A STUDY OF MYXOSPOREA SPP IN RUTILUS RUTILUS L.(ROACH) WITH PARTICULAR REFERENCE TO MYXIDIUM RHODEI LEGER, 1905 IN THE RENAL TISSUE

A thesis presented for the degree of
Doctor of Philosophy to the University of Stirling

By

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DEDICATION

For the women who led me to the volcano,
For the teachers who taught me the language of the birds
For Poulaki, who showed me how to fly
For the one who put into my hand the
Golden thread that leads here.....

.... and to Elena and her heavenly questions, hoping
that she will find satisfactory answers in the future

"And noone does pour the new wine into old skin bag:
otherwise, the new wine will destroy the old skin bag,
and it will run through, and the skin bag will be lost.

But, one must pour the new wine into new skin bag,
so that both will keep well.

And noone who has tasted old wine asks for new straight
away."

I. B.
Thessaloniki 21-5-1987

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CHAPTER 4: INVESTIGATION INTO MORPHOLOGY OF MYXOSPOREA spp SPORES IN RELATION TO SIZE AND ORIGIN

ACKNOWLEDGEMENTS

I would like to thank the people who helped me through the writing of this thesis.

Dr C. Sommerville for the motivation and supervision, for her excellent and respectful criticism, for a lot of encouragement and listening.

Dr M. Beveridge for his help with the water analysis results.

Professor A. Katos and Dr Th. Giannacopoulos, who helped me with the statistical analysis.

Billy Struthers and Mairi Beveridge for their unstinting assistance with the EM and histology processing.

Dr L. Tetley, Christine Skeirow and Dorothy Aitken from Glasgow University, for their generous help with the TEM and SEM.

Giorgos Rigas for providing the fish from Greece.

John McArthur for his help with the drawings, and Willy Thomson for the regular help in repairing the equipment.

Dr Arturo Chacon and Dr L Ross for their assistance in operating the computers.

Joanna Mackay for the difficult task of typing this thesis.

The Greek State Scholarship Foundation for providing the funds.

SDC for making it easier to complete.

Professor G. Pneumaticatos and some of the staff of the Veterinary School of Thessaloniki, Greece, who gave me the opportunity to start this project.

Professor C. Chimonas who made my first introduction to Parasitology so interesting.

Panos Varvarigos for help at the worst and best moments.

Timos and Pineloppi Rallis for coming into my life when they did.

Ian Irvine for being there and helping me work when I could no longer look at these pages.

Elena who inspired me and kept me going all along.

And in stunned amazement at their constancy and deep gratitude
for their unending love, despite the moments of
intolerance,
I thank my parents.

DECLARATION

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

.....FAlwine.....

ABSTRACT

The myxosporean parasites of roach (R. rutilus L) were studied in three different water bodies, two in Britain and one in Northern Greece.

Two Myxobolus species (M. pseudodispar Gorbunova, 1936 and M. ellipsoides Thelohan, 1892) and two Myxidium species (M. rhodei Leger, 1905 and M. pfeifferi Auerbach, 1902) were found.

M. pseudodispar and M. ellipsoides were found in the kidneys and spleen, whereas M. ellipsoides was found in the kidneys, spleen, gill, gill arch and cartilage.

M. rhodei was found in the kidneys, liver, spleen and muscle, whereas M. pfeifferi was found exclusively in the gall bladder and bile ducts.

The seasonality of the two Myxobolus and two Myxidium species was investigated in the present study, and showed that in each of the different habitats all the parasites had at least one peak in prevalence in Springtime. In addition to this, the two myxobolid species found in the Greek lake as well as Myxidium rhodei in all three locations, show another peak during the Winter months (December to February). Myxidium pfeifferi showed only one peak in Spring (April to May) in all three lakes.

The two Myxobolus species studied in the present project were considered well adapted kidney parasites provoking no host response to this tissue. In contrast, M. pseudodispar in the muscles and M. ellipsoides in the gills were found to be very pathogenic.

The spore morphology and variability study of these two species showed that M. pseudodispar has a distinct variability in the spore dimensions between the organs of fish, although M. ellipsoides showed only slight differences.

From the study of the two Myxidium species, evidence was produced that M. rhodei is synonymous with M. pfeifferi. This evidence was produced from the morphological investigation and the experimental infections carried out in two fish hosts and was supported by the seasonal data.

Through three different experimental infections in carp and roach, the life cycle of M. rhodei and its development in the hosts was proposed. No intermediate hosts could be detected in the life cycle of the parasite and the Exposure Method was considered the most effective, and the Intraperitoneal Injection Method the second most successful one.

Two cycles of development of M. rhodei infection were detected in roach. The first, occurring in the bile ducts, had a fast process resulting in the production of mature and maturing spores. The second started with the infection of glomeruli and had a slow progress with the final production of cysts containing mature spores.

Lesions due to degenerating trophozoites were also detected in the interstitial tissue of the kidney, where they were usually calcified, and in the heart. Large mature cysts were also found in the muscles of the fish. In the liver, disturbance of the metabolism of the body was assumed from the extent of the lesions in heavy infections with M. rhodei.

The pathogenicity of the parasite was thought to be serious in both renal tissue and liver/bile ducts. In the kidneys, the pathology concerned both the glomeruli and interstitial tissue. The pathology was assessed for the first time in terms of area of lesions and volume of affected tissues, as well as in relation to the environmental conditions of the habitats.

Other concurrent infections and conditions in fish with M. rhodei were considered to enhance the pathology of M. rhodei in the hosts.

Finally, the infection of M. rhodei was considered important in terms of both Greek economy and fish health monitoring.

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CHAPTER 1

GENERAL INTRODUCTION

In Greece, both aquaculture and the fishing industry are major components of the economy. The commonest and most established aquaculture is generally considered to be the farming of the freshwater species rainbow trout (Salmo gairdneri Richardson), carp (Cyprinus carpio L) and eel (Anguilla anguilla L). Of these species trout (Salmo gairdneri R) and the recently introduced Coho salmon (Oncorhynchus kisutch W) are the most intensively farmed species comprising at least 90% of the freshwater aquaculture industry in Greece. The industry has developed and expanded since 1980 and now operates on a commercial basis.

For reasons of economy, an important part of the diet of cultured salmonids, carps, and even eels to a lesser degree, is raw trash fish. Small marine and freshwater fish of low commercial value are routinely given as a part, but even sometimes as the only source of food to the cultured fish. In Greece, trash fish are very cheap as labour costs are extremely low compared to those of Central and Northern European countries. Unfortunately, these trash fish are fed completely unprocessed, since fish farmers are keen to minimize food costs and commonly refuse or ignore any advice given by specialists.

The most common constituents of the trash fish diet are the following species: Bleak (Alburnus alburnus L), Crucian carp(Carassius carassius) L, Roach (Rutilus rutilus L); among which, the roach is the most commonly used fish. The sources of these fish are three lakes in Northern Greece and the Lake Agios Vassilios, where this study was

carried out, is one of the most important fisheries for small cyprinids, carp (Cyprinus carpio) and perch (Perca fluviatilis L).

Lake Agios Vassilios is a medium-size (surface area: 42Km^2), shallow lake located 25Km NE of Thessaloniki, Northern Greece. The water temperature ranges between 6-7°C in winter and 22-24°C in the summer. The fish fauna is dominated by cyprinids (carp and roach), perch, eels and bleak.

An ecological survey of this lake was carried out in 1984 (Kilikidis, Kamarianos, Fotis, Kousouris, Karamanlis and Ouzounis, 1984) in order to investigate the possibility of the installation of a station for fish breeding and an experimental fishery. According to the results of the survey, the lake is undergoing eutrophication. The water quality has severely deteriorated due to pollution from pesticides and domestic waste. This has become a cause of concern, since it might create future problems in the fishing and fish production of the lake. Natural reproduction of carp in the lake under the present conditions was considered to be not possible by the authors.

Over the past 9 years, a special interest has been aroused in the pathological conditions deriving from the feeding of raw fish to cultured fish because a variety of pathological and fungal infections have been experienced in the cultured freshwater fish, e.g. Ichthyobodo spp., Ichthyophthirius spp., Capillaria spp., Acanthocephala spp. infections (Stamatopoulo s, 1984), Diplostomum infections (Kalfa and Sinis, 1985), Ichthyophonus and Saprolegnia spp.

infections (Stamatopoulos, 1984) and Ligula intestinalis infections (Chimonas, 1977; Athanassopoulou, 1983).

Amongst the parasite fauna, protozoa ectoparasites are commonly found in wild populations but the published data consists of records of incidence without reference to their host relationships. In general, the pathology of these parasites in roach is not very well understood and information on their prevalence in Greek fish is very limited (Athanassopoulou, 1985).

Despite the comparatively larger number of studies that have been carried out on the endoparasitic fauna of cultured fish, far too little of this information is available on Greek fish. The only relevant information remains the study of the disease in roach which was carried out during the period 1984-85 (Athanassopoulou, 1985). According to these results the infections concerned mainly myxosporean and coccidian species. Eimeria spp. infections were detected commonly in Greek roach populations in the Lake A Vassilios and kidneys were the most commonly affected organs (Athanassopoulou, 1985; Athanassopoulou and Vlemmas, 1986).

The myxosporean infections of roach were studied in the Lakes Agios Vassilios and Vistonis, both in Northern Greece, with particular reference to the kidney conditions and pathology (Athanassopoulou, 1985). In the same study, the description and differential identification of lesions found in the renal tissue were also assessed. In particular, Myxidium rhodei Léger, 1905 infections were

considered to be very important because of their high prevalence and pathogenicity. In the light of these preliminary studies and because of the general lack of knowledge of this parasite species a comparative study on its pathogenicity in roach from different habitats was considered to be necessary. Thus, fish from different British locations were examined. Three main sites were selected as sampling sites for the present study, Loch Fad on the Isle of Bute in the West of Scotland, Loch Maben in Dumfries, and a small lake in Yorkshire, because of the high incidence of M. rhodei in the fish from these locations and because the three environments were well separated from each other geographically.

The parasite in fish populations from British habitats have been much better investigated than those in the Greek water bodies. Most of the studies, however, concern mainly helminth data in different fish species including roach. Kennedy, in his checklist of British and Irish freshwater fish parasites, gives an indication of the extent of these studies in Britain up to 1974. The amount of information on myxosporean infections in roach is still very scarce (McGuigan and Sommerville, 1985).

Loch Fad is a freshwater loch situated 2.4Km SW of the town of Rothesay, Isle of Bute. It has a total surface area of about 72ha and a maximum depth of 12m. A total of 75 Kames-type rearing cages for the culture of rainbow trout are usually positioned at the north of the loch. Furthermore, eels are also stocked for a period of six months and wild fish (salmonids, roach and perch) comprise the

permanent fish fauna of this habitat. Although 14 species of macroinvertebrates exist, the benthos is dominated by six oligochaetes, chironomids and gastropod molluscs. The environmental and water quality aspects of this loch are relatively well studied in consecutive years (Beveridge, 1981; Stewart, 1983, 1985, 1986, 1987; Philips, Struthers and Midley, 1989). All these researchers have described the loch as a typical eutrophic lowland Scottish type. Ammonia, nitrate and concentrations of various phosphorous compounds were considered high by Philips et al. (1989) and indicative of a very eutrophic water where significant summer algal growth is possible. Unsatisfactory levels of oxygen at this time of the year may also occur.

The parasites of the cultured wild fish in the Loch were studied by McGuigan and Sommerville in 1985. However, the only myxosporean species recorded in this study was Myxidium giardi Cepède 1906 in the gills of eels. No data is available for Loch Maben but the Loch is a small private water body used mainly for recreational activities.

No information is available on the source of the fish from Yorkshire or details of the type of aquatic habitat. The only source of information on the likely type of habitat may be derived from a survey carried out by the Yorkshire Water Authority (1974). The survey was carried out in reference to a possible programme for intensive roach prop agation. The Authority required basic biological information on roach living under pond conditions in the northern part of England. The lake where the survey was carried out is Sandall Park Boating

Lake, near Doncaster, a small lake of approximately one hectare with an average depth of 2m. The water analysis and the analysis of invertebrate fauna showed that the lake is a typical eutrophic lake but, with some unusual features. Although there was an abundant plant growth, there was not a marked planktonic bloom and thus planktonic crustacea never became particularly abundant; their population was restricted to one species only (Yorkshire River Authority, 1974).

The class Myxosporea is a ubiquitous group of parasites of cold blooded vertebrates, and many have been described from freshwater hosts. These have been listed by Bendele and Klontz (1975), and the Myxosporea were comprehensively reviewed by Mitchell (1977). Virtually all tissues and organs may be infected, but most of the myxosporean show some tissue specificity (Mitchell, 1977).

The taxonomic scheme of the protozoa was developed around the turn of the century, and has been updated continually since then (Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblich, Lom, Lynn, Merinfeld, Page, Poljansky, Sprague, Vavra and Wallace, 1980), and thus the exact position of the class Myxosporea has been under question for some time.

Members of the class Myxosporea have a life cycle that is, in part, essentially amoeboid, hence their original association with the protozoa (Mitchell, 1977), but several workers have produced evidence to show that they have strong affinities with members of other phyla, largely because much of their life cycle shows true multicellularity

and because some similarities between polar morphological capsule and coelenterate nematocyst formation of the phylum Cnidaria exist (Lom, 1969[a], 1973; Lom and De Puytorac, 1965). A separate phylum, Myxozoa, containing these organisms was created in a revision of the taxonomy of the group (Levine et al., 1980). The classification of the class Myxosporea was then further updated by Lom and Noble (1984) to give the present taxonomic position:

Sub-Kingdom	Protozoa
Phylum	Myxozoa Grassé, 1960
Class	Myxosporea Butschli, 1881

The characteristics of the phylum as described by Lom and Noble (1984) are that all members are microscopic parasitic organisms which are pluricellular, and have morphologically and functionally specialized cells. Other major characteristics are those of cells being enveloped within other cells, and multicellular spores containing polar capsules (cnidocysts). These polar capsules contain coiled polar filaments whose function is probably that of attachment. There are one to many amoeboid sporoplasms (germinal elements) within the spore, and two to seven valves form the spore wall.

The class requirements (Lom and Noble, 1984) are that a pluricellular trophozoite should be the main site of proliferation. These trophozoites contain their own vegetative nucleus and generative cells which produce multicellular spores. They range from uninucleate pseudoplasmodia producing one spore to macroscopic plasmodia producing many. The spores contain one or two sporoplasms, one to seven polar

capsules and two to seven valves. These parasites can be histozoic, inter- or intracellular, or coelozoic in poikilothermic vertebrates. Those presented in the present study are included in the following:

Suborder Platysporina Kudo, 1919 emend.
Polar capsules (generally two, sometimes one) at the apex of the spore lie solely in the sutural plane of a bilaterally symmetrical spore. As a rule, histozoic parasites of freshwater fishes, producing large polysporous trophozoites.

Family **Myxobolidae** Thélohan, 1892 - Spores flattened parallel to the straight sutural line; the suture forms an elevated ridge and may be drawn out into long projections. One of the two polar capsules may be smaller; in two general, it has disappeared completely. Most species have an "iodinophilic vacuole". Form as a rule large histozoic trophozoites ("cysts") with numerous spores, mostly in freshwater fishes.

Genus **Myxobolus** Butschli, 1882 - Spores ellipsoid ovoid or rounded in valvular view, and biconvex in sutural view. Shell valves smooth. Two, mostly pyriform polar capsules: exceptionally, one is ostensively missing, in which case the remaining one is not situated axially. Posteriorly, the sutural ridge may extend into a crescentic ledge. Binucleate sporoplasm, often with an "iodinophilic vaculole". Trophozoites as a rule large, polysporous with pansporoblast formation; histozoic in freshwater fishes, some in marine fishes, rarely in amphibians.

Family **Myxidiidae** Thélohan, 1892. Spores spindle shaped, sigmoid or crescentic, sometimes almost semicircular in valvular view, ellipsoidal, have two (in Coccomyxa one is eliminated) polar capsules located in the opposite ends with terminal or slightly lateral capsular foramina; the longitudinal sutural line is straight, curved or sigmoid. Mostly coelozoic, rarely histozoic parasites in marine and freshwater fishes.

Genus Myxidium Butschli, 1882. Spores as a rule fusiform, straight or slightly crescentic or sigmoid, with more or less pointed ends; shell valves smooth or with ridges; sutural line bisecting the spore. Two mostly pyriform capsules are situated one at each end of the spore, capsular foramina lie in the sutural plane, at or near the end of the spore and open mostly in opposite directions. One binucleate sporplasm is located as a rule between the capsules. Typically coelozoic, small or large trophozoites, monosporous, disporous and polysporous, the latter with pansporoblast formation, also histozoic; intracellular stages are known. In marine and freshwater fishes, rarely in amphibians or reptiles.

The aim of this project was to study the biology of M. rhodei and the pathology which the parasite has on the hosts. For this purpose, the biology, life cycle and pathology were studied in the first instance in naturally infected fish.

The occurrence of myxosporean infections in different habitats had previously been established through examination of fish from different sources, in both Greece and Britain, during the period 1981-1986. Therefore, during the project period, the prevalence and seasonality of the Myxidium and Myxobolus species concerned, were investigated in order to establish the time of the year when natural peaks of infections occur and to compare these between species of two different genera, i.e. Myxobolus and Myxidium. Information on seasonality of the specific Myxobolus species found in the project is limited and in the case of M. rhodei and M. pfeifferi, non-existent. As a consequence it was not known whether Myxobolus and/or Myxidium spp. infections show constant seasonal patterns in their life cycle. Thus,

three different and well separated, both geographically and climatically, habitats were selected as sampling sites.

Another important purpose of the project was to assess the morphology of the two Myxobolus and two Myxidium species studied here and to investigate in detail any variability occurring between the same species in relation to the age and size of the hosts, and/or the location of the parasites in the hosts. As information on these particular aspects is also limited, great difficulty was encountered in the comparison of results from different authors. Furthermore, previous lack of a generally agreed taxonomy of this group of parasites made the identification of some species and the subsequent comparison of results even more difficult. For example, the affinity of M. rhodei with other Myxidium species was not certain and was never properly investigated. In response to this question, the possibility of M. rhodei and Myxidium pfeifferi being synonymous was also examined.

Although the previous aspects were examined in depth, the main nature of this study, however, was an assessment of the pathological conditions derived from myxosporean species in Greek and British roach. The pathology of myxosporean species was studied using light and electron microscopy. As M. rhodei was found to be an important parasite in roach from previous studies (Athanassopoulou, 1985), and only limited information was available on its pathology, particular attention was given to its pathogenicity in kidney and other tissues.

Since M. rhodei produces numerous large cysts in the kidneys, the amount of kidney tissue occupied by the cysts was considered to be important and thus, a new method based on image analysis was used to measure the amount of kidney tissue affected. There are a number of image analysis applications in biology requiring the mapping and measurement of two dimensional distributions of cells or particles. Examples are the distribution of colonies in cell culture, particles in freeze-fractured cell membranes, gels and suspensions. In this case, it was used on kidney histological sections to measure the area and volume of the parasitic lesions, as well as the area and volume occupied by normal functional glomeruli, in order to assess and compare the extent of damage caused by M. rhodei on the renal tissue of roach.

The method represents a simple image analysis system based on a microcomputer and digitiser tablet which counts and traces cells or other structures and measures different parameters.

The life cycle and the pathology of M. rhodei was also studied experimentally in order to confirm the findings of the investigations in the naturally infected fish and to gather further information on the life cycle in particular.

Most of the information on experimental infections in Myxosporea relates to Myxobolus cerebralis and data on similar infections of Myxidium spp. was unavailable. Therefore, the first step was to establish the best method for transmission of M. rhodei infections. In

pursuing this, difficulties were encountered in assessing the sampling periods since most of the data was extracted from Myxobolus spp. experimental infections, carried out by other authors whose results were considered to be most encouraging.

After the assessment of the best method, other aspects of the M. rhodei life cycle were studied, such as the existence or non-existence of intermediate hosts, as such intermediate hosts have recently been suggested by some authors for M. cerebralis. Modifications of the best method were used and two species of fish host (carp and roach) were used. The examination of samples comprised both examination of fresh material and histological sections.

Throughout the period of the experimental infections it became obvious that lesions observed in fresh preparations could rarely be detected in the histological sections. Subsequently an effort was made to overcome this problem by cutting a large number of serial sections. This was not always successful. A separate experiment was, therefore, set up almost identical to the first, during which samples were examined only histologically, and in detail, in an attempt to eliminate the possibility of any tissue waste.

Another problem encountered during the experimental period was in the acquisition of myxosporean-free fish as this is a major factor in ensuring the validity of the experimental results. Consequently, only artificially reared fish were used in the experiments in this study and all possible precautions were taken to avoid exposing the fish to

myxosporean parasites other than the experimental introduction of the spores.

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 FISH SAMPLES AND MAINTENANCE

82 roach were collected in January 1986 from the Lake Agios Vassilios using gill nets for different fish sizes. The fish were then transported alive to the laboratory of the Department of Ichthyology and Fish Pathology, University of Thessaloniki, Greece, in plastic bags containing water of the lake. The fish were kept temporarily in small plastic tanks with aeration prior to the post-mortem examination.

A total of 428 roach and 36 perch were caught from the Lake Agios Vassilios throughout the period September 1986 - September 1987. These fish were caught and handled in the same way as above and examined at the Laboratory of Ichthyology and Fish Pathology. The samples were removed throughout the year as follows:

Autumn (September-November 1986 = 45 fish)
Winter (December-February 1986/87 = 185 fish)
Spring (March-April 1987 = 52 fish)
Summer (June-September 1987 = 36 fish).

18 roach from Loch Maben, Scotland, were caught by gill nets, packed in ice inside isothermic boxes and transported dead to the Institute of Aquaculture, University of Stirling, where they underwent prompt full post-mortem examination.

Another 273 roach and 20 perch collected from Loch Fad using gill nets between January 1987-December 1987, were handled and examined in the same way at the Institute of Aquaculture.

62 roach (R. rutilus) and 22 carp (C. carpio) held over in ponds in Yorkshire were sent to the Institute of Aquaculture in a small volume of water in plastic bags filled with oxygen (February and April 1987 samples). As soon as they arrived at the Institute, the fish were placed in a plastic tank (80x70x35cm) filled up with dechlorinated water. The tank was kept outdoors and was intensively aerated. Samples of these fish were removed and examined within 30 days.

30 roach and 5 carp from the same location (July sample) were sent in the same way to the Institute of Aquaculture, held in one plastic tank (80 x 70 x 35 cm) filled up with charcoal filtered water and constantly aerated. The water temperature was 10°C at the time of the examination and the fish were kept with minimal feeding until January 1987 when they were examined as the rest of the fish.

Another 59 roach from Yorkshire were sent on 15 November 1987 to the Institute of Aquaculture. These fish were put in plastic tanks filled up with water. The tanks were kept at 14°C for about one month during which period, they were examined and processed as detailed before.

Another two batches of 168 fish (110+58) were collected from Lake A. Vassilios, N. Greece, as described before, in November and January 1988 respectively. Half of the January fish were immediately processed with a fixative for both histology and EM purposes and the other half were kept in 10% buffered formalin for further histological examination.

2.2 GENERAL POST-MORTEM TECHNIQUES

Each fish was given an overdose of Benzocaine (Sandoz) anaesthetic prior to PM examination, then weighed and the length recorded. Age was estimated by examination of scale rings in the roach and carp and by examination of the spinal rings in the case of rainbow trout. The anaesthetized fish were placed under a dissecting microscope (x20-x45) and examined for the presence of cysts, lesions or parasites on the skin and gills. Squash preparations of skin and gills were also made and examined microscopically (x100-x1000).

Blood smears were taken from the heart and caudal area and stained with two modifications of Giemsa stain (Drury and Wallington, 1980; Kalavati and Narasimhamurti, 1983).

The abdominal wall was then cut and the heart, gastro-intestinal organs and kidneys removed. Squash preparations from all organs were made which were fixed in Schaudinn's fluid and stained with Giemsa (Drury and Wallington, 1980).

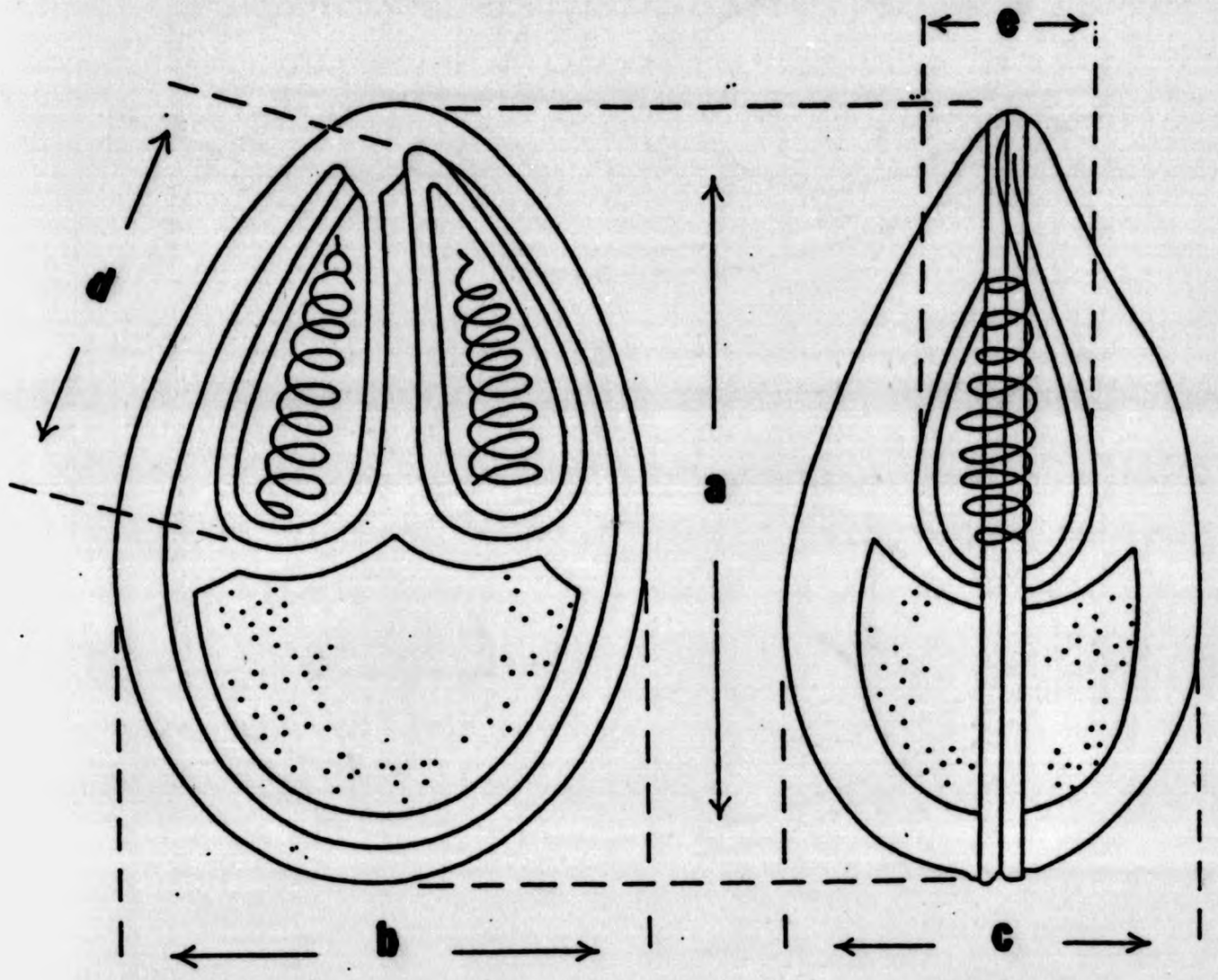
During the PM examination, pieces of tissues from all organs were taken for histological processing.

2.3 PARASITOLOGICAL TECHNIQUES

Parasitological examination was carried out simultaneously with the general PM examination, in all fish samples and the experimental fish prior to the start of the experiment. The parasitological examination was carried out using the method described by Athanassopoulou (1985).

The measurement of the parasites, cysts and spores was made in the fresh state using a standard eye piece graticule calibrated using a micrometer slide. At minimum of 30 parasites, spores, or cysts were measured in each case. The various dimensions measured in each spore are shown in Figs. 1 and 2. All measurements are expressed in micrometres.

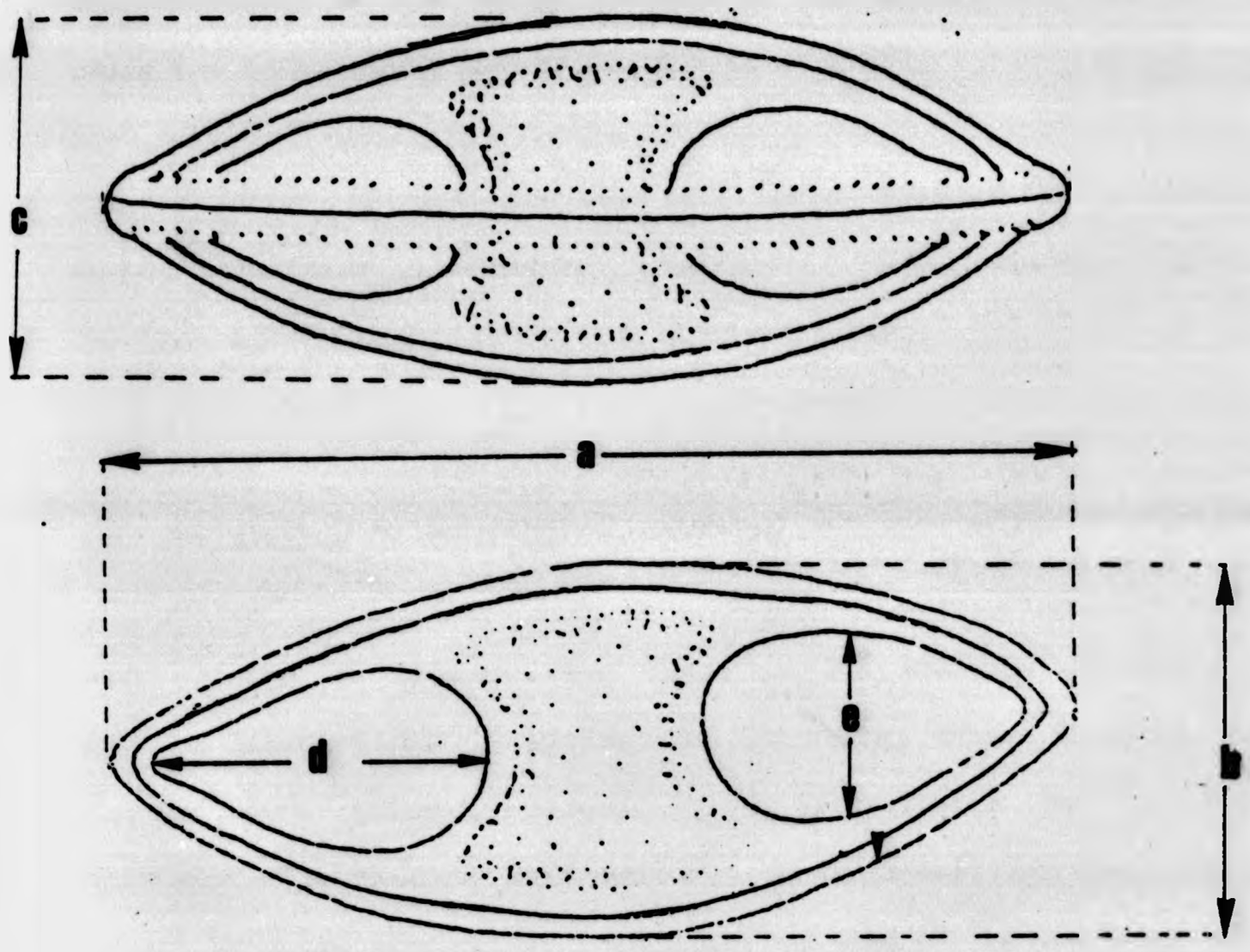
For better observation of myxosporean spp. spore shape and structure, parasitology iodine and Methylene Blue and Trichrome Methylene Blue were used (Appendix II). For the extrusion of polar filaments of the myxosporean spp. spores, the methods described by Kalavati and Narasimhamurti (1983) and Lom (1964) were used.



After Lom and Arthur (1989)

FIGURE 1: Myxobolus spp. spore

a = length of spore
b = width of spore
c = thickness of spore
d = length of polar capsule
e = width of polar capsule



After Lom and Arthur (1989)

FIGURE 2: Myxidium spp. spore

a = length of spore
b = width of spore
c = thickness of spore
d = length of polar capsule
e = width of polar capsule

The existence of the mucus envelope of the spores was detected by using the method described by Lom and Vavra (1963).

Identification of protozoa was made using Bykhovskaya-Pavloskaya, Gusev, Dubinina, Izyumura, Smirnova, Sokolovskaya, Shtein, Schulman and Epshtein (1964), and Schulman (1984).

2.4 PREPARATION OF HISTOLOGICAL MATERIAL

Small pieces of tissues were fixed in 10% buffered formalin, or Bouin's fixative for a minimum of 24 hours. After fixation, the tissues were trimmed, placed in a Histokinette and processed routinely. The tissues were embedded in Paraplast wax and cut at 5-6um. Routine procedures were used to stain the sections with Haemotoxylin and Eosin, Periodic acid Schiff, Von Kossa, Ziehl-Neelson (Drury and Wallington, 1980), Giemsa and Mallory's Trichrome stain (Paul Thompson, personal communication) (Appendix II).

2.5 SPORE CONCENTRATION AND ISOLATION TECHNIQUES

Spores were isolated and concentrated from both Greek and British roach using a modification of the method commonly used for Myxosoma cerebralis (O'Grodnick, 1975) and some Myxobolus spp. (Mitchell, Krall and Seymour, 1983; Athanassopoulou, 1985).

Development of the Method Used

The method used was developed as follows:

10 kidneys from Greek roach were carefully scraped out and blended together in approximately 10ml saline. Kidneys were macerated by hand in a mortar with a pestle. The macerated material was then divided into eight groups. Each group was gravity filtered through different mesh nylon screen sizes. Groups 1 and 5 were filtered through mesh size (1) 300µm, Groups 2 and 6 through mesh size (2), 150µm, Groups 3 and 7 through mesh size (3) 90µm, and Groups 4 and 8 through mesh size (4) 45µm.

The filtrates were placed individually in a separation funnel discharging into the plankton centrifuge. The flow was set at high speed until all saline was removed. The saline from the first run was collected and recentrifuged. The residue of the walls of the plankton centrifuge revolving drum was scraped and the material suspended into a known amount of saline (6-10ml).

The material was then placed in small bottles and shaken thoroughly until it was uniformly suspended (5 min).

A small amount of the above suspension was then placed under a coverslip of the haemocytometer and the spores under 1mm squares were counted. Four chambers were used counting a total of 64 squares. Each sample was counted three times and the mean value was calculated.

The following formula was used to calculate the number of spores in 1ml:

$$\frac{\text{No. spores}}{\text{ml}} = \frac{\text{Total number of spores counted x 10}}{\text{No of 1mm areas counted}}$$

The calculated number was then multiplied by the volume of saline in which the material was suspended giving a total spore count for each group. The outflow of each group was also collected and the number of spores was counted three times in the same way. According to the above procedure, the nylon screen of 4 (45/µm) gave the best results.

In order to try to improve the filtration by inclusion of cotton fibre into the procedure, the method was tried once more using 10 roach kidneys from Greek fish. The kidneys, after mixing, were macerated by hand and divided into eight groups. Groups 1–4 were gravity filtered using simple mesh sizes 45/µm. The groups 5–8 were also gravity filtered using the same mesh sizes and in addition a layer of cottonwool. The samples were thereafter processed in the same way as described previously.

The comparison of the different groups showed that Group 8 which was filtered through mesh size 4 (45/µm) and cottonwool, gave the cleanest and the most dense suspension of spores.

In order to further develop the purity and density of the spores a modification of the method was made, using vacuum filtration. 10 roach kidneys from fish were mixed up, macerated by hand and divided into two groups. Group A was then vacuum filtered through a nylon screen mesh of 4 (45/m) and a layer of cottonwool. Group B was gravity filtered through a nylon screen of mesh 4 (45/m) and a layer of cottonwool without vacuum filtration. The suspensions of Group A were slightly cleaner and more dense in spore numbers.

A further modification was also tried in an attempt to obtain the best results of spore suspensions using an electric homogeniser with a head size of 1.8cm.

20 roach from Yorkshire were used for this modification. The roach kidneys were mixed up and divided into eight groups. Groups 1–4 were homogenised electrically using a head of 1.8cm, and then vacuum filtered through mesh sizes 1–4 respectively, and a layer of cotton wool.

Groups 5–8 were macerated by hand and filtered in the same way. The best results were obtained by electric homogenisation and by filtration through mesh size 4 (45/m) plus cottonwool. The method is schematically presented in Table 1.

TABLE 1

Schematic representation of the modification used for the isolation and identification techniques of Myxosporida spp. spores

STAGE 1					BEST RESULTS
16 kidneys hand ground together and divided into eight groups: gravity filtered	Group 1	Mesh No. 1			Mesh No. 4 (Groups 4 and 8)
	" 2	Mesh No. 2			
	" 3	" No. 3			
	" 4	" No. 4	+ Saline		
	" 5	" No. 1			
	" 6	" No. 2			
	" 7	" No. 3			
	2 8	" No. 4			

STAGE 2					BEST RESULTS
16 kidneys hand ground and gravity filtered divided into eight groups:	Group 1	Mesh No. 1			Mesh No. 4 + Cottonwool (Group 8)
	" 2	" No. 2			
	" 3	" No. 3			
	" 4	" No. 4			
	" 5	" No. 1			
	" 6	" No. 2	+ Cottonwool	+ Saline	
	" 7	" No. 3			
	" 8	" No. 4			

STAGE 3				BEST RESULTS
16 fish hand ground and vacuum filtered divided into eight groups:	Group 1	Mesh No. 4 + cottonwool + vacuum filtered	+ Saline	Mesh No. 4 + Cottonwool + Vacuum Filtered (Group 1)
	Group 2	Mesh No. 4 + cottonwool + gravity filtered		

Table 1 (cont'd)

STAGE 4						BEST RESULTS
16 fish ground by two different methods vacuum filtered divided into eight groups	Group 1 +	Mesh No. 4 +	cottonwool	+ Saline ground by hand		Mesh No. 4 + Cottonwool + Saline + homogenised by electrical grinder (Group 8)
	" 2 +	" " +	"			
	" 3 +	" " +	"			
	" 4 +	" " +	"			
	" 5 +	" " +	cottonwool	+ Saline homogenised by electrical grinder		
	" 6 +	" " +	"			
	" 7 +	" " +	"			
	" 8 +	" " +	"			

2.5.1 Staining Procedures

Spores of Myxobolus spp. and Myxidium spp were stained with different stains in both histological and fresh samples in order to study the morphology and the best staining results for these parasites and their intermediate stages (cysts, trophozoites, etc.).

The spores were stained with the following methods, described in detail in the Appendix II section. Giemsa, Lugol's Iodine stain, Indian Ink method for detecting the spore's envelope (Lom, 1964), Neutral red, Methylene Blue and its modification Polychrome Methylene Blue (Appendix II).

The Polychrome Methylene Blue proved to be the best and most efficient stain for myxosporean spores and their intermediate stages.

2.5.2 Spore viability test

No efficient method has been developed yet for the determination of myxosporean spore viability.

Almost all the previously described methods relied on spore structure and morphology namely the sporoplasm, polar capsule and polar filament morphology and integrity. In particular, extrusion of the polar filament has been thought to be an indicator of spore death by Bond (1938).

The spore viability test using methylene blue stain as an indicator has been developed for testing the viability of spores of Myxosoma cerebralis (Hoffman and Markiw, 1977) but according to the available bibliography, no information exists for spores of other Myxosporea species. In the present study both the methylene blue method and the morphology of the spore's structure were selected to assess the spore viability of Myxobolus pseudodispar and Myxobolus ellipsoides from the roach samples.

Staining was accomplished by adding 0.5% aqueous methylene blue solution to 37µl of spore concentrate, processed as previously described, on a microscope slide, so that the final concentration would be 0.08%. A micropipette (5-40µl) was used to deliver the above amounts of stain and spore aliquots.

