick et al. 1993). However, the search for the presumed definitive fish host (Hedrick et al. 1992, Kent et al. 1995, Morris et al. 1999) and, until recently (see discussion below), for an infective 'spore' stage has been unsuccessful. In addition, attempts to isolate and culture the extrasporogonic stage of the parasite have only been partially successful (R. P. Hedrick and D. Morris pers. comm.). The development and application of several diagnostic tools, including monoclonal antibodies (Adams et al. 1992, Saulnier & de Kinkelin 1996) lectins (Castagnaro et al. 1991) and Polymerase Chain Reaction (PCR) primers (Saulnier & de Kinkelin 1997, Kent et al. 1998), have enabled workers to detect extremely low levels of infection. In addition they have shown that the antigenic profile of the parasite changes as it develops in the fish. Together with light microscopy on fresh or fixed material, the above methods have been used to search for potential fish hosts amongst wild fish populations. In particular, research has concentrated on searching for potential hosts harbouring mature myxospore stages that could be isolated and used in experiments on the transmission requirements of the parasite (Saulnier & de Kinkelin 1997, Kent et al. 1998, Morris et al. 1999). However, until now the only method of infecting susceptible salmonids has been to expose naïve fish to water enzootic for the disease or by the artificial means of injecting infected fish tissue or blood (Clifton-Hadley et al. 1984, Kent & Hedrick 1985, Arkush & Hedrick 1990, Feist & Bucke 1993).

Understanding of the transmission requirements of myxosporean parasites has progressed significantly since the pioneering discovery that an oligochaete is an obligate host in the life cycle of *Myxobolus cerebralis*, the causative agent of whirling disease in

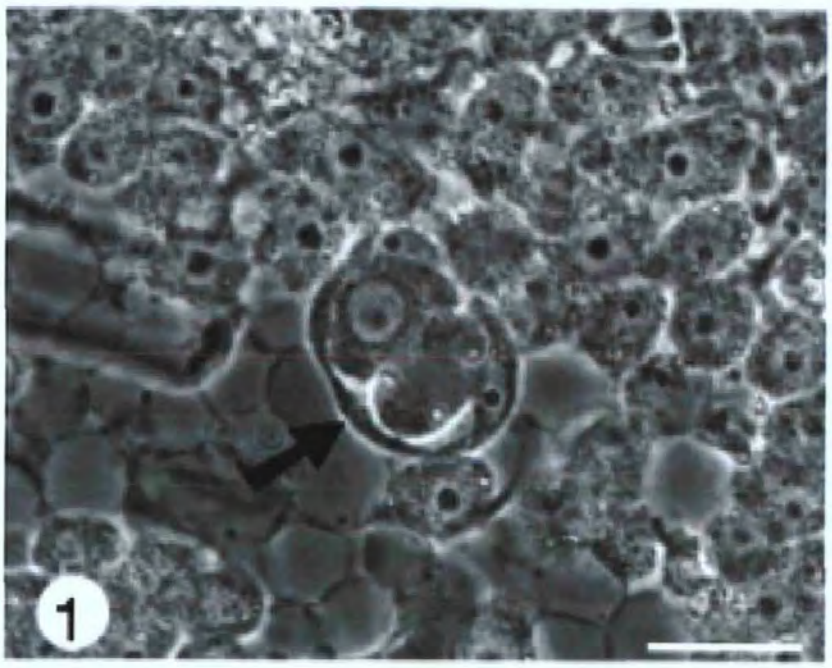
salmonids (Wolf & Markiw 1984). Subsequently, the two-host life cycle was confirmed by El-Matbouli & Hoffman (1989). Similar life cycles have now been demonstrated for other myxosporean genera, including *Hoferellus*, *Ceratomyxa*, *Myxidium* and *Sphaerospora* (e.g. Benajiba & Marques 1993, Troullier et al. 1996, Bartholomew et al. 1997, Molnár et al. 1999). These life cycle studies prompted surveys for actinosporean parasites in waters enzootic for PKD in an attempt to identify the stage infectious to fish (M. Longshaw, unpublished data; D. Morris, pers. comm.). However, a recent discovery (Anderson et al. 1999a, b) revealed that a parasite of bryozoans later named *Tetracapsula bryosalmonae* (Canning et al. 1999), shared near identical partial 18S rDNA gene sequences with the agent of PKD (originally designated PKX by Seagrave et al. 1980). It is now evident that *T. bryosalmonae* is widely distributed. In North America it has been found in lacustrine environments (Anderson et al. 1999a, b), and, in the U.K., in rivers enzootic for PKD (Longshaw et al. 1999). These findings have opened up new avenues for investigation in PKD research. Here, we report in detail the first study to test and achieve successful transmission of *T. bryosalmonae* from a bryozoan host, *Fredericella sultana,* to rainbow trout, with the induction of clinical PKD, first reported in a footnote to Canning et al. (1999).

Materials and methods

Sample sources

Infected colonies of the bryozoan *Fredericella sultana* were obtained on July 26th 1999 from a river feeding a rainbow trout farm enzootic for PKD (site B of Longshaw et al. 1999). Colonies or portions of colonies were collected, together with the willow (*Salix* sp.) roots on which they were growing. They were transported to the laboratory in river water and, on arrival, colonies were examined for the presence of myxozoan sacs similar to those of *T. bryozoides* described by Canning et al. (1996) and Okamura (1996). Sacs were released from some colonies by breaking apart the colony branches. The released sacs (Fig.

Figure 9.1. Immature spore of *T. bryosalmonae* (arrowed) from a ruptured parasite sac released from a *F. sultana* zooid. Unfixed specimen. Phase contrast. Bar = 20 μm


1

9.1) were then collected and maintained in sterile, de-mineralised water at 4°C until required. Infection trials with these sacs were initiated within 24 h of collection. Additionally, willow roots with attached, infected *F. sultana* colonies were used for cohabitation trials. Although willow branches supporting infected colonies were suspended in a tank with flow-through water throughout the experiment, no attempt was made to maintain the bryozoans. Finally, kidneys from four fish showing clinical signs of PKD (including pronounced renal hypertrophy) were used as positive controls. These fish were obtained from a fish farm, which experiences annual epizootics of PKD. The site was approximately 80 km distant from the collection site for bryozoan material. The kidneys were coarsely homogenised, allowed to settle, and the supernatant was used in infection trials (Clifton-Hadley et al. 1984).

Rainbow trout (*Oncorhynchus mykiss*) were imported as eyed eggs from a site on the Isle of Man, which is known to be free of PKD. The eggs were hatched and reared in the laboratory, to an average weight of 18 g in flow-through water. The tank water was dechlorinated drinking water from boreholes and could not therefore be a source of infection. Prior to the start of the experiment a subsample of ten fish was used to confirm that the fish stock was not infected with *T. bryosalmonae* by examination of histological sections, kidney imprints and PCR, as described below.

Experimental procedure

Ten fish were used per trial. Naïve, susceptible fish were exposed by: (a) intraperitoneal (I/P) injection with 0.2 ml of a suspension of 20 disrupted *T. bryosalmonae* sacs from *F. sultana* in 3 ml distilled water; (b) exposure to disrupted bryozoans and *T. bryosalmonae* sacs in approximately 30 l of aerated water for 90 min without flow-through water (short-term exposure); (c) long-term cohabitation with infected *F. sultana* colonies for the duration of the experiment. Although oral gavage would have been a desirable additional route of infection, fish were not exposed by this route owing to lack of material.

Additionally, control groups were established which received either no treatment or I/P injection with 0.2 ml sterile phosphate buffered saline (PBS) (negative controls) or I/P injection with 0.2 ml of the supernatant of kidney homogenate from rainbow trout clinically infected with PKD (positive control group). Injection challenged fish were lightly anaesthetised with 3-aminobenzoic acid ethyl ester (MS222) solution (Sigma) prior to injection, then allowed to recover in aerated water before being placed in experimental tanks.

Exposure of fish to bryozoan material was carried out on July 27th 1999 and the positive controls were established on July 28th 1999. This was designated as week 0. Single fish were sampled from each trial approximately every two weeks on Aug 5th 1999 (week 2), August 20th 1999 (week 4), September 1st 1999 (week 6), September 16th 1999 (week 8), September 30th 1999 (week 10) and finally the experiment was terminated on October 14th 1999 (week 12) when the remaining fish were sacrificed for examination. Fish were maintained in 50 l tanks with flow-through water at 16°C ± 0.5°C and held under a 12 h light (200 lux), 12 h dark regime with a transition period of 30 min subdued lighting. Water flow rates were adjusted to give a complete exchange every 70 min for the duration of the trial. Fish were killed by terminal anaesthesia in MS222. At each sampling point samples were taken for molecular biology, electron microscopy and histology.