2.6 PHOTOGRAPHY - DRAWINGS

Microscopic photographs were taken with an Olympus BH-2 research microscope fitted with a photomicrographic camera (C.35AD) using direct light and interference contrast techniques.

Drawings were made using a drawing tube on an SH Lux Compound microscope, Leitz Wetzlar, Germany. Free hand drawings were also made.

For photography purposes, the myxosporean spores were embedded in 1.5% agar according to the method described by Lom (1965) and covered with coverslips No. 00.

CHAPTER 3

INVESTIGATION INTO SEASONALITY, PREVALENCE AND INTENSITY OF MYXOSPOREA spp. INFECTIONS IN ROACH

3.1 INTRODUCTION

Seasonal changes in nature are clearly reflected in organic life. It is therefore natural to expect that parasites, even endoparasites, although they have no direct ties with the external environment, should not remain indifferent to annual cycles of climatic change.

It is also apparent that cyclical changes in the susceptibility between various groups of hosts and symbiotes exist and this may be altered by different factors. Although there is considerable data on annual rhythms of parasites of mammals (Hawking, 1975) there is little information on the seasonal occurrence of fish parasites. The seasonal changes in the incidence of some species have been reported in the literature (Dogiel, 1964). Chubb (1977, 1979, 1980, 1982), recognising the fact that treatment and elimination of many helminths in fish farming conditions will be effective only if related to the knowledge of the seasonal biology of the species, produced a review of the seasonal occurrence of helminths in freshwater fish. According to this research, many parasite species when in their fish host, exhibit seasonal cycles and such cycles have been reported in monogeneans, digeneans, acanthocephalons, copepods and leeches in freshwater and marine fish (Chubb, 1977, 1979, 1980, 1982). Iqbal (1984) described a seasonal migration and reproduction in the blood fluke Sanguinicola inermis Plehn which occurred in the carp even in an aquarium where

environmental parameters were constant throughout the year, demonstrating endogenous rhythms relating to external factors in the natural environment. Seasonal cycles almost certainly also occur in Protozoans, although these have not been studied in such detail, and in regions other than the sub-Arctic and temperate latitudes (Kennedy, 1977).

In general, the factors known to induce changes in the parasite population's development may be grouped into three major categories according to Schad (1977):

(1) External environmental factors;
(2) Host factors which determine the host's suitability as an environment for further development, and
(3) Parasite-related factors either genetic or density-dependent.

Other authors (Chubb, 1977) describe the factors as abiotic and biotic.

Bauer (1959) also discussed these factors and amongst the abiotic factors included the amount of light entering the water, the depth, pH, oxygen content, salinity and temperature of the water. The biotic factors included the host feeding habits, the density of fish populations, reproductive behaviour, growth rates and migratory patterns.

Kennedy (1977) studied the density-dependent and independent factors affecting the parasite populations in fish. Density-independent factors such as the temperature and feeding patterns are known to be

associated with cyclic manifestations of susceptibility in animals (Reid, 1970). In fish, according to Kennedy (1977) only density-independent factors have been shown to affect parasite populations. Of these, the most widespread and important appear to be the water temperature and the fish behaviour, both dietary and social.

The density-dependent factors (such as the host immune response) is considered by Kennedy (1977) to be less important. Mammals and birds are capable of mounting effective immune responses against their parasites. Among different fish species investigated, most have been shown capable of producing circulating antibodies mainly belonging to the IGM group (Houghton, 1987). Therefore, fish seem to be incapable of producing skin sensitizing antibodies or systemic anaphylactic shock. However, although fish are not able to mount complete immune responses involving antibodies against their parasites, some species are able to produce responses involving mucous changes that are effective against some ectoparasites (Graves, Evans and Dawe, 1985), including the protozoan parasite Ichthyophthirius multifiliis (Houghton, 1987). These responses may be general and short-term or specific and long-term but in both cases confer resistance to reinfection.

In the studies of fish immunology it has been known from an early stage that antibody response is dependent upon the temperature of the environment. At low temperatures (8-12°C) the response is slow or not detected, but at higher temperatures it occurs more rapidly (Avtalion, 1969; Rijkers, Frederix-Wolters and Muiswinkel, 1980). Such a

variation in activity of antibody production, reflecting changes in water temperature, has been used by Chubb (1977) to explain seasonal variations in the incidence of parasites .

Other factors described by Kennedy (1977) as density-dependent factors, such as inter- and intra-specific competition in parasite populations as well as other specific mechanisms, have also been reported in fish but these refer mainly to helminths (Kennedy, 1977).

Seasonal epizootics and outbreaks of different myxosporean infections have often been reported in the literature according to the review by Mitchell (1977). These seasonal cycles are thought to have an influence on the transmissibility and rate of development in the hosts. The literature reveals little information, however, about species other than these which affect commercially important hosts. There is even less data available concerning myxosporeans from sub-tropical countries like Greece. Reports from such countries exist mainly from Spanish reservoirs and other freshwater habitats (Gonzales-Lanza and Alvarez-Pellitero, 1985; Alvarez-Pellitero, Gonzales-Lanza and Pereira-Bueno, 1983).

As in the case of other fish parasites, a variety of factors may be responsible for the seasonal fluctuations of the myxosporean infections. Aquatic conditions which provide concentrations of hosts and infective stages promote massive development of the parasites and increase frequency and intensity of epizootics. It is thought, however, that myxosporean disease most often results from cumulative

hyperinfection (McCraren, Landolt, Hoffman and Meyer, 1975).

Seasonal cycles of myxosporean infections have also been related to the environmental temperature, and strict temperature requirements of some species may determine the intermittent occurrence of disease (Schafer, 1968).

Lom (1970) suggested that seasonal cycles in some myxosporean species may be strongly influenced by host antibody response. During cold periods when antibody titres are low, massive infections may develop. On the other hand, tolerance levels of hosts for environmental stress, such as oxygen depletion, are reduced by gill infections of Myxosporea (Mitchell, 1977).

Incidence of infection with Ceratomyxa shasta has been directly related to the distance covered by the fish during migration in freshwater and there are indications that natural, specific foci for myxosporean infections do exist and these may be sharply delineated as with C. shasta. It is also possible that genetic strains of hosts are being cultured in hatcheries for this parasite (Schafer, 1968). The incidence of infections with Myxosporea is extremely variable and in many hosts 100% infection is not uncommon.

Several species appear more frequently or exclusively in mature hosts and it is thought that older fish usually carry greater total numbers of spores than juvenile specimens. On the other hand, very young hosts in general are often most susceptible to disease and major

epizootics most frequently occur in hatcheries in juvenile fish. It is also suspected that significant disease may result as previously unexposed host species become infected (Mitchell, 1977).

This part of the study is an extensive assessment of the prevalence, seasonality and intensity of the myxosporean infections in roach in both Greek and British environmental conditions which represent typical temperate and sub-tropical climates. It also examines the above parameters in relation to the different biological characteristics of the fish populations, namely the age, sex and length, taking into consideration also the environmental conditions of the habitats during the period January 1986-January 1988 when this research project was carried out.

3.2 MATERIALS AND METHODS

The study covers the period January 1986 to January 1988. During this period, the Lake A Vassilios was sampled in January 1986 and again in January 1988. Samples were taken more regularly, i.e. one per month, during the period September 1986 to September 1987.

British roach samples were taken from Loch Fad (Scotland) during the period January 1987-December 1987 and from Yorkshire, N England, during the period February 1987 to December 1987.

A total of 486 Greek and 442 British fish were examined. The fish were categorised according to their length and age as follows:

Origin of Fish	Nos. Fish		Standard Length (cm)	Age (years) (scale reading)
Lake Agios Vassilios (Greece)	Small	105	10-16	$0^{+}-3^{+}$
	Large	381	16.5-24	$3^{+}-7^{+}$
Loch Fad (Scotland)	Small	116	10-15	$0^{+}-3^{+}$
	Large	157	19.5-26	$3^{+}-7^{+}$
Loch Maben (Scotland)	Small	0	12.5-15.5	$0^{+}-3^{+}$
	Large	18	16-23	$3^{+}-8^{+}$
Yorkshire (N England)	Small	68	0	$2^{+}-3^{+}$
	Large	83	18.5-32.5	

It should be noted that no samples were available for Lake A Vassilios during the months of May and June since fishing in the lake was prohibited due to the reproductive activity of the fish.

Length refers to standard length and is expressed in centimetres. The fish were aged using scales and/or opercular bones. The fish were measured in a fresh state. All measurements were taken between verticals in accordance with standard methods (Lagler, Bardach and Miller, 1962).

Statistical Analysis

In order to determine the influence of age and length of fish on the infections with Myxosporea spp, the Chi square (X^2 test) was used.

Host, sex influence was also studied using the Chi square test (2x2 table) for the prevalence.

The intensity of the infections was semi-quantitatively evaluated and thus not subjected to statistical analysis. Since it was impossible to count all the spores, the intensity was graded according to the number of spores per viewing field and categorised as different levels of infection. These are as follows :

Intensity Level	Myxobolus spp.	Magnfcn.	M. rhodei	Magnfcn.
Level 1 (IL1)	5-10 spores/field	250x	1-3 cysts/field	100x
Level 2 (IL2)	10-50 " "	"	3-5 " "	"
Level 3 (IL3)	50-100 " "	"	5-8 " "	"
Level 4 (IL4)	>100 " "	"	8 " "	"

The Mean Intensity was calculated by summing the index for the intensity level (IL) and dividing by the number of infected fish.

Environmental Parameters

The description of the environmental conditions of the habitats was outwith the scope of this study. Thus, the information was taken from previous studies where possible. In the case of Lake A. Vassilios data from the biological and water parameters are presented in the Appendix I (Tables 1-8).

Loch Fad (Scotland): The water quality data of the different workers are given in Tables 9 and 10 of Appendix I.

No data is available for Loch Maben, Scotland. No data was available for some of the fish from Yorkshire since they were obtained from a commercial fishery, other than that fish were from a local, unnamed source but were held over in overwintering ponds on the farm site in Humberside.

The results of the water analysis of Sandall Park Boating Lake in Yorkshire which was considered to be a habitat similar to the environment where the fish for this study originated, are given in Table 11 of Appendix I. These data were obtained from the Yorkshire Water Authority (1977).

3.3 RESULTS

The most commonly-occuring myxosporeans found in the fish from all the sampling locations examined in the present study are presented in Table 2. Two Myxobolus species, M. pseudodispar Gorbunova, 1936 and M. ellipsoides Thelohan, 1892, and two Myxidium species, M. rhodei Leger, 1905 and M. pfeifferi Auerbach, 1908, were found. In addition, Sphaerospora spp was found but on a few occasions only. Data was not collected for this species.

The sites of infection and the details of the parasitic infections found in the fish from the different locations are shown in Tables 2 and 3.

TABLE 2

Prevalence, sampling periods and parasites found in roach (R. rutilus) from Greece and Britain

Lake	Sampling Periods	NOS. FISH EXAMINED Small	Large	Parasites Found	Sampling dates when parasites were found	PREVALENCE (%) Small	Large	Overall
Agios Vassilios (Greece)	Jan. 1986	36	46	Myxobolus pseudodispar Gorbunova 1936	All samples	5.96	11.52	17.48
	Sep. 1986	3	9					
	Nov. 1986	6	27	Myxobolus ellipsoides Thelohan 1892	" "	8.23	19.96	28.19
	Dec. 1986	14	16					
	Jan. 1987	14	35	Myxidium rhodei Leger 1905	" "	6.86	22.63	29.40
	Feb. 1987	7	17					
	March 1987	13	19	Myxidium pfeifferi Auerbach 1908	" "	2.26	8.23	10.49
	April 1987	7	13					
	July 1987	22	14					
	Nov. 1987	42	68					
	Jan. 1988	18	40					
	Total	182	304					
Loch Fad (Scotland)	Jan. 1987	5	18	Myxobolus pseudodispar Gorbunova 1936	March 1987	0	0.36	0.36
	March 1987	2	21					
	May 1987	3	20	Myxobolus ellipsoides Thelohan 1892	March 1987	0	3.33	3.33
	July 1987	5	18					
	Sept. 1987	6	17	Myxidium rhodei Leger 1905	All samples	7.32	26.01	33.03
	Dec. 1987	95	63					
	Total	116	157	Myxidium pfeifferi Auerbach 1908	" "	5.13	10.99	16.12
Loch Maben (Scotland)	March 1987	0	18	Myxobolus pseudodispar Gorbunova 1936	March 1987	0	22.22	22.22
Yorkshire (N England)	Feb. 1987	7	12					
	April 1987	22	21	Myxobolus pseudodispar Gorbunova 1936	April 1987	5.96	13.90	19.18
	July 1987	12	18	Myxidium rhodei Leger 1905	All samples	11.92	21.85	34.77
	Nov. 1987	27	32	Myxidium pfeifferi Auerbach 1908	" "	8.61	15.23	23.84
	Total	68	83					

TABLE 3

Parasites and their site of infection found in roach (R. rutilus) from Greece and Britain

(MMC = melanomacrophage centres)

LAKE	Parasite Species	Sites of Infection	Details
Agios Vassilios (Greece)	Myxobolus pseudodispar	Kidneys, Spleen	free spores in MMC
		Muscles	cysts
	Myxobolus ellipsoides	Kidneys, Spleen	free spores in MMC
		Gills, Gill Arch, Cartilage	cysts
	Myxidium rhodei	Liver, Spleen, Muscles	cysts (containing mature spores)
		Interstitial tissue (Kidney)	" " " "
		Glomeruli (Kidney)	trophozoites
	Myxidium pfeifferi	Gall Bladder	free mature spores
		Bile ducts	trophozoites
Loch Fad (Scotland)	Myxobolus pseudodispar	Kidneys	free spores in MMC
		Muscles	cysts
	Myxobolus ellipsoides	Kidneys	free spores in MMC
		Muscles	cysts
	Myxidium rhodei	Kidneys (interstitial tissue)	cysts/trophozoites
		" (glomeruli)	" "
	Myxidium pfeifferi	Gall Bladder	free mature spores
		Bile ducts	trophozoites
Loch Maben (Scotland)	Myxobolus pseudodispar	Kidneys	free spores in MMC
Yorkshire (N England)	Myxobolus pseudodispar	Kidneys	free spores in MMC
		Muscles	cysts
	Myxidium rhodei	Liver, Spleen, Muscles	cysts
		Interstitial tissue (Kidney)	cysts
		Glomeruli (Kidney)	trophozoites
	Myxidium pfeifferi	Gall Bladder	free mature spores
		Bile ducts	trophozoites

3.3.1 Seasonality, Prevalence and Intensity of the Genus Myxobolus

Parasite: Myxobolus pseudodispar Gorbunova. 1936

In all fish examined, the parasite was found in the kidneys, spleen and muscles. In the kidneys and spleen only, free spores forming small groups were found concentrated in the melanomacrophage centres. In a few cases, scattered spores were also found in the renal parenchyma. In the muscles, the spores were found in cysts, well demarcated by connective tissue (Table 3).

The seasonality of the parasite was studied only in the Lake A. Vassilios (Greece) and in the Yorkshire (N England) environments, since in the two Scottish lochs the parasite was found only in the March samples and in only one fish from each location, as noted in Table 2.

Prevalence

Figure 3 shows the seasonal pattern of the prevalence of M pseudodispar in Lake A. Vassilios, Greece. The figure suggests a greater prevalence in the warm months (March through September) but high levels were also recorded in the colder month of January. The highest values were observed in January 1986, September 1986 and March 1987. Three different very variable values, however, were observed in the January samples during the years 1986, 1987 and 1988 (53.66%,

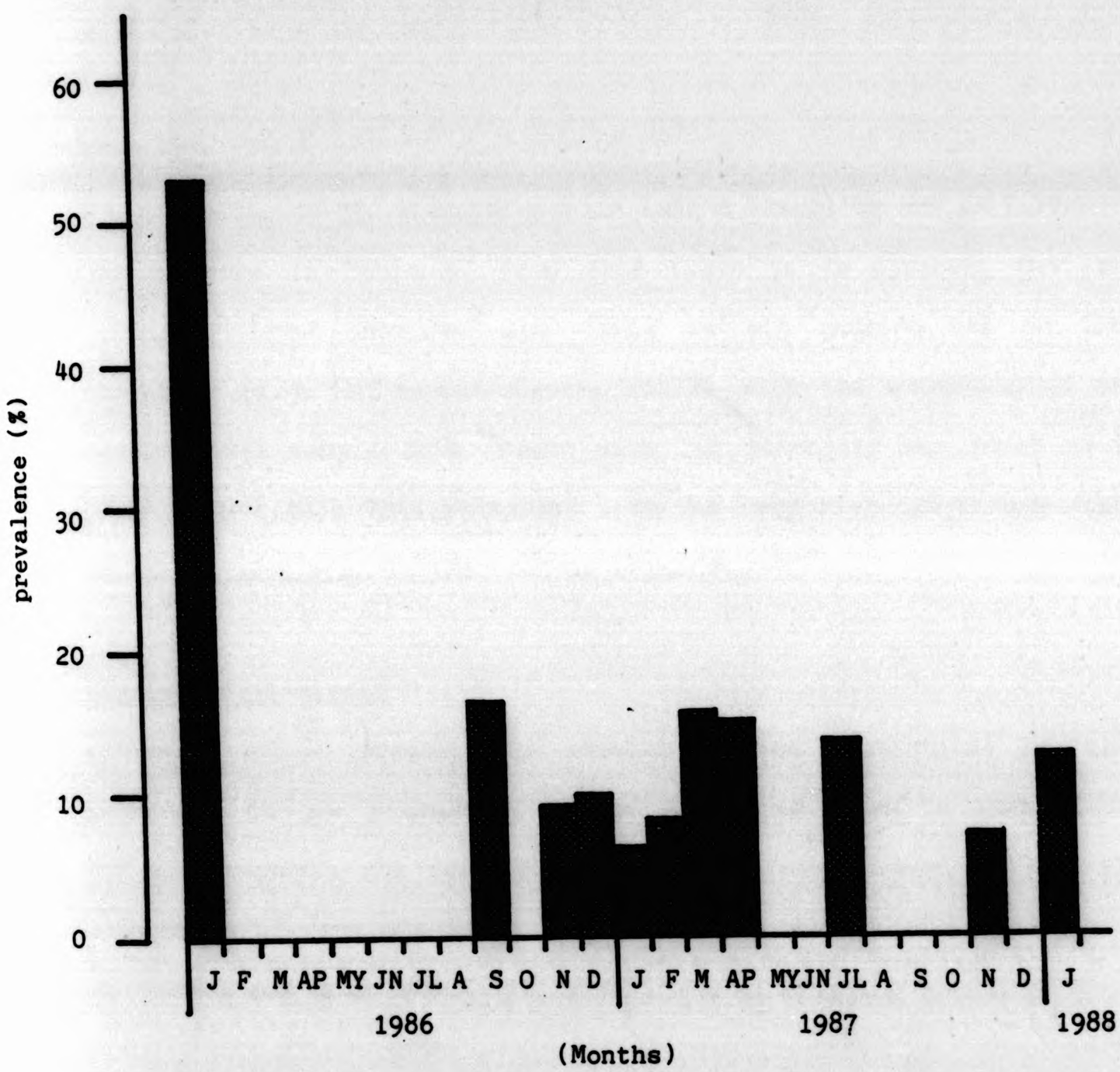


FIGURE 3: Showing months during which samples were taken and prevalence of Myxobolus pseudodispar in Lake A.Vassilios. A zero recording denotes no sample taken

6.12%, 12.07%), suggesting a non-constant seasonal pattern for consequent years for this parasite.

In Yorkshire, the higher prevalence of the parasite was found in April but in the rest of the sampling periods the prevalence was 0 (Table 2).

Intensity

The intensity of M. pseudodispar in Lake A Vassilios and in Yorkshire can be seen in Table 4. From this table it is apparent that the intensity level remained low (IL1) in all samples and in both locations (i.e. 10 spores/viewing field) with the exception of the January 1986 sample from Greece when the intensity was found to be quite high (IL3). This coincided with the very high prevalence found for M. pseudodispar in that sample (Fig. 3).

Statistical Analysis

Tables 6 and 7 present the results of the statistical analysis performed on the prevalence of the parasites found in this study. Table 6 presents the results of the prevalence between the two age categories of fish and Table 7 presents the results of prevalence in the female and male fish.

Significant differences between the prevalence of the infection in male and female fish were not found in either Greece or Yorkshire (Table 7). In contrast, there was a significant difference between

TABLE 4

Intensity of M. pseudodispar found in roach from Lake A. Vassilios (Greece) and Yorkshire

Lake	Sample	Intensity Level (IL)
Agios Vassilios (Greece)	Jan. 86	IL3
	Sept. 86	"1
	Nov. 86	"1
	Dec. 86	"1
	Jan. 87	"1
	Feb. 87	"1
	March 87	"1
	April 87	"1
	July 87	"1
	Nov. 87	"1
	Jan. 88	"1
Yorkshire	Feb. 87	IL1
	April 87	"1
	July 87	"1
	Nov. 87	"1

TABLE 5

Intensity of M. ellipsoides found in roach from Lake A. Vassilios (Greece)

Lake	Sample	Intensity Level (IL)
Agios Vassilios (Greece)	Jan. 86	IL1
	Sept. 86	"1
	Nov. 86	"1
	Dec. 86	"1
	Jan. 87	"1
	Feb. 87	"1
	March 87	"1
	April 87	"1
	July 87	"1
	Nov. 87	"1
	Jan. 88	"1

TABLE 6

Statistical analysis of the prevalence of old/young fish from Greece and Great Britain

Parasite species	Origin of fish	x^2 ($p < 0.05$)
Myxobolus pseudodispar	Lake A. Vassilios	9.39 (S)
" "	Yorkshire	5.32 (S)
Myxobolus ellipsoides	Lake A. Vassilios	27.6 (S)
Myxidium rhodei	Lake A. Vassilios	(48.8) (S)
" "	Loch Fad	26.73 (S)
" "	Yorkshire	
Myxidium pfeifferi	Lake A. Vassilios	6.62 (S)
" "	Loch Fad	18.3 (S)
" "	Yorkshire	34.21 (S)

(S) denotes significant values for $P < 0.05$

TABLE 7

Statistical analysis of the prevalence of female and male fish from Greece and Great Britain

Parasite species	Origin of fish	X^2 (P< 0.05)
Myxobolus pseudodispar	Lake A. Vassilios	1.59 (NS)
" "	Yorkshire	1.30 (NS)
Myxobolus ellipsoides	Lake A. Vassilios	3.34 (NS)
Myxidium rhodei	Lake A. Vassilios	0.0063 (NS)
" "	Loch Fad	1.49 (NS)
" "	Yorkshire	1.36 (NS)
Myxidium pfeifferi	Lake A. Vassilios	1.14 (NS)
" "	Loch Fad	1.08 (NS)
" "	Yorkshire	2.05 (NS)

(NS) denotes non-significant values for $P < 0.05$

the prevalence in small and large fish in both geographic locations (Table 6). The greater prevalence was found in the large fish.

Parasite: Myxobolus ellipsoides Thélohan, 1892

Free spores of this parasite were found forming small groups in the interstitial tissue of the kidney. The spores were found encysted in the gills, gill arch and cartilage.

As shown in Table 2, the parasite was found only in Greece and Loch Fad, but the seasonality could be studied only in Lake A. Vassilios because in Loch Fad the parasite was only detected in the kidneys of nine large fish (3.33%).

Prevalence

Figure 4 shows the seasonality of M. ellipsoides in Lake A. Vassilios. As in the case of M. pseudodispar, higher levels were apparent during a cold month i.e. January 1986, and during the warmer periods, March 1987, July 1987 and September 1986. Two very different values for the prevalence were observed in January 1986 and 1987, as was also observed for M. pseudodispar. The highest prevalence occurred in September 1986 and the lowest in April 1987.

Intensity

The intensity is presented in Table 5 and was found to be constantly low throughout the year (IL1).

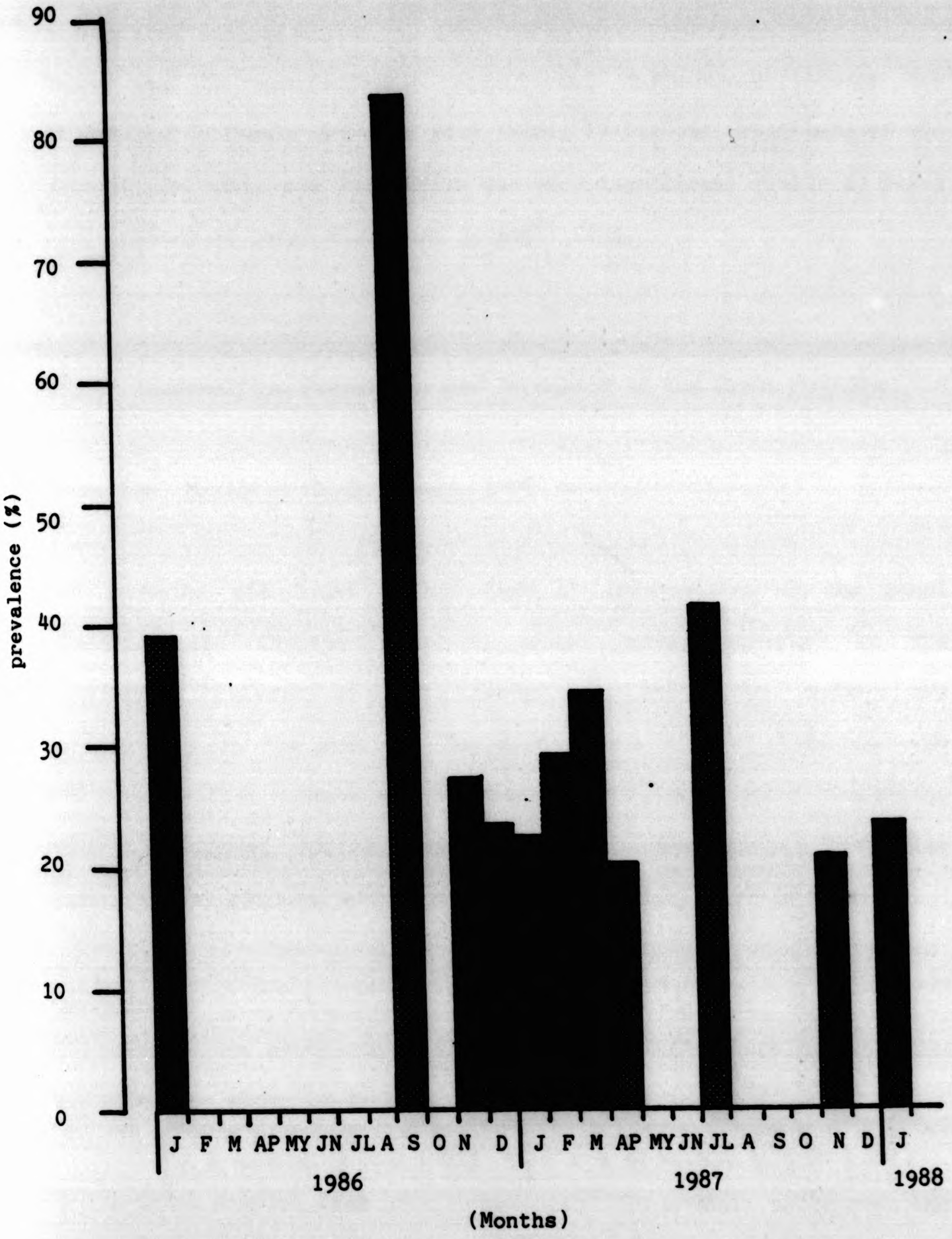
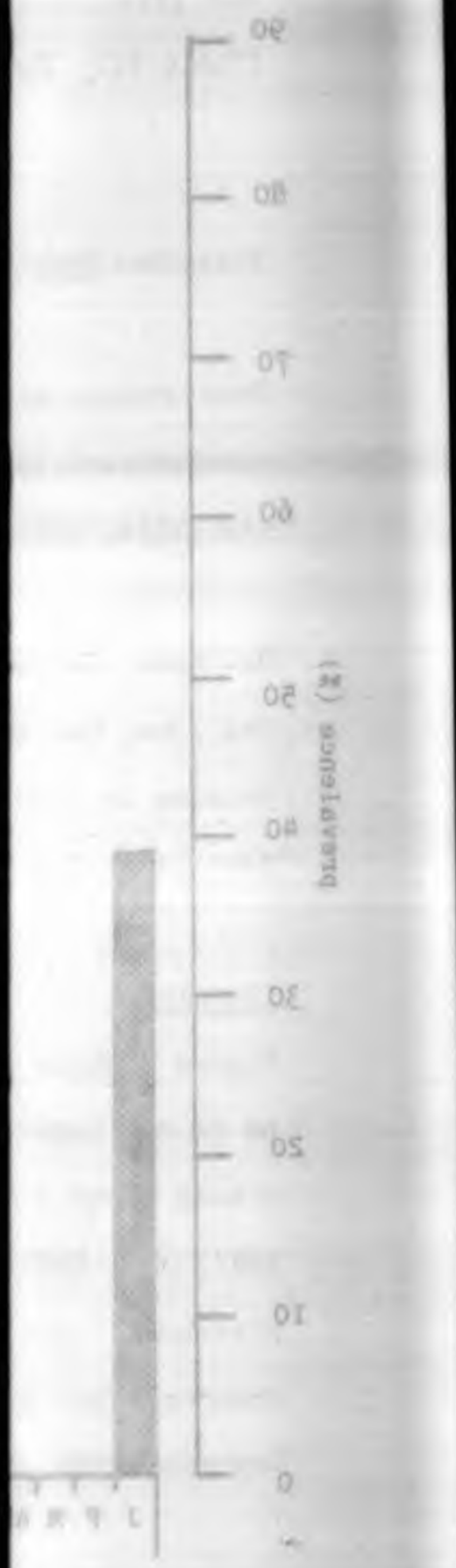


FIGURE 4: Showing months when fish were sampled and prevalence of Myxobolus ellipsoides in Lake A.Vassilios



Statistical Analysis

There was no statistically significant differences between the prevalence in female and male fish (Table 7) but the prevalence of the parasite in small and large fish was very significant (Table 6) being much greater in large fish than in small fish.

3.3.2 Seasonality, Prevalence and Intensity of the Genus Myxidium

Parasite: Myxidium rhodei Léger, 1905

The parasite was found in the form of trophozoites in the renal glomeruli and in the form of mature spores encysted in the interstitial tissue of the kidneys, in the liver, spleen and occasionally in the muscles. The most common site of infection was the interstitial tissue of the kidney where large cysts containing mature M. rhodei spores were observed. In the British fish, the muscle tissue was also a common site of infection for this parasite.

Prevalence

The parasite was prevalent in all samples taken from Loch Fad and from Yorkshire, as shown in Table 2.

In the Greek samples from Lake A Vassilios, the overall prevalence was found to be 29.4% (143/486), the prevalence of the small/young fish

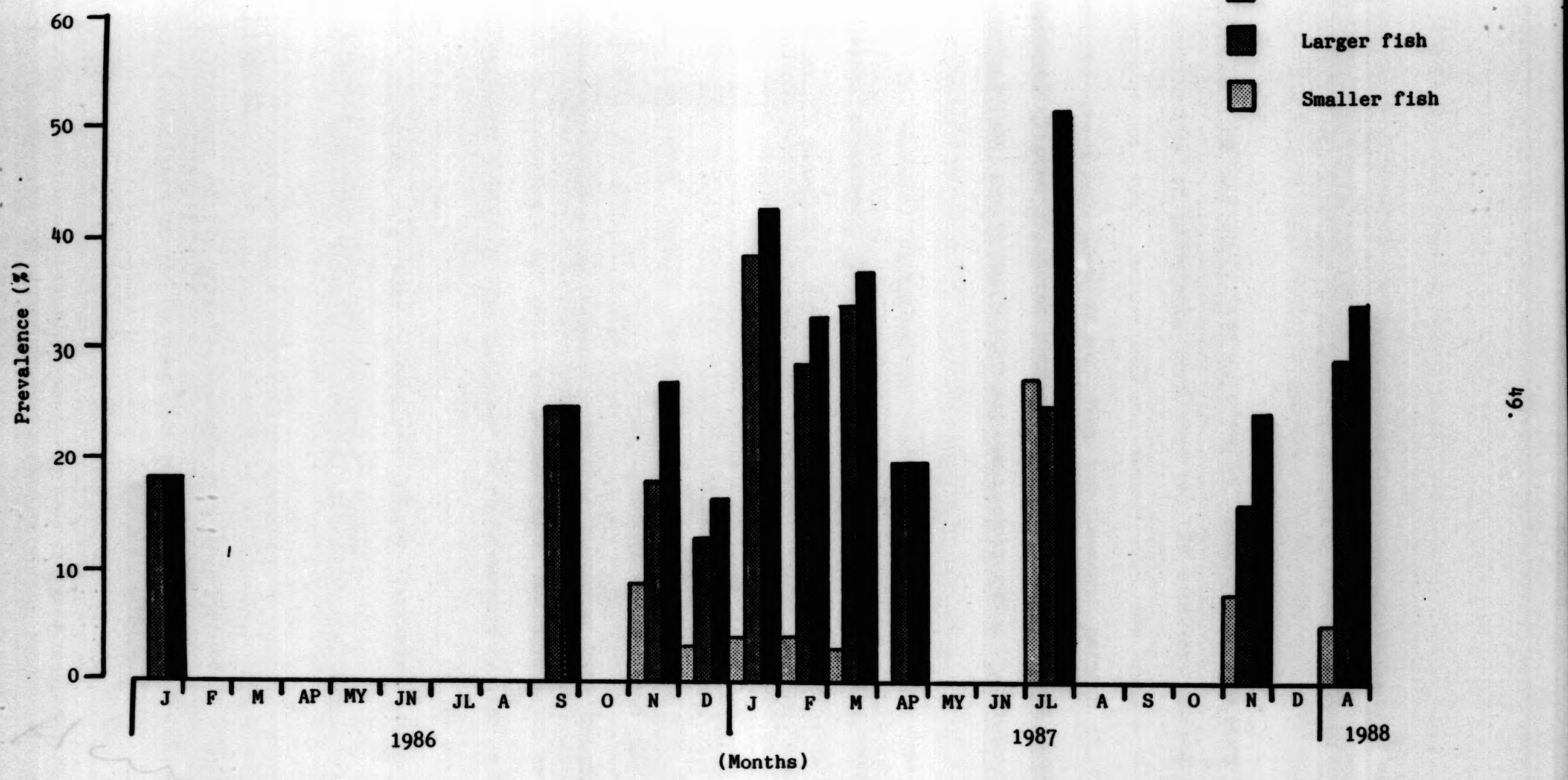


FIGURE 5: Showing months when fish were sampled and prevalence of Myxidium rhodei in Lake A. Vassilios

6.82% (33/486) and in the large/old fish 22.63% (110/486). The prevalence for each sample is shown in Figure 5.

December 1986 and April 1987 were the two sampling periods when the lowest values of prevalence were observed. In contrast, during the months of July 1987, January 1987 and January 1988, the highest values of the prevalence were found. From the seasonal pattern of this parasite (Figure 5), three peaks could be detected: one in January 1987 and 1988, one in March 1987 and one in July 1987.

When the seasonal variation in prevalence in the large fish is examined separately, it is apparent that this follows the overall pattern of prevalence. However, only two peaks were detected in the large fish, namely January 1987 and March 1987.

The prevalence remains constantly low in the small/young fish throughout the year with only one distinct peak in July 1987 when the maximum prevalence was found (27.7%). In September 1986 and April 1987 the prevalence for these small fish was 0, but this might be due to the small number of fish collected in these samples (3 fish, 4.5cm, Age 0+). In January 1986 no small fish were collected in the samples and therefore the same prevalence figure represents both the prevalence of large fish and the overall figure.

Intensity

The intensity of the parasite in this lake is presented in Figure 6. From this figure it can be seen that the highest values were observed

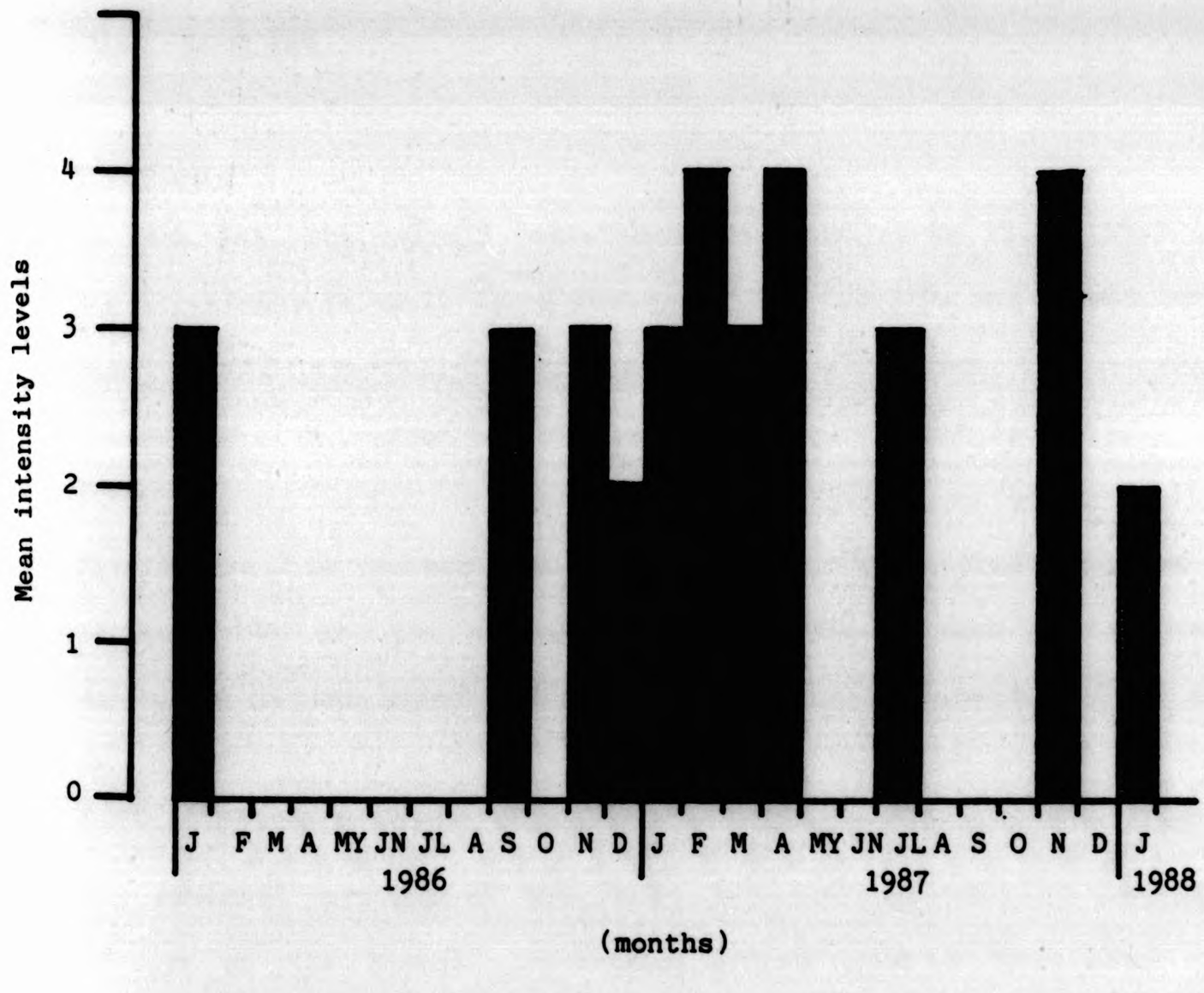


FIGURE 6: Showing the intensity of Myxidium rhodei in Lake A. Vassilios

during the months of February 1987, April 1987 and November 1987. It is interesting to note that the intensity was not high when the higher values for the prevalence were observed. For April, in particular, the intensity is high (IL4) whereas the overall prevalence has its lowest value (Figs 5 and 6).

Lake: Loch Fad

Prevalence

In Loch Fad, the overall prevalence was found to be 33.3% (91/273). The prevalence in small/young fish was 7.32% (20/273) and in old/large fish 26.01% (71/273) (Table 2). Figure 7 shows the seasonal prevalence of M. rhodei in this loch, and Figure 8 its intensity.

From Figure 7 we can see that the lowest prevalence value was observed in March 1987 and the highest in December 1987. In Loch Fad two peaks were seen in the prevalence of this parasite - in December 1987 and May 1987.

The seasonal pattern of the large fish follows more or less the pattern of the overall prevalence. Unlike Lake A. Vassilios, the seasonal prevalence of the small fish is also similar to the overall prevalence. In December 1987, however, the prevalence of small fish drops from 8.59% to 6.32% in contrast to the value of the overall prevalence for that month which rises (39.87%).

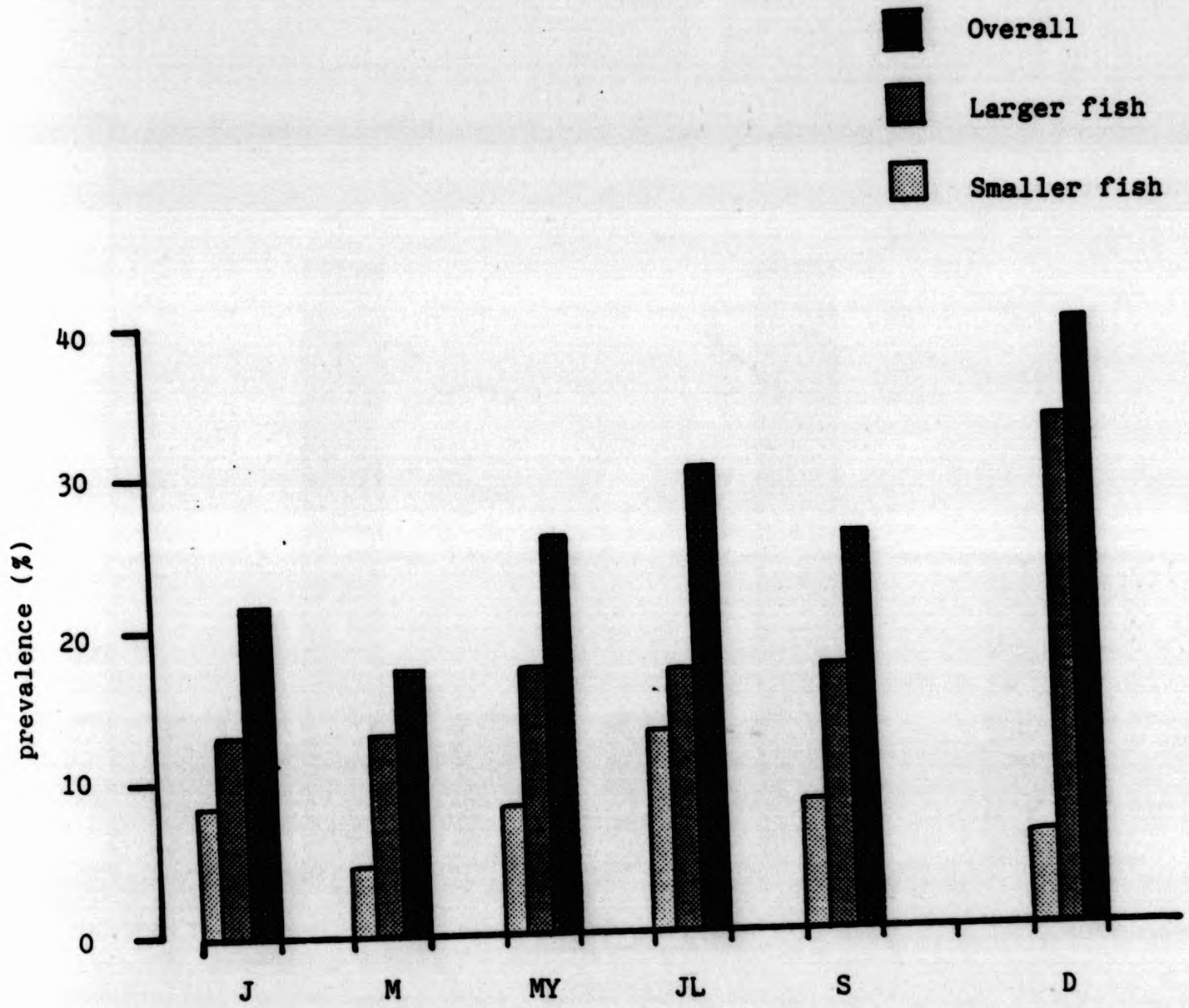


FIGURE 7: Showing months when fish were sampled and prevalence of Myxidium rhodei in Loch Fad

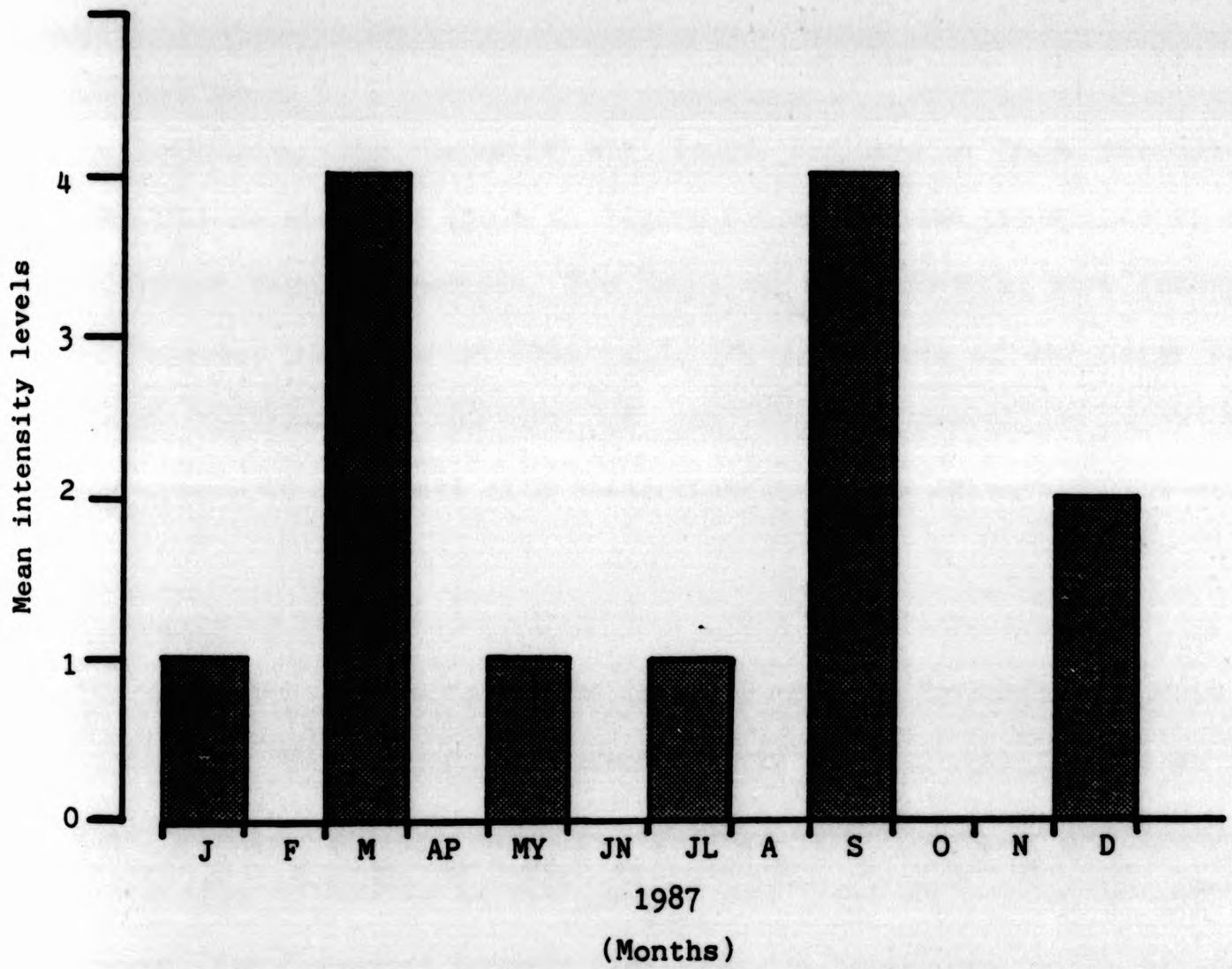


FIGURE 8: Showing the intensity of Myxidium rhodei in Loch Fad

Intensity

The higher value for the intensity was observed in March and September 1987. The intensity peaks (Figure 8) do not correspond to the highest prevalence values (Figure 7).

Lake: Yorkshire

Prevalence

In Yorkshire, the parasite was found to have a high prevalence (34.77%) as shown in Table 2. Figure 9 presents the prevalence in the different sampling periods. Two peaks of the infection were detected in February 1987 and in July 1987. The prevalence of the large fish again follows the pattern of the overall prevalence with the prevalence of the small fish being constantly low throughout the year.

Intensity

The intensity of the infection in the fish from Yorkshire is shown in Figure 10. The intensity remains low in most of the samples during the year, with the exception of the April sample when the intensity is quite high (IL4). As in the case of the Greek fish infected with M. rhodei, the intensity appears high when the prevalence is low (Figures 9 and 10).

Statistical Analysis

In all geographic locations no significant differences were observed in the parasite prevalence between male and female fish. The

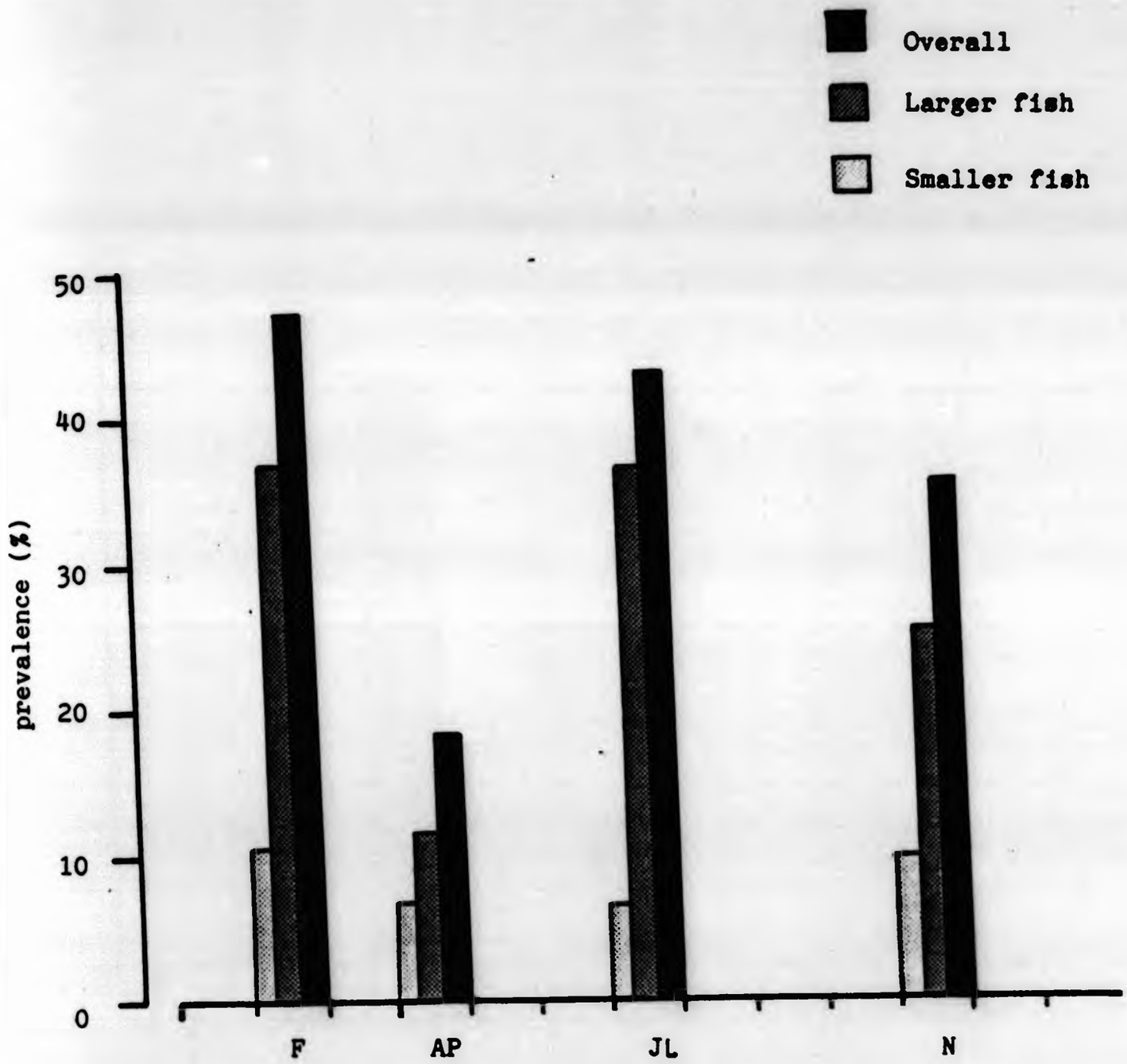


FIGURE 9: Showing months when fish were sampled and prevalence of Myxidium rhodei in Yorkshire

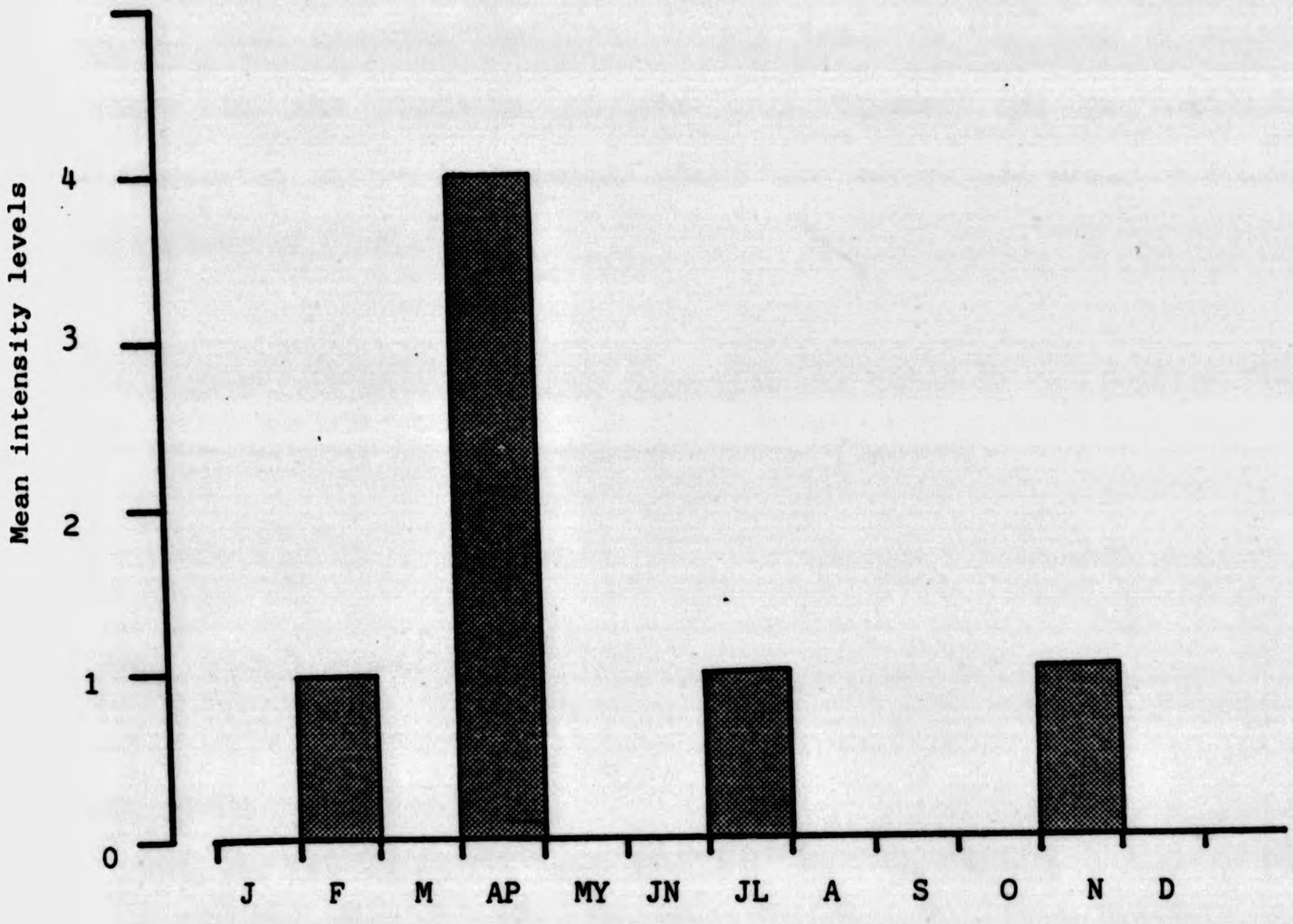


FIGURE 10: Showing the intensity of Myxidium rhodei in Yorkshire

prevalence between large/old and small/young fish, however, differed significantly in all samples examined. The statistical analysis of the prevalence of the samples from the three different locations is shown in Tables 6 and 7.

Parasite: <u>Myxidium pfeifferi Auerbach. 1902</u>

In all fish examined the parasite was found in the form of mature spores in the contents of the gall bladder and as coelozoic trophozoites in the bile ducts, where both mature and immature spores were observed (Table 3).

The highest prevalence of the infection was found in the British fish and in particular in the Yorkshire habitat (Table 2).

Lake: Lake A Vassilios

<u>Prevalence</u>

As shown in Figure 11, in Lake A. Vassilios, the highest values of the prevalence were observed in the months September 1986, April 1987 and January 1988. However, only a single peak was detected in the prevalence pattern of <u>M. pfeifferi</u> in this lake, in April 1987. When the prevalence of the large fish was examined separately, it was shown that this was identical with the overall pattern until April 1987. In the last three samples (July, November 1987 and January 1988) the

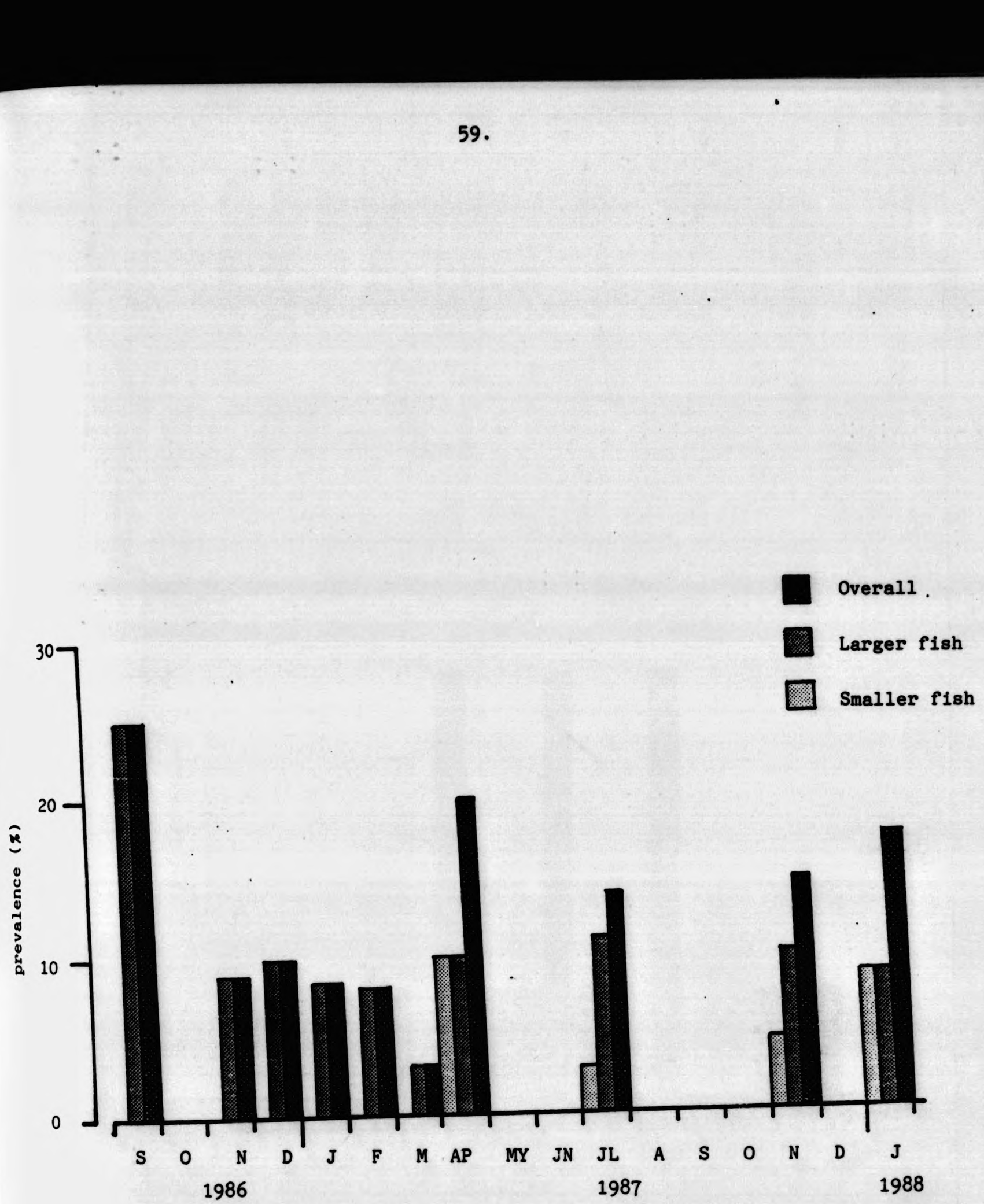


FIGURE 11: Showing months when fish were sampled and prevalence of M. pfeifferi in Lake A Vassilios, Greece

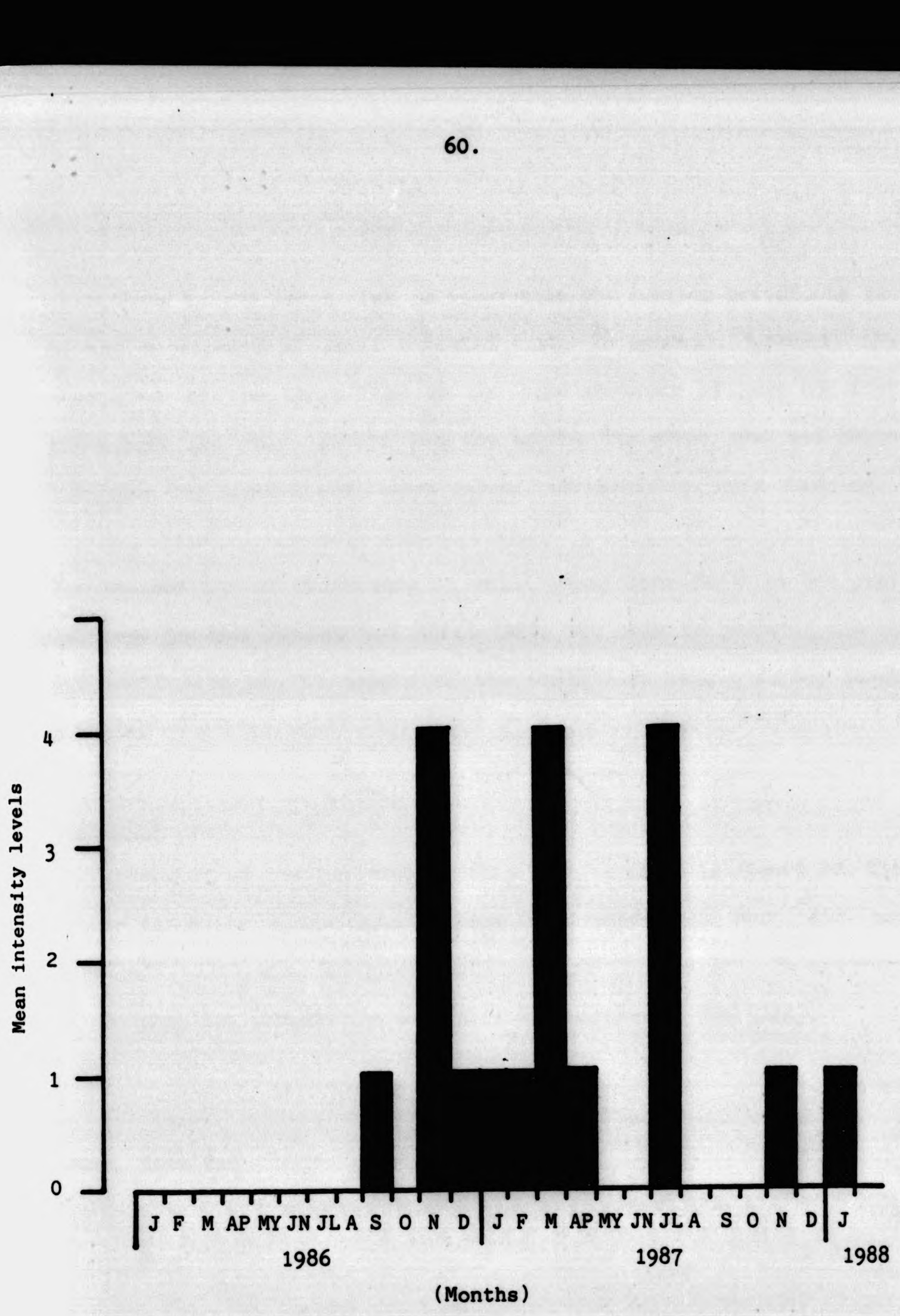


FIGURE 12: Showing the intensity of Myxidium pfeifferi in Lake A. Vassilios

prevalence in the large fish is lower than the overall prevalence due to the appearance of small infected fish. In general, however, the prevalence of the large fish in the lake was more or less constant throughout the year, except for the months September 1986 and March 1987 when the highest and lowest values, respectively, were observed.

The seasonality of prevalence in small/young fish is 0 in the year 1986 and between January and April 1987, but this is probably due to the small size of the sample of the small fish caught during these periods.

Intensity

The intensity of the parasite in this lake is shown in Figure 12. The higher intensity values observed were in November 1986, March 1987 and July 1987 (IL4) when the prevalence was low (Figure 11). In the rest of the sampling periods the intensity was constantly low (IL1).

Lake: Loch Fad

Prevalence

In Loch Fad, the highest prevalence observed was in May 1987 (Figure 13) and the lowest in January and March 1987. One peak was detected (May 1987). The prevalence in small/young fish was 0 during the months January and March 1987, constant for the rest of the samples, with one peak in May 1987. In the large fish, the prevalence follows the pattern of the overall prevalence.

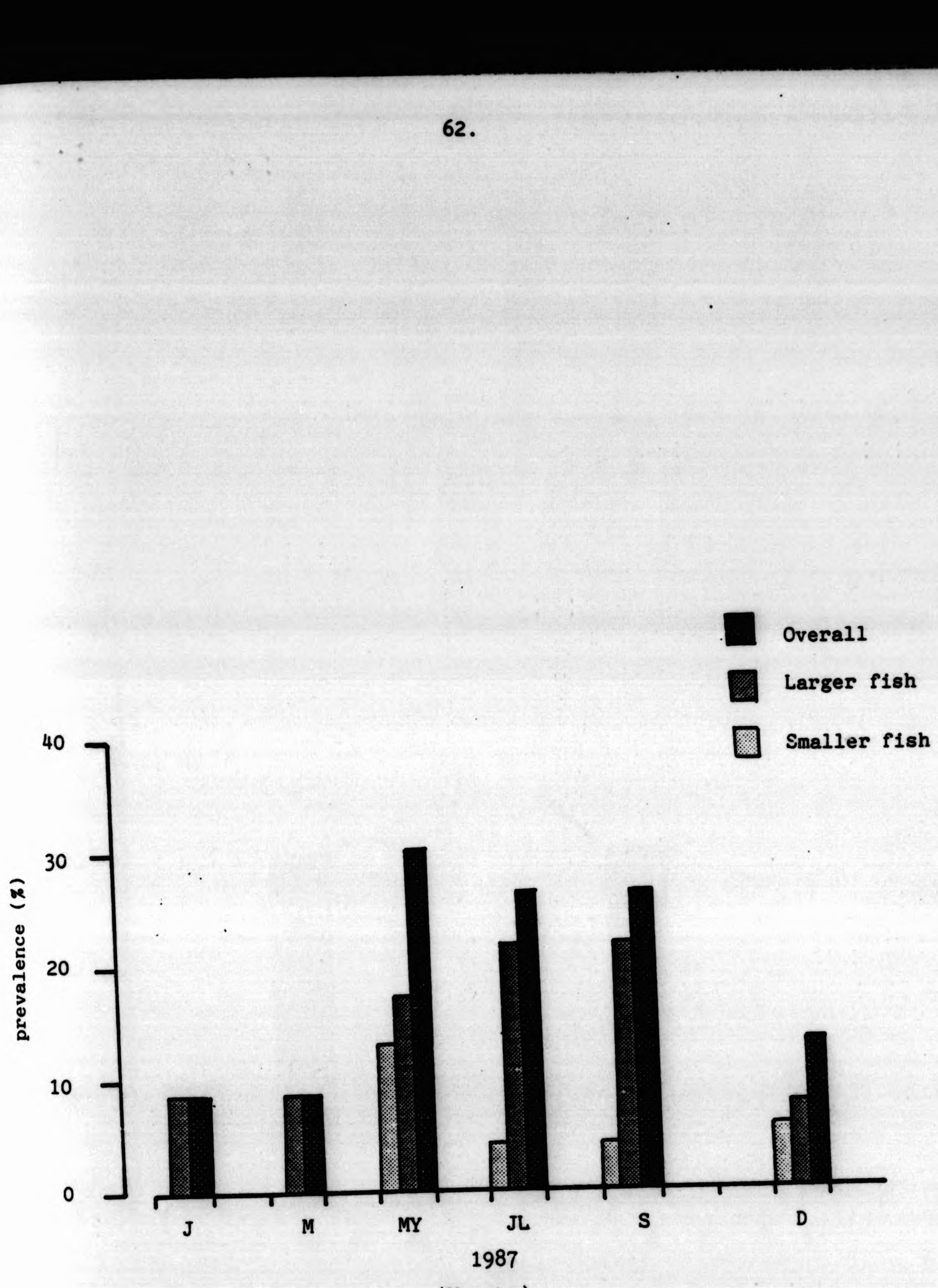


FIGURE 13: Showing months when fish were sampled and prevalence of Myxidium pfeifferi in Loch Fad

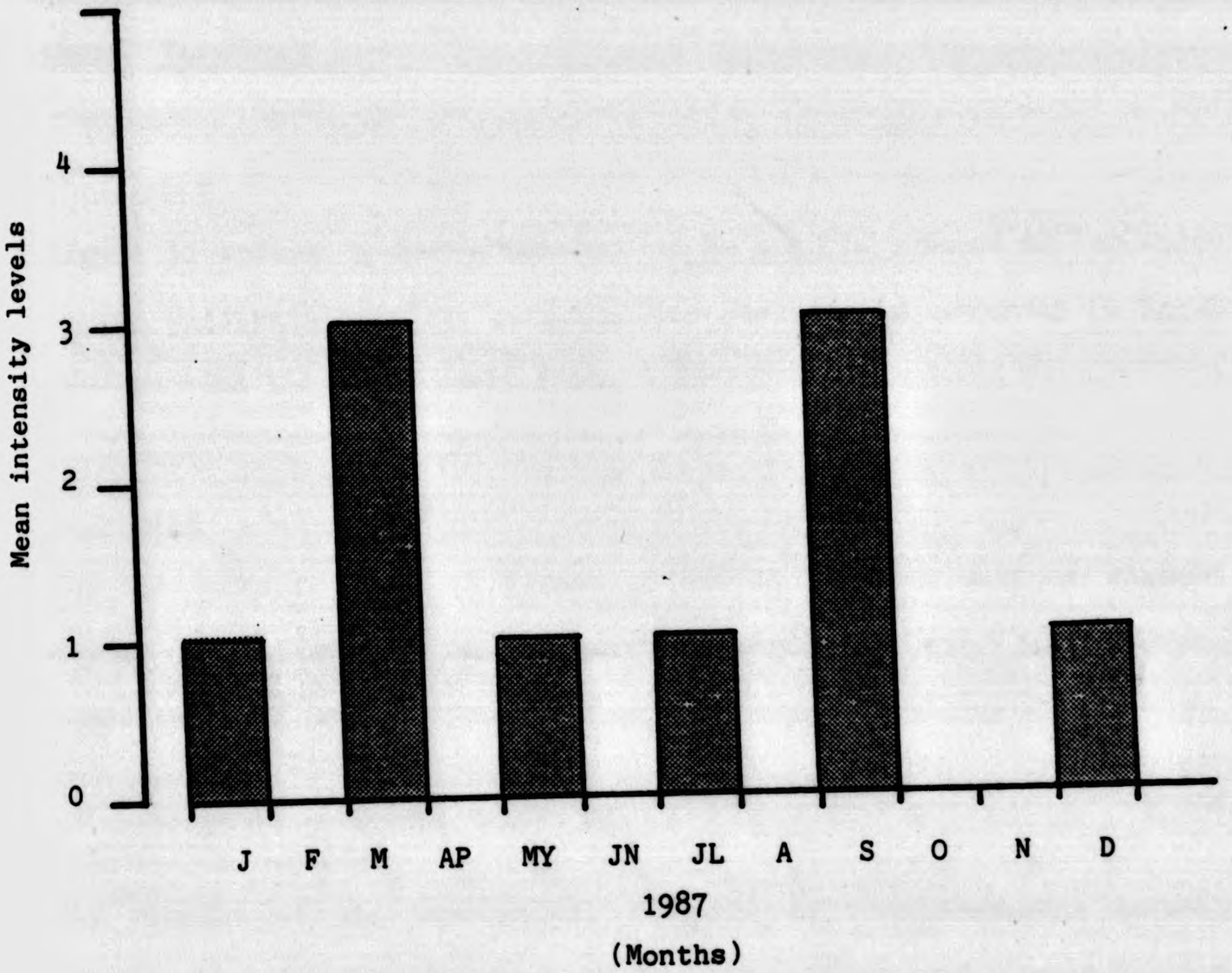


FIGURE 14: Showing the intensity of Myxidium pfeifferi in Loch Fad

Intensity

The intensity of the parasite in this loch is presented in Figure 14. It is apparent that during March and September 1987 the intensity had its highest values. In relation to this in March 1987 the prevalence was quite low (Figure 13).

Lake: Yorkshire

Prevalence

Figure 15 refers to the prevalence of M. pfeifferi found in Yorkshire during different sampling periods. One peak can be observed in April 1987 in both large and small fish.

Intensity

The intensity is shown in Figure 16 and it is clear that the highest levels were observed in February and November 1987. Again during these sampling periods the prevalence, as shown in Figure 15, is high.

Statistical Analysis

The results of the statistical analysis of the prevalence between female and male fish were not proven to be significant (Table 7), but those of old and young fish were found to be very significant (Table 6) being constantly greater in older fish.

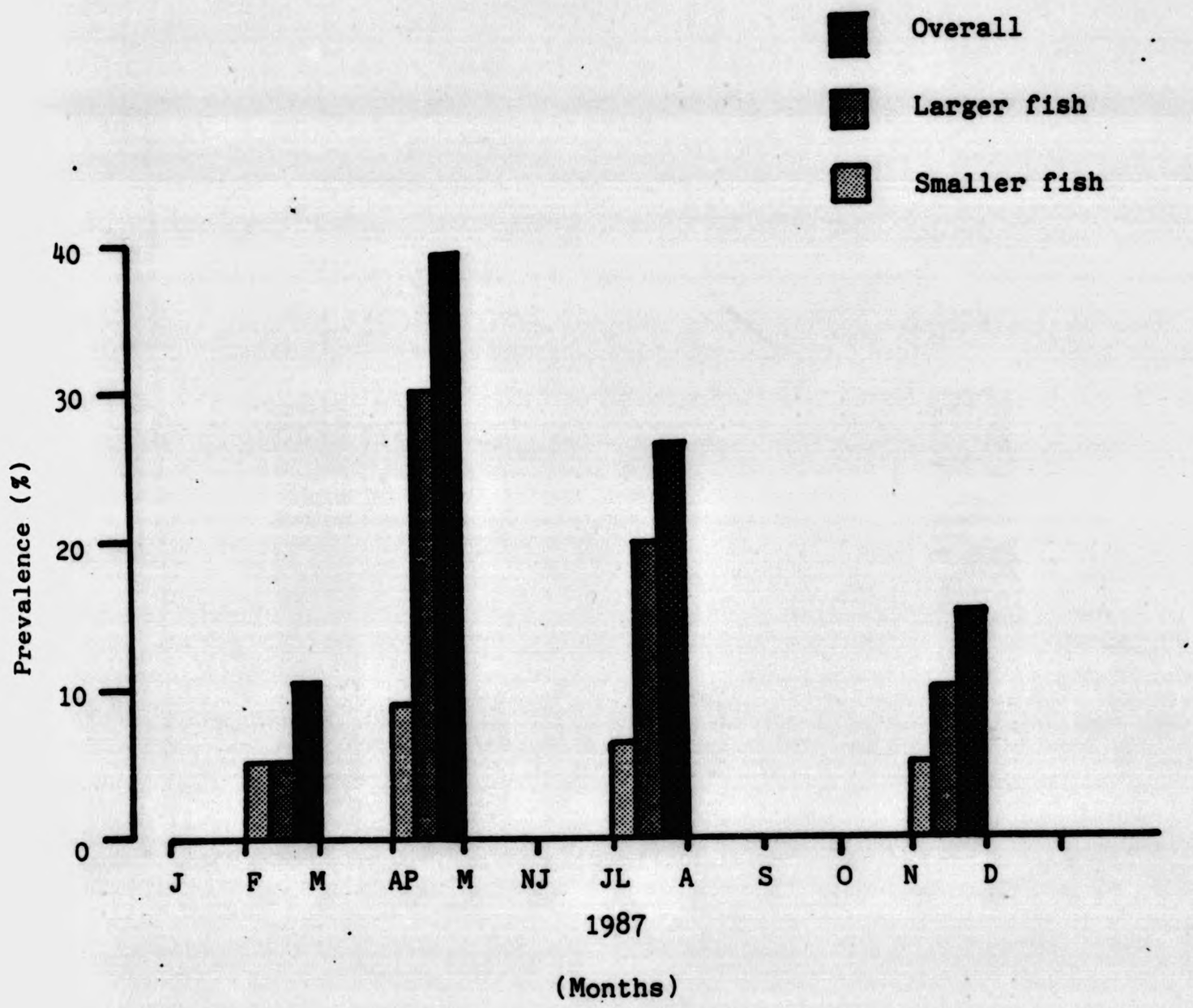


FIGURE 15: Showing the months when fish were sampled and prevalence of Myxidium pfeifferi in Yorkshire

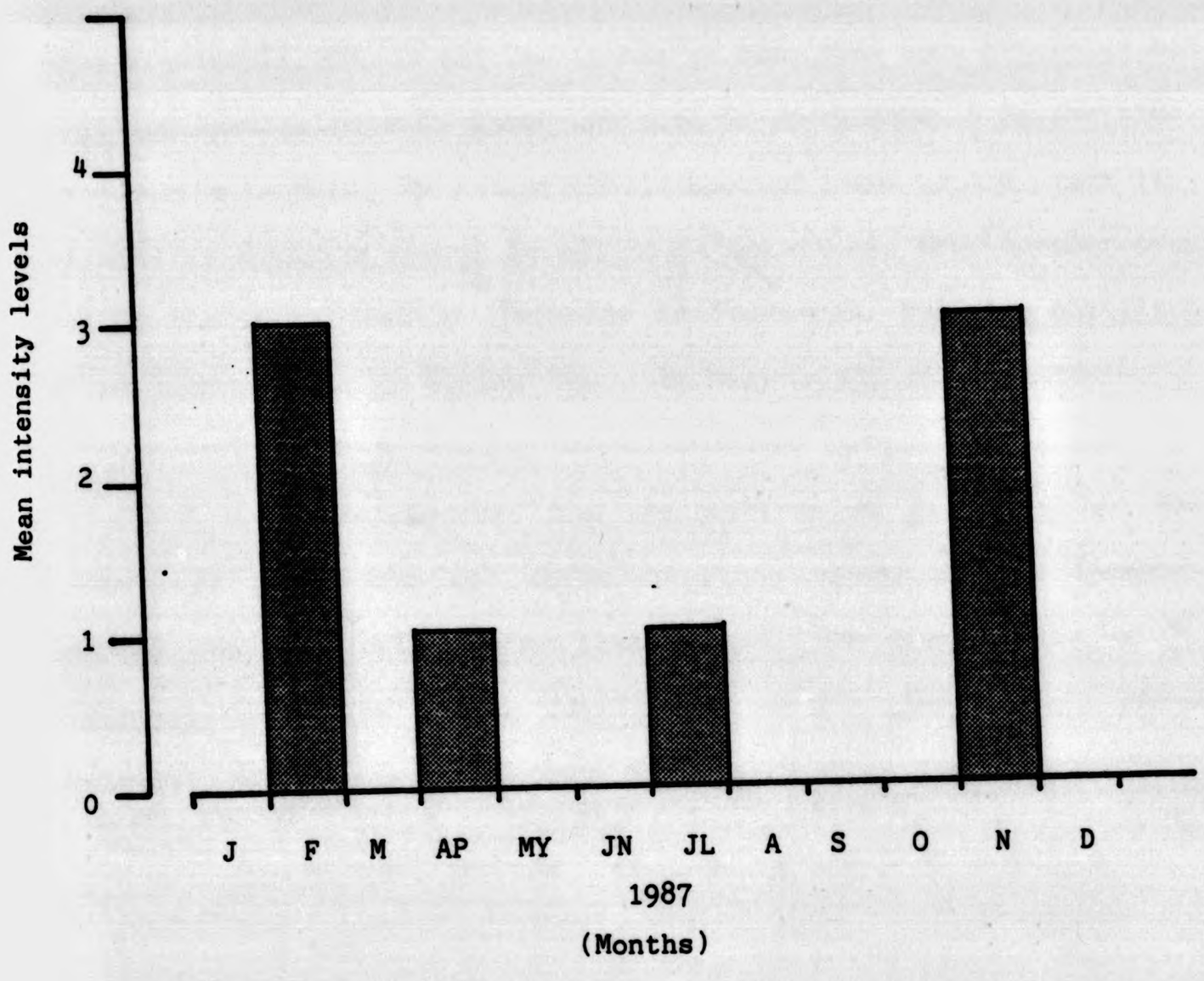


FIGURE 16: Showing the intensity of Myxidium pfeifferi in Yorkshire

SUMMARY

A summarised profile of the myxosporean infections found in each geographic location are given in Figures 17, 18, 19 and 20. The points in the figures represent presence of mature spores, TB represents the presence of trophozoites in the bile ducts, and TK represents the existence of trophozoites in the kidneys.