Light microscopy

Samples of brain, gill, muscle, skin, heart, liver, kidney, spleen and gut were fixed in 10 % neutral buffered formalin (NBF). Samples of skin, muscle and gill were further treated with 10 % formic acid for 24 h and all tissues were then transferred to 70% industrial methylated spirits (IMS) and processed to wax blocks on a vacuum infiltration tissue processor. Sections were cut at 4 µm and stained with haematoxylin & eosin (H&E). Blood smears and kidney imprints from all fish were air-dried, fixed in 0.5 % acetic acid in methanol and stained with May-Grünwald Giemsa.

DNA extraction, polymerase chain reaction and DNA sequencing

Samples of spleen and kidney were placed in 100 % ethanol. Prior to DNA extraction, samples were washed twice in TE buffer (10 mM TRIS, 1 mM EDTA) and then soaked in TE buffer for one hour. Samples were lysed in 44 µl 1 % sodium dodecyl sulphate (SDS), 5 µl proteinase K (20 µl/ml) and 400 µl TE buffer for 1 h at 60°C. They were then extracted twice with 400 µl phenol/chloroform/isoamyl alcohol (50:50:1) and 40 µl 3 M sodium acetate. Genomic DNA was precipitated in absolute ethanol, centrifuged at 13000×g for 15 min and the ethanol removed. The resultant pellet was air-dried, resuspended in 20 µl of RNAase/DNAase free water and stored at -20°C until required.

The myxozoan 18S rDNA was amplified by a single round PCR using primers PKX 5F and PKX 6R of Kent et al. (1998). PCRs were performed in standard 100 µl reactions containing 10 µl reaction buffer IV (AB gene) (10×), 10 µl 25 mM magnesium chloride, 0.5 µl of each primer (1 µg/µl), 1 µl Red Hot DNA polymerase (5 U/µl), 1 µl 25 mM dNTP's, 67 µl of RNAase/DNAase free water and 10 µl of myxozoan DNA. Positive and negative controls in the form of PKD affected kidneys and RNAase/DNAase free water respectively were used in all amplifications. The samples were overlaid with two drops of mineral oil and the temperatures were cycled in a Stratagene Robocycler 40. Thirty-five cycles of 1 min at 94°C, followed by 1 min at 55°C, followed by 1 min at 72°C were used in the amplification. This was ended by a 5 min extension at 72°C. PCR products were resolved for 20 min at 140 v on a 2 % agarose gel with ethidium bromide by loading 20 µl of the 100 µl reaction volume with Blue/Orange 6× loading dye (Promega). Fragment size was determined by running 5-10 µl of a 100 bp DNA ladder (Promega). The DNA was visualised with UV light. The DNA fragment of interest was excised from the agarose gel and separated from the agarose using the Geneclean Spin system (Bio101 Inc.), following the manufacturer's protocol. Purified PCR products were eluted in 16-20 µl RNAase/DNAase free water and frozen at -20°C until required.

PCR products were cloned into the pGEM-T Easy Vector II system (Promega). At least two clones with inserts for each sample were picked out using sterile toothpicks and inoculated in 10 ml Lennox B broth containing 100 µl of 10 mg/ml ampicillin. Samples were incubated overnight at 37°C for 16-24 h with shaking. DNA from clones was extracted using an alkaline lysis mini preparation. Cells were lysed in 100 µl GTE solution (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0) for 5 min at room temperature, followed by 200 µl of NaOH/SDS (0.2 M NaOH, 1 % SDS) on ice for 5 min, then 150 µl of 3 M potassium acetate solution (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml DNAase free water) on ice for 5 min. DNA was precipitated in ethanol by centrifuging at 13000×g for 20 min. The ethanol was removed by vacuum suction and the pellet washed in 250 µl of 70 % ethanol. Pellets were air-dried, and 60 µl of RNAase A (10 µg/ml) added. To confirm presence of an insert, 5 µl of each sample was added to 2 µl buffer II (Promega), 0.5 µl EcoRI (Promega) and 12.5 µl water. Samples were incubated at 37°C for up to 1 h and were then run on a 2% agarose gel with ethidium bromide at 140v for 20 min. DNA was visualised using UV light. Template DNA was cycle sequenced on a GeneAmp PCR System 9600 using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and 3.2 pmol of either PKX5F or PKX6R. Extension products were purified using an ethanol/sodium acetate precipitation and stored in Template Suppression Reagent (PE Biosystems) prior to electrophoresis on an Applied Biosystems ABI 310 Genetic Analyser.

Results

Results are summarised in Table 9.1. Sequence information showed that there were only minor differences (0.5 %) in the 18S rDNA sequence of *T. bryosalmonae* obtained from *F. sultana* and from naturally infected rainbow trout kidney material.

Table 9.1 Summary of results from *Tetracapsula bryosalmonae* transmission trials showing PCR results, presence or absence of parasite in renal imprints, gross clinical signs of PKD, and presence (+) or absence (-) of the parasite cells in stained tissue sections of liver, kidney, spleen or gills.

Treatment group	Week No.	PCR	Renal Imprints	Clinical PKD	Presence of *T. bryosalmonae* cells in histological sections.			
					kidney	spleen	liver	gill
Short-term exposure	2	-	-	-	-	-	-	-
	4	+	+	-	+	-	-	-
	6	+	+	-	-	-	-	-
	8	-	+	-	-	-	-	-
	10	+	-	-	+	+	-	-
	12	+	+	+	-	+	+	+
	12	+	+	+	+	-	-	-
	12	-	-	-	-	-	-	-
	12	+	+	+	+	+	-	-
	12	+	-	-	-	-	-	-
Long-term cohabitation	2	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	6	+	+	-	-	-	-	-
	8	+	+	-	+	-	-	-
	10	-	+	-	-	-	-	-
	12	+	-	-	-	-	-	-
	12	+	+	-	+	+	-	+
	12	+	-	-	+	-	-	-
	12	+	+	+	+	+	-	-
	12	+	-	-	-	-	-	-
I/P bryozoan *Tetracapsula*	2-12	-	-	-	-	-	-	-
I/P kidney homogenate	2	-	-	-	-	-	-	-
	4	-	+	-	+	+	-	-
	6	+	+	-	+	+	+	-
	8	+	+	-	+	+	+	+
	10	+	+	+	+	+	+	-
	12	+	+	+	+	+	+	-
	12	+	+	+	+	+	+	-
	Mort*	+	+	+	NE**	NE	NE	NE
	Mort	+	+	+	NE	NE	NE	NE
	Mort	+	+	+	NE	NE	NE	NE
I/P PBSa	2-12	-	-	-	-	-	-	-
Negative control	2-4	-	-	-	-	-	-	-

* Mortalities. Fish not sampled for histology. ** Not examined.

Short-term exposure

Extrasporogonic *T. bryosalmonae* cells were detected by at least one method in all fish challenged by short-term exposure with the exception of the fish sampled in week 2 and in one fish from week 12 (see Table 9.1). No sporogonic stages were detected within renal tubules. During the short-term exposure to disrupted bryozoans and *T. bryosalmonae* sacs, fish showed agitated behaviour, including twitching and flashing. This was apparent throughout the 90 min of exposure. These behavioural changes were not observed in other challenge fish groups (see below).

Successful amplification of a 435 bp PCR product was achieved in fish sampled in weeks 4, 6 and 10 and in 4 of 5 fish sampled in week 12. The nucleotide sequences obtained for the stages of *T. bryosalmonae* from bryozoans and experimentally infected fish were 100 % homologous.

Gross clinical signs of PKD, including exophthalmia, splenomegaly, renal hypertrophy with pale foci, gill pallor, and abdominal distension (ascites), were observed in 3 out of 5 fish sampled in week 12. Mild renal signs of PKD were seen the fish sampled at week 10. Histologically, fish exhibited pathology typical of PKD with numerous parasites present in the kidney and spleen (Figs. 9.2, 9.3, 9.4 & 9.5) and in small numbers in the liver (Fig. 9.6). *T. bryosalmonae* cells were visible in the gill of a single fish and were associated with inflammation of the primary lamellae (Fig. 9.7). Inflammatory foci in the liver, spleen and heart were also noted in two fish sampled at week 12. Parasites were not observed within these foci.

No blood stages of the parasite were noted in any fish from this challenge group.