From Figures 17, 18, 19 and 20, it can be seen that both Myxobolus and Myxidium species have at least one peak in prevalence in Spring time. In addition to this, the two myxobolid species found in the Greek lake as well as Myxidium rhodei in all three locations, show another peak during the winter months (December to February). Myxidium pfeifferi shows only one peak in Spring (April to May) in all three habitats.

As shown by these figures, the trophozoites of M. rhodei in the kidneys of roach from all three locations appear in the December samples and last only during that period. The trophozoites of M. pfeifferi appear in Spring (April and March) also in all three habitats. Mature spores of both Myxidium species appear constantly throughout the year (Figures 18, 19 and 20).

TK = trophozoites in the kidney

TB = trophozoites in the bile duct

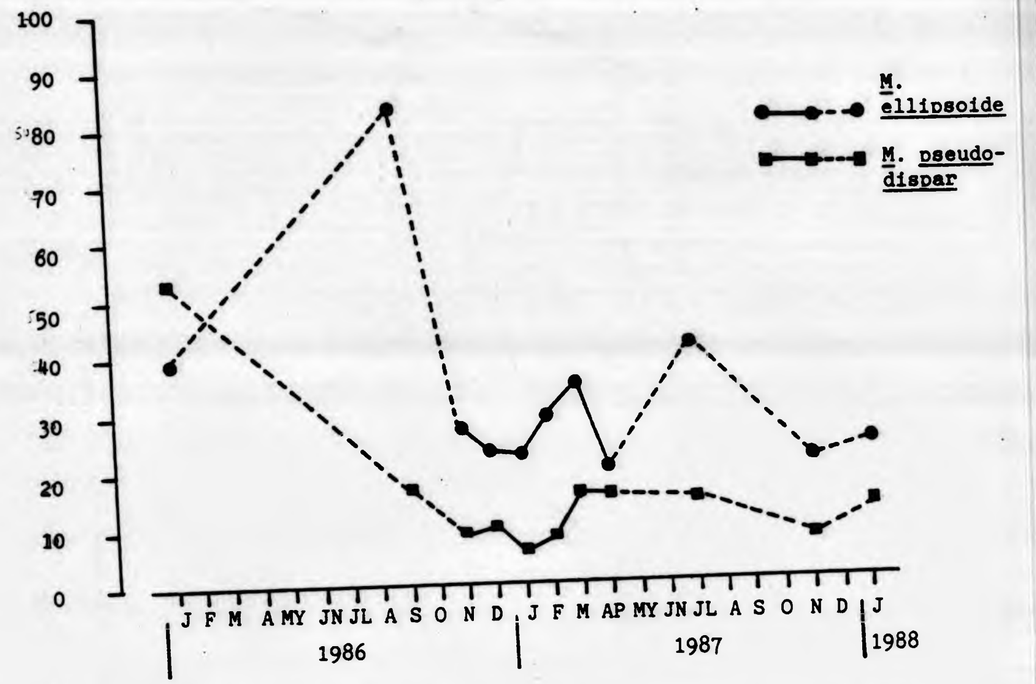


FIGURE 17: Showing the seasonal variations of the two Myxobolus species found in roach from Lake A Vassilios

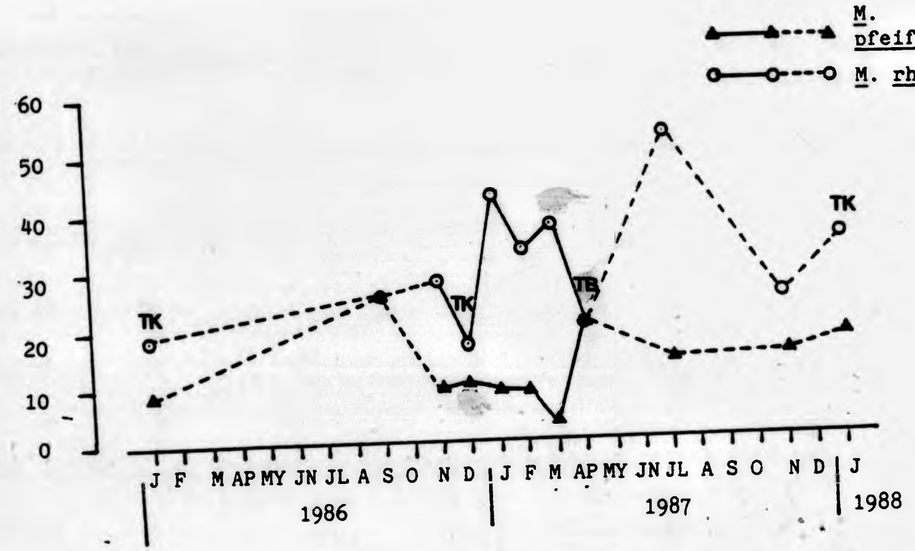


FIGURE 18: Showing the seasonal variation of the two Myxidium species found in roach from Lake A Vassilios

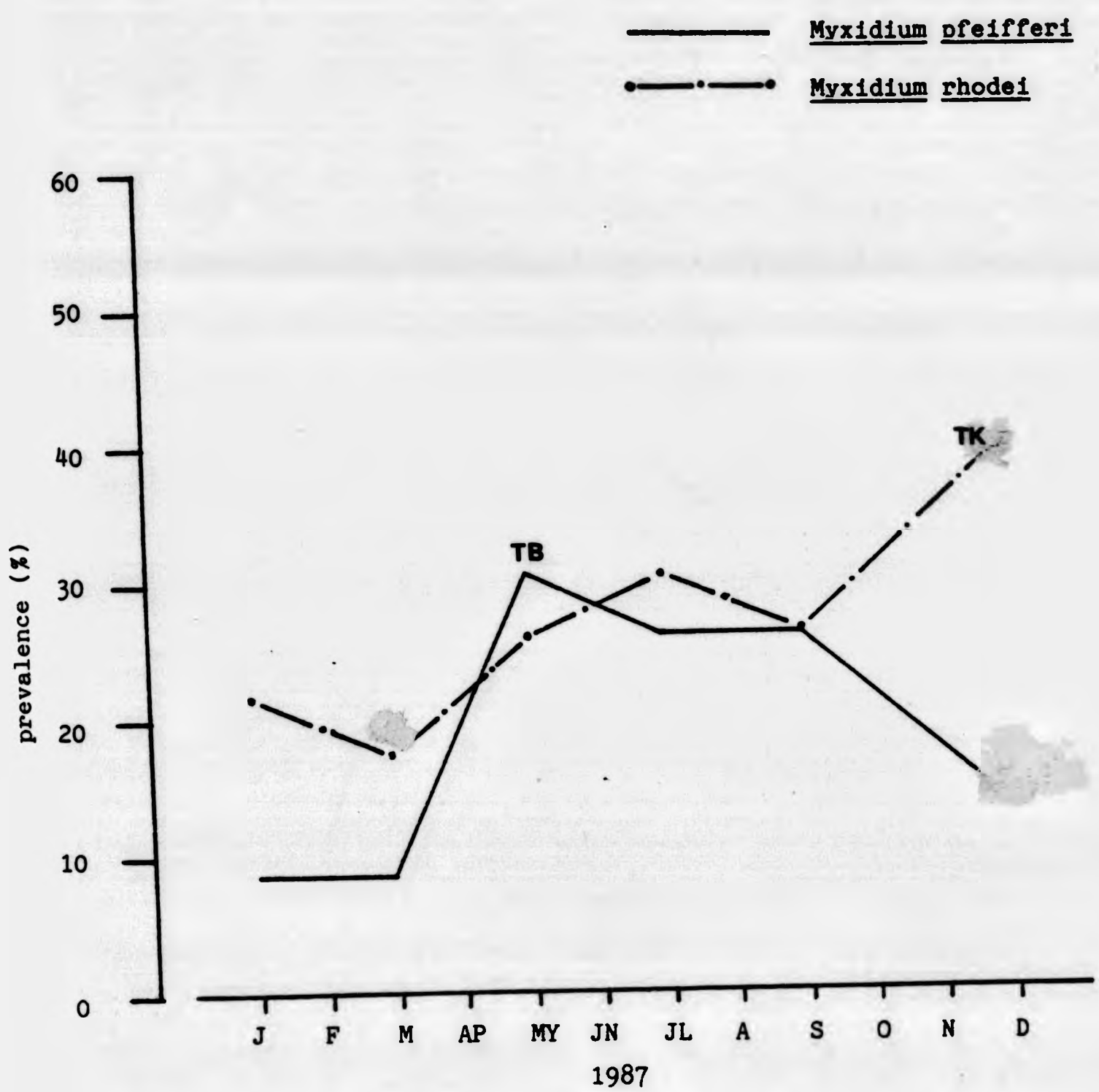


FIGURE 19: Showing the seasonal variations of Myxidium species prevalence found in roach from Loch Fad

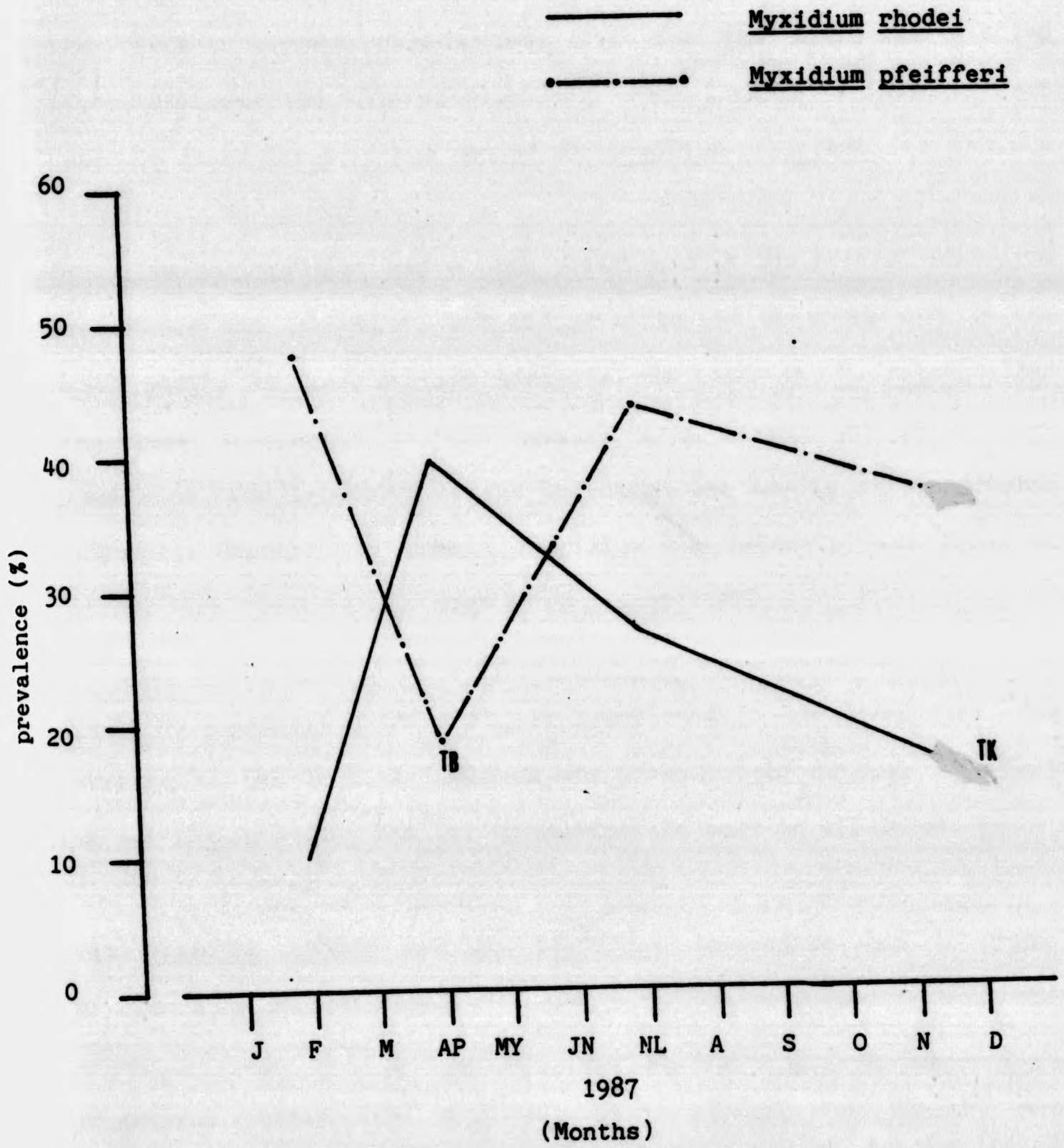


FIGURE 20: Showing the seasonal variations of the two Myxidium species prevalence found in roach from Yorkshire

3.4 DISCUSSION

Information on the seasonality of the myxosporean species studied in the present project exists only for the two myxobolid species (Conzalez-Lanza and Alvarez-Pellitero, 1984, 1985). No seasonal data could be found for Myxidium rhodei and Myxidium pfeifferi.

Myxobolus pseudodispar had a high prevalence in the British fish from Loch Maben and Yorkshire, but the parasite was detected only in the March sample in both British habitats. In contrast, M. pseudodispar was found constantly in all samples from Greece but at a lower prevalence. The intensity of the infection was low in all geographic locations. However, in Lake A Vassilios the intensity was found at quite a high level in one sample, in January 1986.

Myxobolus ellipsoides had a higher prevalence in the Greek fish. The parasite in the British habitats was present only in fish from Loch Fad but the intensity was low throughout the year in all locations.

Both Myxidium rhodei and M. pfeifferi infections had a higher prevalence in British fish.

In general, three main periods (Winter, Spring and Summer) were observed in the seasonality of the Myxosporea species studied in this survey. The Winter (December/January) and the Spring (March/April) peaks were common for all parasite species and in all locations. The

Summer peak was observed in Greece for M. rhodei and the myxobolid infections, and in Yorkshire for M. rhodei.

Similar bimodal seasonality has been observed in other Myxosporea species. The evidence in the literature on Myxobolus spp. is, however, contradictory. Alvarez-Pellitero et al. (1985) examined different Myxobolus species (including the two species found in this study) in a number of fish hosts from Spanish reservoirs. According to their results, M. pseudodispar showed no seasonal variation, the prevalence being 100% all year round. The intensity, however, was found to be higher in December. The prevalence of M. ellipsoides was higher during Summer (July) and in September.

Davies (1968) did not observe a clear seasonal pattern for Myxobolus mue lleri in Leuciscus leuciscus and Rutilus rutilus. Mitchell (1989) reported high infection prevalence of Myxobolus muelleri in muscles and kidneys throughout the year but the seasonal prevalence in gills and subcutaneous tissue was markedly fluctuating. The prevalence of myxobolids within the different organs was not recorded for all species in this study. George, Harrison and Hadley (1977) observed an increase of the prevalence of Myxobolus dentium in Esox masquinongy from June to August, and a decrease until October. On the other hand, Mitchell (1977) reported the maximum infections of Myxobolus dujardini in northern squawfish during cold months.

Taking into account the findings of all these researchers, however, there does appear to be a seasonal pattern for the Myxobolus species;

the main peak periods described are either the warmer months or the Winter time. The main three peak periods for the seasonality of the myxosporean species observed in the present study are therefore in accordance with the findings of the above-mentioned workers.

These peaks in the seasonality pattern of the myxosporean parasites could be related to a range of different biological and/or environmental conditions. The two major contributing factors in the present study are considered to be the spawning period and the temperature. The spawning period for roach in Lake A Vassilios is from April to June, depending on the temperature. During this period fishing is prohibited, hence there were no samples taken during May/June 1987. The peak of the prevalence in all myxosporeans observed in March/April in Lake A Vassilios, therefore, may be due to the pre-spawning activity of the fish. In general, fish during spawning may be more susceptible to disease since this activity may influence the immunological process of the hosts, as much as any other stress factor. The hormonal state of the fish, besides, is considered as a highly significant factor in fish parasite biology by Bauer (1959).

A relationship between infections with parasitic helminths and reproductive activity of fish has been reported in the literature by Rompus (1975), who observed that maturing and gravid female hosts, Cottus gobio, showed a higher level of infection with the nematod worms Nicolla gallica. The evidence suggested that this was due to the retention of worms already established for longer periods by these fish than by fish in non-productive conditions. The author was unable,

however, to explain the precise manner in which the reproductive condition of C. gobio affected the parasite population.

Although there is no data on the precise period when spawning occurs in Loch Fad and in the Yorkshire environment, it is generally believed that roach require water temperatures of 14°C and above before reproduction can occur (Goldspink, 1978). Information from other locations, that roach spawning season is between March and May for Southern England habitats (Goldspink, 1978) and between June and July for Yorkshire (Yorkshire River Authority, 1974). Thus the peak observed during Springtime in the British fish may also be due to the spawning activity, as in the case of Greek fish from Lake A. Vassilios. Thus, spawning fish may be rendered more susceptible to infection as a result of the suppression of the immune system.

The Summer peak in the prevalence of Myxidium rhodei seen in both Yorkshire and the Greek lake, may be due to a pre-spawning susceptibility. In Yorkshire it is possible that this is due to the spawning time of the roach, since this period has been reported as being the spawning season for roach in this area (Yorkshire River Authority, 1974).

On the other hand, the peak during Winter may be correlated to the influence of the water temperture on the immunological activity of fish. Antibody production in fish is temperature-dependent (Harris, 1973) and during cold periods the titres are low and thus there is potential for higher infections to develop. The temperature effect on

the antibody formation was suggested by Lom (1970) to explain the increased infection of fish with Henneguya spp. during low temperatures.

In Greece, the increased prevalence during this sample may be attributed to the existence of an increased number of larger/older fish in the catches which were more infected. The July sample consisted mainly of fish aged more than 1+, since smaller fish (0+) could not be caught. It is interesting to note here that the seasonal pattern of all myxosporean infections which emerged when the data from all the fish from each lake was analyzed, was wholly attributed to the larger/older fish. This was still evident where the small fish were removed from the samples.

According to the literature (Mitchell, 1977) very young hosts are usually most susceptible to the disease but several myxosporeans appear more frequently or exclusively in mature hosts. The latter situation has also been observed for different Myxobolus spp. by Lucky (1978) and by Cone and Anderson (1977). In contrast, Alvarez-Pellitero et al. (1985) found that M. pseudodispar did not vary with age of the hosts. The prevalence of M. ellipsoides increased from 1+ age group to 2+ age group fish but decreased in the two oldest fish groups. This was not statistically significant, however.

Many factors may contribute to the occurrence of higher prevalence in older fish. In the first instance, older fish have a longer period

of exposure to the infective stages of myxosporeans. This suggests that the parasite is long-lived within the host and the parasite accummulates with time. Autoinfection may also occur, thus fish exposed to the infection at an earlier stage may become heavily infected later in life.

Older fish are exposed to different environmental and disease conditions and the concurrent presence of other infectious agents may also contribute to myxosporean infections.

There are, also, other factors which may play an important role in these infections and these may or may not be related to the age and size of fish. For example, the feeding habits, the nature of the diet and possible intermediate hosts must also be taken into consideration when studying the seasonal peaks of these parasitical infections.

Tubifex spp. worms have been shown by Wolf and Markiw (1984) to be intermediate hosts for Myxobolus cerebralis, a parasite of the cartilage of salmonids. There is some doubt as to the significance of this with respect to other myxosporean. Nevertheless, the presence of an intermediate host in the life cycle of Myxobolus or Myxidium species in roach would have a major impact on seasonal considerations.

It is generally known that feeding activity in roach is increased in temperatures between 15°-25°C (Hofer, 1979) and thus, consumption of possible intermediate hosts may increase during summer months, thus explaining the peaks observed in summer months.

On the other hand, observation of some researchers (Yorkshire River Authority, 1974) showed that roach feed on tubificids all year round and benthos is a typical diet of large fish during the Spring time. During Winter, roach consume more detritus (Yorkshire River Authority, 1974; Dimitriadou, personal communication). This might contribute to the higher infection levels observed during this period. Clearly the role of invertebrate hosts is crucial in interpreting these results. However, one might suppose that even if an intermediate host is not involved, an invertebrate might devour spores from sediment and thus act as a vector. If not, then it must be assumed that the fish become infected passively during feeding on detritus. The greatest percentage observed during Winter and the results of the experimental infections carried out during this project (Chapter 6) suggest the latter route of infection.

Another interesting aspect in the study of the seasonality of myxosporean infections from the different locations is the fact that at least two habitats (Lake Agios Vassilios and Loch Fad) are eutrophic environments. Lake A. Vassilios is heavily polluted by domestic waste, causing great concern for the cyprinid populations (Kilikides et al., 1984). Such conditions are stressful to fish and together or not with other disease conditions in the lakes which are likely to lower the host resistance and make the fish more susceptible to myxosporean infections. For example, tuberculosis in carp and roach from this lake was observed during this period of investigation

as well as in previous years (Athanassopoulou, 1985; Athanassopoulou, unpubl. data).

Furthermore, parasites such as Ligula intestinalis and Diplostomum spp. have also been reported (Athanassopoulou, 1983; Kalfa-Papaioannou and Sinis, 1985). Of particular importance are coccidial infections which are present in the kidneys of the roach and cause considerable damage (Athanassopoulou, 1985; Athanassopoulou and Vlemmas, 1986).

In Loch Fad, ectoparasites, BKD, ERM (Stewart, 1985) and cestodes (McGuigan and Sommerville, 1985) also have been reported in roach. The loch is considered to be very eutrophic by different workers (Beveridge, 1981, Philips et al., 1989) since it has supported cage culture of rainbow trout for several years and is of small size.

The seasonal occurrence of parasites is only one aspect of the biology of the host-parasite relationship as a whole and other general topics of the dynamic aspects of parasite biology population and regulation of fish parasite population must always be kept in mind with respect to the seasonality. Further long-term investigations and research on the life cycle of these parasites would contribute greatly to the interpretation of the seasonal data.

CHAPTER 4

INVESTIGATION INTO MORPHOLOGY OF MYXOSPORIDA SPORES IN RELATION TO FISH SIZE, ORIGIN AND SITE OF INFECTION

4.1 INTRODUCTION

Identification of myxosporeans is currently based on the morphological character of the spores together with the host species and specific tissues which they invade. The measurements most commonly used for the identification of the Myxobolus and Myxidium species are shown in Figure 21. Unfortunately, many descriptions of the species are inadequate and limited only to the dimensions presented in the Figure 21. This has resulted in confusions of identification when comparing data from different authors.

The limitations in the use of the dimensions of the spore in myxosporean taxonomy have long been realised and some attempts have been made to fully utilise other morphological features of this stage (Lom, 1969; Donetz and Shulman, 1973). The disadvantages have been compounded by the large number of studies in which the sole description is based on little material from one host or site of infection and a new species described. Little attention has been paid to variability on spore dimensions. In some general (e.g. Myxobolus) species have been distinguished on the basis of very small differences in morphology or size which in some cases, are, perhaps products of natural variability. Mitchell's (1977) observation

After Lom and Arthur (1989)

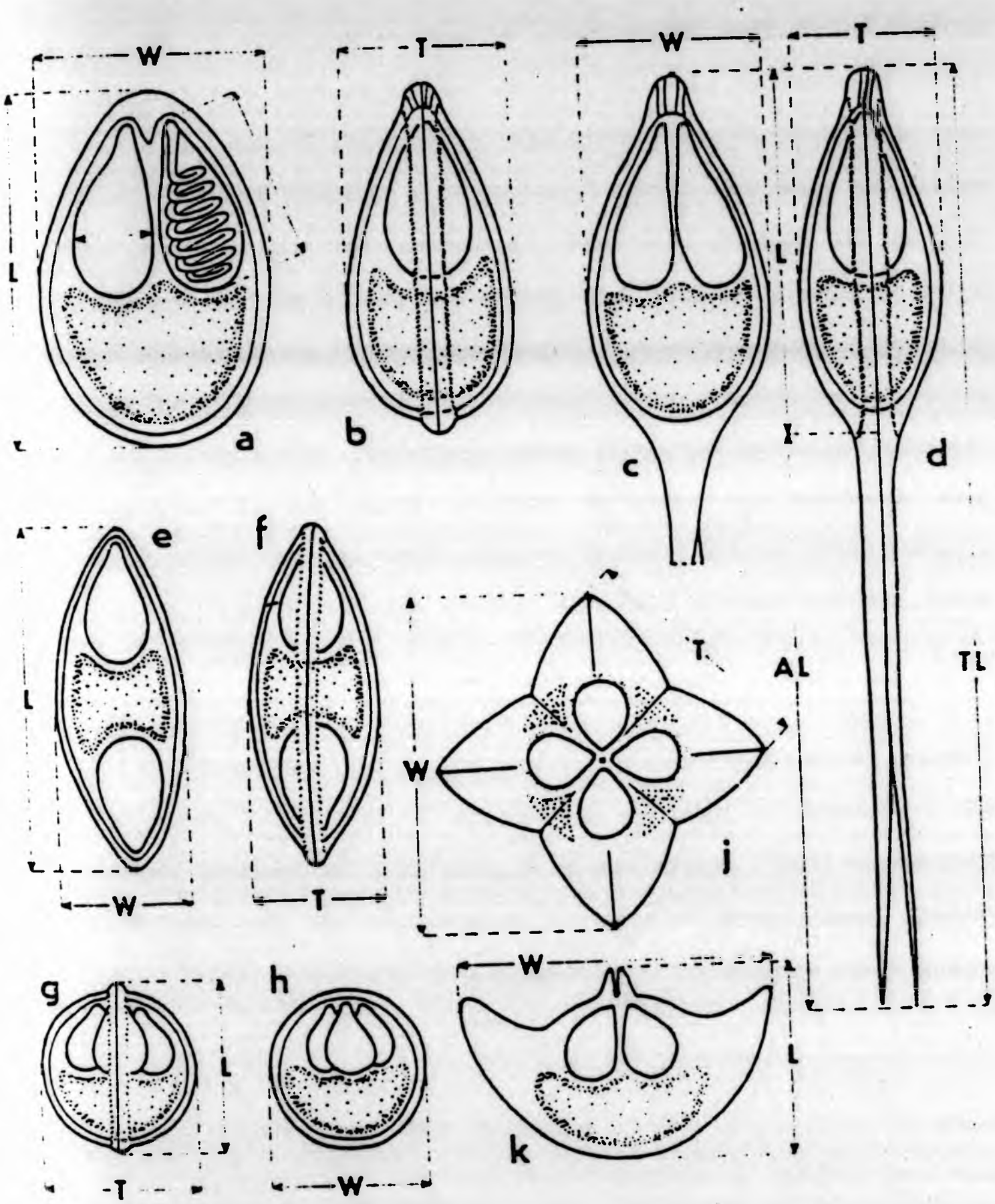


FIGURE 21: Methods of measurement of myxosporean spores of various genera: (a&b) Myxobolus in frontal (a) and sutural (b) or side view: (c&d) Henneguya in frontal and side view: (e&f) Myxidium in frontal and side view: (g&h) Chloromyxum in side or sutural (g) and frontal (h) view: Kudoa in apical (j) and in one of the possible side views (h) which is the diagonal one. Measurement of the polar capsule is indicated in (a): L=length of the spore: W=width of the spore: T=thickness of the spore: in spores with caudal appendages such as Henneguya, AL=length of the caudal appendage, TL=total length of the spore.

"Species descriptions are based mostly on spore morphology and the validity of many species remains questionable" reflects a commonly expressed view amongst current workers. For many species established around the turn of the century, the spores were so poorly described that the identification by subsequent workers was quite subjective and arbitrary. In other instances, identity of the host was the only guideline for the identification and thus when the present criteria for identification are applied several species are quite at variance with the original descriptions of their spores, vegetative stages and sites of infection. The actual number of species so far encountered, therefore, is not quite accurate as there are doubtless many unrecognised synonyms, many cases of distinct species under a single name and many cases with species described without specific names (Lom and Arthur, 1989).

It is evident that there is a need for accurate and uniform criteria for proper description of myxosporean species. In response to the renewed interest of this group, Lom and Arthur (1989) established guidelines for the preparation of species descriptions. Recent publications, therefore, give more information also on other stages of the life cycle of these parasites.

Several authors have drawn attention to the variability in spore dimensions which so often makes determination of species even more difficult. However, detailed studies of spore dimension variability have been made only in a few cases (Hine, 1975, 1978, 1980; Mukherjee

and Haldar, 1981; Athanassopoulou and Sommerville, 1987). The spore variability has been variously ascribed to:

a) the biochemical/physiological character of the organ/tissue or of the outside environment.

b) the suitability of the site for sporogony, and

c) the suitability of the host (Hine, 1979).

Kalavati and Narasimhamurti (1983) suggested that various factors such as the host species, host size, site of infection and the cyst size affect the spore size and shape. His observations showed that the physiochemical factors of the environment do play a role in influencing the morphology of the myxosporean spore of Myxosoma microspora when this parasite is found in the gills of Mugil cephalus.

Moser (1977) observed that internal factors such as the confines of the space control the size of the myxosporean spores whereas external factors, such as the host environment select their shape.

Species of Myxidium have also been distinguished on the basis of spore shape and size (Hine, 1975; Hoshina, 1952; Meglitsch, 1937) and striation number or lack of striations on the valve surfaces (Fujita, 1927; Hine, 1975). Early studies placed emphasis on cyst size and shape and site of sporogony (Fujita, 1929; Hoshina, 1952; Ishii, 1915).

Hine (1978) showed that Myxidium zealandicum may infect different organs of Anguilla australis Richardson and Anguilla dieffenbachii Gray and that variations in spore size and maturity may be related to the site of sporogony.

Spore size and shape may also vary with species of host and individual characteristics of the host and parasite (Hine, 1979). Therefore, cysts from different sites of infection in different hosts might be expected to contain spores having considerable variation in size and shape. This hypothesis has, however, been rarely tested out (Hine, 1978).

With the growing interest in myxosporeans as important pathogens of fish, especially in intensive aquaculture, along with the discoveries of formerly unexpected stages in their life cycle, electron microscopical studies appeared in the literature which greatly advanced the existing knowledge of these parasites.

Even now, we do not know to what extent some features of the vegetative stages and the existence or absence of intermediate hosts will affect the taxonomic criteria at the generic or suprageneric levels. Nevertheless there is some information now available on special structures. For example, some spores bear sporal mechanisms and structures serving for an improved dispersal of the species in the aquatic environment. Such mechanisms include an increased spore surface or projections and mucous envelopes as seen in the Myxobolus species. Only limited information on scanning electron microscopy

(SEM) studies of Myxidium species is available and most of the existing SEM studies concern the morphology of individual species with no comparison of the morphological variability of the spores within the host. The study of Hine (1980) on different Myxidium spp. from eels is the only SEM information available for species of this genus in fish.

In view of these reports and of the limited information concerning the morphology and dimensions of myxosporean parasites in roach in general, and specifically in comparison to hosts from two habitats with different latitudes, a preliminary report was presented (Athanassopoulou and Sommerville, 1987). The data concerned two Myxobolus species, M. ellipsoides and M. dispar and showed that M. dispar had a marked variability in spore size and morphology, but M. ellipsoides did not.

In the present study, morphometric data from two different myxosporean groups, a) Myxobolus with a wide host and site specificity was collected, and b) Myxidium with a narrower host/site specificity was collected in order to compare the variability of the spores of the same species from different organs, as well as from hosts of different age/size and geographic location. To supplement light microscopical data, scanning electron microscopy level studies were also undertaken to overcome the morphometric problems and to support them.

4.2 MATERIALS AND METHODS

Spores were extracted from the tissues of freshly killed roach from Lake A Vassilios, Greece and from roach from Yorkshire, N England, as described in Chapter 2.

Spores were released from the tissues using the parasitological techniques described in 2.3 and 2.5.

An average of 30 spores were measured from each organ sample from each fish by using an eye-piece graticule to measure the dimensions shown in Figure 21. In the case of the Greek samples, 14 fish were used, and 15 fish from the Yorkshire sample.

The fish were further divided into age/size and seasonal groups for comparison. The younger/smaller fish being 0+–2+, 10–14cm, and the older/larger fish being 3+–6+, 16–18cm.

The spores were measured in the fresh state according to the method described by Lom (1965) and stained according to the procedures described in 2.5.1 and based on the guidelines given by Lom and Arthur (1989). The morphology of the spores and other parasitical stages was also studied and described in fresh preparations. Measurements were carried out on both polar capsules. In the case of Myxidium spp. both polar capsule dimensions were measured for the first 10 samples and were found to be equal. Subsequently only one

polar capsule was measured in each sample, the other was assumed to to be equal.

The Myxobolus spp. data was processed by a Bull Computer using the Factor 3b program. This is a modification of the Analysis of Variance Test described by Katos (1984). For this method of statistical analysis to be valid, fish infected simultaneously in all organs with either M. pseudodispar or M. ellipsoides had to be used. Therefore, six Greek fish infected with M. pseudodispar and seven Greek fish infected with M. ellipsoides were available for analysis.

Since only five British fish infected simultaneously in all organs were available, for the analysis of the morphometric data from the two different habitats, five fish from each geographic location were used for each parasite species.

The processing of the data of the present study was partly done in Greece (Department of Statistics, School of Veterinary Medicine) and partly in Scotland (Institute of Aquaculture, University of Stirling). Inevitably two different statistical programs were used, because the Factor 3b program was not available at the University of Stirling. As a result, the analysis of the Myxidium species was performed by using the t test and the analysis of variance using Minitab.

Scanning Electron Microscopy

For scanning electron microscopy the spores were processed according to the methods referred to by Glauert (1981) and Tetley, L. (pers. comm.) at the Histopathology Unit, Department of Zoology, University of Glasgow.

Definitions

Because of the confusion that presently exists in the literature on the terminology used by different researchers for the developmental stages of myxosporean parasites, a clarification of the terminology used in the present study is given below and is based on that used by Lom and Dykova (1987) and Larsson (1989).

The term 'plasmodium' refers to the large multinucleated structure developed from the spore sporoplasm. Large plasmodia are polysporic with the formation, or not, of sporoplasts; small plasmodia often produce two spores only.

'Pansporoblast' is the union of the pericyte and sporogenic cell present in the large plasmodia. Large plasmodia are polysporic in the formation of pansporoblasts, small plasmodia produce two spores only. It is not known if these stages have also formed pansporoblasts.

'Trophozoite' refers to the more advanced stage of plasmodia containing immature, maturing or mature spores.It is often used simultaneously with the term 'plasmodium'.

The term 'cyst' determines trophozoites which are encapsulated by host tissue. The host reaction may be minimal or severe and the cysts may or may not contain visible spores or structural features. When measured the internal diameter of the cysts was taken as the parasite size. The thickness of the cyst was measured as measurement of host reaction

4.3 RESULTS

4.3.1 Morphology of Myxobolus spp. in Greek and British Roach

4.3.1.1. Morphology of M. pseudodispar Gorbunova, 1936

Spores: elongatedly oval in shape with two unequal pyriform polar capsules with the apices shifted to one side so that they open to the side of the anterior pole (Figs. 22,22A). The polar filaments form 4-5 coils in the large polar capsule and 3-4 in the small one. No mucous envelope was observed after treatment with Indian Ink.

M. pseudodispar spores were oval or pyriform in shape with a smooth surface. Spores had shrunk appearance (Fig. 23), especially in the central part of the valve surface, and were thickened along the

sutural line (Figs. 23, 24). The two valves were distinctly separated by a wide ridge (Fig. 24). The sutural line showed an opening which is presumably the mouth of the discharging canal for the polar filament (Fig. 24).

Vegetative stages: oval cysts up to 1.5µm in size in the muscles of roach (Fig. 25). No cysts were observed in the renal tissue. Spores of M. pseudodispar were found in kidneys and spleen, most often adjacent to or in the melanomacrophage centres; in the intestine, swim-bladder and the gills.

The measurements of M. pseudodispar spores in the different organs of roach from the two different habitats, Lake A Vassilios in Greece and Yorkshire in England, are shown in Table 8.

The measurements of M. pseudodispar in the different organs of young (0-2 years old) and older (more than 2+ years of age) fish are shown in Table 9.