Long-term co-habitation

T. bryosalmonae was successfully transmitted to rainbow trout by long-term co-habitation with infected bryozoans. A 435 bp PCR product was amplified in fish sampled in weeks 6 and 8 and in all five fish sampled in week 12. Again, nucleotide sequence

Figure 9.2 Renal imprint showing characteristic proliferative stages of *T. bryosalmonae* (PKX) (arrowed) surrounded by host lymphocytes and phagocytes. May-Grünwald Giemsa. Bar = 20 μm.

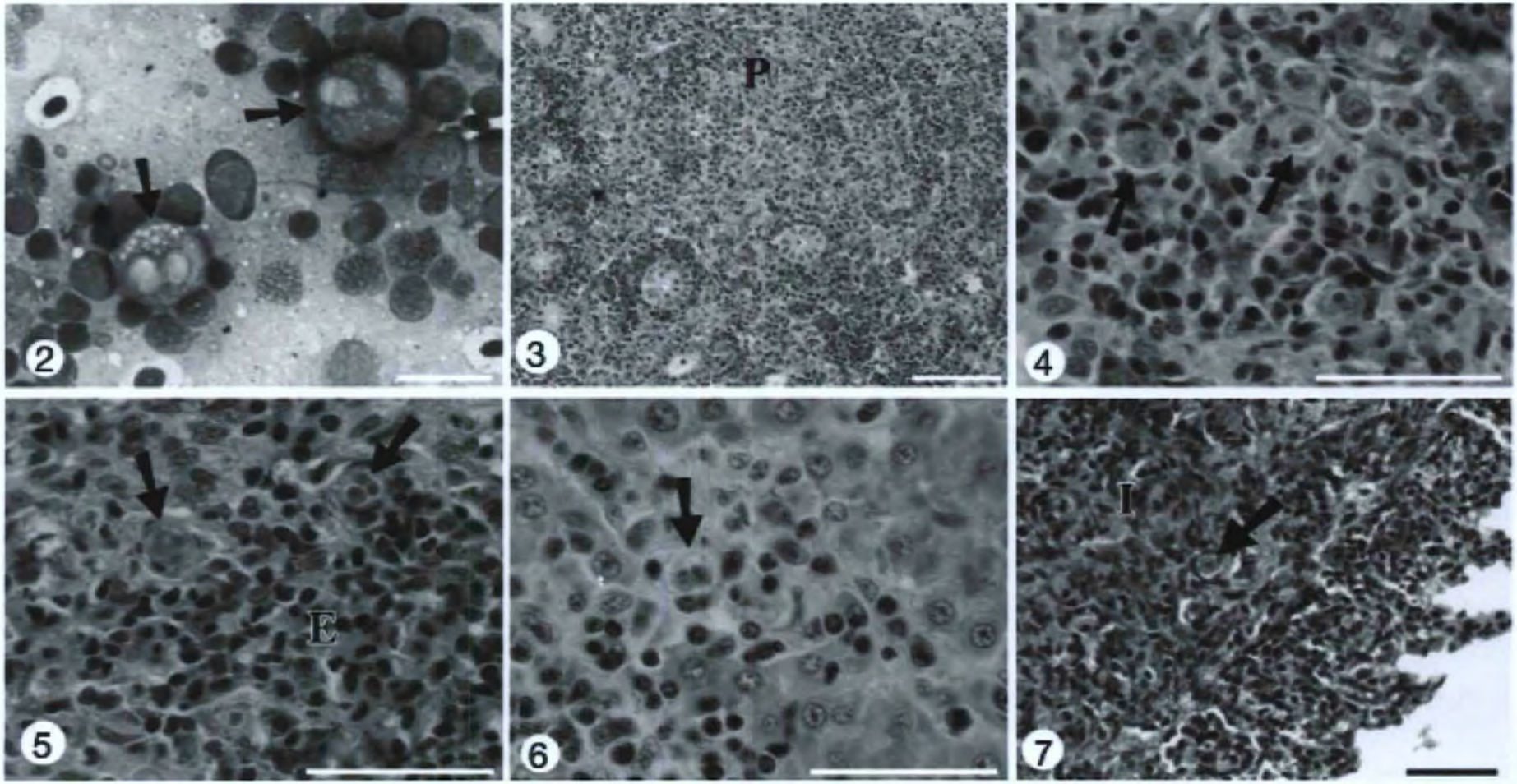
Figure 9.3 Section of kidney exhibiting renal hypertrophy. There is a typical lack of excretory elements in areas of interstitial cell proliferation (P). Haematoxylin and eosin. Bar = 80 μm.

Figure 9.4 High power view of Fig. 3 with numerous *T. bryosalmonae* cells (arrowed) eliciting a vigorous inflammatory response. Haematoxylin and eosin. Bar = 40 μm.

Figure 9.5 Section of spleen with several *T. bryosalmonae* present (arrowed) amongst haematopoietic tissue and numerous mature erythrocytes (E). Haematoxylin and eosin. Bar = 40 μm.

Figure 9.6 Focal hepatic inflammation surrounding a single parasite (arrowed). Haematoxylin and eosin. Bar = 40 μm.

Figure 9.7 Marked branchitis with hyperplasia of the respiratory epithelium and diffuse infiltration of the primary lamellae (I) associated with the presence of *T. bryosalmonae*. Haematoxylin and eosin. Bar = 40 μm.


2
P
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E
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information showed a 100 % homology between bryozoan and fish stages. Pale foci on the kidney were observed at post mortem in a single fish sacrificed at week 12, which also displayed frayed fins and haemorrhaging posterior to the adipose fin. Parasites were detected in all fish from week 6 onwards by at least one method and histopathological evidence of PKD was noted in samples taken at weeks 8 and 12. Again, no blood or sporogonic stages of *T. bryosalmonae* were detected in fish from this challenge group.

Intraperitoneal injection of *T. bryosalmonae* spores

T. bryosalmonae was not transmitted nor was PKD induced in those fish that were exposed to the bryozoan parasite via intraperitoneal injection as determined from PCR, kidney imprints, blood smears and histological examination of all tissues.

Positive controls (injection of PKD kidney homogenate)

Moderate to severe renal hypertrophy was noted in all fish after and including week 10. Typical extrasporogonic *T. bryosalmonae* cells were observed in kidney imprints and histological sections in fish from week 4 onwards. PCR results also confirmed that parasite transmission had occurred in all fish from week 6 onwards. Induction of PKD in this group confirmed that the fish used for experimentation were susceptible to *T. bryosalmonae.* Mortalities attributed to *T. bryosalmonae* infections occurred on 4/10/99 (two fish) (week 11) and on 13/10/99 (week 12) (confirmed by the presence of many *T. bryosalmonae* cells in kidney imprints and by PCR). Nucleotide sequence data showed 100 % homology between the PCR products from fish kidneys used for challenge and the PCR products from spleen and kidney samples taken during infections that subsequently developed. No blood or sporogonic stages of *T. bryosalmonae* were detected in fish from this challenge group.

Negative controls

T. bryosalmonae was not detected in either of the two negative control groups, i.e. the untreated group or those fish which had received an intraperitoneal injection with PBS. No evidence of *T. bryosalmonae* was found in these fish by PCR, histology, or examination of kidney imprints and blood smears. However, all fish in the untreated control group had died of an *Aeromonas hydrophila* infection by week 6. The bacterial infection was not encountered in any other tanks and since no mortalities occurred in the stock fish from which this group had been taken, this was considered an isolated incident.

Discussion

Both short-term exposure to *T. bryosalmonae* sacs and disrupted bryozoans and long-term cohabitation with bryozoans infected with *T. bryosalmonae* resulted in the successful transmission of *T. bryosalmonae* from one of its bryozoan hosts to naïve rainbow trout and subsequent induction of PKD in fish. In both trials, fish were exposed to sacs containing spores and possibly also to early proliferative stages of *T. bryosalmonae*, the bryozoans themselves, and the associated invertebrate fauna. This invertebrate fauna typically included naidid oligochaetes, dipteran larvae (especially members of the family Chironomidae), *Hydra* spp., *Ithytrichia* sp., and a wide variety of free living protists. However, through sequencing the 18S rDNA of the stages in the fish and in the bryozoan hosts and by examining morphological characteristics of the parasite, we have unequivocally linked the two stages and confirmed the conclusions of previous molecular studies (Anderson et al. 1999a, b) that bryozoans are hosts of the causative agent of PKD. In addition, it should be noted that over 60 species of invertebrates, including representatives of the above species of associated fauna, have tested negative for *T. bryosalmonae* by PCR with *T. bryosalmonae*-specific primers (Longshaw & Feist unpublished). This provides evidence that the fauna associated with bryozoan colonies examined to date are not hosts of *T. bryosalmonae*.

The group of rainbow trout exposed to colonies of *F. sultana* infected with *T. bryosalmonae* may not have been exposed to *T. bryosalmonae* spores for more than a few days as no attempt was made to maintain the bryozoans and information on their longevity under experimental conditions is lacking. It is likely that the infected colonies died under these laboratory conditions after several days, releasing *T. bryosalmonae* spores into the tank only at the beginning of the experiment. Nonetheless, clinical PKD was induced in fish in this experiment. This suggests that only a few *T. bryosalmonae* spores are needed to cause infection in susceptible fish since the spores were released in a flow-through system. This apparent efficiency in achieving infection with few spores may explain why, in field situations, morbidity of infected fish can reach 100 %. However, we cannot dismiss the possibility that fish may have grazed on infected *F. sultana* colonies and obtained a larger dose of infective stages. Gavage experiments are planned to test this possibility.

T. bryosalmonae cells were first detected in the gills with an associated inflammatory response during week 12 in the short-term exposed and long-term cohabited fish. Had this been the initial site of infection, it might be expected that *T. bryosalmonae* cells and pathological changes would have been apparent in the gills soon after exposure. This suggests that the primary site of infection into the fish may be elsewhere, perhaps the skin.

Injection challenge with homogenate of PKD-infected kidneys appears to be more effective at inducing an intense systemic infection than challenge by cohabitation or by short-term exposure to infective stages. One factor influencing this could be that a minimum infective dose is required in the initial stages of infection. Whirling disease has been induced in rainbow trout and brown trout that were exposed to a minimum dose of ten *Myxobolus cerebralis* actinospores (Hedrick et al. 1999). Parasites injected into the peritoneal cavity as PKD-infected kidney homogenates are likely to be both numerous and adapted to conditions within the fish host. Thus they may be more likely to induce a rapid

and vigorous cellular response within the host. In contrast, parasites released from bryozoans must first gain entry into the fish host, adapt to new conditions, migrate to the target organs and tissues, and proliferate within the fish before initiating sporogony in the renal tubules. Our data indicate that when spores released by bryozoans gain entry to fish, *T. bryosalmonae* can reach the kidney in four weeks. El-Matbouli et al. (1995) showed that *M. cerebralis* took 21 days to migrate from its point of first entry into the fish to the head cartilage. During this migration, the parasite underwent several multiplications, as is known to occur in *T. bryosalmonae* (Kent & Hedrick 1986).

Amplification of a 435 bp product using *T. bryosalmonae*-specific primers and sequence matching of the resultant products from the pre- (bryozoan) and post- (fish) challenge stages provided further evidence that the parasite had been transmitted. In some samples, PCR positive results were obtained, although *T. bryosalmonae* cells were not detected in imprints, smears or in stained tissue sections. There are several possible explanations for this. False positives may have resulted if the primers amplified a sequence other than that of *T. bryosalmonae*. However, this is unlikely since the 18S rDNA sequences, even for the two known *Tetracapsula* species, are about 20 % dissimilar and these differ significantly from other myxozoans and from fish DNA. An apparent false positive result might have been obtained if individual fish mounted an immune response strong enough to suppress the development of the parasite, such that it could not be detected by histological methods. This is also unlikely since the naïve fish stock used is one known to be highly susceptible to the parasite. There is also a possibility that the primers were amplifying DNA from very low numbers of *T. bryosalmonae* cells, or from non-viable *T. bryosalmonae* cells, or fragments of *T. bryosalmonae* DNA. These latter explanations are reasonable possibilities since Kent et al. (1998) reported a sensitivity of eight *T. bryosalmonae* cells per gram of fish kidney when using the primers PKX5F and PKX6R.

There were three samples that were negative by PCR but in which *T. bryosalmonae* cells were visible in either tissue sections or in kidney imprints. Since the primers are very sensitive and would have detected low numbers of parasites the negative result is surprising. The parasites observed could have been of a closely related *Tetracapsula* species with a different 18S rDNA sequence. However this was regarded as unlikely since the DNA sequence of the bryozoan stage showed 100% homology with the DNA sequence in both *Plumatella* and *Fredericella* hosts examined previously (Longshaw et al. 1999). Another explanation is a possible non-uniform distribution of the parasite within the fish kidney or spleen since samples for histology or for smears were taken from slightly different parts of the organ from those used for molecular studies. It is possible that the parasite was in very low numbers or absent in the samples for molecular biology. This was also considered unlikely since examination of histological sections of the donor kidneys showed the parasite to be relatively evenly distributed. These three samples were re-amplified but were again negative. We are currently unable to provide an adequate explanation for this result.

In conclusion, a combination of diagnostic methods allowed the detection of successful transmission of *T. bryosalmonae* from bryozoan to fish hosts, including PCR, kidney imprints and blood smears, and histology. Such a multidisciplinary approach is recommended, particularly in view of the occasional erroneous PCR results. The application of other techniques, such as serology (monoclonal antibodies), *in-situ* hybridization, and lectin binding, should also be considered when appropriate. Further studies to establish the routes of entry of the parasite into the fish host and the timing and early pathogenesis of the disease are in progress.

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DEVELOPMENT OF PROLIFERATIVE KIDNEY DISEASE (PKD) IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, FOLLOWING SHORT-TERM EXPOSURE TO *TETRACAPSULA BRYOSALMONAE* INFECTED BRYOZOANS

Abstract

The initial site of infection for *Tetracapsula bryosalmonae*, causative agent of proliferative kidney disease (PKD) is poorly understood. Following the recent recognition that freshwater bryozoans harbour the infective stages to salmonid fish, experimental transmission studies were undertaken to investigate 1) the route of entry of the parasite into the fish host and, 2) the minimum exposure time required to induce clinical signs of PKD. *In-situ* hybridisation studies were carried out on naïve rainbow trout (*Oncorhynchus mykiss*) exposed to naturally infected bryozoans *Fredericella sultana* for up to ninety minutes. The sporoplasm of *T. bryosalmonae* was detected entering the fish via mucous cells in the skin epithelium within the first minute of exposure. In addition, *T. bryosalmonae* cells were infrequently detected in the skeletal musculature of exposed experimental fish up to 72 hours post-exposure. The route of migration through the fish to the kidney and spleen was not determined. All fish exposed to infected, disrupted bryozoans for 10, 30 and 90 minutes and maintained for up to eight weeks developed clinical PKD.

Introduction

Proliferative Kidney Disease (PKD) of salmonids is caused by the degenerate metazoan parasite *Tetracapsula bryosalmonae* Canning, Curry, Feist, Longshaw & Okamura (1999). Anderson, Canning & Okamura (1999) and Longshaw, Feist, Canning & Okamura (1999) demonstrated by PCR and sequencing of the 18S rDNA that the parasite utilises bryozoans as an alternate host. Subsequently, Feist, Longshaw, Canning & Okamura (2001) induced PKD in naïve rainbow trout exposed for 90 minutes to the phylactolaemate bryozoan *Fredericella sultana* harbouring *T. bryosalmonae* developmental stages and spores. The authors confirmed parasite transmission by observation of *T. bryosalmonae* cells in histological sections and by PCR as well as clinical signs of the disease. A number of techniques to visualise and characterise the parasite in the fish host and in potential alternate hosts have been developed. These include the use of lectins (Marin de Mateo, Adams, Richards, Castagnaro & Hedrick 1993), monoclonal antibodies (Adams, Richards & Marin de Mateo 1992), PCR and sequencing of the 18S rDNA (Kent, Khattra, Hervio & Devlin 1998; Saulnier & de Kinkelin 1997). Most recently Morris, Adams & Richards (1999) have developed *in-situ* hybridisation (ISH) methods for the detection of *T. bryosalmonae*. ISH has also been developed and utilised to track infections of *Myxobolus cerebralis,* the agent of whirling disease in salmonid fish, from the site of infection to the cranial cartilage, the main site of *M. cerebralis* sporogony (Antonio, Andree, McDowell & Hedrick 1998). These authors showed that the skin and gills were the portals of entry with subsequent migration of the parasite via the peripheral nervous system to the cartilage. Similarly, Morris, Adams & Richards (2000) provided evidence that *T. bryosalmonae* utilises the gill as a portal of entry into the fish host. However, routes of infection to other tissues were not confirmed and a haematogenous spread appears the most likely mechanism for the dissemination of the parasite (Fernández-de-Luco, Peribáñez, García & Castillo, 1997). The aims of the

current study were to extend the results of Feist *et al.* (2001) on the transmission of the bryozoan stage of *T. bryosalmonae* to rainbow trout, to determine the minimum length of exposure required to induce PKD in fish and to provide preliminary data on *T. bryosalmonae* migration within the fish host.