4.3.1.2 Comparison of the dimensions of M. pseudodispar spores between the different organs of roach from Lake A Vassilios

In order to study the variability of the dimensions of M. pseudodispar between the different organs of the host the Factor 3b program was used. The factors involved were:

TABLE 8

Mean dimensions (μm ± SD) of Myxobolus pseudodispar spores

	LAKE AGIOS VASSILIOS					LAKE IN YORKSHIRE, ENGLAND				
	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Swim bladder n=30	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Swim bladder n=30
Spore length	10.27 1.05	9.96 1.01	10.72 0.95	9.96 0.81	9.94 0.85	10.90 0.64	10.12 0.70	10.57 0.57	11.05 0.46	10.52 0.42
Spore width	7.53 0.46	7.07 0.34	7.49 0.35	7.66 0.26	7.50 0.42	7.99 0.05	7.65 0.32	7.78 0.29	7.83 0.22	7.76 0.42
Polar capsule length (L)	4.91 0.22	4.65 0.23	4.72 0.31	4.86 0.20	4.97 0.25	4.01 0.13	4.92 0.34	3.64 0.05	4.02 0.04	4.14 0.20
Polar capsule width (L)	3.65 0.41	3.50 0.50	3.67 0.44	2.71 0.42	3.67 0.43	2.88 0.12	2.86 0.29	3.04 0.05	3.06 0.05	3.87 0.14
Polar capsule length (S)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Polar capsule width (S)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Thickness	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70

L = largest; S = smallest

TABLE 9

Mean dimensions (µm ± SD) of Myxobolus pseudodispar spores from Lake Agios Vassilios according to fish age

	0+ - 2+ R. rutilus					2+ R. rutilus				
	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Swim bladder n=30	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Swim bladder n=30
Spore length	10.29 0.20	9.27 0.50	9.08 0.20	9.13 0.27	9.22 0.32	10.25 0.30	8.66 0.89	9.37 0.98	9.26 0.79	9.66 0.49
Spore width	7.10 0.11	7.10 0.17	7.28 0.16	7.52 0.18	7.17 0.15	7.96 0.08	7.16 0.60	7.66 0.38	7.81 0.27	7.83 0.32
Polar capsule length (L)	4.79 0.25	4.50 0.33	4.70 0.25	4.78 0.24	4.85 0.28	5.03 0.08	4.77 0.23	4.90 0.17	4.95 0.13	5.09 0.14
Polar capsule width (L)	3.31 0.31	3.06 0.10	3.32 0.35	3.33 0.30	3.33 0.37	2.99 0.02	2.93 0.31	3.09 0.20	3.05 0.05	4.09 0.14
Polar capsule length (S)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Polar capsule width (S)	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Thickness	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70	6.70

L = largest; S = smallest

A = The size of the fish host (A_1 = small fish, A_2 = large fish; A = 2)

B = The location of the parasite in the host: (B_1 = kidneys, B_2 = gills, B_3 = spleen, B_4 = intestine, B_5 = cartilage; B = 5).

C = The dimensions of the spores (C_1 = length of spore, C_2 = width of spore, C_3 = length of the large polar capsule, C_4 = width of the large polar capsule; C = 4).

The dimensions of the other polar capsule were not processed since these were constant in all organs (2 x 1 µm).

The results of the statistical analysis are given below in Table 10.

TABLE 10

Variance Analysis of Factor 3b

Factors	F value
AB	1.0068
AC	27.06032*
BC	1.6446
ABC	-

*indicates significance for $P < 0.05$

The above Table shows that there are statistically significant differences in the dimensions of the parasite spores (C) and the size of the host (A).

Further analysis of the factors AC using the test of the least significant difference (LSD) (Katos, 1984) had shown that there are differences in the dimensions of the spores between large and small fish but not between the different organs of the host. However, when comparing the parasite dimensions in the different organs within each age category of the fish, differences were shown only between the organs of the large fish (Table 11).

The thickness of the spore was consistent in all cases.

4.3.1.3. Morphology of M. ellipsoides Thélohan, 1892

Spores: regularly ellipsoidal in shape; occasionally narrowed posteriorly. Polar capsules pyriform occupying less than the half of the spore length, with filaments forming 3-5 loops. Small inter-capsular process (Fig. 26). After treatment with Indian Ink a very thin mucous envelope of 1-1.5um is visible only in the posterior part of the spore. The spores when seen in the scanning electron microscope showed almost no shrinkage (Figs. 27, 28). Furrows along the sutural line, openings for the polar filament, or mucous stands on the valve surface, could not be detected.

No vegetative stages were observed in the organs, but the spores were found in groups in the kidneys and spleen associated with the melanomacrophage centres, in the intestine, the gills and cartilage.

TABLE 11

Statistical analysis of the dimensions of M. pseudodispar in the different organs of large fish

(Test of least significant difference)

Dimensions	Kidneys	Gills	Spleen	Intestine	Swim bladder
Length of spore	b	a	c	c	d
Width of spore	b	a	d	c	c
Length of large polar capsule	d	c	d	a	b
Width of large polar capsule	c	a	b	db	ac

Within each horizontal row a different letter indicates a significant difference (P 0.05)

The thickness of the spore and the small polar capsule measurements were consistent in all cases

TABLE 12

Mean dimensions ($\mu m \pm SD$) of spores of Myxobolus ellipsoides

	LAKE AGISO VASSILIOS					LAKE IN YORKSHIRE, ENGLAND				
	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Cartilage of gills & gill arch n=30	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Cartilage of gills & gill arch n=30
Spore length	16.74 0.36	16.21 0.25	16.34 0.25	16.26 0.26	16.07 0.33	16.31 0.59	15.90 0.17	16.07 0.04	15.89 0.16	15.76 0.04
Spore width	10.27 0.33	9.55 0.35	10.26 0.24	10.25 0.16	9.91 0.10	9.86 0.48	9.74 0.41	9.84 0.42	10.01 0.15	9.84 0.11
Polar capsule length	5.19 0.21	4.88 0.43	5.19 0.10	4.70 0.32	4.39 0.36	4.80 0.37	4.49 0.47	4.92 0.39	4.52 0.44	4.13 0.07
Polar capsule width	3.45 0.38	3.32 0.23	3.34 0.25	3.36 0.26	3.32 0.36	3.20 0.32	3.16 0.04	3.29 0.34	3.15 0.05	3.13 0.08
Thickness	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6

TABLE 13

Mean dimensions (µm ± SD) of Myxobolus ellipsoides spores from Lake Agios Vassilios according to fish age

	0+ R. rutilus					1+ R. rutilus				
	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Cartilage of gills & gill arch n=30	Kidney n=30	Gills n=30	Spleen n=30	Intestine n=30	Cartilage of gills & gill arch n=30
Spore length	16.59 0.37	16.01 0.13	16.17 0.08	16.06 0.17	15.78 0.05	16.80 0.30	16.40 0.17	16.50 0.25	16.44 0.19	16.36 0.19
Spore width	10.00 0.18	9.80 0.32	10.15 0.17	10.12 0.07	9.85 0.08	10.50 0.19	9.79 0.16	10.37 0.26	10.38 0.10	9.97 0.07
Polar capsule length	5.06 0.11	4.58 0.41	5.07 0.05	4.53 0.33	4.14 0.07	5.30 0.22	5.19 0.11	5.25 0.11	4.86 0.24	4.65 0.37
Polar capsule width	3.24 0.25	3.26 0.29	3.17 0.03	3.19 0.08	3.14 0.07	3.24 0.25	3.26 0.29	3.17 0.03	3.19 0.08	3.14 0.07
Thickness	5.60	5.60	5.60	5.60	5.60	5.60	5.60	5.60	5.60	5.60

The dimensions of M. ellipsoides spores in the different organs of roach from the two different habitats are shown in Table 12.

The dimensions of the spores in the organs of young (0^+-2 years old) and older (more than 2^+ years of age) are shown in Table 13.

4.3.1.4. Comparison of M. ellipsoides dimensions between the different organs of roach from Lake A Vassilios

Applying the Factor 3b program for the variability of the dimensions between the organs the F values for the factors A B C were found as follows (Table 14):

TABLE 14

Variance Analysis of Factor 3b

Factors	F value
AB	0.5681971
AC	1.284267
BC	3.587441*
ABC	3.320054*

*indicates significance for $P < 0.05$

The above significant F values mean that there are significant differences in the parasite dimensions, the size of the host and in the organs of the host.

Further analysis of the factors B, C and A, B, C using the test of the least significant difference (LSD) has shown the following results (Table 15). Table 15 shows that there are differences in the dimensions of the spores between large and small fish but not between the different organs of the host. When comparing the parasite dimensions in the different organs of each age category of the fish, there were differences between the organs but only in the large fish.

4.3.1.5 Comparison of the dimensions of M. pseudodispar and M. ellipsoides between the different organs of roach from Greece and from Britain

Using the same 3b Factor program on the spore dimensions from British and Greek sources, and taking as factors:

A = origin of fish (A_1 = English, A_2 = Greek),

B = organs of fish (B_1 = kidneys, B_2 = gills, B_3 = spleen, B_4 = intestine, B_5 = swim-bladder or cartilage) and

C = dimensions of the spores (C_1 = length of spore, C_2 = width of spore, C_3 = length of polar capsule and C_4 = width of polar capsule);

the F values were found to be as follows (Table 16):

TABLE 15

Statistical analysis of the dimensions of M. ellipsoides in the different organs of roach

(Test of least significant difference)

Dimensions (um)		Kidney	Gills	Spleen	Intestine	Cartilage of gill & gill arch
Length of spore	Small fish	b	a	a	a	a
	Large fish	a	a	a	a	a
Width of spore	Small fish	b	a	b	b	b
	Large fish	b	a	b	b	b
Length of polar capsule	Small fish	b	c	b	ac	a
	Large fish	b	bc	bc	ac	a
Width of polar capsule	Small fish	a	a	a	a	a
	Large fish	b	a	a	ac	a

Different letters in the same row are significantly different (P 0.05)

TABLE 16

Variance analysis of the dimensions of Myxobolus spp spores from Greek and British roach (R. rutilus)

FACTORS	F VALUE	
	M.ellipsoides	M.pseudodispar
AB	0.5141	2.329
AC	1.787	1.2618
BC	2.6067*	3.0612*
ABC	0.9734	1.1692

*indicates significance for $P < 0.05$

The above Table shows that the Factor A (origin) has no effect on the dimensions of the spores as ABC is not significant.

The significance in the factors BC shows that there are differences in the measurements in the different organs of the fish as this has already been shown in the analysis of the spores from the Greek fish.

4.3.2 Morphology of Myxidium spp. in Greek and British roach

4.3.2.1. Morphology of Myxidium rhodei Léger, 1905

Spores: Spindle shaped with poles pointed and polar capsules equal in size, lying at either end of the longitudinal axis of the spore. The polar filaments form 4-5 coils in the polar capsules. Spores have 21-23 longitudinal surface ridges and some spores were observed to be narrower centrally (Figs. 29, 30).

Scanning electron microscopy results showed that the surface of the spores has narrow striations which overlap at the poles. The spores are elongated with occasional infusions centrally and with pointed apices. The striations are thicker along the sutural line. No mucous strands or other structures (furrows, papules, etc.) were observed. Immature spores did not show any thickening along the sutural line (Fig. 31).

Immature spores were observed within disporous pansporoblasts and in the large cysts (trophozoites) in the interstitial tissue of the kidney. These were smaller and wider than the mature spores and had less pointed apices. Immature spores were only observed in the renal tissue (Figs. 31, 32, 33).

Cysts: Round, oval or discoid measuring from 62µm to 800µm in diameter were found in the interstitial tissue of the kidneys, in the muscles and on a few occasions, in the liver parenchyma. The cysts more often contained mature spores, but cysts containing immature M. rhodei spores in different stages of development were also seen. The cysts in the muscles and liver contained only mature spores. In some cases, the cysts in the kidneys appeared to be subdivided into smaller cysts (2-6) (Fig. 34), all containing mature Myxidium rhodei spores.

Trophozoites: Early developmental stages of M. rhodei were observed in Bowman's capsules but only in a few cases (Figs. 35, 36). The

plasmodia were elongated with a middle fold (Fig. 37). The plasmodia were transparent with highly vacuolated cytoplasm. When stained with Giemsa, densly stained nuclei were also seen. Cilia were always present along the periphery of the plasmodial body.

4.3.2.2 Measurements of M. rhodei spores, cysts and trophozoites

A. GREEK ROACH

Early developmental stages were found only in a small number of fish, therefore the measurements were taken in a limited number of parasites and are shown in Table 17. However, sample size would not be satisfactory for accurate statistical analysis (Table 17).

The measurements of these stages showed that the pansporoblasts were oval and the plasmodia elongated, transparent with a number of vacuoles and cilia around the external surface (Figure 37). The immature spores were found to be smaller than the mature ones from the kidneys or from any other organ of roach (Tables 18, 19).

The dimensions of both immature and mature spores did not differ in the two age categories of fish (Tables 18, 19).

The measurements of mature M. rhodei spores in the different organs of roach from Lake A Vassilios are shown in Table 19. Statistical analysis using the analysis of variance test showed differences

TABLE 17

Measurements of developmental stages of M rhodei from the renal tissue of roach from Lake A Vassilios (Greece)

Dimensions (μm)	Pansporoblasts (μm)			Plasmodia (μm)		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	8-18	9.09	2.84	60-80	71.8	10.42
Width	4-10	5.27	1.62	15-23	18.0	4.12
n		11			5	

TABLE 18

Measurements of immature M. rhodei spores in the renal tissue of roach from Lake A Vassilios (Greece)

	YOUNG FISH n=10			OLD FISH n=10		
Dimensions (μm)	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	8-10	8.6	0.74	8-10	8.63	0.70
Width	4-6	4.5	0.74	4-6	4.6	0.74
Polar capsule Length	3-4	3.69	0.48	3-4	3.58	0.51
Polar capsule Width	3-4	3.66	0.50	3-4	3.68	0.51

TABLE 19

Measurements of mature M. rhodei spores from the different organs of roach from Lake A Vassilios (Greece)

(μm)

Dimensions	KIDNEY			MUSCLE			LIVER		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
YOUNG FISH n=30									
Length	9-12	9.65	0.91	9-10	9.46	0.43	9-11	9.50	0.74
Width	3-5	3.90	0.83	3-5	3.67	0.75	3-5	3.66	0.81
Polar Capsule									
Length	3-4	3.66	0.50	3-4	3.60	0.51	3-4	3.50	0.52
Width	3-4	3.45	0.52	3-4	3.33	0.50	3-4	3.40	0.51
OLD FISH n=30									
Length	9-13	10.08[a]	1.18	9-10	9.30[ab]	0.40	3-10	9.20[ab]	0.38
Width	3-5	4.10[a]	0.75	3-5	3.58[ab]	0.74	3-5	3.60[ab]	0.73
Polar Capsule									
Length	3-5	3.85	0.89	3-4	3.85	0.52	3-4	3.50	0.52
Width	3-4	3.45	0.52	3-4	3.45	0.52	3-4	3.45	0.52

Different letters in the same row are significantly different ($P < 0.05$)

between the spore dimensions in the organs of older fish. Both dimensions (length and width) were significant and the dimensions of the spores in the kidneys were always bigger than in the other two organs. No significant differences were observed in the dimensions of spores between liver and muscles.

The measurements of the cysts formed by M. rhodei in the organs of roach are shown in Table 20. From this table it can be seen that there were significant differences in the size of cysts between the organs in both small and large fish. Significant differences were also found when comparing the cysts of larger and smaller fish. The cysts in the kidneys (and especially of the larger fish) were always larger than the ones in the muscles (Table 20).

The measurements of mature M. rhodei spores from roach of this lake during the different seasons are presented in Table 21. No significant differences were found in the spore dimensions (length and width) between the seasons either in younger or older fish (Table 21A)

B. BRITISH ROACH

The measurements of immature spores and developmental stages of M. rhodei from the renal tissue of roach from Yorkshire are shown in Table 22. Spores were collected from 5 older group fish.

TABLE 20

Measurements of the cysts produced by M. rhodei in the kidneys and muscles of roach from Lake A Vassilios (Greece)

(μm)

YOUNG FISH*								
Dimensions	Kidney*				Muscle*			
	Range	$\bar{x}$	SD	n	Range	$\bar{x}$	SD	n
Length*	70-260.4	108.9	41.2	39	62-145	79	17.5	30
Width*	58.8-252	97.0	35.5	39	51-99.6	68	11.4	30
Thickness of connective tissue	2.8-11.2	6.58	3.38	39	2.8	2.8	0	30

OLD FISH*								
Dimensions	Kidney*				Muscle*			
	Range	$\bar{x}$	SD	n	Range	$\bar{x}$	SD	n
Length*	300-800	455	165	32	75-110	92.3	10.3	30
Width*	100-300	130	54.8	33	62-95	74.7	11.2	30
Thickness of connective tissue	10-15	12.56	2.02	31	2.8	2.8	0	30

*denotes statistically significant differences between the dimensions' length and width and all the organs as well as in similar organs between young and olf fish ($P<0.05$)

TABLE 21

Measurements of mature spores of M. rhodei from roach originating from Lake A Vassilios (Greece) during the different seasons

(/µm)

YOUNG FISH n=30												
Dimensions	Autumn			Winter			Spring			Summer		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	9-11	9.7	0.70	9-11	9.5	0.74	9-10	9.4	0.64	9-11	9.7	0.69
Width	3-5	3.5	0.71	3-5	3.48	0.63	3-5	3.63	0.71	3-5	3.7	0.71
Polar Capsule												
Length	3-4	3.5	0.53	3-4	3.5	0.51	3-4	3.8	0.63	3	3	0
Width	3-4	3.4	0.516	3-4	3.4	0.516	3-4	3.2	0.46	3-4	3.2	0.42

OLD FISH n=30												
Dimensions	Autumn			Winter			Spring			Summer		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	9-12	9.7	0.96	9-10	9.5	0.77	9-13	9.6	1.07	9-11	9.5	0.68
Width	3-5	3.5	0.59	3-5	3.6	0.75	3-5	3.6	0.69	3-4	3.6	0.69
Polar Capsule												
Length	4	4	0	3-4	3.8	0.65	3-4	3.6	0.52	4	4	0
Width	3	3	0	3	3	0	3	3	0	3	3	0

TABLE 21A

Statistical analysis of the dimensions of M. rhodei from roach from Lake A Vassilios (Greece) during the different seasons

	YOUNG FISH n = 30		OLD FISH n = 30	
Dimensions	F value	P	F value	P
Spore length	1.36 (NS) (n = 120)	0.26	0.41 (NS) (n = 120)	0.74
Spore width	0.68 (NS) (n = 120)	0.50	0.14 (NS) (n = 120)	0.93

NS = Non significant difference for $P < 0.05$

TABLE 22

Measurements of immature spores and developmental stages of M. rhodei from the kidneys of roach from Yorkshire (N England)

(µm)

Dimensions		IMMATURE SPORES YOUNG FISH			IMMATURE SPORES OLD FISH			PANSPOROBLASTS			PLASMODIA		
		Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length		8-9	8.5	0.41	8-9	8.51	0.42	10-25	16.3	5.01	55-83	65.66	15.14
Width		4-6	4.6	0.60	4-6	4.56	0.55	5-13	8.38	2.65	13-28	20.00	7.54
Polar Capsule	Length	4	4	0	4	4	0		-			-	
	Width	3	3	0	3	3	0		-			-	
	n		30			30			10			3	

The measurements of cysts formed by M. rhodei in the interstitial tissue of kidneys and in the muscles of roach from Yorkshire are shown in Table 23. Statistical analysis of the measurements of length and width in the organs of fish as well as in the two age groups, showed differences in the kidneys of young and older fish but not in the muscles of young and older fish. Furthermore, differences in the young fish existed between kidneys and muscles only for the length (larger diameter) of the cysts (Table 23A). For the older fish, both dimensions differed significantly (Table 23A).

The measurements of mature M. rhodei spores from the kidneys, muscles and liver of young and older fish from Yorkshire are shown in Table 24. The spores were collected from the organs of 10 young and 10 older fish. At least 30 spores were measured in each case. Using the analysis of variance test no significant differences on the spore dimensions (length and width) were found between the organs of both young and older fish (Table 24A).

4.3.2.3. Morphology of Myxidium pfeifferi Auerbach, 1908

Spores: spindle shaped with poles less pointed than M. rhodei. Polar capsules equal in size lying in either end along the longitudinal axis of the spore. The polar filaments form 4-5 coils in the polar capsules. Mature spores were found only in the bile ducts and gall-bladder contents. In scanning electron microscopy, M. pfeifferi spores were similar to M. rhodei with no distinct differences (Figs.

TABLE 23

Measurements of M. rhodei cysts in the organs of roach from Yorkshire (N England)

(/µm)

Dimensions	YOUNG FISH n=10						OLD FISH n=10					
	Kidney			Muscles			Kidney			Muscles		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Large diameter	33.6-78.4	53.2	14	28-98	44.5	18	70-182	100.4	37.6	36.4-56	44.25	6.95
Small diameter	36.4-84	51.9	15	39.2-95	45.1	14.3	70-1456	99.4	21.8	30.8-59	47.9	12.20
Thickness of connective tissue	2.8-16.8	9.09	5.13	2.8	2.8	0	2.8-24	9.95	4.59	2.8	2.8	0
n		30			30			30			30	

TABLE 23A

Statistical analysis of the dimensions of M. rhodei cysts in the organs of roach from Yorkshire (N England)

Dimensions	Organs	T value	P
Length	K_1K_2	6.45 (S)	0.000
	M_1M_2	0.06 (NS)	0.950
	K_1M_1	2.10 (S)	0.040
	K_2M_2	8.05 (S)	0.000
Width	K_1K_2	9.80 (S)	0.000
	M_1M_2	0.81 (NS)	0.420
	K_1M_1	1.79 (NS)	0.078
	K_2M_2	11.27 (S)	0.000

K_1 = kidney of young fish

K_2 = kidney of old fish

M_1 = muscle of young fish

M_2 = muscle of old fish

NS = non significant values for $P < 0.05$

S = statistically significant difference for $P < 0.05$

TABLE 24

Measurements of mature spores of M. rhodei in the different tissues of roach from Yorkshire (N England)

(µm)

Dimensions		KIDNEY			MUSCLE			LIVER		
		Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
YOUNG FISH n=10										
Length		9-13	10.03	1.05	9-11	9.9	1.05	9-10	9.6	0.54
Width		3-5	3.6	0.66	3-5	3.5	0.68	3-5	3:55	0.68
Polar Capsule	Length	3-4	3.37	0.52	3-4	3.4	0.52	3-4	3.55	0.52
	Width	3-4	3.77	0.45	3-4	3.36	0.50	3-4	3.42	0.51
OLD FISH n=10										
Length		9-13	9.7	0.70	9-11	9.5	0.95	9-11	9.5	0.53
Width		3-5	3.4	0.45	3-5	3.3	0.58	3-5	3.6	0.64
Polar Capsule	Length	3-4	3.46	0.52	3-4	3.4	0.52	3-4	3.37	0.52
	Width	3-4	3.38	0.51	3-4	3.42	0.52	3-4	3.32	0.52

TABLE 24(A)

Statistical analysis of the dimensions of mature M. rhodei spores in the different tissues of roach from Yorkshire (N England)

	ANOVAR TEST				T TEST		
	YOUNG FISH n=30		OLD FISH n=30				
Dimns.	F value	P	F value	P	Organs	T value	P
Length	1.45 (NS) n = 90	0.24	0.85 (NS) n = 90	0.43	K_1-K_2	1.41 (NS)	0.16
					M_1-M_2	1.28 (NS)	0.21
					L_1-L_2	0.96 (NS)	0.34
Width	0.11 (NS) n = 90	0.9	1.73 (NS) n = 90	0.18	K_1-K_2	1.37 (NS)	0.18
					M_1-M_2	1.01 (NS)	0.32
					L_1-L_2	0.49 (NS)	0.63

K_1 = measurements of M. rhodei spores in the kidneys of young fish
K_2 = " " " " " " " " old fish
M_1 = " " " " " " muscles " young fish
M_2 = " " " " " " " " old fish
L_1 = " " " " " " liver " young fish
L_2 = " " " " " " " " old fish

NS = non significant differences for $P < 0.05$

ANOVAR = Analysis of variance test

38, 39) in shape and morphology. The pattern of the striations and the number was identical as in the case of M. rhodei spores. The sutural line was thickened in the mature M. pfeifferi spores but not in the immatures (Figs. 38, 39).

Vegetative Stages: Plasmodia occurred in bile ducts containing large number of mature and immature spores (Figs. 40, 41). No early developmental stages were found in the gall-bladder.

4.3.2.4 Measurements of M. pfeifferi spores and trophozoites

A. GREEK ROACH

The measurements of mature and immature spores of M. pfeifferi from the gall bladder and bile ducts of roach collected from Lake A. Vassilios (Greece) are shown in Tables 25 and 26 respectively. The spores were collected from 10 young and 10 older fish. Using the two sample t-test, no significant differences were detected in the organs of young and old fish (Tables 25A and 26A respectively).

B. BRITISH ROACH

The measurements of the mature and immature spores of M. pfeifferi from the gall bladder and bile ducts of roach from Yorkshire are shown in Tables 27 and 28 respectively. The spores were collected

TABLE 25

Measurements of mature spores of M. pfeifferi from the gall bladder of roach from Lake A Vassilios (Greece)

	YOUNG FISH n=10			OLD FISH n=10		
Dimensions (µm)	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	10-14	10.08	1.22	10-14	10.18	1.32
Width	4-5	4.10	0.41	4-6	4.18	0.58
Polar capsule Length	4-5	4.55	0.51	4-5	4.71	0.46
Polar capsule Width	3-4	3.80	0.41	3-4	3.75	0.44
n		30			30	

TABLE 26

Measurements of immature spores of M. pfeifferi from the bile ducts of roach from Lake A Vassilios (Greece)

	YOUNG FISH n=10			OLD FISH n=10		
Dimensions (µm)	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	8-10	8.60	0.41	8-10	8.61	0.49
Width	4-6	4.50	0.41	4-6	4.61	0.61
Polar capsule Length	3-4	3.66	0.48	3-4	3.71	0.47
Polar capsule Width	3-4	3.68	0.47	3-4	3.61	0.51
n		30			30	

TABLE 25(A)

Statistical analysis of the measurements of mature M. pfeifferi spores from the gall bladder of old and young fish from Lake A Vassilios (Greece)

Dimensions	T value	P
Length	L_1-L_2 = 0.56 (NS)	0.58
Width	W_1W_2 = 0.14 (NS)	0.38

L_1 = length of M. pfeifferi spores in young fish

L_2 = " " " " " " old fish

W_1 = width " " " " " young fish

W_2 = " " " " " " old fish

NS = non significant value for $P < 0.05$

S = significant difference for $P < 0.05$

TABLE 26A

Statistical analysis of the measurements of immature spores of M. pfeifferi from the bile ducts of young and old fish from Lake A Vassilios (Greece)

Dimensions	T value	P
Length	L_1-L_2 = 1.55 (NS)	0.13
Width	W_1-W_2 = 0.61 (NS)	0.54

L_1 = length of M. pfeifferi spores in young fish

L_2 = " " " " " " old fish

W_1 = width of " " " " young fish

W_2 = " " " " " " old fish

NS = Non significant value for $P < 0.05$

S = Significant difference for $P < 0.05$

TABLE 27

Measurements of mature spores of M. pfeifferi from the gall bladder of roach from Yorkshire (N England)

	YOUNG FISH n=10			OLD FISH n=10		
Dimensions (µm)	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	10-14	10.09	0.96	10-14	10.05	0.65
Width	4-5	4.00	0.41	4-6	4.00	0.42
Polar capsule Length	4-5	4.23	0.43	4-5	4.60	0.52
Polar capsule Width	3-4	3.73	0.46	4-5	4.45	0.51
n		30			30	

TABLE 28

Measurements of immature spores of M. pfeifferi from the bile ducts of roach from Yorkshire (N England)

	YOUNG FISH n=10			OLD FISH n=10		
Dimensions (µm)	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Length	8-9	8.60	0.72	8-10	7.90	1.71
Width	4-5	3.88	0.84	4-6	3.90	0.76
Polar capsule Length	3-4	3.67	0.51	3-4	3.60	0.47
Polar capsule Width	3-4	3.70	0.47	3-4	3.69	0.48
n		30			30	

from 10 old and 10 young fish. No significant differences were observed in the organs of young and old fish, using the two sample t-test (Tables 27A and 28A respectively).

4.3.2.5. Comparison of the dimensions of M. rhodei and M. pfeifferi spores in the organs of Greek and British roach

A. M. pfeifferi

Statistical Analysis (using the analysis of variance test) of the measurements of mature M. pfeifferi spores showed no differences in both young and old fish (Table 29).

Using the same test and the two sample t-test for the analysis of the measurements of immature spores of M. pfeifferi, significant differences were found in the width of Greek and British fish (both young an old). The length dimensions were significant only in the old fish (Table 30).

B. M. rhodei

Statistical Analysis using the two sample t-test of the measurements of mature M. rhodei spores in the tissues of roach from Lake A. Vassilios (Greece) and Yorkshire (N. England, showed that in the young fish, no significant differences were observed, except for the

TABLE 27A

Statistical analysis of the measurements of mature M. pfeifferi spores from the gall bladder of old fish from Yorkshire (N England)

Dimensions	T value	P
Length	$L_1 L_2$ = 1.18 (NS)	0.24
Width	W_1-W_2 = 0.15 (NS)	0.88

L_1 = length of M. pfeifferi spores in young fish

L_2 = " " " " " " old fish

W_1 = width " " " " " young fish

W_2 = " " " " " " old fish

NS = non significant value for $P < 0.05$

S = significant difference for $P < 0.05$

TABLE 28A

Statistical analysis of the measurements of immature M. pfeifferi spores from the bile ducts of young and old fish from Yorkshire (N England)

Dimensions	T value	P
Length	L_1-L_2 = 2.11 (NS)	0.04
Width	W_1-W_2 = 0.24 (NS)	0.81

L_1 = length of M. pfeifferi spores in young fish

L_2 = " " " " " " old fish

W_1 = width " " " " " young fish

W_2 = " " " " " " old fish

NS = non significant value for $P < 0.05$

TABLE 29

Statistical analysis of the measurements of mature M. pfeifferi spores from the gall bladder of fish from Greece and Britain
(Lake A. Vassilios and Yorkshire, respectively)

Analysis of Variance Test		
Dimensions	F value	
Length	$L_1L_2L_3L_4$ = 1.81 (NS)	0.15
Width	$W_1W_2W_3W_4$ = 0.53 (NS)	0.66

L_1 = length of M. pfeifferi spores in Greek young fish
L_2 = " " " " " " " old fish
L_3 = " " " " " " British young fish
L_4 = " " " " " " " old fish

W_1 = Width of M. pfeifferi spores in Greek young fish
W_2 = " " " " " " " old fish
W_3 = " " " " " " British young fish
W_4 = " " " " " " " old fish

NS = Non significant value for $P < 0.05$

TABLE 30

Statistical analysis of the measurements of immature M. pfeifferi spores from the bile ducts of fish from Greece (Lake A Vassilios) and Yorkshire (N England)

Dimensions	T value	P
Length	L_1-L_2 = 0.76 (NS)	0.45
	L_3-L_4 = 2.11 (S)	0.04
Width	W_1-W_2 = 3.58 (S)	0.009
	W_3-W_4 = 2.70 (S)	0.009

L_1 = length of immature M. pfeifferi spores in the organs of young Greek fish

L_2 = " " " " " " " " organs of young British fish

L_3 = " " " " " " " " organs of old Greek fish

L_4 = " " " " " " " " organs of old British fish

W_1 = width " " " " " " " organs of young Greek fish

W_2 = " " " " " " " " organs of young British fish

W_3 = " " " " " " " " organs of old Greek fish

W_4 = " " " " " " " " organs of old British fish

NS = non significant values for $P < 0.05$

S = significant difference for $P < 0.05$

muscles, but in the old fish significant differences were observed in the liver, muscles (for the length) and in the kidneys (for the width) of Greek and British fish (Table 31).

When examining the measurements of the immature M. rhodei spores in the kidneys of roach from the two geographic locations, significant differences were found only in the spore length dimensions in both young and old fish (Table 32).

Both dimensions (length and width) of the cysts produced by M. rhodei in the tissues of Greek fish were statistically different from the cysts found in the British fish (Table 33).

4.3.3 Comparison of the morphology and dimensions of M. rhodei versus M. pfeifferi spores

Statistical analysis (two sample t-test) of the measurements of mature M. rhodei and M. pfeifferi spores from Greece showed no significant differences in both young and old fish (Table 34). The same results were observed when comparing the dimensions of the immature M. rhodei and M. pfeifferi spores (Table 34).

Analysis of the dimensions of mature M. rhodei and M. pfeifferi from Yorkshire, however, showed statistically significant differences (Table 35), M. rhodei being always larger than M. pfeifferi.

TABLE 31

Statistical analysis of the measurements of mature M. rhodei spores in the tissues of Greek and British roach

Dimensions	YOUNG FISH		OLD FISH	
	T value	P	T value	P
Length	K_1-K_2 = 1.05 (NS)	0.30	K_1-K_2 = 1.93 (NS)	0.05
	M_1-M_2 = 2.87 (S)	0.006	M_1-M_2 = 2.68 (S)	0.010
	L_1-L_2 = 0.40 (NS)	0.69	L_1-L_2 = 2.21 (S)	0.03
Width	K_1-K_2 = 1.97 (NS)	0.050	K_1-K_2 = 4.23 (S)	0.0001
	M_1-M_2 = 0.62 (NS)	0.53	M_1-M_2 = 1.16 (NS)	0.25
	L_1-L_2 = 0.60 (NS)	0.60	L_1-L_2 = 0.19 (NS)	0.85

K_1 = measurements of the spores in the kidneys of Greek roach

K_2 = " " " " " " " " British roach

M_1 = " " " " " " muscles of Greek roach

M_2 = " " " " " " " " British roach

L_1 = " " " " " " liver of Greek roach

L_2 = " " " " " " " " British roach

NS = Non significant values for $P < 0.05$

S = Significant difference for $P < 0.05$

TABLE 32

Statistical analysis of the measurements of immature M. rhodei spores in the kidneys of roach from Greece (Lake A Vassilios) and Britain (Yorkshire)

Dimensions	T value	P
Length	L_1-L_2 = 2.68 (S)	0.010
	L_3-L_4 = 2.61 (S)	0.012
Width	W_1-W_2 = 0.10 (NS)	0.92
	W_3-W_4 = 0.20 (NS)	0.85

L_1 = length of M. rhodei spores in the kidneys of young Greek roach
L_2 = " " " " " " " " " " British roach
L_3 = " " " " " " " " " old Greek roach
L_4 = " " " " " " " " " " British roach

W_1 = width of M. rhodei spores in the kidneys of young Greek roach
W_2 = " " " " " " " " " " British roach
W_3 = " " " " " " " " " old Greek roach
W_4 = " " " " " " " " " " British roach

NS = non significant values for $P < 0.05$

S = significant difference for $P < 0.05$

TABLE 33

Statistical analysis of the measurements of the cysts produced by M. rhodei in the tissues of Greek and British roach

	YOUNG FISH		OLD FISH	
Dimensions	T value	P	T value	P
Length	K_1-K_2 = 7.00 (S)	0.00	K_1-K_2 = 16.59 (S)	0.000
	M_1-M_2 = 7.54 (S)	0.00	M_1-M_2 = 21.21 (S)	0.000
Width	K_1-K_2 = 6.40 (S)	0.00	K_1-K_2 = 2.85 (S)	0.007
	M_1-M_2 = 6.85 (S)	0.00	M_1-M_2 = 8.89 (S)	0.000

K_1 = measurements of the cysts in the kidneys of Greek roach

K_2 = " " " " " " " " British roach

M_1 = " " " " " " muscles " Greek roach

M_2 = " " " " " " " " British roach

S = significant difference for $P < 0.05$

TABLE 34

Statistical analysis of the dimensions of M. rhodei and M. pfeifferi spores from Lake A Vassilios (Greece)

MATURE SPORES n=30				
	YOUNG FISH n=20		OLD FISH n=20	
Dimensions	T value	P	T value	P
Length	L_1L_2 = 1.02 (NS)	0.99	L_1L_2 = 0.75 (NS)	0.67
Width	W_1W_2 = 0.83 (NS)	0.85	W_1W_2 = 0.45 (NS)	0.79
IMMATURE SPORES n=30				
	YOUNG FISH n=20		OLD FISH n=20	
Dimensions	T value	P	T value	P
Length	L_1L_2 = 1.13 (NS)	0.08	L_1L_2 = 1.43 (NS)	0.31
Width	W_1W_2 = 1.53 (NS)	0.2	W_1W_2 = 0.92 (NS)	0.42

L_1 = length of mature or immature M. rhodei spores
L_2 = " " " " " M. pfeifferi spores
W_1 = width " " " " M. rhodei spores
W_2 = " " " " " M. pfeifferi spores

NS = non significant difference for $P < 0.05$

TABLE 35

Statistical analysis of the dimensions of M. rhodei and M. pfeifferi spores from Yorkshire (N England)

MATURE SPORES n=30				
	YOUNG FISH n=20		OLD FISH n=20	
Dimensions	T value	P	T value	P
Length	L_1L_2 = 1.25 (NS)	0.31	L_1L_2 = 1.38 (NS)	0.42
Width	W_1W_2 = 1.2 (S)	0.04	W_1W_2 = 1.43 (S)	0.01

IMMATURE SPORES n=30				
	YOUNG FISH n=20		OLD FISH n=20	
Dimensions	T value	P	T value	P
Length	L_1L_2 = 4.3 (S)	0.01	L_1L_2 = 5.3 (S)	0.02
Width	W_1W_2 = 6.2 (S)	0.00	W_1W_2 = 5.4 (S)	0.00

L_1 = length of mature or immature M. rhodei spores
L_2 = " " " " " M. pfeifferi spores
W_1 = width " " " " M. rhodei spores
W_2 = " " " " " M. pfeifferi spores

NS = non significant values for $P < 0.05$

S = significant difference for $P < 0.05$

4.4 DISCUSSION

Myxosporea were generally very common in R. rutilus, especially Myxobolus pseudodispar and Myxidium rhodei. Site specificity was only observed in M. rhodei and Myxidium pfeifferi, the latter only occurred in the bile ducts. M. rhodei was considered to be site specific to the kidney because it was only rarely observed in other organs (spleen, liver, etc).

The dimensions of the spores referred to in the literature represent values of mature spores only, thus a comparison of other developmental stages of the spores, which were also measured in the present study, is very difficult (Alvarez-Pellitero et al., 1983; Dykova, Lom and Grupcheva, 1987). However, the dimensions of the spores of both M. pseudodispar and M. ellipsoides were in accordance with the range of dimensions quoted in previous descriptions, although M. pseudodispar was generally slightly smaller in this survey.

M. ellipsoides showed only slight differences in the spore dimensions in the different organs and specifically, between gills and the rest of the organs; the spores were smaller in the gills and gill cartilage.

In the case of M. pseudodispar marked variability in the spore dimensions was observed between the different organs of larger fish.

Differences in size and shape of myxosporean spores call into question the factors or mechanisms controlling these morphometric parameters.

Several researchers have studied the variability of the spore morphology and dimensions in other myxosporean species. Moser (1977) proposed that in Myxosporea

a) spore size is determined by factors located primarily within the host (i.e. confines of space);

b) spore shape is determined by the presence of physiologically and behaviourally suitable fish hosts, and

c) there is a constancy in spore size that is independent of geographic location, water depth or season.

The author compared spore dimensions of histozoic and coelozoic species and finding that coelozoic species tend to be larger, he concluded that space is an important factor. He also considered that shape is critical in determining spore settlement rates in the water column and is selected to maximise the time over which the host might ingest spores. His experimental evidence, however, was not considered convincing by other authors (Hine, 1979).

Hine (1978) suggested that spore shape, size and maturity were largely determined by the biochemical and/or physiological suitability of the environment in which the parasite develops; or more specifically, in which sporogony occurs.

In the present study, the fact that the spores of M. ellipsoides were found to be longer in the internal organs (and in particular in the kidney) and smaller in the gills may be due to the fact that biochemical and/or physiological conditions in the gill tissue are less suitable for the development of this species. In addition to this, the spores in the gills are subjected more often to external environmental changes and this might contribute to spore size. On the other hand, the nature and structure of the cartilage of the gill arch does form a strict confinement of space for the trophozoites in this location and thus perhaps this is the reason why the spores remain smaller.

However, M. pseudodispar showed variability in the internal organs and, in both species, the origin of the fish made no difference to the spore dimensions. Consequently, it seems unlikely from these data that the variability in the spore dimensions of these two species is due to the biochemical/physiological nature of the immediate or outside environment per se, as suggested by Hine (1979) for other myxosporean species. Though these factors might have an influence in some cases, the variability is more marked in some myxosporean species than others, and thus might be a feature of that particular species. Such a feature might be considered to have survival advantage and might be due to the retention of a high degree of genetic polymorphism maintained by some form of selection. This trait could be related to their ability to make use of a large variety of host species, mainly cyprinids. It is interesting to note

here that M. pseudodispar is considered identical with M. cyprinii Doflein 1898 by Dykova and Lom (1984), a species showing a great variability in the shape of spores.

A better understanding of the life history of Myxobolus species would clarify some of these differences observed.

Comparison of the mature M. rhodei spores shows that in both geographic locations, the spores were smaller than those referred to in the literature (Bykhovskaya-Pavloskaya et al., 1964; Dykova, Lom and Gruphcheva, 1987).