Materials and Methods

Experimental exposure and sampling

Naïve susceptible rainbow trout were reared from eggs under quarantine conditions at CEFAS Weymouth to an average weight of between 2 and 4 grammes. Colonies of naturally infected *Fredericella sultana* were collected from the inlet channel to a PKD enzootic rainbow trout farm in the south of England in September 2000 and returned to the laboratory in river water in plastic containers. Sub-samples of the bryozoan colonies were examined for the presence of typical *T. bryosalmonae* sacs. Immediately prior to the experimental trials, the bryozoans were coarsely homogenised in 100 ml of deionised water and diluted in 10L of water in a tank with aeration at 16°C. A total of 99 trout were added to the tank. Sub-samples of 4 fish (2 for histology and 2 for Scanning Electron Microscope studies) were taken at 1, 2, 5, 10, 30 and 90 minutes post-exposure. Subsequently, a batch of 15 fish were transferred to clean, aerated flow through water and sub-samples of 5 fish each were sampled for histology at 24, 48 and 72 hours post-exposure. The remaining fish, in batches of 20 fish each, were removed after 10, 30 and 90 minutes exposure and transferred to separate tanks with aerated flow-through water and maintained at 16°C for up to 8 weeks. Two fish from each of these batches were sampled every two weeks for histology and PCR to detect *T. bryosalmonae* DNA sequence and monitored for clinical signs of PKD. All fish were sacrificed by over-dose of the anaesthetic MS222 followed by severance of the spinal column. The visceral cavity of the

fish was opened to allow penetration of the fixative and whole fish were preserved in neutral buffered formalin (NBF) for histological studies and in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer for SEM studies. Fish were fixed in NBF for a minimum of 24 hours then transferred to 70% industrial methylated spirits (IMS) and subsequently de-calcified for 2-4 days in 10% formic acid. Transverse steaks approximately 5mm thick were taken along the length of the fish and these were processed to wax blocks using standard protocols. Tissue sections were cut at approximately 4 µm and floated onto either glass slides and stained with Haematoxylin & Eosin (H&E) or onto Vectabond reagent (Vector) treated slides for ISH studies.

In-situ hybridisation

Kidneys from fish known to be infected with *T. bryosalmonae* were used to provide template DNA for the PCR reactions. Samples were fixed in 100% ethanol and prior to DNA extractions were washed twice in TE buffer then soaked for 1 hour in fresh TE buffer. DNA was extracted using a standard SDS/proteinase K extraction followed by a phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The Digoxigenin-labelled probes were produced by amplification of plasmid DNA using a single round PCR in standard 100µl reactions containing reaction buffer IV (AB gene), 2.5mM $MgCl_2$, 500ng of each forward and reverse primers, 5U Red-Hot DNA polymerase (AB gene), 10µl of PCR-Dig labelling mix (Roche) and 50-100ng/µl template plasmid DNA. The *T. bryosalmonae* specific primers were PKX5F (5'-CCTATTCAATTGAGTAGGAGA-3') and PKX6R (5'-GGACCTTACTCGTTTCCGACC-3) of Kent *et al.* (1998). Positive controls to confirm the performance of the PCR reaction were included in standard 100µl reactions. These last were prepared as above except that the PCR-Dig labelling mix was replaced by non-labelled dNTP's (0.25mM each). Three negative controls in standard 100µl PCR reactions were also utilised as above but the template DNA was replaced with

RNAase/DNAase free water and the PCR-Dig labelling mix was replaced by non-labelled dNTP's in two of the negative controls. PCR was run for 35 cycles of 1 minute denaturation at 95°C, followed by 1 minute annealing at 55°C, followed by 1 minute elongation at 72°C. This was ended with a 10-minute elongation step at 72°C. 2µl of PCR products were visualised under UV light, after electrophoresis in a 2% agarose gel and remaining probe stored at -20°C until required.

Tissue sections prepared on Vectabond reagent treated slides (Vector) were allowed to dry at 60°C for 24 hours and stored at 4°C until required. Slides were dried at 60°C overnight prior to the ISH and sections de-waxed in Clearene (Surgipath) for 2 × 5 minutes, followed by 100% IMS for 2 × 5 minutes. Sections were then air-dried and target DNA and RNA in tissues unmasked by proteinase K. A hybridisation mix (25% formamide, 2x SSC), consisting of 60µl of probe or control and 60µl of hybridisation buffer was added to each slide, contained by a Gene Frame (Advanced Biotechnologies). Target DNA and RNA present in the tissue sections and probe were denatured at the same time by incubating the slides for 5 minutes at 95°C and rapidly cooling down on ice. Sections were then hybridised overnight at 42°C. A stringency wash in 1× SSC, 0.2% Bovine serum albumin and DEPC treated distilled water was carried out at 42°C for 10 minutes after hybridisation. Tissue sections were then blocked in 6% skimmed milk at room temperature for 1 hour. Specific probe binding in tissue sections was visualised by means of a sandwich of an anti-digoxigenin antibody (Boehringer Mannheim) and an alkaline phosphate conjugate antibody (Sigma) revealed by BCIP/NBT (Vector), substrate of the alkaline phosphatase Slides were counterstained using 0.5% Bismark Brown Y (Sigma) for 10 seconds, washed well in deionised water, dehydrated, cleared and mounted in Eukitt resin (BDH-Merck). Stained sections were examined using a Nikon Eclipse E800 (Nikon, UK Ltd) microscope and representative images were captured using the Lucia Screen Measurement software (Nikon UK Ltd).

Results

Results are summarised in Tables 9.2 & 9.3. Following short term exposure, up to 10 cells per fish, which stained positive for *T. bryosalmonae* by ISH, were detected in the epithelial mucous cells of the skin in fish exposed to disrupted bryozoans (Figure 9.8). In one fish sampled at 90 minutes post-exposure, a single *T. bryosalmonae* cell was visualised in the gill epithelium. Parasite cells were visualised in tissue sections of all fish exposed to bryozoans with the exception of one fish sampled at 10 minutes in which the parasite was not detected by ISH. In one fish sampled at 1 minute post-exposure, a single *T. bryosalmonae* cell was observed in an apparently intercellular location within the uppermost layer of the epidermis adjacent to a mucous cell (Figure 9.9). In another fish sampled after 5 minutes exposure, a single *T. bryosalmonae* cell was seen in amongst the basal cells of the epidermis. All *T. bryosalmonae* cells in the mucous cells of the fish were spherical and measured approximately 9µm ± 1µm. Multicellular *T. bryosalmonae* cells morphologically indistinguishable from the extrasporogonic forms described from the kidney (previously designated PKX) were detected in the skeletal muscle of one of each fish sampled at 90 minutes, 24, 48 and 72 hours post-exposure.

All fish examined in weeks 6 and 8 post-challenge showed typical clinical signs of the disease including mild to moderate nephromegaly. In fish that were exposed to disrupted bryozoans for 10, 30 and 90 minutes, *T. bryosalmonae* cells were first detected 4 weeks post-exposure in the kidney and spleen (Table 9.3). In the fish initially exposed for 10 minutes to disrupted bryozoans, parasites were detected in gill, kidney, spleen and heart after 6 weeks and additionally in the liver after 8 weeks. In fish exposed to bryozoans for 30 minutes, parasites were detected in kidney, spleen and heart after 4 weeks and additionally in the gill and liver after 6 weeks. In fish exposed to bryozoans for 90 minutes, parasites were detected in kidney, spleen and liver after 6 weeks and additionally in the gill after 8 weeks. No *T. bryosalmonae* spores were detected in the skin or musculature of any fish sampled in weeks 2, 4, 6 and 8 post-challenge.

Table 9.2. Summary of results from *Tetracapsula bryosalmonae* transmission trial to demonstrate route of entry into the fish host showing positive staining of *T. bryosalmonae* cells by the *in-situ* hybridisation technique

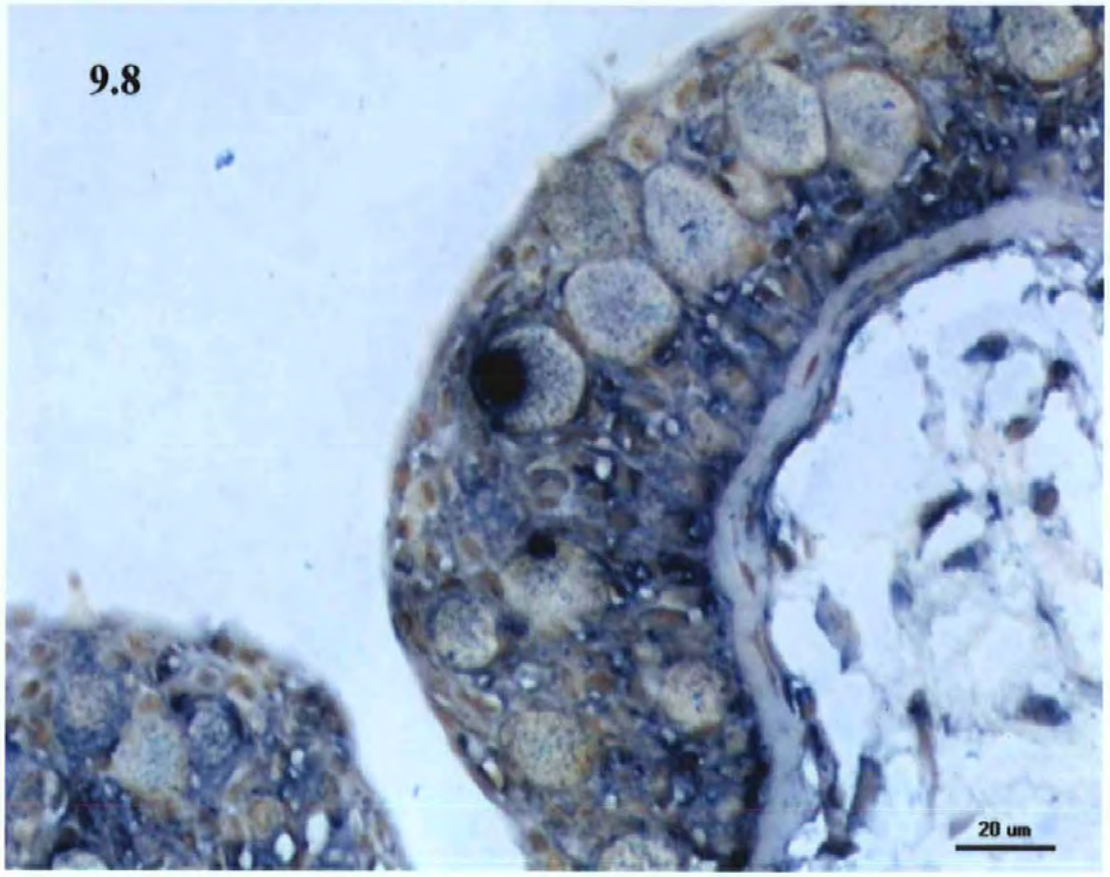
Exposure time (in minutes)	Sampling time post-exposure	Presence of *T. bryosalmonae* cells in tissue sections determined by *In-situ* hybridisation			
		Skin	Muscle	Gills	Fins
1	1 min	+	-	-	-
2	2 min	+	-	-	-
5	5 min	+	-	-	-
10	10 min	+	-	-	-
20	20 min	+	-	-	-
30	30 min	+	-	-	-
90	90 min	+	+	+	+
90	24 hrs	+	+	-	-
90	48 hrs	+	+	-	-
90	72 hrs	+	+	+	-

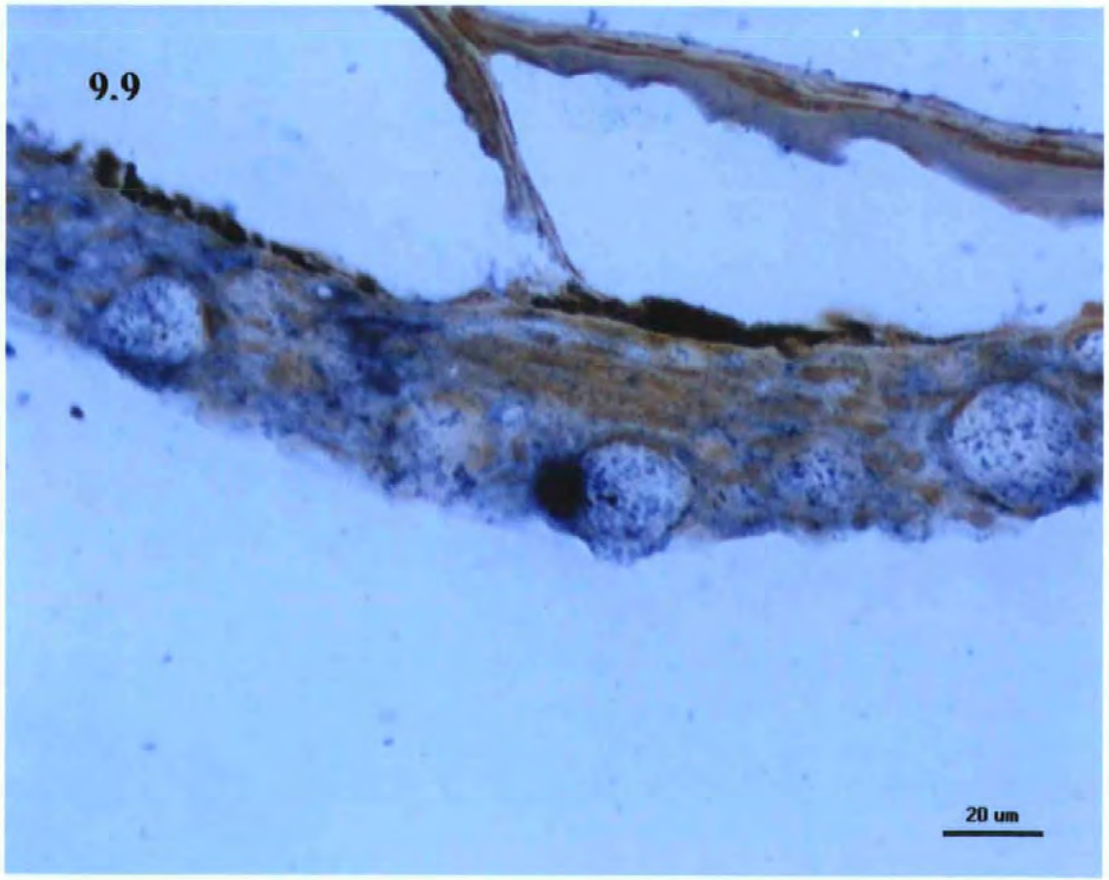
Table 9.3. Summary of results from *Tetracapsula bryosalmonae* transmission trials to determine the length of exposure to the parasite on development of PKD.

Time of exposure (in minutes)	Number of weeks post-exposure	Clinical PKD	Presence of *T. bryosalmonae* cells in histological sections detected by *in-situ* hybridisation				
			Gill	Kidney	Liver	Spleen	Heart
10	2	N	-	-	-	-	-
	4	N	-	+	-	+	-
	6	Y	+	+	-	+	+
	8	Y	+	+	+	+	+
30	2	N	-	-	-	-	-
	4	N	-	+	-	+	+
	6	Y	+	+	+	+	-
	8	Y	+	+	-	+	-
90	2	N	-	-	-	-	-
	4	N	-	+	-	+	-
	6	Y	-	+	+	+	-
	8	Y	+	+	-	+	-

Figure 9.8 Staining of a *Tetracapsula bryosalmonae* sporoplasm cell by the *in-situ* hybridisation technique within a mucous cell of the skin epithelium of rainbow trout after 1-min exposure to *T. bryosalmonae* infected *Fredericella sultana*.

Figure 9.9 *Tetracapsula bryosalmonae* sporoplasm cell adjacent to a mucous cell in the skin epithelium of a rainbow trout 1 min post-exposure to infected bryozoans.