Differences of the spore dimensions were observed between the different organs of large fish only in Greece, suggesting that, at least in the case of young fish, all three infection sites within the host are equally suitable for initial settlement of the infective stages of M. rhodei, which gradually stimulate tissue reaction resulting in the formation of cysts. This observation is most probably related to the fact that the cysts produced by M. rhodei were always larger in the kidney tissue and in older fish, especially in the Greek hosts, where the cyst dimensions differed significantly between the kidney and the rest of the organs. The cysts located in the muscles were smaller and rounder than the kidney ones. In both muscle and liver, the layer of connective tissue surrounding the cysts was found to be thin and early developmental stages of the parasites were not observed. In contrast, in the renal tissue the

host reaction varied from 2.8µm to 11µm (Greek fish) or 2.8µm to 16.8µm (British fish).

Hine (1979) also observed a great variation in cysts of Myxidium zalandicum in different species of eels in which the restraints imposed by the surrounding tissues, in this case gill intramellar space, were the same; and suggested that the cyst shape and position within one host species may be of taxonomic value as the site of sporogony can determine the cyst shape.

The critical factors at the site of sporogny are thought to be biochemical/physiological, contributing nutrients to or disposing of the metabolic wastes of the cyst, and that availability of space is of relatively little importance (Hine, 1979). This may explain the larger size of the cysts observed in the kidneys of roach in the present study, in comparison to the cysts found in the muscles and liver.

On the other hand, the size of the cysts and spores may be related to the age of the spore and trophozoite thus, the spores and cysts of younger fish are smaller than in the 2-3 year old fish. Consequently, the age of trophozoites and cysts might be significant if the infection is perennial. In relation to this, differences in the temperatures found between the Greek and British habitats may have a direct influence on the duration of the developmental period of the trophozoites/spores; thus, trophozoites in the Greek fish may

prolong their development even during the winter months and produce the larger cysts and spores found.

It is generally believed that the renal tissue in fish plays a more important role in host defence and immunity than in higher animals (Roberts, 1978) and the melanomacrophage centres have a substantial role in relation to this. Therefore, the renal tissue is apparently more efficiently equipped to resist any infection and the host reaction in this particular tissue could be anticipated.

Trophozoites, disporous pansporoblasts and immature spores of M. rhodei were also observed in this location. Trophozoites were found in the bowman's capsules of the glomeruli, the pansporoblasts within the cysts, and the immature spores (which were always smaller and wider than the mature ones) were found within the cysts and/or in conjunction with the pansporoblasts. The cysts produced by M. rhodei in the kidneys of British fish were smaller than the cysts of the Greek fish but the host reaction was more intense and may account for the smaller size if this could be seen to act as a constraint.

All the findings of the present study suggest that not only is the renal tissue the most commonly affected organ by M. rhodei, but the ability of this parasite to reproduce and expand in the renal parenchyma is facilitated in the kidneys of older fish. This may be due to different reasons. The maturation of the spores and their ability to reproduce and infect might be age-dependent as this is already suggested for the spores in the environment (Hoffman and

Putz, 1971). Older fish might be expected to be exposed for longer periods to the infecting stages of M. rhodei as with the other infective agents (parasitical, microbial, viral, etc. - Chapter 5) that may lower the host defence ability. Spores then accumulate in larger numbers in older fish. Furthermore, rupture of cysts may lead to autoinfection resulting in higher infection levels of larger fish.

It is also possible that released spores from ruptured cysts in other organs (liver and muscles) go directly into the kidneys as a result of the high vascular supply of this tissue. It is interesting to note here that Copland (1983) reported the presence of Myxidium spp. spores in the vessels and metastatic lesions in the organs of eels. Spores of M. oviforme were seen within macrophages in blood and tissue of S. gairdneri; following experimental infection with spores of M. oviforme (pers. comm. of C. Sommerville).

The dimensions of the mature spores were not found to be different amongst the four seasons in the case of Greek fish, suggesting that slight and short-term external environmental conditions do not contribute to M. rhodei spore variability. This was also suspected from data collected during the years 1984-85 (Athanassopoulou, unpubl. data).

The spore dimensions between the two geographic locations were different in both mature and immature spores, the Greek spores being always wider or larger than the British spores. Also taking into consideration that the M. rhodei cysts in Greek fish were much larger

than in British fish, it is feasible to assume that the development of the M. rhodei infection in the Greek lake is much more favourable than in the Yorkshire habitat.

The most obvious reason would be the differences in the environmental conditions, especially in the temperature, which may influence directly the development of the parasite. As mentioned before, the higher temperatures usually present in the Greek habitat may prolong the development of the trophozoites and cysts, thus producing more and larger cysts and spores. The temperature may also have an effect on the feeding activity of the fish, increasing the consumption of a larger number of intermediate hosts or detritus for longer periods (even during winter months) in Greece. As a result, a larger number of spores may be consumed. Intermediate hosts have been suspected to be implicated in the life cycle of other myxosporeans and in particular, Myxobolus cerebralis (Markiw and Wolf, 1963).

Other factors may also be present in the Greek environment which may influence the host susceptibility. Infections reported in the area (Ligula and coccidial infections) may have a key role in the development and expansion of M. rhodei in the hosts (Athanassopoulou, 1981; 1985; Athanassopoulou and Vlemmas, 1986) by debilitating the fish.

No trends were noted in variation in striation number and pattern as this was demonstrated by the scanning electron microscopy study on spores from different organs of the hosts and from different

geographic locations. It seems that striation number and pattern are probably genetically determined and not influenced by biochemical/physiological factors that modify shape and size, and thus as consistent features, more reliable for taxonomic identification. Indeed Hine (1979) has recommended that this be developed as one of the criteria for taxonomic purposes of Myxidium species. This is also endorsed by Lom and Arthur in their 1989 guidelines.

The dimensions of mature M. pfeifferi were within the range given in the literature (Bykhovskaya et al., 1964). The mature spores of M. pfeifferi showed no differences between older and young fish, in both geographic locations.

When comparing between Greek and British fish, significant differences were observed only in the spore width dimensions, the Greek spores being wider. The dimensions of immature spores, however, were longer in the Greek fish in both age categories.

All the above findings show a strong similarity to the variations on spore dimensions observed in the case of M. rhodei. In both species the age of the fish had no effect on the spore dimensions but the origins of the fish did; the spores from Greek fish being always larger.

Furthermore, no differences were observed in the dimensions of spores (mature and immature) of M. rhodei and M. pfeifferi in the Greek

fish. In British fish, however, the immature spores of M. rhodei were generally larger than M. pfeifferi immature spores.

These differences are probably due to two reasons:

a) either because the samples measured in each case probably represented different stages of development, or

b) as the immature M. pfeifferi spores were measured in the trophozoites in the bile ducts, these were probably constricted to a smaller space. The fact that no differences were observed between the mature spores of the two Myxidium species supports the above suggestions.

The variability of the spore dimensions between the different geographic locations may be due to several factors as discussed in the case of M. rhodei. It is interesting to note that differences in the dimensions of M. rhodei spores from Greece also differed from those quoted by Bykhovskaya et al. (1964) from USSR habitats, despite being well within the range given for the species.

The morphology and dimensions of mature M. rhodei and M. pfeifferi spores were found to be identical in both Greek and British samples by both light and scanning electron microscopy observations, including striation. It has been reported before that M. rhodei is morphologically quite similar to M. pfeifferi (Dykova et al., 1987) and that the only difference is the site of infection in the fish. In light of this, the results of the present study are quite significant. The identification of M. rhodei, an histozoic species, and M. pfeifferi, a coelozoic species, fits the description of other

authors for the same species. Further evidence is needed, however, either by experimental infection or histopathological study to demonstrate the possibility of these two species being identical.

FIGURE 22. Myxobolus pseudodispar spore. Fresh preparation. Interference phase contrast

x1000

FIGURE 22A Myxobolus pseudodispar spores in muscle demonstrating the polar capsules smear. Methylene blue

x400

FIGURE 23. Myxobolus pseudodispar spores in the kidney tissue. Note the shrinkage of the valves (arrow) and the thickening of the sutural line (double arrow)
SEM x5000

FIGURE 24. Myxobolus pseudodispar spore from muscle tissue or roach. Note the thickened sutural line (double arrow) and the opening (arrow) of the discharging canal of the polar filament
SEM x10,000

FIGURE 25. Myxobolus pseudodispar trophozoite. Some spores (arrows) are released

SEM x350

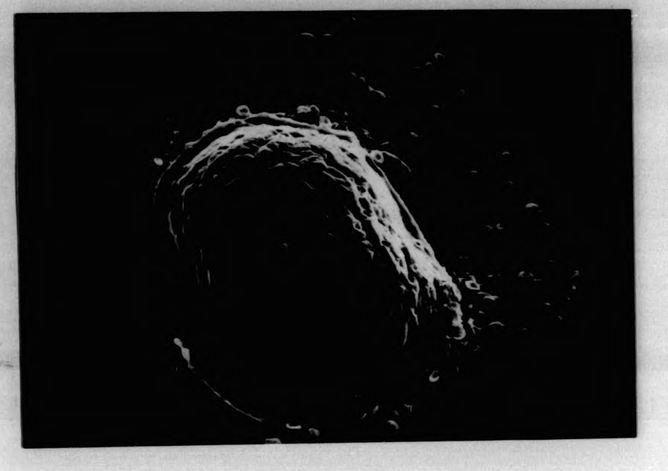


FIGURE 26. Myxobolus ellipsoides spores (arrows). Fresh preparation

x400

FIGURE 27. Myxobolus ellipsoides spores. Note that the valves are not shrunk and the polar filament (arrow) is extended.

SEM x3500

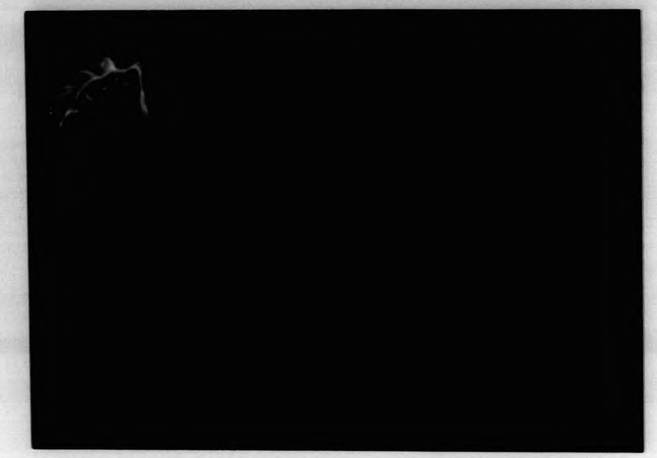


FIGURE 28. Myxobolus ellipsoides spore

SEM x5000

FIGURE 29. Myxidium rhodei spore, narrower centrally. Phase contrast x400

FIGURE 30. M. rhodei mature spore. Note the thickening of the sutural line (arrow) SEM x7500

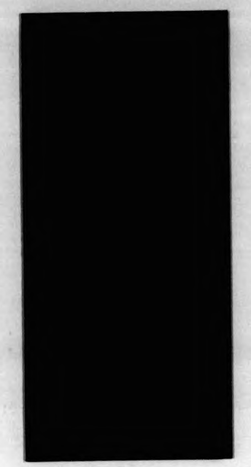


FIGURE 31. M. rhodei immature spore. Fresh preparation x400

FIGURE 32. M. rhodei immature spore from renal tissue. Note the absence of thickening in the sutural line (arrow) SEM x7500

FIGURE 33. M. rhodei immature spore (early stage of development) Fresh preparation. Phase contrast

x400

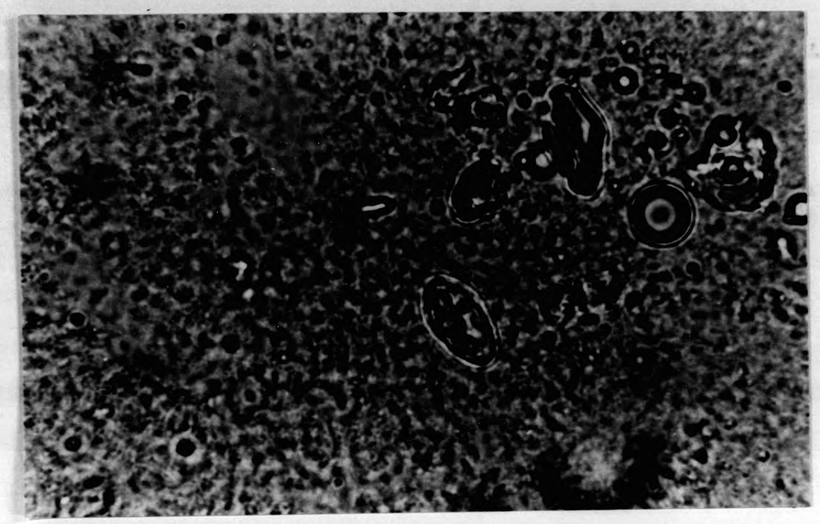


FIGURE 34. Cysts (Type A) containing mature M. rhodei spore (arrow). Note the internal sub-division. Fresh squash preparation

x250

FIGURE 35. Cyst (Type A) containing mature M. rhodei spores. The cyst is burst and some spores are apparent (arrow)
SEM x350

FIGURE 36. Higher magnification of Figure 35, showing the mature M. rhodei spores within the trophozoite
SEM x1500

FIGURE 37. Trophozoites of M. rhodei in glomeruli of roach. Note the cilia-like projections on the surface of the trophozoite and the two vacuoles in the cytoplasm. Semi-thin section
x1500 Methylene blue

FIGURE 38. Mature Myxidium pfeifferi spores. Note the thickening of the sutural line (arrow)

SEM x7500

FIGURE 39. Immature M. pfeifferi spores

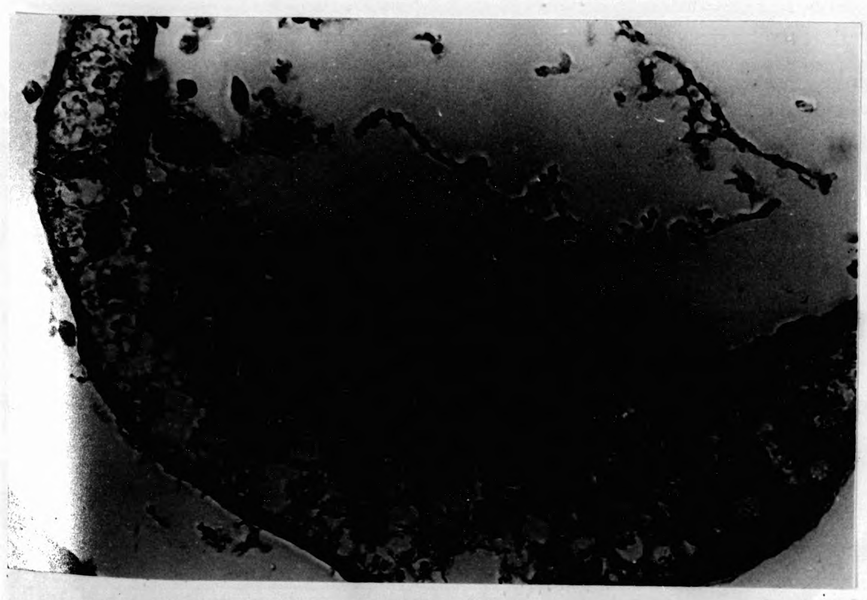
SEM x7500

FIGURE 40. Myxidium pfeifferi trophozoite in the bile duct. Note the deep-staining of polar capsules of the spores (arrow)

x400 Giemsa stain

FIGURE 41. Myxidium pfeifferi trophozoite in bile duct at an earlier stage of development. Immature spores are apparent (arrow)

x400 H&E



CHAPTER 5

INVESTIGATION INTO PATHOLOGY OF MYXOSPORIDA spp. AND IN PARTICULAR OF MYXIDIUM RHODEI USING LIGHT AND ELECTRON MICROSCOPY

5.1 INTRODUCTION

Most of the research on Myxosporea until 1980 concerned aspects of the biology of this parasite group, such as the life cycle, transmission studies and sporogenesis, whilst studies on pathology were neglected.

Even today, research on the pathology and host-parasite interaction of myxosporeans only reveals information on the species of major importance, in particular, Myxobolus cerebralis and Ceratomyxa shasta infections in salmonids. Although there is an extensive literature on other Myxobolus species comprising reports on incidence and records of new species, specific references to M. pseudodispar and M. ellipsoides in roach have only been made by Conzalez-Lanza and Alvarez-Pellitero (1985) and Athanassopoulou (1985), and they did not address the question of pathogenicity.

Infections of myxosporean genera such as Myxobolus, Henneguya, etc on gills, are very common in freshwater fishes and result in the production of many cysts which are white or yellowish in colour. These range from microscopic to those readily visible to the naked eye. The cysts may develop within and/or between lamellae or filaments of gills. According to Minchew (1973), in the intralamellar form, the cysts develop within blood capillaries of lamellae or gill

filaments. Interlamellar cysts which develop among epithelial cells between lamellae seem to be less restricted in growth potential. These may retain a definite shape or may become large masses surrounded by a relatively weak host tissue capsule. Tissue reaction to the interlamellar cysts is highly variable. Severe inflammation may induce fusion of lamellae of adjacent filaments. Heavy infections of either inter or intralamellar cysts may seriously impair gill function. Myxobolus exiguus infections in the gills of mullet in the Black Sea were reported by Shulman (1957) which caused significant losses by general impairment of the gill function and tissue damage resulting in bleeding and asphyxia.

Many myxosporeans, but especially Henneguya and Myxobolus species, frequently infect integumentary tissues, and underlying muscles and connective tissue of freshwater fish. Cysts may develop anywhere on the body surface and are often prominent and visible to the naked eye. It is sometimes impossible to distinguish the primary site of tissue invasion for all layers of the body wall may be invaded. In many cases there is little or no inflammatory response to the trophozoites. Sometimes, small blood vessels may be included in the capsule and some haemorrhaging may occur as the parasite grows. The overlying epidermis is often thin and may appear to slough or rupture as a result of pressure from the growth of the cysts. Spores may be released abruptly or gradually from such sites. In cases of diffuse infiltration, spores may be spread internally from the main site of infection to a variety of other tissues where further development may or may not occur (Mitchell, 1977), or they may find their way out of

the body into the environment, e.g. through the kidney (Molnar, 1989).

Myxobolus pfeifferi found in barbels (Barbus barbus L) primarily infects the connective tissue of the body musculature and may produce "boil" or "tuberoulcer disease" in these hosts (Mitchell, 1977).

There are numerous other species that cause similar, although somewhat less conspicuous, conditions in the integument, musculature, subcutaneous and visceral connective tissue of freshwater fish. In other cases, superficial infections are characterised by a temporary thickening of the overlying epidermis. Phagocytic cells are frequently found in the connective tissue capsule of older cysts.

Several definite cases of necrosis in different organs have been described in relation to histozoic species. Of particular interest is necrosis and lysis due to enzymes (proteolysis) which have been described by Patashnik and Groninger (1964) in the muscle tissue. Such necrosis and liquefaction are characteristic of certain marine myxosporean species, e.g. Hexacapsula nesthunni in tunas and several species of Kudoa in a variety of fish species. However, similar effects in freshwater fish are also known, especially in anadromous trout and salmon. In these cases, Henneguya and Myxobolus species are involved and intermuscular cysts in living hosts are often accompanied by local tissue necrosis known as "milky pockets". In harvested fish, softening and liquefaction of the tissue may occur soon after death, but most often these effects appear after several days of cold and frozen storage. Many of these conditions have not

been investigated fully and the "milkiness" described may be due either to debris of necrosed tissue or massive numbers of spores, or a mixture of both. Many muscle inhabiting myxosporeans may develop cytozoically within muscle fibres (Mitchell, 1970, 1977). Cross sections show that these trophozoites replace the sarcoplasm and may eventually fill the space within the sarcolemma. These intrafibrillar infections often occur with concurrent infections of intermuscular connective tissue (Mitchell, 1977).

In contrast to histozoic myxosporeans, coelozoic species are generally thought to be less harmful to hosts than histozoic forms. It is possible that the gall bladder is the oldest site of myxosporean infection and Myxidium spp are the most common parasites of this location.

Myxidium minteri has been described from salmonids and may be found in the gall bladder and liver (Sanders and Fryer, 1970) but may also concentrate within the kidney tubules and cause tubular degeneration. Myxidium oviforme causes a proliferation and dilation of the bile ducts of salmon due to the large numbers of trophozoites present in the lumen (Walliker, 1968; Sommerville, pers. comm.).

Histozoic species of Myxidium also exist but studies on their pathogenicity only scarcely appear in the literature; these studies mainly deal with new host records or records of infection sites for various species of Myxidium. In particular, Myxidium rhodei is considered to be a common parasite of freshwater fish known to occur

in 32 host species (Schulman, 1984). However, this parasite has been reported to have only a rare occurence in carp (Dykova and Lom, 1988).

A preliminary study of M. rhodei concerning its prevalence and pathology in roach from two lakes in Northern Greece was made by the present author (Athanassopoulou, 1985) but Dykova et al. (1987) recently provided information on the pathology of M. rhodei in Czechoslovakian and Bulgarian roach.

The tissue localization of M. rhodei had long been thought to be only the kidneys until Schulman (1984) suggested that M. rhodei also occurs in the liver and muscles albeit rarely. Further observations of Kerp (1987) confirmed these two additional sites of infection of M. rhodei in roach.

The kidney damage, according to the study of Dykova et al. (1987), was considered to be severe in heavy infections and was due to the loss of functional glomeruli as well as to the atrophy of the tissues surrounding infected glomeruli. These results were assessed by the authors by examination of infected material using light microscopy, and was based on descriptions of the nature of the tissue damage as seen histologically. No measure of the extent or significance to the whole organ was made.

Much of the available information on myxosporeans within tissues, however, results from studies aimed primarily at understanding the structural features of sporogonic development of myxosporean

parasites. Only two recent reports (Dykova et al., 1987 and Paperna, Hartley and Cross, 1987) deal with host-parasite relations in conjunction with the ultrastructure of the myxosporean parasites. Furthermore, these authors include the descriptions of the plasmodium as well as pathogenicity of members of the genus Myxidium.

Until 1959, the knowledge of myxosporean ulstrastructure had been quite neglected and confined to light microscope studies. Grasse (1960) published a preliminary report on Sphaeromyxa, stressing the pleuricellular character of the myxosporean trophozoite and the evidence that they do not form gametes, a fact which, together with other characters, prevents inclusion of this group among Metazoa.

Chessin, Schulman and Vinnitchenko (1961), Lom and Vavra (1963, 1964) and Lom (1964) investigated the ultrastructure and morphogenesis of the polar capsule of Myxobolus and Henneguya species and pointed out the resemblance between the polar capsule and coelenterate nematocyst formation, thus supporting the views of previous authors, i.e. Grell, (1956) and Chapman and Tilney (1959) who had excluded myxosporeans from the protozoan realm. These workers had proposed to treat myxosporidea as belonging to Mesozoa rather than to the phylum Protozoa on the basis of the phylogenetic relations between coelenterates and myxosporeans. A substantial study by Lom and De Puytorac (1965) on myxosporean ultrastructure and polar capsule development established the unity in the polar capsule development in all myxosporean orders. These authors also suggested that the sporoblast formation, starting by union of two cells is a uniform

process in both coelozoic and histozoic species; trophozoites, however, differ greatly in terms of size but also in aspects of morphology.

Further ultrastructural studies on myxospor ea appeared in the literature thereafter, concerning new data on the fine structure of different parasites of this group and their sporogenesis in fish (Desser and Paterson, 1978; Current, 1979; Lom, Dykova and Lhotakova, 1982) or invertebrates (Weidner and Overstreet, 1979). Even so, until the early 1980s, it was thought that relatively few myxosporean species were pathogenic. This however, probably reflected the lack of knowledge of host-parasite relationships for the majority of the species. As more information accumulates about the dynamics of wild fish populations and as more fish become commercially significant, detrimental effects of particularly, muscle and gill-inhibiting myxospor ea are being more frequently recognised. Indeed, during the period 1980-1989 much more information has become available on the pathogenicity of many species (Lom et al., 1982; Desser, Molnar and Horvath, 1983; Azevedo, Lom and Corral, 1989) reflecting the renewed interest and importance of this group of organisms.

In the present study, a specific interest in Myxidium rhodei infections was developed through the routine examination of commercially important roach populations in Northern Greece. As the extent of the lesions and the prevalence of the parasite in these fish was found to be high, a further follow-up of these cases was evidently becoming essential. Through a search of the relevant

literature, it became obvious that although M. rhodei was considered to be a common parasite of cyprinids, its pathology and effects on the hosts had never been seriously assessed. This aspect of the project was therefore initiated, aiming primarily to assess its pathology and pathogenicity in this fish species.

During the course of this study, other aspects of the life cycle and biology of the parasite were also examined as information on such aspects was very limited. Thus, it was possible to relate pathology to environmental and biological factors.

The relationship between coelozoic and histozoic species of Myxidium was never questioned despite the fact that morphological information suggested that no basic differences existed between some species, such as Myxidium rhodei histozoic in the kidney, and Myxidium pfeifferi coelozoic in the gall bladder (Dykova et al., 1987). Thus, it became more and more evident that interrelations with other myxosporean species should be included in the study, and as a result, Myxobolus infections and other lesions were also investigated. It was also necessary to take these into account when considering the impact of infection on the kidney.

The pathology was studied primarily using light microscopy. Electron microscopical examination was only performed when this was needed to elucidate inconclusive cases, or in order to provide more information on host-response where it was particularly lacking at the light level.

Through the examination of this pathological material over a period of time, it became evident that a method for assessment of the extent of the lesions would be essential because the cysts produced by M. rhodei were often very large and covered a considerable area of the small sections examined microscopically. After several trials the digital method described in 5.2.3 was found to be the most effective, since it could provide results expressed in terms of area and volume. It would appear that no such method has ever been used before to assess pathological conditions in fish tissues. Consequently, this is the first time that histopathological studies on myxosporean species have been investigated in detail and in relation to information on quantitative pathology. In addition, this is the first report on pathogenicity of M. pfeifferi in roach in general, and in the Greek habitats in particular.

As roach, and to a greater extent carp, represent an important component of the fishing industry in Greece, it is clear that diseases that may render the fish undesirable to consumers or even decrease their numbers in catches, have serious economic implications for the Greek economy. Therefore, any knowledge on pathology of such diseases, as presented in the present study, may contribute towards a better management control and conservation of fishing stocks.

5.2 MATERIALS AND METHODS

5.2.1 Light Microscopy Techniques

Tissue for examination by light microscopy was taken in the form of two 3-5mm transverse sections from each fish kidney after dissection, such that one section included anterior kidney and the other posterior kidney. In addition to these, sections of all internal organs and muscles were also taken. The tissues were fixed in 10% neutral buffered formalin for 24 hours (Disbrey and Rack, 1970) and then embedded in paraffin wax using a Reichert-Jung Histokinette 2000 automatic processor (Appendix II). The wax embedded blocks were cut to give 5-7µm thick sections using a Leitz, Wetzlar microtome and the sections were floated onto glass slides and then stained routinely with Haematoxylin and Eosin (Appendix II).

Any tissue producing sections showing evidence of parasitic infection with the Haematoxylin and Eosin stain, had further sections cut which were stained with Giemsa, PAS and Von Kossa methods (Appendix II). The Giemsa stain was used, as in the impression smears, in an attempt to demonstrate the presence of myxosporean polar capsules, and the nuclei of potential developmental stages of the parasite.

The PAS stain was used because it was thought to demonstrate successfully the sporoplasms more clearly than the Haematoxylin and Eosin method (Branson, 1987).

The Von Kossa method was used in an attempt to identify the nature of the calcareous deposits found often in the kidneys and to demonstrate any calcium deposits.

Light microscopy was carried out using an Olympus SH-2 with a 35mm camera.

5.2.2 Electron Microscopy Techniques

Samples of tissue taken for transmission electron microscopy were fixed in 2.5% glutaraldehyde in cacodylate buffer (Bullock, 1978). Samples from fish showing signs of myxosporean parasites under light microscopy were then used as samples for electron microscopy.

These samples were post fixed in 1% Osmium tetroxide and embedded in Emix or Lemix resin (Appendix II).

Sections were cut from the resulting resin blocks of 2µm thickness using an LKB Paratome. These sections were stained for one minute using Toluidine Blue, and then examined under a light microscope. Semi-thin sections of Lemix embedded material were also stained with Haematoxylin and Eosin. Areas of interest were identified and related to the blocks which were then trimmed down in order to leave a relevant area small enough for ultrathin sectioning.

Ultrathin sections of approximately 0.2µm thickness were cut using an LKB Ultratome and these sections were floated onto coated, 200 mesh ultra high transmission, electron microscope copper grids. These sections were stained using Uranyl acetate and lead citrate (Appendix II). The samples were examined under a JEQL, J 100G transmission electron microscope.

5.2.3 Method for the Quantitative Assessment of the Renal Pathology

The method used was a BBC-based image analysis system described by Hayes and Fitzke (1987) and Hayes (1988), for other biological applications.

Sections of the mid and posterior kidney at specific levels were examined in roach both infected and non-infected with M. rhodei. A special effort was made to ensure that the sections were dissected always from the same part of the mid or posterior kidney. 30 fish of each category were used and three sections, 5um thick, were cut from each fish. The cysts or glomeruli of these sections were digitised, according to the method referred to by Hayes (1988). The area under study (in μm^2) was then measured using Minitab software in a BBC microcomputer. The mean values of the measurements were calculated and these were compared to a marked standard area of the section. The marked standard area was randomly selected before the digitising

procedure in order to obtain accurate and statistically valid results. The volume of the tissue and cysts was calculated from the area and thickness of sections.

5.2.4 Water Analysis

Water Samples

The samples were collected from the lake in January and May 1987 using a Friedinger water sampler. Samples were taken from the surface and bottom at each station. Stations are shown in Fig. 1 of the Appendix I.

Samples were decanted from the sampler into approximately 100ml polyethylene containers previously rinsed in 50% nitric acid. The bottles were rinsed several times in distilled water and in the sample water before being filled and returned to the shore.

pH and temperature measurements were taken at the time of the sampling using a portable pH meter and mercury thermometer respectively.

Laboratory Analysis of Samples

1. Filtration of water for nutrients: The samples were filtered through 0.45 mmGF/C filter, previously washed with distilled water immediately after sampling. Then the samples were stored

in the fridge and processed in the same day.

2. Water analysis: Water analysis was carried out using a Hach DR-EL/2 system supplied by a spectrophotometer with conductivity, buret and tiration stand system.

Alkalinity: Alkalinity was measured using the titration method (APHA Standard Methods, 1980).

Carbon dioxide: Carbon dioxide was determined using the titration method described in APHA Standard Methods (1980).

Ammonia: Nitrogen, ammonia levels were determined using the cadmium reduction method (Hach DR-EL/2, 3 and 4 Methods Mannual).

Nitrite: Nitrogen, nitrite was measured using the Diazotization Method described in the Hach DR-EL/2, 3 and 4 Methods Mannual.

Nitrate: Nitrogen, nitrate was measured using the cadmium reduction method described in the Hach DR-EL/2, 3 and 4 Methods Manual.

Phosphorus: Phosphorus reactive was measured using the Ascorbic Acid Method (APHA Standard Methods, 1980).

Sulphate: Sulphate levels were determined using the Turbidimetric Method (APHA Standard Methods, 1980).

5.3 RESULTS

5.3.1 Histopathology of Myxobolus ellipsoides

M.ellipsoides spores were found in the kidneys, gills, spleen and cartilage of both Greek and British roach. In the kidneys, these were few and scattered in the renal parenchyma and in particular in the melanomacrophage centres. Only mature spores were observed in the kidneys, but no evident pathology associated with M. ellipsoides was found in the renal tissue. Mixed infections with M. rhodei, Eimeria rutili and Myxobolus pseudodispar were also observed.

In the gill arch, the spores were in groups and were located in the fibrous zone between the gill cartilage and the ceratobranchial element of the branchial arch (Fig. 42). In this location both mature spores and large trophozoites were found resulting in the formation of large, well-demarcated cysts. The pathological effect consisted of dislocation of the surrounding periosteum due to the size of the cysts and stenosis of the neighbouring vessels. In severe cases, necrosis of the cartilage occurred which was totally replaced by the trophozoites. In some specimens, after the maturation of the spores, these were released in the periosteum.

In the gill filaments, the parasites formed small, round trophozoites initially in the primary lamellae between the epithelial cells. As the development continued the size of the trophozoites enlarged and the primary lamellae were totally degenerated and the adjacent lamellae dislocated from the presence of the large trophozoites. A thin layer of connective tissue surrounding the infected primary

lamellae was also seen (Fig 43) as an attempt by the host to isolate the lesion.

The host response around the branchial mature cysts was also studied under electron microscopy. In these sections, at the electron microscopy level, a distinct zone between the limit of the parasitic cyst wall and the normal tissue was seen (Fig. 44). This zone seemed to represent the area where most of the metabolic activity of the parasite was taking place, such as nutrient absorption by the parasitic cyst or where metabolic wastes are dispersed into the surrounding zone. This area probably corresponds to the so-called "ectoplasm"; a term used by Current (1979) and Uspenskaya (1969).

Pinocytic vesicles were often present in this area indicating a major nutrient function. In latest stages of development the fibres of connective tissue could also be seen (Fig. 44). The host cells surrouding this zone of metabolic activity then often appeared stretched and flattened and encysted the spores which were located centrally (Fig. 45).

Trophozoites showing earlier stages of development were also seen under electron microscopy in the gill and gill arch, in which underdeveloped, immature spores were found. Inside the spores, the sporoplasm and early signs of the polar filament were present (Fig. 46). In more mature spores, the polar capsule contained a well formed polar filament forming four coils (Fig. 46A).

In severe cases, fusion of the primary lamellae was evident under light microscopy, necrosis of the epithelial cells and formation of very large cysts containing maturing and mature spores was observed. In some cases the filaments were totally destroyed and replaced by the massive group of free spores (Fig. 47).

In the spleen, the spores were concentrated in the melanomacrophage centres and no host reaction was usually detected. However, in only a few cases of the Greek roach, where there were heavy infections with M. ellipsoides also in the gills, the melanomacrophage centres of the spleen were enlarged and demarcated by a thin layer of connective tissue (Fig. 48). On these occasions Myxidium rhodei infection in the kidneys was also coincident.

5.3.2 Histopathology of Myxobolus pseudodispar

Spores of M. pseudodispar were found in the kidneys and muscles of both Greek and British roach. In the kidneys, the spores were scattered singly in the parenchyma but more often confined to the melanomacrophage centres of the parenchyma. No host reaction was observed in the renal parenchyma.

In the muscles, large trophozoites with developing spores and smaller cysts containing mature M. pseudodispar spores were found. The trophozoites size ranged from small to very large.

Large numbers of small multinucleated trophozoites were located between the muscle fibres (Fig. 49) and were easily detected by H & E stain. The muscle fibres showed longitudinal splitting and, in some cases, loss of striation (Fig. 49).

Cellular infiltration into the damaged area was also observed. In the most advanced stages granular degeneration occurred in either the entire fibre or in a portion of it, resulting in the collapse and disappearance of the fibres.

Large masses of free mature spores, occupying large areas of muscle tissue and totally replacing the fibres, were seen as the final process of the infection (Fig. 50). On one of these occasions, tubular bags, as these were described by Hibiya (1982), were also observed (Fig. 51). These bags represent fibres with phagocytes filling up their degenerated portions.

In older fish and especially in the British fish, the host response to the parasite appeared milder with formation of oval, medium-sized cysts containing maturing and mature M. pseudodispar spores. In sections examined under transmission electron microscopy, no inflammatory cells were observed in the immediate vicinity of these cysts. Inside the cysts spores of M. pseudodispar in different stages of development were observed.

In the early stages of spore development, the sporoplasm contained two large nuclei in the polar cell. The primordium of the polar

capsule and external tube which gives rise to the polar filament, were lying next to each other (Fig. 52). The nucleus of the valvic cell was also well formed.

During the maturation process of the spores, the polar capsules were found in different stages of the development (Fig. 53). In each polar cell the nucleus lay behind the polar capsule (Fig. 53) In older fish and especially in the British fish, the pathogenicity of the parasite appeared milder with the formation of oval, mediumsized cysts containing maturing and mature M. pseudodispar spores. The cysts were well demarcated by connective tissue with the formation of epithelioid cells in the periphery of the lesions (Fig. 54). Spores escaping from the host demarcated area were further surrounded by inflammatory cells.

In the British fish the small trophozoites were also surrounded by a mild infiltration of host inflammatory cells but appeared to be easily destroyed by the host, as evidenced by their degenerated appearance and the presence of melanin within the inflammatory area (Fig. 55).

Both Myxobolus spp. were stained slightly with H & E but well demonstrated by Giemsa and Mallory Trichrome stains.

5.3.3 Histopathology of Myxidium rhodei

No macroscopic findings were obvious in necropsy apart from a slight discolouration of the middle part of the kidney, obvious only in heavy infections. The parasite was found in the form of cysts and trophozoites.

I. **Cysts**

The cysts were embedded in the tissue of the mid and posterior kidney only. Two types of cysts were found in the interstitium, the Type A and the Type B cysts.

1. Type A:

The Type A cysts were large in size, oval or circular in shape, bounded by a distinct but thin layer of connective tissue. In some cases the Type A cysts were extremely large and subdivided into two or three internal masses of M. rhodei spores. This was particularly apparent in fresh material (Fig. 34) but was seen also in histological sections. The Type A cysts most often contained mature spores (Fig. 56). Less frequently, only amorphous material was present in the Type A cysts (Fig. 57). Calcification of the lesions was observed only in a few cases and was demonstrated by staining of the sections with Von Kossa. In a few cases, cysts containing a few mature M. rhodei spores, together with amorphous material, were also seen in histological sections (Fig. 57A).

In order to further determine the relationship between the mature spores and the presence of amorphous material in the cysts, all kinds of Type A cysts were subjected to tansmission electron microscopy. From this examination the following features could be recognised:

(a) In electron microscopy the wall of the Type A cysts which contained mature spores appeared stratified and consisted of layers of epithelioid cells joined by desmosomes (Fig. 58). Phagocytic cells were also seen between the connective tissue fibres surrounding the cysts, but their identity could not be classified in the material examined (Fig. 58).

(b) The cysts containing only amorphous material also showed the same stratified wall structure (Fig. 59).

(c) Cysts containing both amorphous material and spores were also similar in morphology to the rest of the cysts described above. In addition to the typical wall structure of the Type A cysts, the spores found in these types of cysts (c) were undergoing degeneration and the polar capsules were the last structures to degenerate.

From the above electron microscopy examination it was concluded, therefore, that all the Type A cysts were related and represented a sequence of events in the life cycle of the parasite as influenced by the host tissue. It would appear that after maturation and the production of mature M. rhodei spores by the trophozoite, the spores underwent degeneration. The exclusive presence of amorphous material in the cysts was considered, therefore, as one of the latest stages of development of the Type A cysts.

Pathology of M. rhodei Type A Cysts in the Kidney

In histological sections, the Type A cysts were only rarely associated with the melanomacrophage centres and did not normally exhibit serious host response. When in association with melanomacrophage centres, the Type A cysts were often seen in close contact with two or three Type B cysts (Fig. 60). Melanin deposition around large Type A cysts containing amorphous material was also observed in some cases (Fig. 61).

Only rarely did the renal Type A cysts stimulate a granulomatous inflammatory reaction and where this occurred, it coincided with a loss in stainability of the polar capsules within. The granulomas were characterised by the present of a wide zone of epithelioid cells but no giant cells of the foreign body type (Fig. 62).

In heavy infections, where the type A of cyst were very numerous and very large, the tissue surrounding the cysts was undergoing atrophy. Tubules in the close vicinity were found to be degenerated with sloughing of endothelial cells or vacuolation of their cytoplasm (Fig. 63).

Glomeruli were often compressed and dislocated and even reduced in size due to the present of the Type A cysts adjacent to them (Fig. 64). No other pathological changes in the interstitial tissue were observed at the light microscopy level.