9.8
20 um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Discussion

The results of the current study confirm those of Feist *et al.* (2001) who demonstrated experimentally that bryozoans were an alternate host for *T. bryosalmonae.* In the present study, fish exposed to infected bryozoans for 10, 30 and 90 minutes and then maintained for up to eight weeks became infected with *T. bryosalmonae* and developed clinical signs of PKD. The results indicate that fish exposed to the parasite for as little as 10 minutes may develop the disease. Additionally, infective stages could be detected in the fish after only 1 minute exposure to disrupted bryozoans. Since fish exposed to the parasite for less than 10 minutes were sacrificed, it was not possible to confirm that these fish would have developed clinical PKD. It is well recognised that extrasporogonic *T. bryosalmonae* stages rapidly proliferate within the fish host (Hedrick, MacConnell & de Kinkelin, 1993). It follows that exposure to a low parasite dose for as little as 1 minute could induce clinical PKD. In this study, *T. bryosalmonae* sacs were not seen within or naturally released from *F. sultana* zooids despite careful searching. Since the fish became infected, it is reasonable to conclude that infective stages of *T. bryosalmonae* were present in only low numbers and that when released from the bryozoan host they are highly effective at locating and infecting fish. Whether all the infective stages invaded the fish within the first few minutes of exposure was not determined in this study. Spore stages of the two described species of *Tetracapsula* are approximately 20µm in diameter and contain two sporoplasms each measuring approximately 10µm in diameter. The cells visualised in tissue sections in this study were approximately 9µm, slightly larger than those reported by Morris *et al.* (2000) and most likely represent sporoplasm cells of *T. bryosalmonae.* Canning *et al.* (2000) suggested that autogamous fusion of the sporoplasm cells may occur in the epidermis of the fish.

Morris *et al.* (2000) exposed rainbow trout to natural infections in PKD-enzootic water for three days and located *T. bryosalmonae* cells in the gills by using *in-situ*

hybridisation but did not detect further *T. bryosalmonae* cells until 10 days post-exposure. These results, together with those of the current study suggest that *T. bryosalmonae* is non-specific in its route of entry, utilising both gills and skin as a portal. Both of these routes would facilitate the transport of the parasite to the kidney via the blood or lymphatic systems. Entry portals of actinospore stages of myxozoans have rarely been studied. Those of *M. cerebralis* enter the fish through all epithelial surfaces (El-Matbouli, Hoffman & Mandok 1995), whereas actinospores of *M. cultus* and *M. arcticus* enter fish via the fins and skin and *Thelohanellus hovorkai* enter fish via the gills (Yokoyama & Urawa 1997). Using SEM, El-Matbouli, Hoffman, Schoel, McDowell & Hedrick (1999) demonstrated that the actinospore stage of *M. cerebralis* entered the fish via the epithelial surfaces of the skin, gills and buccal cavity through the mucous cells. In the current study, the use of the mucous cell as a specific portal of entry by the parasite has also been demonstrated. Although it is established that *T. bryosalmonae* spores from bryozoans are not actinospore stages and in fact belong to a different myxozoan class (Malacosporea) (Canning *et al.* 2000), the behaviour of the spores and release of a sporoplasm into the mucous cell appears to be similar to that described for actinospores. Actinospore stages of myxozoans may require mechanical and/or chemical stimuli, such as are present in mucus in order to locate and attach to a host and to release the sporoplasm (El-Matbouli *et al.* 1999; Xiao & Desser 2000). Whilst some actinospores may take a few minutes to respond to fish mucus, others such as Raabeia B of Xiao & Desser (1998a) and Aurantiactinomyxon form of Xiao & Desser (1998b) instantaneously release their sporoplasms in response to fish mucus (Xiao & Desser 2000). The presence of *T. bryosalmonae* cells within the mucous cells of the skin epithelium after 1 minute exposure suggests that the location of host attachment and penetration by the parasite is rapid. Entry via the mucous cells may be beneficial to the parasite as it provides easy access into the fish through the mucous cell pore. Host recognition factors and the reasons for the apparent unsuitability of various epithelial surfaces as routes of entry by the infective stages of these parasites require further study.

The utilisation of specific techniques such as *in-situ* hybridisation and other cytochemical tests may assist in determining the chemical cues within the mucus that the parasite responds to.

Although no parasites were detected directly within veins or arteries, they were occasionally visualised by *in-situ* hybridisation within the skeletal muscle in fish sampled up to 72 hours post-exposure. It is possible that these parasites may have been within capillaries associated with the muscle. These parasites were multicellular and appeared to be similar to the extrasporogonic forms usually observed in the kidney of fish with clinical PKD. The lack of any obvious inflammatory response may be expected since during the early pre-clinical phase of the infection the host has yet to mount an effective cellular response to the parasite. This contrasts with the marked chronic inflammatory and granulomatous response reported during the recovery phase of PKD by Fernández-de-Luco *et al.* (1997). Understanding the mechanisms by which the parasite apparently evades the host immune response and macrophages within the fish 72 hours of infection will be crucial in the development of vaccines and targeted chemotherapeutants to combat the disease. The role, if any, of the sporoplasmosomes in parasite protection or presentation of antigens to the host immune system has yet to be elucidated but may assist in understanding the mode of avoidance of the host immune system by the parasite.

A typical management strategy employed by fish farmers to minimise the impact of the disease and induce immunity in farmed fish is to maintain naïve salmonids at sites known to be free of PKD through the summer months. When the water temperatures begin to decrease in the autumn, these fish are transferred to PKD-enzootic farms and become infected with *T. bryosalmonae.* However, they generally only develop sub-clinical infections. The presence of the surviving colonies of *F. sultana* at PKD enzootic sites throughout the winter and development of PKD in fish transferred to higher temperatures after exposure to water from a river in France through the winter months has previously

been demonstrated (Okamura, Anderson, Longshaw, Feist & Canning, 2001). The development of PKD in fish exposed to bryozoans in September in the current study confirms that water temperature is a major factor affecting the development of clinical PKD.

To date successful transmission of *T. bryosalmonae* from bryozoans to fish has only been achieved by exposure to naturally infected bryozoans. Isolated *T. bryosalmonae* parasites have not been obtained in sufficient numbers to undertake detailed studies to investigate behaviour of parasites outside the bryozoan host. A laboratory based culture system similar to that developed by El-Matbouli *et al.* (1999) and other workers for *Myxobolus cerebralis* is required to assist in targeted and controlled studies to understand the mode of action of potential chemotherapeutants and vaccines to combat the disease in farmed fish.

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CHAPTER TEN

GENERAL DISCUSSION

Previous studies on myxozoans in the UK have been limited, with the majority of efforts being directed towards those of economic or ecological significance such as *Tetracapsuloides bryosalmonae* and often in fish and oligochaetes collected in a small spatio-temporal range. Indeed, many of these studies appear to be straightforward descriptive studies, with a few describing the pathology associated with any infections. Exceptions to this have been reports of rare epizootics associated with mass mortalities (Williams, 1964) or pathological deformities (Bucke and Andrews, 1985; Lom *et al.*, 1991). No new species of myxospores have been described from UK freshwater fishes since the checklist of parasites of freshwater fish by Kennedy (1974). There have however, been new records of myxospores which have been described in other European countries (e.g. *Myxobolus pfeifferi*). Actinospore studies have however provided new data on spatio-temporal trends of these parasites and provided descriptions of previously unrecorded types (McGeorge *et al.*, 1997; Özer *et al.*, 2002). This paucity of information is at odds with the relative importance of myxozoans as disease agents in both wild and farmed fish. It is recognised that diseases such as PKD and whirling disease in salmonids can lead to mortality (Hedrick *et al.*, 1993, 1998) but there has been a lack of suitable data available to demonstrate an effect of other myxozoan diseases on individual and population success. This has in part been due to the unavailability of long-term data sets necessary to demonstrate this phenomenon. The current study has attempted to redress this balance by providing long-term data of myxozoan infections in both fish and oligochaete hosts and a thorough description of both myxospores and actinospores (Chapters 3, 4 and 5). It is clear that there are differences in the spatio-temporal trends of these important diseases and highlights the need to collect long-term data in order to ensure that a true representation of the disease status of fish is achieved. The lack of data concerning the disease status of juvenile fish also gives rise for concern. It is during the early stages of life when fish are immunologically naïve that disease agents are able to more successfully invade. Unfortunately many studies of myxozoan infections, and indeed other disease agents have

focussed on adult fish that invariably represent survivors of infections. In order to better understand the role of disease in determining population success and to gain a true representation of the types of pathogenic disease agents present in wild and farmed fish, future studies should consider juvenile fish. The current study has demonstrated that disease is an important component of freshwater fish populations (Chapter 6). Whilst some free-living ruminant populations have been shown to be regulated by parasites (Stein *et al.*, 2002), this is the first study that has demonstrated the role of disease in fish population success. Evidence from the USA has suggested that whilst whirling disease has been responsible for declines of wild salmonids, this has not been substantiated using modelling techniques and there is some suggestion that its impact on some salmonid populations has been negligible (Modin, 1998). There is a need to incorporate disease as a measure of natural mortality in future population models to ensure that realistic management decisions are made to preserve exploited stocks.

Myxozoan infections in both oligochaete and fish hosts can vary between years and between sites which reflect a number of biotic and abiotic factors, including sediment type and host availability (Xiao and Desser, 1998). At a smaller, seasonal scale, patterns of release and infection are also apparent. Release of actinospores from oligochaetes is clearly seasonal, with the majority being released in spring and summer. These releases appear to be linked to the availability of fish hosts even though they can be released through the winter. The release of actinospores in spring and summer would be expected in order to maximise the chance of encountering a naïve host. At the time of actinospore release, juvenile hosts will be concentrated in the margins of the river (Cowx, 2001). The timing of release to coincide with the wide availability of fish hosts would ensure the successful survival of myxozoans. Whilst this idea is intuitive, no studies of myxozoan life cycles have incorporated this idea. Future studies should consider the ecology of both hosts and of the parasite in order to provide a holistic approach to better understanding the life cycle and biology of these important parasites.

Several myxozoan life cycles have now been elucidated, but with the exception of *Myxobolus cerebralis*, none have considered the genetic susceptibility of the invertebrate hosts. Some early studies on myxozoans did consider the cues that actinospores responded to in host selection. These however were restricted to identifying mucus as one of the major factors (Yokoyama *et al.*, 1993). Subsequent studies have accepted this view and not considered it further. Given the advances in molecular techniques in recent years, there appears to be merit in revisiting these studies to assess which are the major factors involved in host recognition and resistance. Given that during the current study there was a wide variation in host specificity of myxospore and actinospore stages, it appears that the cues that they respond to are equally variable. For example, whilst *M. pseudodispar* and *M. buckei* apparently have a wide host specificity extending beyond individual fish genera, others such as *Sphaerospora* spp. have a very narrow host specificity. Knowledge of the factors that determine parasite success in either host would allow the development of specific treatments to control infections in farmed and wild fish. Since few chemical methods have proved to be effective at eliminating or reducing the impact of myxozoan diseases in fish and treatment of wild fish is impractical, the development of such methods to disrupt host-finding mechanisms may prove fruitful.

However, treatment of wild fish is impractical and is best achieved through careful management of wild stocks. A thorough understanding of the role of disease in population success is, as already suggested, imperative. In addition, understanding of the seasonality of infection and critical phases of the lifecycle can also assist in protecting wild stocks. For example, since it is now recognised that *M. buckei* preferentially infects fish prior to ossification, any restocking programmes must be done outside of the critical period of infection and post-ossification in order to minimise the impact of the disease on juvenile fish. There is also a need to ensure that infected individuals are not moved from areas known to be enzootic for the disease to areas considered free of the parasite. The minimising of the spread of this important pathogen is essential to avoid a similar scenario

to that shown by whirling disease in the USA. It has been suggested that the seeding of *M. cerebralis* resistant oligochaetes may alleviate the impact of *M. cerebralis* in wild salmonids (Beauchamp *et al.*, 2002). However, since no other data is available on genetic susceptibility of other oligochaete-myxozoan systems, this is unlikely in the near future to be a viable option for control. Alternatively, the production of resistant fish hosts through genetic manipulation or through the production of resistant strains or hybrids of fish may be applicable to reduce or negate the impact of disease, particularly in aquaculture.

The control of PKD in farmed fish has been problematic since the inception of freshwater salmonid farming. Due to a lack of knowledge concerning the lifecycle of the parasite, chemical control methods were utilised. Clifton-Hadley and Alderman (1987a) and Alderman and Clifton-Hadley (1988) demonstrated that repeat treatment with malachite green of sub-clinical fish affected by PKD controlled the development of the disease and the presence of the extrasporogonic parasites in susceptible rainbow trout. However, repeated doses of malachite green can lead to pathological alterations of livers and gills in exposed fish (Gerundo *et al.*, 1991) and its use in food fish is banned due to the accumulation of the chemical or its metabolites in fish tissues and its potential carcinogenic properties. The oral administration of Fumagillin or its analogue TNP-470 have been shown to be efficacious in the treatment of *T. bryosalmonae* in Chinook salmon and sockeye salmon (Hedrick *et al.*, 1988; Higgins and Kent, 1998) but toxic effects have been reported in fish exposed to higher doses of Fumagillin, including reduced growth rates, loss of appetite, direct mortality and reductions in the size of kidney and spleen (Wishkovsky *et al.*, 1990; Le Gouvello *et al.*, 1999). Morris *et al.* (2003) suggested that whilst a treatment regime of 1.0mg TNP-470 kg^{-1} fish day^{-1} for 6 days was successful at removing parasites from rainbow trout, there was conflicting evidence regarding the mortality effects of the drug and further work would be required to test the effects of the drug on different host species. Many farm managers now have a controlled exposure programme in place to expose naïve salmonids to the infective agent of PKD during

August and September in order to produce immune fish for the following summer (Longshaw *et al.*, 2002). Detailed understanding of the immune responses will assist in the development of strategies towards negating the impact of PKD on the host. Studies investigating the molecular basis of immune response via expression of immune-regulatory genes during the pathogenesis of myxozoan diseases will identify specific dynamic changes occurring during the disease process (Holland *et al.*, 2003). Ultimately, techniques to boost the immune system to combat important myxozoan infections and the development of vaccines against these diseases will provide a successful means of reducing the impact of these parasites.

The breakthrough in understanding of the lifecycle of *M. cerebralis* provided the impetus to carry out further research on myxozoans, including the agent for PKD. Since it was recognised that the PKD agent was a myxozoan and that myxozoans utilised oligochaetes, early life cycle studies on the PKD agent were directed towards examining these invertebrate hosts (Hedrick *et al.*, 1992). However, the isolation of actinospores from oligochaetes and attempts to transmit these stages to fish were unsuccessful at inducing PKD in naïve hosts. The description of a new genus of myxozoan in bryozoans by Canning *et al.* (1996) led to calls by Feist (1997) and Kent (1998) to consider these hosts as a possible source of infection. During the current study, the discovery of bryozoans as one of the hosts has led to a renewed interest in the parasite (chapter 8). New avenues of research have been developed, including further understanding of the immune responses in infected hosts (Holland *et al.*, 2003). Additionally, given that at least one of the hosts have been identified, measures can be taken in farms to limit the growth or completely eliminate bryozoans from water inlets. The route of entry by the parasite and minimum length of exposure required to induce the disease in naïve hosts has allowed greater understanding of the early pathogenesis of the disease (Feist *et al.*, 2001; Longshaw *et al.*, 2002), including measures directed at enhancing the host response in the initial phase of infection. However, whilst the elucidation of part of the life cycle is considered

as a major step forward in our understanding of this pathogen, there are still a number of gaps in our understanding. Experiments to transmit the parasite between bryozoan hosts and from infected fish back to bryozoans have been unsuccessful, thus Koch's postulate has not been fulfilled. The current study would suggest that neither oligochaetes nor selected, common invertebrates are implicated in the life cycle (chapter 8). However, these hosts cannot be completely precluded from a role in transmission since limited numbers of invertebrates were examined. Whilst it is possible that an alternate fish host, such as the geographically widespread stickleback may be involved in the lifecycle, this seems unlikely since no fish species commonly occur at sites where PKD is enzootic. However, given that carp are known to harbour PKX-like cells, this option cannot be excluded (Voronin, 1993; Voronin and Chernysheva, 1993). The completion of the life cycle of *T. bryosalmonae* should be considered as a priority area for research in order to better understand and control the infection in wild and farmed fish.

This thesis has provided new data on myxospores and actinospores of ecological importance (chapters 3, 4 and 5), demonstrated the role of disease in regulating fish populations (chapter 6) and elucidated part of the lifecycle of *T. bryosalmonae*, including its route of entry into the salmonid host (chapter 9), and suggested new areas of research in myxozoan taxonomy and ecology. Future work, specifically in the freshwater environment should consider the role of other invertebrates in the lifecycle of *T. bryosalmonae*. Given the current state of knowledge, it is highly likely that a second invertebrate host is involved, although the exact species continues to elude researchers. The current author would suggest that research should be directed towards free-living copepods as a potential source of infection to naïve bryozoans. Elucidation of further myxozoan lifecycles and consideration of the genetic susceptibility of the invertebrate host will be needed, along with phylogenetic and taxonomic studies of myxozoans. Given the global increase in mariculture, there will be a need to gain greater understanding on the pathogenesis, lifecycles and taxonomy of marine myxozoans that may have pathogenic potential.

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