Type A cysts containing M. rhodei spores in different stages of development were also examined under electron microscopy in order to study pathology in the surrounding tissue. In such examinations, a distinct zone was always seen between the Type A cysts and the host cells (Figs. 65A,B), similar to that observed for M. ellipsoides, which was characterized by the presence of pinocytic vesicles. In most mature cysts connective tissue fibres were present and in increased numbers (Fig. 65C).

The spores found in the centre of the renal Type A cysts always showed the characteristic arrangements of the two polar capsules (Figs. 66, 67) and spores in different stages of development were also observed. In the mature spores, the filaments formed four coils in the polar capsules.

Pathology of M. rhodei Type A cysts on other organs

When the intensity of the infection was high (IL.4) M. rhodei Type A cysts were also found in the adipose tissue surrounding the external surface of the kidney (Fig. 68).

Type A cysts were also observed in the liver, spleen (Fig. 69) and muscles of a few Greek fish. Some of these cysts contained mature M. rhodei spores and some degenerating material. In the spleen, melanomacrophage centres contained M. rhodei spores, demarcated by a thin layer of connective tissue without, however, any host reaction

(Fig. 70). In liver (Fig. 71) and muscles, the cysts were also demarcated by a thin layer of connective tissue without any significant host response.

When the Type A cysts in the liver tissue were examined in electron microscopy sections, these showed a similar appearance to the Type A cysts observed in the renal interstitial tissue. However, the "absorption" zone was narrower and the activity of the cells located in this area less marked. Only a few secretory cells were present but large numbers of connective tissue fibres were evident. The rest of the cells between the newly formed fibres usually underwent degeneration as this was observed in most sections (Figs. 72, 73).

2. Type B:

The Type B cysts were small, round and located also in the interstitial tissue of the kidney. They were readily distinguished from the Type A cysts by the presence of concentric layers of host reaction around them (Fig. 74).

These cysts only rarely contained M. rhodei spores, but more often they contained amorphous material which, in most cases, was calcified as this was demonstrated by the Von Kossa staining of the tissue (Fig. 75).

In histological sections these cysts were seen to provoke a serious host response, forming granulomas with the typical inflammatory cell presence. However, no giant cell formation or new capillaries were observed in any of the sections.

The Type B cysts were also related to melanomacrophage centres (Fig. 76) and in some cases, were also near blood vessels but no increase in melanomacrophage centre number or size was observed.

The Type B interstitial cysts were more often observed in young fish and in particular, in the Greek fish. Large fish from Greece and Britain showed similar lesions and host reaction.

The histological examination of the kidney lesions using different staining techniques and in particular, the Von Kossa staining, revealed the presence of calcium in both Type A and Type B kidney lesions (Figs. 75, 77). However, the calcification of the Type A lesions was less often observed. Calcification of the lesions found in organs other than the kidney was not observed.

On other occasions, however, small granulomas were found in the heart (Fig. 78). The local inflammatory reaction around them was characterized by a zone of epithelioid cells or macrophages. At the periphery, fibroblasts and lymphocytes were present. These lesions resembled the Type B cysts found in the interstitial tissue of the kidney and were always associated with infection of both cyst types,

but were never calcified and, therefore, did not fit readily into Type A or B categories.

II Trophozoites

In the kidneys, plasmodia of M. rhodei were found only in the Bowman's space of the renal corpuscles. This form of M. rhodei infection was limited and present only at specific times during the season and thus were not easily observed. Consequently, early trophozites in the glomeruli were found only in light microscopy and in semi-thin sections where serial sections could be made. Here the trophozoites appeared to be limited by a smooth, thin membrane. The endoplasm was highly vacuolated and fibrillar strands were expanded from the surface of the plasmodium and extended to both the capillary tuft and the Bowman's capsule (Fig. 79).

In histological sections, the infected glomeruli were enlarged and the Bowman's space, occupied by the plasmodium, was increased in size (Fig. 80). The walls of the capillaries were thickened and the capillary tuft compressed and shrunk (Fig. 81). The glomeruli appeared to undergo gradual atrophy and this was followed by the appearance of large cysts full of mature spores. The parietal layer of the Bowman's capsule was transformed to a thin membrane and the whole structure giving the impression of an interstitial cyst. In some cases debris-like material was found in the enlarged Bowman's space (Fig. 82). The parasite was found either in the periphery of

the Bowman's space or within the capillary tuft where it resulted in the division into two-three parts. In more advanced cases the tuft was completely shrunk, the surrounding membrane absent and the degenerating capillary tuft free without encapsulation in the interstitial tissue.

All the above noted lesions found in the kidneys of roach were asynchronous, different types of lesions occurring concurrently in the same fish. Furthermore, mixed infections with Eimeria rutili, Trichodina spp. and Sphaerospora were also seen together with M. rhodei cysts (Type A and B) in the kidneys of roach. Eimeria rutili was found in small cysts, almost always within the melanomacrophage centres. Trichodina spp. were always found in the urinary ducts, and Sphaerospora spp. mature spores and early developmental stages were observed in the renal tubules of some fish.

5.3.4 Histopathology of Myxidium pfeifferi

M. pfeifferi was found in the form of large trophozites containing pansporoblasts and maturing spores in the bile ducts, and in the form of mature spores found free in the bile fluid.

M. pfeifferi never coincided with the presence of M. rhodei trophozoites in the kidneys but, in some cases, M. rhodei mature spores in the liver and kidneys were found together with infections of M. pfeifferi in the bile ducts. In heavy infections the liver

around the gall bladder was found to be paler in colour and the gall bladder contents were thickened and lighter in colour.

The infection ranged from mild, with only one hepatic duct per viewing field infected, to massive infections destroying the surrounding hepatic cells (Fig. 83). The hepatic ducts containing large trophozoites were very enlarged and their epithelial cells showed signs of degeneration.

Small granulomas were also seen in the hepatic parenchyma next to the infected hepatic ducts (Fig. 84) but their origin was not clear. In a few cases of heavy infection, inflammation of the hepatic parenchyma around the ducts was also present (Fig. 83). Newly formed blood vessels, close to the infected ducts, were observed and these were associated with the existence of granulomas around the lesions (Fig. 85). In heavy infections too, the hepatic cells close to the ducts were seen to undergo vacuolar degeneration and even necrosis.

Despite the extensive sampling of infected material for electron microscopy, M. pfeifferi was not found in electron microscopy sections. Attempts to study the ultrastructure of the parasite in the bile ducts as well as the host response, by means of processing paraffin histological sections did not give satisfactory results.

5.3.5 Other Lesions

Another type of lesions in the renal tissue ("Type C" lesions) were also observed during the study period. These lesions were found only in Greek fish and were characterised by deposits of variable size and shape in the renal tissues. These were irregularly lobed and resembled inorganic material (Figs. 86A, B). The lesions were evident only microscopically and occurred in the tubules of the posterior and middle part of the kidney. In a few cases only debris-like material was present in the kidney tubules. When different staining techniques were used, the lesions proved to be positive with Von Kossa staining, indicating that calcium deposits were present. As the origin of these lesions was not evident, a further analysis of the water chemistry was considered essential in order to contribute to an explanation of the deposits within the tubules. Furthermore, the relationship between the calcified lesions was not clear particularly, since no evidence of a parasite presence was seen in the "Type C" lesion. In an attempt to examine the possibility that all types were related and to shed some light on their relationship, an analysis of their occurrence on a seasonal basis was also performed. This is presented in Fig. 87.

Records of the presence of "Type C" lesions appear only in the winter months (January, 1986, 1987, 1988 and February 1987). As the "Type C" lesions were found only in Lake Agios Vassilios in this study, the seasonality of the renal lesions is described only in this location.

Lake Agios Vassilios

Type A and B Lesions

These lesions were found constantly in all samples throughout the year 1987 and also in the January 1986 and January 1988 samples. The overall prevalence for both A and B lesions was 23.66% (115/486). These fish were also infected with M. rhodei in the kidneys.

Type C Lesions

The lesions were found only in the months of January (1986, 1987 and 1988) and of February 1987. The overall prevalence was 5.3% (26/486) and it remained low in all samples where they were found.

5.3.6 Environmental Parameters

The presence of the calcified lesions in the roach kidneys suggested that a closer look at the water chemistry might contribute to an explanation of the deposits within the tubules. The water samples were taken during two sampling periods and these are shown in Tables 36 and 37. The temperature results showed that Lake A Vassilios was well mixed at both times of sampling. The pH and alkalinity measurements showed that the lake contained very alkaline water; the measurements were similar at both sampling times.

The level of inorganic nitrogen (ammonia, nitrite and nitrate) was within the limits approved for cyprinid aquaculture. Nitrite and

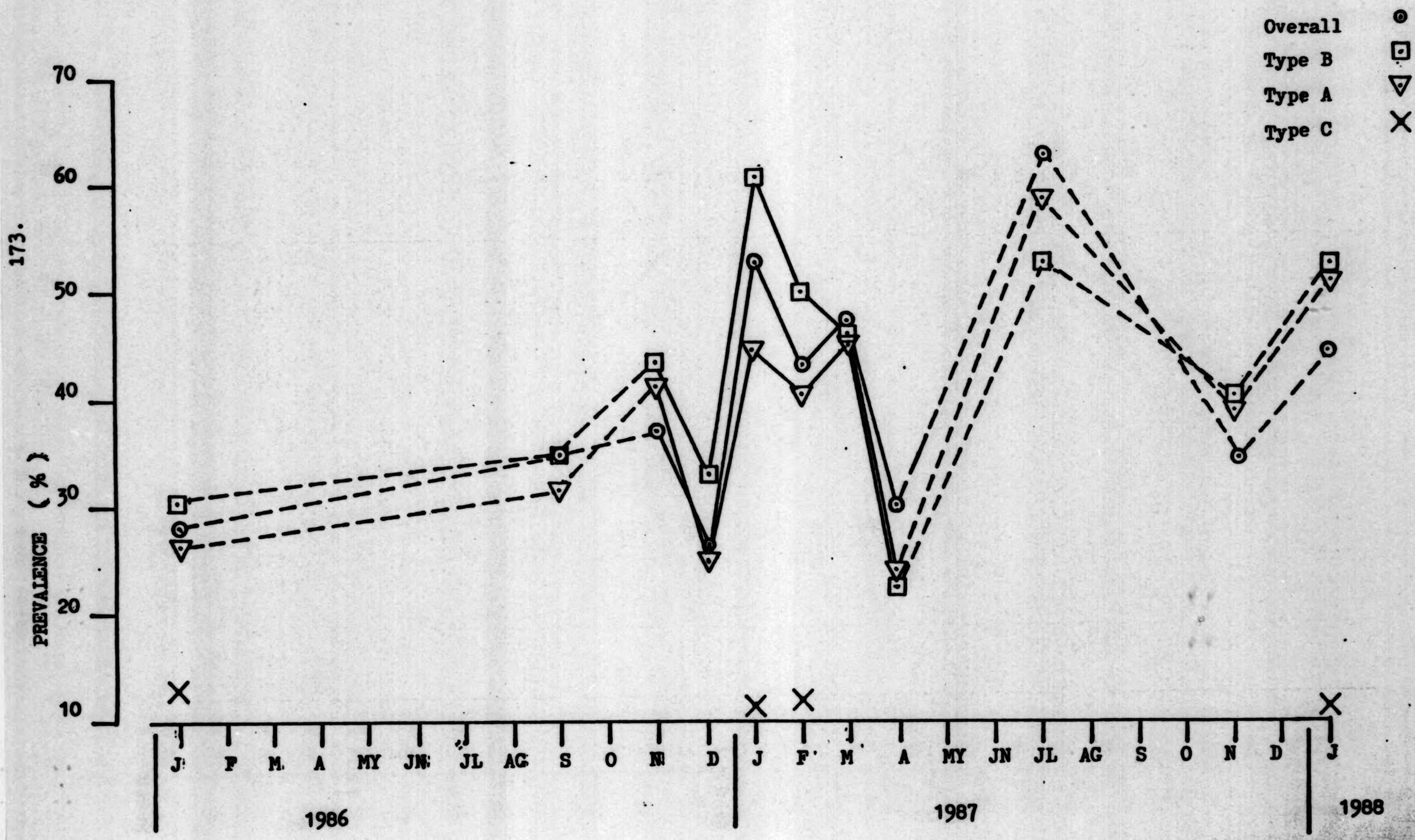


FIGURE 87: Showing months when fish were sampled and the prevalence of type A, B and C lesions found in Lake A. Vassilios

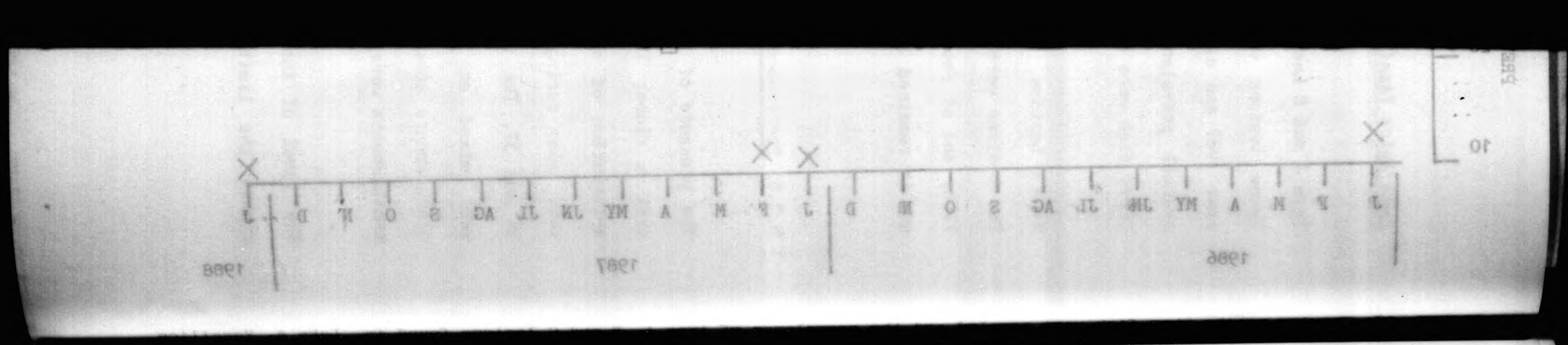


TABLE 36

Water analysis results from the Lake Agios Vassilios during May 1987

Sample No.		Alkalinity ($CaCo_3$ mg/l)	CO_2 $mgCO_2/L$	N-NH $mgNH_3/L$	N-nitrate $mgNO_3/L$	N-nitrite $mgNO_2/L$	P $mgtSO_4/L$	S $mgPO_4/L$	pH	t (°C)
1	S	325	1.3	>0.1	0.1	0	0.1	50	7.16	11
	B	328	1.35	> 0.1	0.1	0	0.2	50	6.91	11
2	S	350	1.2	0	0	0	0.8	58	7.24	11
	B	348	1.2	>0.1	0	0	1.2	58	7.60	11
3	S	392	2.4	0	0.15	0	1	52	7.20	11
	B	400	2.4	0	0.15	0	1.2	52	7.27	11
4	S	410	1.2	0	0	0	1	51	7.31	11
	B	420	1.2	0	0	0	1.1	51	7.31	11
5	S	420	1.1	0	0.2	0	1.2	50	7.65	11
	B	420	1.1	0	0.2	0	1.2	50	7.77	11

S = surface sample

B = bottom sample

TABLE 37

Water analysis results from the Lake A Vassilios during January 1987

Sample No.		Alkalinity $CACO_3$mg/l	CO_2 $mgCO_2$/L	N-NH $mgNH_3$/L	N-nitrite $mgNO_2$/L	N-nitrate $mgNO_3$/L	S $mgtSO_4$/L	P $mgPO_4$/L	pH	t (°C)
1	S	310	20	0	0.0221	2.20	60	2.5	6.5	6
	B	310	21	0	0.021	2.25	60	2.5		
2	S	315	20	0	0.968	2.64	68	1.79	6.5	6
	B	315	20	0	0.0168	2.64	68	1.80	6.7	6
3	S	300	20	0	0.0271	2.20	66	2.36	6.5	6
	B	301	20	0	0.0271	2.20	66	2.65	6.6	6
4	S	310	21	0	0.0174	2.20	70	1.45	6.5	6
	B	310	20	0	0.0174	2.20	70	1.45	6.6	6
5	S	310	20	0	0.0232	1.78	72	1.38	6.7	6
	B	310	20	0	0.0232	1.78	72	1.49	6.7	6

S = surface sample

B = bottom sample

nitrates were low and well below any toxic thresholds (EIFAC Tech. Paper, 1984).

Phosphate values ranged between 0.1-2.65 mg/PO_4/1 and these are considered high and indicative of a eutrophic water (Kilikidis et al., 1984; M Philips, pers. comm.; J Tallig, pers. comm).

5.4 RESULTS OF THE QUANTITATIVE ASSESSMENT OF THE RENAL PATHOLOGY

5.4.1 Image Analysis Results of M. rhodei cysts in the kidney tissue

The results of the image analysis of kindeys infected with M. rhodei in Greek and British fish are shown in Tables 38 and 39, respectively.

The results showed that the area and volume occupied by the M. rhodei cysts in the posterior part of the kidneys was larger than the area and volume of the cysts located in the middle part of the organ in both Greek and British hosts (Tables 38 and 39). The posterior kindey was more intensively parasitised (four cysts/area unit) than the mid-kidney (three cysts/area unit) in both Greek and British fish. The percentage of the area and volume occupied by the cysts in the posterior kidney was slightly higher than that in the mid kidney, in both Greek and British fish (Tables 38 and 39).

The area occupied by cysts in the mid-kidney in Greek roach was found to be 5.9 times bigger than the respective area occupied by the

TABLE 38

Results of Image Analysis of M. rhodei cysts in the kidneys of Greek roach

	Mid-Kidney	Posterior Kidney
Mean area of M. rhodei cysts (mm^2)	15.598×10^{-3} ($\pm$ 3.12)	16.384×10^{-3} ($\pm$ 3.8)
Minimum area of M. rhodei cysts (mm^2)	10.183×10^{-3}	10.004×10^{-3}
Maximum area of M. rhodei cysts (mm^2)	22.334×10^{-3}	21.43×10^{-3}
Mean number of M. rhodei cysts observed per area unit	3	4
Mean area of tissue infected (%)	0.37	0.40
Mean volume of tissue infected (mm^3)	48.04×10^{-3}	52.8×10^{-3}
Ratio between M. rhodei cysts and glomeruli area	5.9	6.67
Ratio between volume of cysts and volume of viewing field	1:7.29	1:6.7

TABLE 39

Results of Image Analysis of M. rhodei cysts in the kidneys of British roach

	Mid-Kidney	Posterior Kidney
Mean area of M. rhodei cysts (mm^2)	11.368×10^{-3} ($\pm$ 4.1)	13.442×10^{-3} ($\pm$ 3.8)
Minimum area of M. rhodei cysts (mm^2)	6.443×10^{-3}	6.992×10^{-3}
Maximum area of M. rhodei cysts (mm^2)	14.843×10^{-3}	14.774×10^{-3}
Mean number of M. rhodei observed per area unit	3	4
Mean area of tissue infected (%)	0.25	0.26
Mean volume of tissue infected (mm3)	32.10×10^{-3}	35.8×10^{-3}
Ratio between M. rhodei cysts and glomeruli area	3.57	3.71
Ratio between volume of cysts and volume of viewing field	1:10.9	1:10

glomeruli and 6.67 times in the posterior kidney (Table 38). This indicates that, apart from the functional damage to the glomeruli which would correspond to an area of $2.69x10^{-3}mm^2$ of normal glomeruli, an additional pathology must be assumed to exist due to the enlargement of 5.9 or 6.67 times of the glomeruli due to the infection with M. rhodei. Furthermore, damage to the surrounding interstitial tissue also exists due to the pressure of the enlarged glomeruli. This was confirmed in the histopathological study and can be seen in Fig. 81.

The same comparison for the British roach, however, showed that the cysts' area in the mid and posterior kidney, was 3.57 and 3.71 times bigger, respectively, than the normal glomeruli area in the same organ locations (Table 39). The volume of the cysts in the mid and posterior kidney was found to be $48.04x10^{-3}mm^3$ and $52.8x10^{3}mm^3$ respectively, for the Greek fish. For the British fish this was $32.10x10^{-3}mm^3$ and $35.8x10^{-3}mm^3$ respectively. When comparing the ratios between volume of normal glomeruli and the volume of cysts in relation to the volume of their viewing field, then it was found that these were 1:72 and 1:573 for the Greek fish and 1:10.9 and 1:593 for the British fish (Tables 38-42). This means that, for example, in the case of the posterior kidney, the normal glomeruli in this area have increased their volume 78 times (for the Greek fish) and 54 times (for the British fish).

5.4.2 Results of Image Analysis of Normal Glomeruli in the Kidney Tissue

These results are presented in Tables 40 and 41.

The mean area and volume occupied by normal glomeruli in the renal tissue is almost the same in the posterior and mid kidney of the fish (Tables 40 and 41).

The number of glomeruli found per unit area was always constant in both locations of the kidney tissue as well as in both Greek and British fish.

The percentage of area occupied by the normal glomeruli in mid and posterior kidney was found to be 0.06% and 0.07% for Greek and British roach, respectively (Tables 40 and 41).

The volume occupied by normal glomeruli in mid and posterior kidney of Greek and British fish was found to be $6.6x10^{-3}mm^3$ and $6.4x10^{3}mm^3$ and $5.9x10^{-3}mm^3$ and $6.14x10^{-3}mm^3$ respectively.

Statistical Analysis

A statistical comparison of the rea/volume of kidney tissue occupied by the *M. rhodei* cysts with the area/volume occupied by normal glomeruli was carried out in order to give some indication of the

TABLE 40

Results of Image Analysis of normal glomeruli in the kidneys of Greek roach

	Mid-Kidney	Posterior Kidney
Mean area of glomeruli (mm^2)	2.757×10^{-3} ($\pm$ 0.95)	2.794×10^{-3} ($\pm$ 0.99)
Minimum area of glomeruli (mm^2)	1.093×10^{-3}	1.098×10^{-3}
Maximum area of glomeruli (mm^2)	4.664×10^{-3}	5.338×10^{-3}
Mean number of glomeruli observed per area unit	6	6
Mean area of glomeruli (%)	0.06	0.06
Mean volume of glomeruli (mm3)	6.4×10^{-3}	6.4×10^{-3}
Ratio between volume of glomeruli and volume of viewing field	1:573	1:546

TABLE 41

Results of Image Analysis of normal glomeruli in the kidneys of British roach

	Mid-Kidney	Posterior Kidney
Mean area of glomeruli (mm^2)	2.693×10^{-3} (± 0.88)	2.784×10^{-3} (± 0.85)
Minimum area of glomeruli (mm^2)	1.126×10^{-3}	1.257×10^{-3}
Maximum area of glomeruli (mm^2)	4.694×10^{-3}	4.824×10^{-3}
Mean number of glomeruli observed per area unit	5	6
Mean area of glomeruli (%)	0.07	0.07
Mean volume of glomeruli (mm^3)	5.9×10^{-3}	6.15×10^{-3}
Ratio between volume of glomeruli and volume of viewing field	1:593	1:569

impact of the parasite on the kidney in terms of area/volume of normal tissue replaced by the parasite.

According to this statistical analysis of the areas occupied by the cysts and normal glomeruli in mid and posterior kidney they showed that these differed significantly (F=755.19, P=0 and F=789.6, P=0, respectively). Similarly, the differences in the values of the volume between normal glomeruli and cysts in mid and posterior kidney were also significant (F=638, P=0 and F=695, P=0, respectively).

5.5 DISCUSSION

The infections of roach with the two Myxobolus species studied in this project, showed that the kidney was not the most important site of infection. No pathological changes were detected in this location and the parasite spores seemed to be concentrated and limited to the melanomacrophage centres and it might be assumed that they had been deposited in this site by macrophages from a source elsewhere in the body. Spores of Myxobolus pseudodispar in the melanomacrophage centres have also been reported by other authors (Roberts, 1975; Dykova and Lom, 1988; Dykova, 1984). In the melanomacrophage centres, the spores are believed to be digested and completely eliminated (Dykova and Lom, 1988).

The pathology, however, in the other sites of infection of these parasites was quite severe, and in heavy infections was estimated to result in severe functional disturbances of the hosts. However, the pathology of M. ellipsoides was considered less serious than that of M. pseudodispar since extensive lesions were only rarely seen. In contrast, in the case of M. pseudodispar, the infections of early trophozoites in the muscles would be very important in young fish, resulting in degeneration of a large part of the myomeres.

As roach can be a desirable species for human consumption in particular areas of Greece, this infection may have some economic importance in the local market. Although there is a lack of data concerning transmission of these parasites from one fish species to

another, it might be suspected that carp populations would be infected too since the host range of M. pseudodispar is wide (Bykovskaya et al., 1964).

If carp populations are implicated in these infections, the economic losses would be more severe. Taking also into consideration that the carp population in this lake has recently declined (Kilikidis et al., 1984) and research has shown that natural reproduction of carp in the lake is not possible any more because of the pollution of the environment, it is clear that an additional Myxobolus spp. infection would further reduce the financial losses. This is the first report on the pathogenicity of these two Myxobolus spp. apparently in roach.

The study of the pathology of M. rhodei in roach populations showed that this parasite has a more severe pathogenicity for the fish fish hosts than it was indicated by other researchers (Dykova et al., 1987; Kerp, 1987). A description of the pathogenicity of this parasite has recently been published (Dykova et al., 1987) following histological observations of renal tissue. However, their study was confined to the kidney whereas it has been shown here that the parasite is also present in liver, spleen and muscle tissues.

In the present study the most common site of infection was the interstitial tissue of the kidney, where both Type A and Type B parasite cysts were observed. The Type A cysts appeared to originate from infected glomeruli after degeneration and disappearance of the capillary tuft due to the presence of early stages of M. rhodei. The

most likely origin of the Type B interstitial cysts is the appearance of trophozites which initially locate in the interstitium and thus provoke serious host reaction which results in the rapid death of the parasite and the final formation of small granulomas. These results are in accordance with the findings of previous authors (Dykova et al., 1987) who demonstrated the existence of early trophozoites in both glomeruli and interstitial tissue. In this study, however, early trophozoites were observed very rarely and only in the glomeruli, although a thorough seasonal examination of material was carried out. This must be due to the speed of the development of early stages of M. rhodei, especially in Lake A Vassilios, where the most detailed study was carried out. This has also been suggested by Dykova et al. (1987). Another explanation for their rarity would be the different factors involved in the habitat which may result in a slightly different development of the life cycle of the parasite in the Greek fish.

In this study the pathology of M. rhodei and M. pfeifferi have been considered separately. However, it was suggested in Chapter 4 that these two species are indistinguishable. In addition, in 5.3.4 it was mentioned that M. pfeifferi trophozoites never coincided with M. rhodei trophozoites in the glomeruli; thus supporting the suggestion that these two species are identical. The pathology, therefore, of this single species is more serious for the host because it involves several organs.

The presence of M. rhodei cysts in other organs, namely spleen, liver, muscles and heart, is very interesting. M. rhodei cysts in muscles and liver have been reported before (Kepr, 1987) but no account of the pathology was given. There is no other report of M. rhodei in spleen or heart. The lesions in these organs were well demarcated with (in the case of the heart) or without (in the other organs) host reaction. The lesions in these organs might possibly be metastatic due to haematological spread of spores or perhaps, generative cells from ruptured mature M. rhodei cysts in the kidney. The presence of spores in the vascular system was not demonstrated, however, in this study, but the M. rhodei lesions in the different organs were occasionally found close to vessels. Copland (1983) also suggested a similar process for Myxidium giardi in eels and did observe spores in blood. On the other hand, if spores require to leave the host before further development, as for example when an intermediate host is required (Wolf and Markiw,1984; Markiw, 1989), then spread of the infection into different organs must be at a presporogenic stage followed by asynchronous development in the different organs.

M. pfeifferi trophozoites were found to be quite pathogenic in heavy infections and serious damage to functional liver parenchyma may occur. There are only a few detailed studies on the pathogen of coelozoic Myxidium spp. in the bile ducts and gall bladder. Zschokkella russelli recorded from the gall bladder of British rocklings was found to produce proliferation, enlargement and thickening of the hepatic ducts, lowering of the duct epithelium and perichólangitis (Davies, 1985). Sinusoidal dilation and thickening

around hepatic ducts was also shown by the same author in a further study of the same parasite (Davies and Sienkowski, 1988).

Most information on coleozoic myxosporeans concerns reports of Myxidium occurrence, distribution and prevalence in different fish species and areas. For example, Myxidium minteri was found primarily in the gall bladder by Sanders and Fryer (1970) but cysts containing spores were occasionally found in the liver tissue. No pathology was described, however.

Some other reports only include observations of a clinical nature, i.e. an enlarged but not discoloured gall bladder was observed by Morrison and Pratt (1973) during an infection with Sphaeromyxa but no details of histopathology were given. In other coleozoic myxosporean species, macroscopic changes in some gall bladder contents have been reported and reviewed by Mitchell (1970). These concern mainly abnormalities in colour (light yellow, orange or cloudy).

The only papers describing the pathology of coelozoic myxosporean species is that of Dykova and Lom (1988). The authors described necrosis in the liver of carp in intensive culture due to Chloromyxum cyprini.

More recently Paperna, Hartley and Cross (1987) studied the ultrastructure of Myxidium giardi and its attachment to the epithelium of the urinary bladder of Anguilla mossambica. According to the authors, this was the only paper describing the form of attachment of

coleozoic plasmodia to the host tissue up to 1987. However, in 1980, Mitchell, Listebarger and Bailey also described, although not in detail, the method of attachment of Chloromyxum trijugum and the related histopathology of this myxosporean parasite in centrarchid fishes.

Most of the remaining and even more recent publications describe the ultrastructure of trophozoites or investigations of their life cycles (Azevedo, Lom and Corral, 1989; Molnar, 1989). According to the available literature this present study appears to be the only report on M. pfeifferi pathogenicity in roach.

The final stage of both Type A and Type B cysts was found to be the calcification. A similar process is commonly found in advanced lesions in higher mammals and has also been suggested in fish (Leger, 1931, cited by Dykova et al., 1987). However, Dykova et al. (1987) did not observe this in their study.

Calcium deposits have, however, been reported to be associated with a number of different conditions in fish; notably nephrocalcinosis was reported to be related to high levels of free CO_2 in hatchery water by Harrison and Richards (1979). Nutritional factors were also implicated in this condition (Landolt, 1975). Dietary factors were thought to be the cause of Visceral Granuloma which also involves renal lesions containing calcium (Landolt, 1975). High levels of dietary selenium were responsible for nephrocalcinosis in rainbow trout in experiments carried out by Hicks, Hilto and Ferguson (1984),

Cowey, Knox, Audron, George and Pirie (1977), produced renal calcinosis by feeding rainbow trout a magnesium-deficient diet. A syndrome described by Blaker and Wolke (1983) included renal tubular calcium oxalate crystals in cultured Amphiprion ocellaris which was associated with deficiency of the fat soluble vitamins A and E and lipid peroxidation in the diet. Dietary factors as well as environmental factors were thought to interact in the systemic granulomatous condition which involved calculi in uriniferous ducts described by Paperna, Harrison and Kissil (1980).

Infections agents are more rarely implicated in lesions containing calcium deposits in fish. However, they were found unusually in tuberculosis of aquarium fish by Mageed, Gopinath and Jolly (1981) and in lesions resulting from experimentally induced PKD by d'Silva, Mulcahy and De Kinkelin (1984). It is also interesting to note here that Wooten and McVicar (1986) found considerable amounts of debris in the kidney tubules and evidence of degeneration of these tubules in farmed sea trout and Atlantic salmon, due to an intracellular renal parasite. However, the authors did not notice any calcification, but macroscopic changes of the kidneys and an increase of lymphocytes and neutrophils were observed in the haemopoietic tissue.

Taking into consideration the life cycle of M. rhodei and the pathological lesions found by Dykova et al. (1987) in the kidneys or roach from Bulgaria and Romania as well as the results of this study, the following events are suggested.

- The Type A lesions found in the renal interstitial tissue possibly arose from the later stages of M. rhodei trophozoites which were initially located in the glomeruli which were subsequently destroyed by the parasite and disappeared, leaving the parasite surrounded by interstitial tissue. The host response, obstructing the further development of the trophozoite or, following death due to senility, could result in degeneration and calcification as happens commonly in higher mammals (Dunne and Leman, 1975; Cheville, 1983).

- The Type B lesions found in the renal interstitial tissue were possibly the remains of M. rhodei trophozoites which located directly in the interstitial tissue, such as those interstitial forms observed by Dykova et al. (1987). In this location, the host response was possibly very effective and resulted in the death and rapid degeneration and encapsulation of the early trophozoites. However, such early trophozoites in the renal interstitial tissue were not observed in the Greek and British samples examined in the present study, but it is possible that these stages last for only a short period and only in specific seasonal samples, and therefore not represented in the fish samples examined. It is worth noting here that the immature (i.e. without mature spores) trophozoites of M. rhodei in all samples of this study were rarely observed and in only a small number of fish, in contrast with the findings of Dykova et al. (1987) who found them very often in their samples.

- The few nephrocalcinosis-like lesions in the tubules of the fish (Type C) were thought not to be related to M. rhodei infections and were possibly due to environmental conditions.

The Lake A Vassilios in Northern Greece from where the tubular lesions of the roach were found, is considered to have undergone eutrophication as a result of pollution from domestic waste. The fish populations in this lake are a cause for concern and growth and production are affected, particularly in carp (Kilikidis et al., 1984). The lake has a high alkalinity compared to most European lakes and the salinity is generally high, with Na^+ being the dominant cation (J Tallig, pers. comm).

There is a paucity of data on carbon dioxide levels of natural water bodies; the values obtained from the Greek lake samples in the past reached a maximum of 20 µg/l in winter time (Athanassopoulou, 1985). That this is an elevated level of CO_2 is evidenced by the fact that the lowest pH values in winter are lower than would be expected at air equilibrium (from the alkalinity) by about two pH units (J Tallig, pers. comm). In highly productive waters such as this, CO_2 levels often reach higher values. The CO_2 levels found experimentally to induce nephrocalcinosis in rainbow trout by Smart, Knox, Harrison, Ralph, Richards and Cowey (1979) ranged from 12-60 µg/l. It is possible, therefore, that in winter time in Lake A Vassilios when the CO_2 levels are high, roach becomes susceptible to environmental conditions during the time the Type C lesions are formed in the kidney tubules. This is further supported by the fact that Type C lesions

were never found in the British lakes. Further experimental evidence is needed, therefore, to prove the direct effect of the high CO_2 levels in water on the roach kidney metabolism.

An alternative explanation would be that the calcification was due to the calcification of tubule-invading parasites such as Sphaerospora spp., Hoferellus spp. etc. Such parasites have been reported to produce calcified casts in the lumen of the tubules (Molnar, 1980). Sphaerospora spp. was also found in the Greek and British samples in this study.

The results of the Quantitative Method using Digital Analysis showed that glomeruli infected with M. rhodei can be enlarged up to seven times in the case of Greek fish and up to four times in the British fish. The extent of the damage, therefore, in terms of glomeruli function is quite severe. The reasons for the difference in size effect between the Greek and British infections is not clear but is likely to be the temperature effects on size/growth relations in a sub tropical versus a temperature habitat. In addition to the replacement of normal tissue by these enlarged glomeruli, the atrophy and pathological changes observed in the area surrounding the cyst tissues, as described by the histological examination, further contributes to the overall impact of this parasite on the kidney. The overall kidney tissue area affected, therefore, is quite extensive and almost certainly results in severe functional disturbances in the host.

The method used for quantitative assessment of the kidney pathology also confirmed that the infections in the Greek habitat is more severe than in the British environment where this study was carried out. This is supported by the seasonal studies of prevalence and intensity and the results of the morphological studies (Chapters 3 and 4).

This is the only report on quantitative assessment of pathology using image analysis in fish tissues.

FIGURE 42. Group of Myxobolus ellipsoides spores located in the fibrous zone between gill cartilage and ceratobranchial element of the branchial arch

x200 H&E

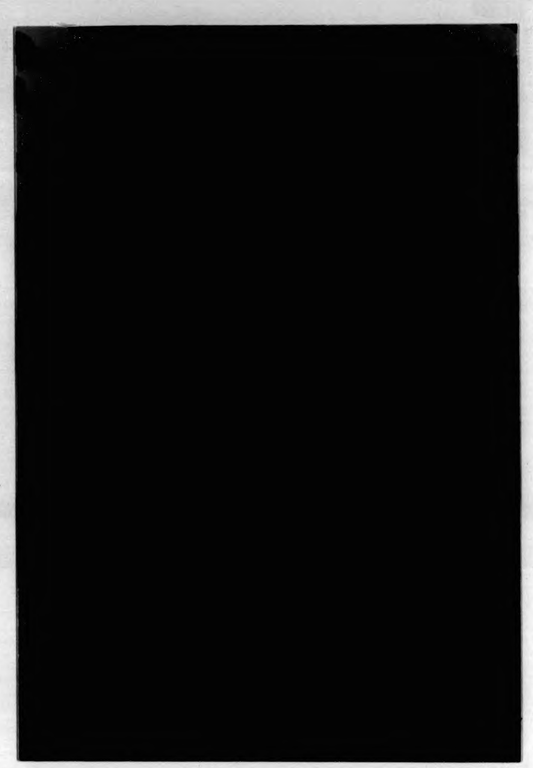


FIGURE 43. M. ellipsoides cyst showing thin layer of connective tissue (arrow) surrounding the infected primary lamellae

x200 H&E

FIGURE 44. Zone between limit of M. ellipsoides parasitic cyst wall and normal tissue. Pinocytic cells (PC) and fibres of connective tissue are present (F)

TEM x11,700

PC = pinocytic cell
Ch = cell of the host
F = fibres
D = ddesmosomes
Sp = M. ellipsoides spore
Lt = limit of trophozoite
Za = zone of absorption
G = golgi apparatus

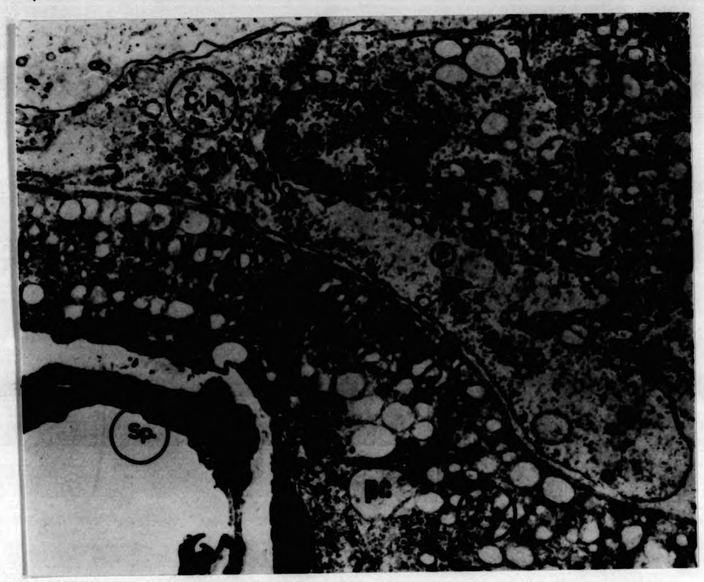


FIGURE 45. Interface between Myxobolus ellipsoides cyst. The host cells surrounding the zone of absorption are stretched and flattened and encyst the spores which are located centrall (Sp)

TEM x18,700

Sp = M. ellipsoides spores
Ch = cell of the host
Tr = trophozoite

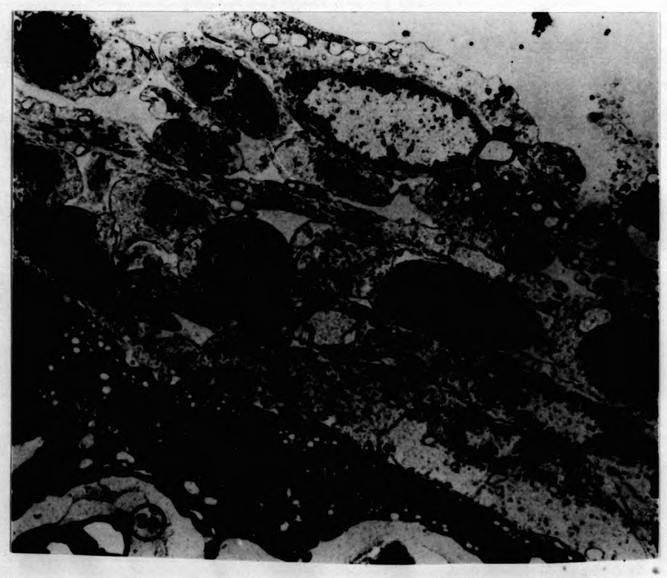


FIGURE 46. Immature M. ellipsoides spore

TEM x18,700

S = sporoplasm
Fi = filament (early signs)

FIGURE 46A Polar capsule of M. ellipsoides spore. The polar filament forms four coils

TEM x44,700

FIGURE 47. Primary lamellae totally replaced by masses of M. ellipsoides spores

x 400 H&E

FIGURE 48. M. ellipsoides spores in the melanomacrophage centres of spleen. The centres are demarcated by a thin layer of connective tissue

x400 H&E

FIGURE 49. Myxobolus pseudodispar trophozoite (arrow) between the muscle fibres of roach. The fibres show double longitudinal slitting and loss of striation (arrow)
x400 H&E

FIGURE 50. Mature M. pseudodispar spores occupying large areas of muscle and replacing large areas of muscle fibres
x250 H&E

FIGURE 51. Granular degeneration of muscular fibres due to the presence of trophozoites of M. pseudodispar in roach. Note the infiltration of inflammatory cells and the formation of tubular bags (arrow)
x400 H&E

FIGURE 52. Myxobolus pseudodispar immature spore. The sporoplasm contains two large nuclei in the polar cell. The primordium (P) of the polar capsule (PC) and the external tube are lying next to each other
TEM x11,700

Bs = basic suture
M = microtubules
Te = external tube
P = primordium
Ncv = nucleus of the valvonic cell
S = sporoplasm
Cp = polar capsule

FIGURE 53. M. pseudodispar immature spore. The two polar capsules (Cp) are in a different stage of development. In each polar cell the nucleus (N) lies behind the polar capsule

TEM x12,600

FIGURE 54. M. pseudodispar cyst in the muscles of roach. The cyst is demarcated by connective tissue with formation of epithelioid cells in the periphery of the lesions (arrow)

x400 H&E

FIGURE 55. Inflammatory area in the muscles of British roach due to the presence of M. pseudodispar degenerating trophozoites. Melanin deposition can also be seen in the area (arrow)

x400 H&E

FIGURE 56. Myxidium rhodei cyst (Type A) in the kidney of roach, containing mature spores (arrow)

x400 H&E

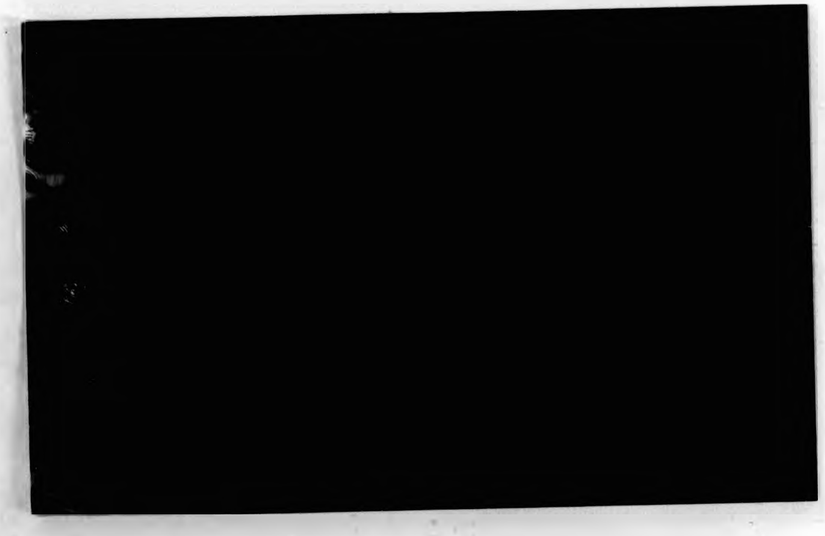


FIGURE 57. M. rhodei cyst (Type A) containing amorphous material (arrow)

x400 H&E

FIGURE 57A M. rhodei cyst (Type A) containing mature spores (arrow) and amorphous material (double arrow)
x400 H&E

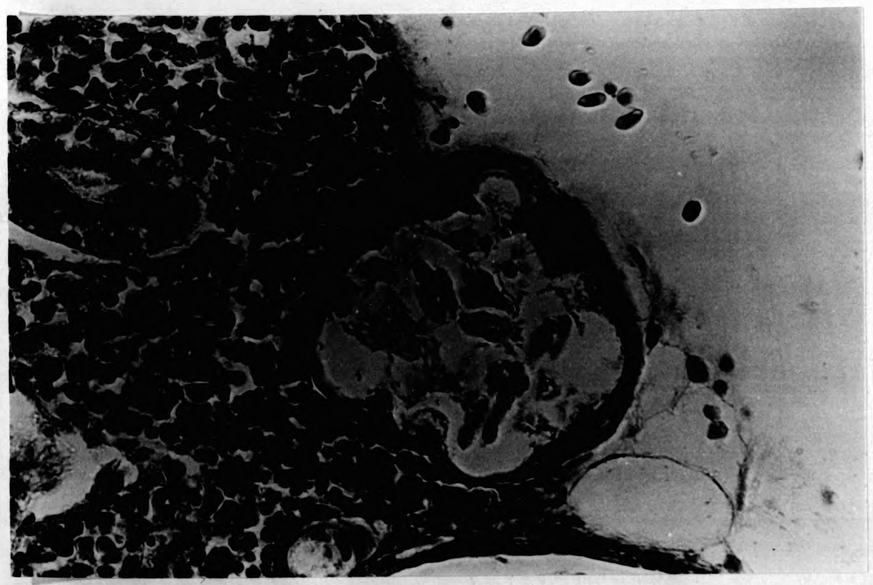


FIGURE 58. M. rhodei cyst (Type A) containing mature spores. At the periphery of the cyst, epithelioid cells are joined by desmosomes (arrow). A phagocytic cell is also present (double arrow)

TEM x5000

FIGURE 59. M. rhodei cyst (Type A) containing only amorphous material. The cyst shows similar appearance with the one in Figure 58

TEM x2000

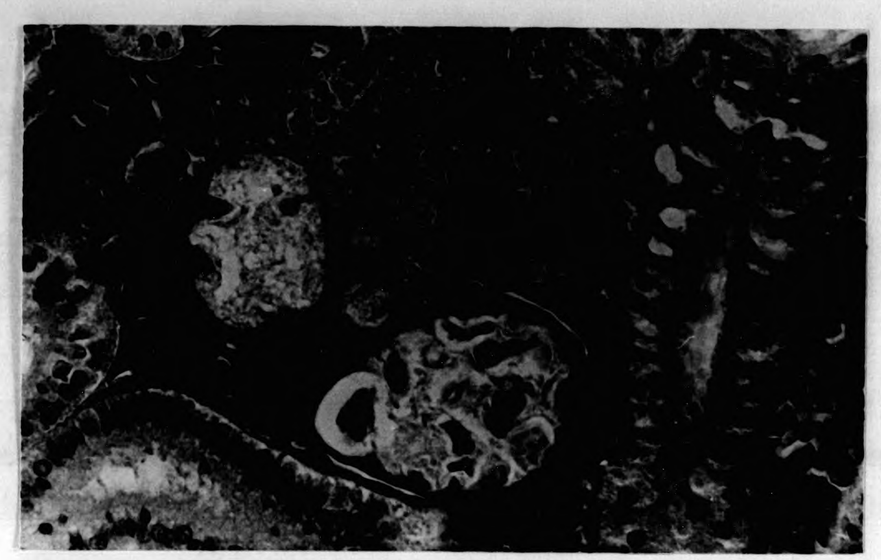


FIGURE 60. M. rhodei cyst (Type A) (arrow) associated with Type B (double arrow) inside a melanomacrophage centre in the renal tissue of roach

x400 H&E

FIGURE 61. Melanin deposition around a M. rhodei (Type A) cyst in the renal tissue of roach

x200 H&E

FIGURE 62. Granulomatous inflammatory response around a M. rhodei cyst (Type A) showing a characteristic presence of epithelioid cells (arrow)

x250 H&E

FIGURE 63. Tubules in close vicinity with M. rhodei cysts (Type A) showing degeneration of endothelial cells (vacuolation of cytoplasm) and sloughing of the cells in the lumen

x400 H&E

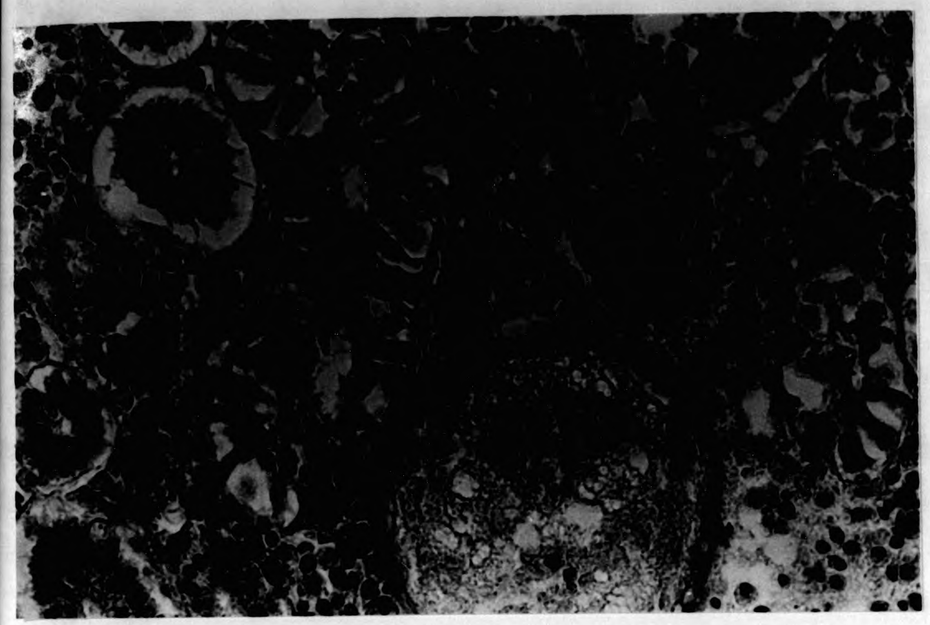


FIGURE 64. Compressed and dislocated glomeruli due to the presence of M. rhodei (Type A) cysts adjacent to them (arrow)

x200 H&E

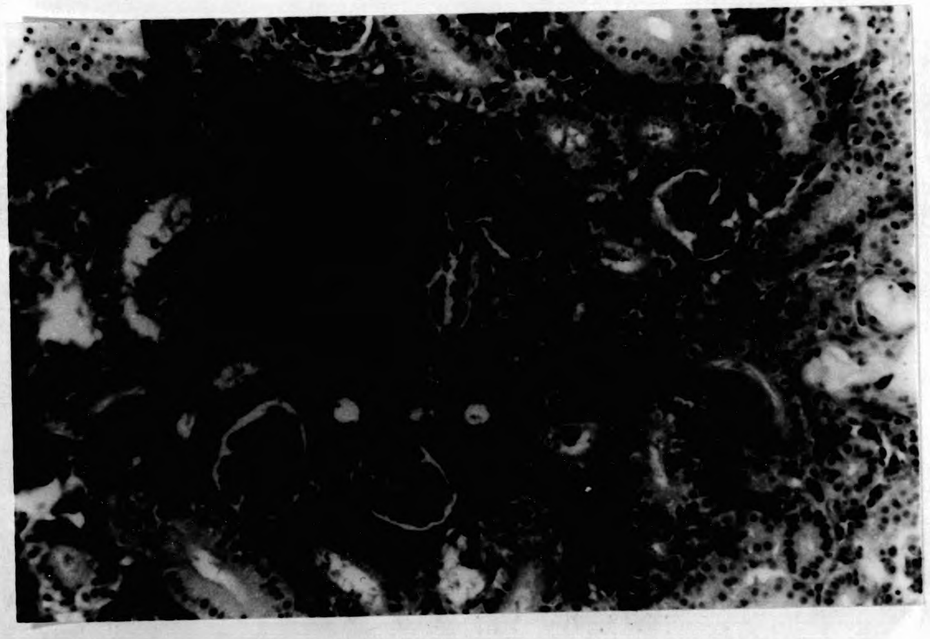


FIGURE 65A A M. rhodei (Type A) cyst showing a distinct zone (Za) between the parasites (Sp) and the renal cells (Ch). Secretive cells (Cs) and connective tissue fibres (F) are also present

TEM x9000

B Higher magnification of the structure of the membrane of the trophozoite. Arrows 1 and 2 show the parallel arrangement of the membrane, and the rest of the arrows show the granular layer of the membrane

TEM x31,500

C Fibres of connective tissue around the parasite cyst

TEM x15,300

Sp = M. rhodei spore
Za = zone of absorption
Lt = limit of trophozoite
Ch = cell host
F = fibres
Cs = secretory cell

FIGURE 66. M. rhodei spores showing characteristic arrangement of the polar capsules (arrows)

TEM x8000

FIGURE 67. Spores of M. rhodei in a different stage of development. The polar filament form four coils in the polar capsule (arrow)

TEM x10,000

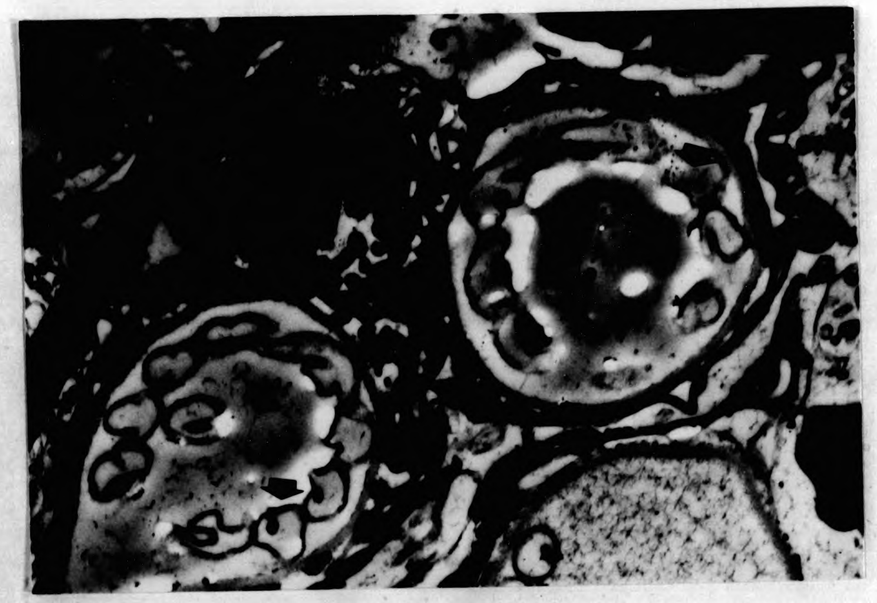


FIGURE 68. M. rhodei (Type A) cysts (arrows) in adipose tissue surrounding the kidney

x250 Giemsa stain

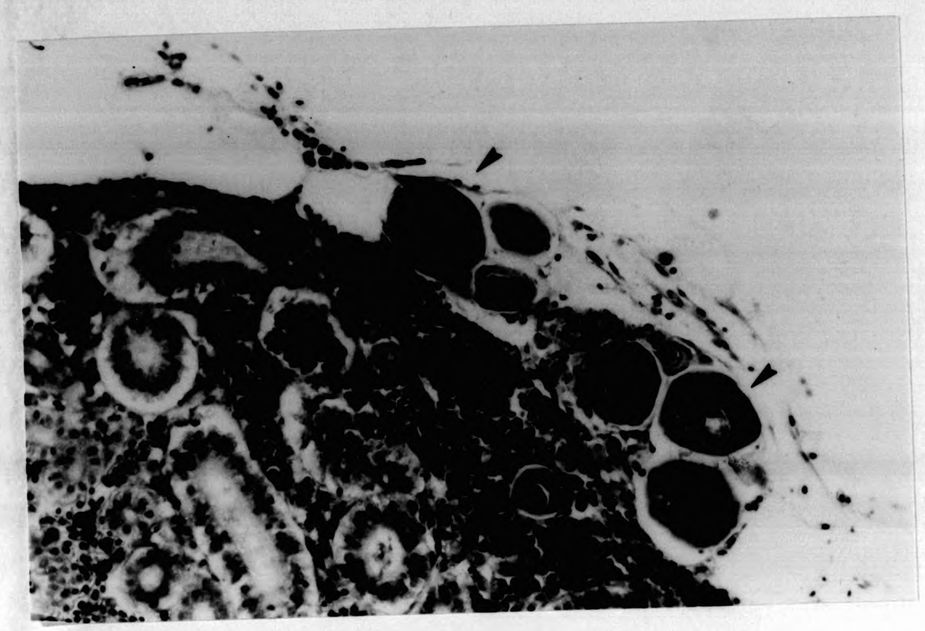


FIGURE 69. M. rhodei cyst in the spleen containing mature spores (arrow) and degenerating material (double arrow)

x250 H&E

FIGURE 70. M. rhodei spores in the melanomacrophage centres of spleen. The whole melanomacrophage centres are demarcated by a thin layer of connective tissue (arrow)

x400 H&E

FIGURE 71. M. rhodei cyst (Type A) in the liver of roach. The cyst is demarcated by a thin layer of connective tissue (arrow) surrounded by a layer of inflammatory cells (lymphocytes) (double arrow)

x200 H&E

FIGURE 72. M. rhodei cyst (Type A) in liver. Around the parasitic cyst (My), the host cells are detached and stretched. Among the hepatic cells (Hp) numerous fibres appear (F). The cells around the fibres gradually degenerate (Cd)

TEM x9000

Ca = hepatic cell (stretched)
Cd = cell degenerating
CK = Keepffer cells
F = fibres
Hp = hepatocyte
My = Myxidium cyst

FIGURE 73. Cyst wall of Myxidium rhodei in the liver. The host cells around the parasitic cyst degenerate. Note the presence of fibres (F)

TEM x9000

My = Myxidium cysts
F = fibres
Hp = hepatocyte
Cd = degenerating cell

FIGURE 74. Myxidium rhodei (Type B) cysts in roach characteristically surrounded by concentric layers of host reactions (arrow).

x400 H&E

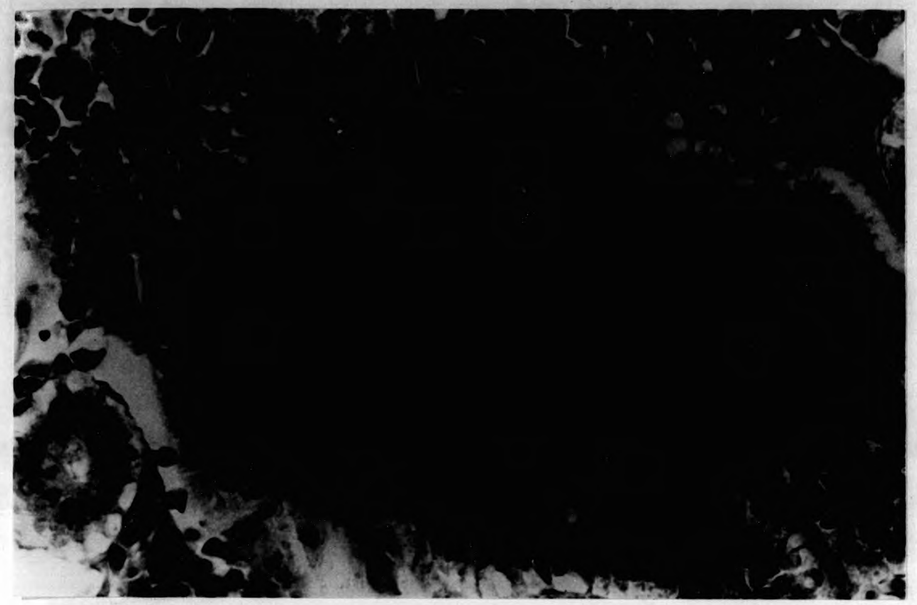


FIGURE 75. Calcified M. rhodei cysts (Type B) in the renal interstitial tissue of roach showing the dense black reaction of the Von Kossa Stain

x200

FIGURE 76. M. rhodei cyst (Type B) (arrow) adjacent to melanomacrophage centre

x400 H&E

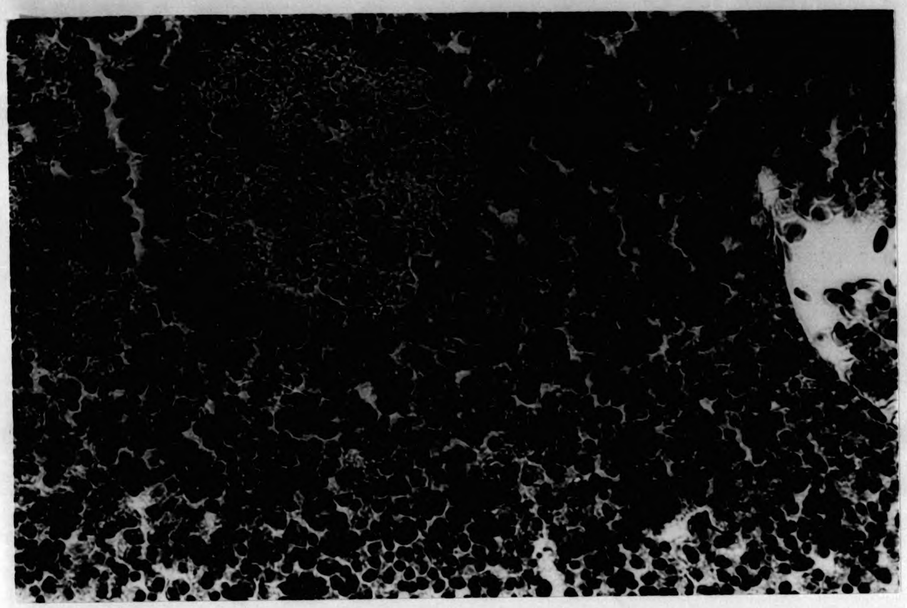


FIGURE 77. Calcified M. rhodei (Type A) (arrow) cyst in the renal interstitial tissue of roach

X100 Von Kossa

FIGURE 78. Granulomas in the heart musculature. The local inflammatory reaction is characterised by epithelioid cells (E) and macrophages (Cm)
x400 H&E

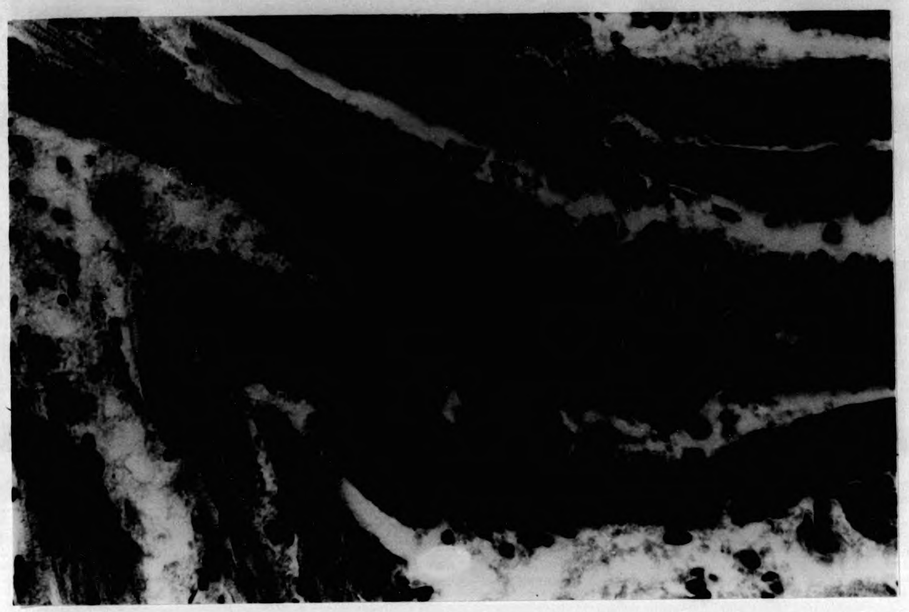


FIGURE 79. Trophozoite of M. rhodei (arrow) in a glomerulus of roach. The trophozoite has a vacuolated endoplasm
x400 H&E

FIGURE 80. Two M. rhodei trophozoites (arrows) invading a glomerulus. The glomerular tuft is shrunk and the Bowman's space increased in size

x400 H&E

FIGURE 81. Glomeruli enlarged and capillary tuft shrunk due to the presence of M. rhodei trophozoites (arrow)

x400 H&E

FIGURE 82. Debris-like material (arrow) in an enlarged Bowman's space of a glomerulus

x200 H&E

FIGURE 83. Massive infection of trophozoites of Myxidium pfeifferi in the bile ducts, occupying a large area of the parenchyma. Note the inflammation around the hepatic ducts (arrows).

x200 H&E

FIGURE 84. Small granulomas (g) in the hepatic parenchyma around bile ducts infected with M. pfeifferi pansporoblasts (arrow)

x200 H&E

FIGURE 85. Newly formed vessels (arrow) close to infected bile ducts

x400 H&E

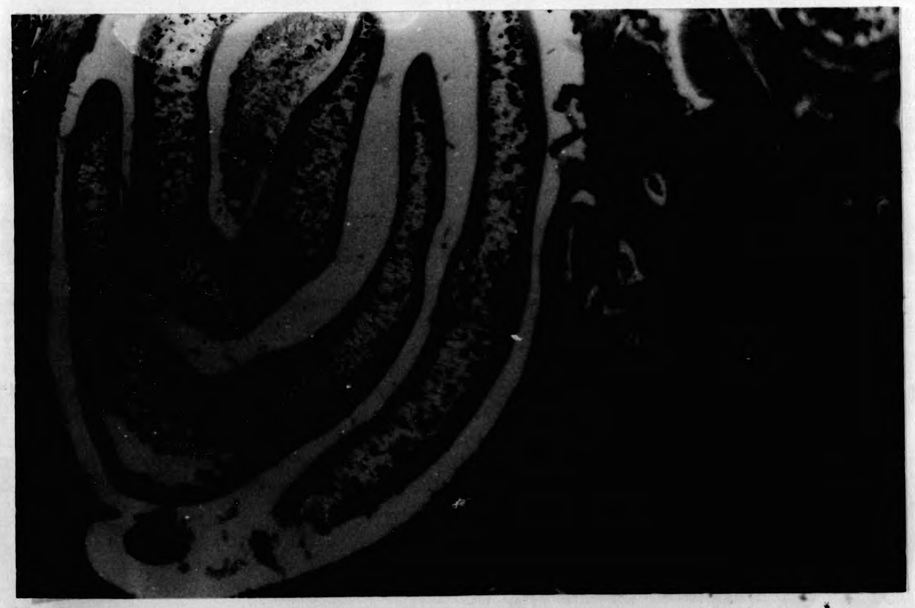


FIGURE 86A Type C lesion in the renal tubules of roach

x400 H&E

FIGURE 86B Irregularly lobed inorganic material free in the renal interstitial tissue of roach. Fresh preparation

x200 H&E

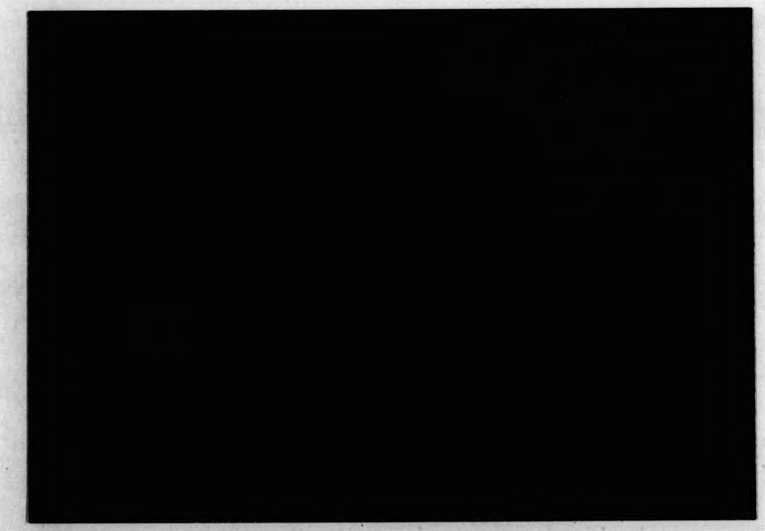


FIGURE 88. Oval vacuolated and transparent trophozoites (arrow) of M. rhodei (1st sampling, Exposure Method). Fresh preparation

x400

FIGURE 89. Pansporoblasts of Myxidium infection in the bile ducts of roach (3rd sampling, Exposure Method)

x400 H&E

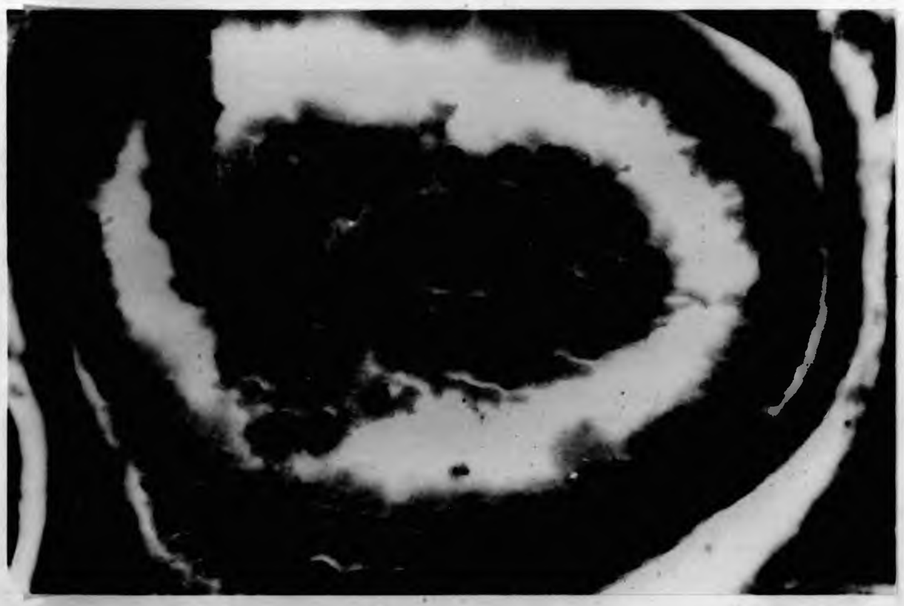


FIGURE 90. Disporous pansporoblast containing immature M. rhodei spores. Fresh preparation of kidney tissue (4th sampling, Exposure Method)

x400 H&E

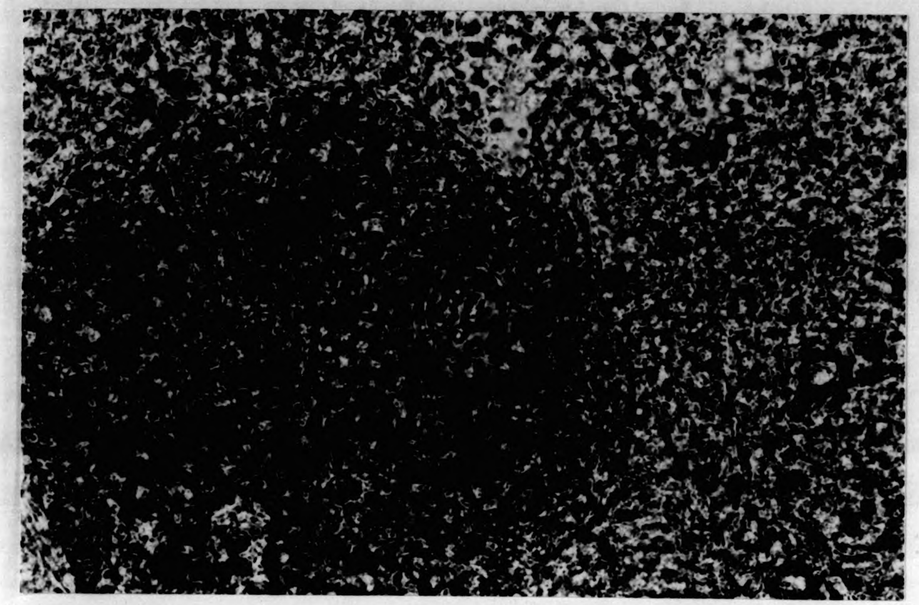


FIGURE 91. Trophozoites of Myxidium in bile ducts of carp containing mature and maturing spores

x100 H&E

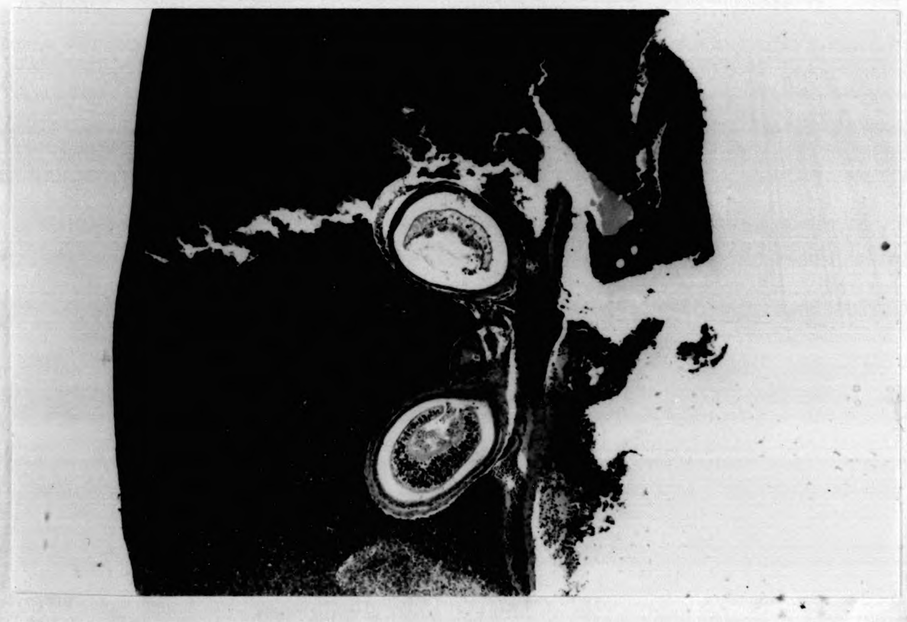
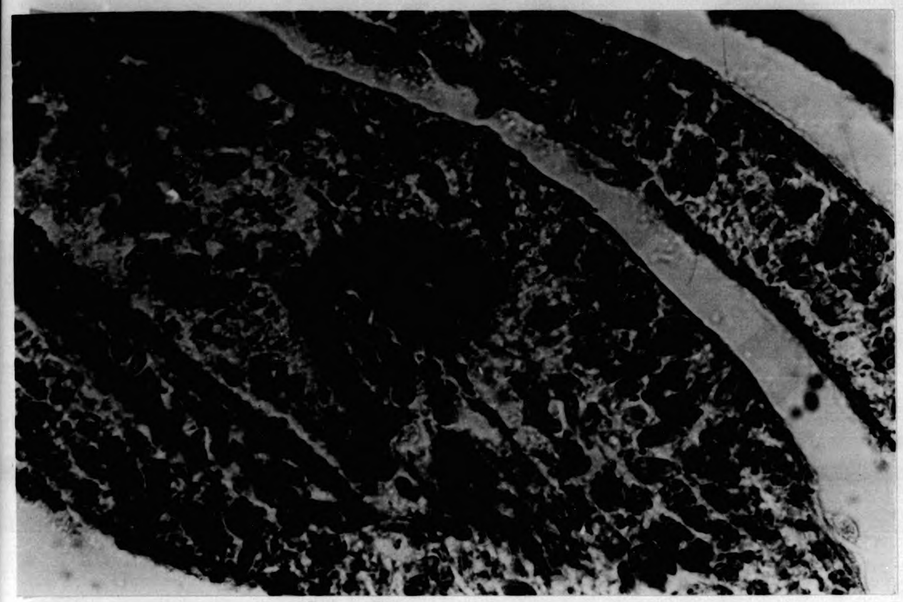


FIGURE 92. Higher magnification of Figure 91 showing the immature and mature spores (arrows)

x400 H&E



92

FIGURE 93 Cyst containing material of undefined origin in the kidneys of roach showing concentric structure (5th sampling, Exposure Method). Fresh preparation

x400 H&E

FIGURE 93A Histological examination of the above (Figure 93) cyst showing typical granulomatous appearance with epithelioid cells and macrophages surrounded by connective tissue

x400 H&E

93

93A

CHAPTER 6

INVESTIGATION INTO THE LIFE CYCLE OF MYXIDIUM RHODEI

6.1 INTRODUCTION

Whilst a considerable quantity of information is available on the occurrence, location and morphology of spores of Myxosporea spp., there is only limited information available on the life cycle and mode of transmission of this parasite group. However, it is generally accepted that the myxosporea progress through different stages during their life cycle, four of which are already known and are as follows:

The spore or the enveloped condition is considered as characteristic of Myxosporea (Lom and Dykova, 1986). The pansporoblast formation is considered typical of species with large plasmodia, including Myxidium spp. Large plasmodia are polysporic, though in some cases such as in Myxidium spp., the plasmodia produce only two spores. The formation of the pansporoblast in Myxidium rhodei shows a peculiarity since early stages of the pansporoblast have been reported to stay in close contact with each other (Lom and Dykova, 1986), unlike in other myxosporeans, which remain separated. Extrasporogenic stages which are morphologically different from the sporogenic ones also exist in Myxosporea and occupy other sites than the primary ones within the fish host. These have been described as the "Csaba" or "UBO" stages in the bloodstream (Csaba, 1976; Molnar, 1980). The swimbladder stages described by Csaba, Kovans-Gayer, Bekesi, Buscek, Szakolczai and Molnar, 1984 and Hermanns and Korting (1984) of Sphaerosphora renicola are also considered to be extrasporogonic. Although the

precise sequence of these cycles is not known, it is believed that they probably precede the spore formation (Lom and Dykova, 1986).

Intracellular development has also been reported in Myxosporea as in the cases of Myxobolus cyprini (Molnar and Kovacs-Gayer, 1985), Mitraspora cyprini and Sphaerospora renicola (Ahmed, 1973; Dykova and Lom, 1982) and Myxidium lieberkuhni in the renal tubules (Dykova and Lom, 1986) and in Myxobilatus legeri in the renal tubules of cyprinid fishes (Molnar, 1989).

During the early research history of myxosporean parasites, and until the last decade, it was generally agreed that the spore was the infective stage and that only a single host was involved in the life cycle. The mode of transmission of Myxosporea species, however, always remained uncertain and a source of contraversy. The main problem has been the extreme difficulty in successfully transmitting infection directly from spores experimentally, thus no sequential study of development has so far been achieved.

Experimental infections have been tried mainly with the histozoic species Myxosoma cerebralis and Ceratomyxa shasta in salmonids and some have been claimed to be successful, but, according to Mitchell (1977), complete life cycles have not been maintained under controlled laboratory conditions. Reports of such experimental infections in earlier literature have not been repeated and were in later years, critically questioned by Mitchell (1977).

It was well established, however, through the experimental trials of M. cerebralis, that the spores required ageing or maturation outside the fish hosts before they became infective. According to Mitchell (1977), Uspenskaya was able to infect fish hosts by pipetting spores aged 4 months in spring water into the stomach. Other workers have not been successful using this method, but have been able to produce infections of M. cerebralis and C. shasta by holding hosts in contaminated water or in mud-bottom ponds (Mitchell, 1977; Schafer, 1968).

It was upon this information that Markiw and Wolf based their experimental efforts and produced subsequent evidence (Wolf, Markiw and Hiltunen, 1986; Markiw and Wolf, 1983; Wolf and Markiw, 1984)of the existence of an intermediate host, for M. cerebralis in salmonids. Tubificid worms harbouring the actinosporean Triactinomyxon gyrosalmo were said to be effective in successful transmission of M. cerebralis. These authors claimed that the actinosporeans, which they described from the Tubifex, and myxosporeans might be alternate life stages of a single organism, and provided their evidence as an alternative model for investigating other myxosporean life cycles (Wolf and Markiw, 1984).

Following these findings, Corliss (1985) suggested that fundamental changes in the classification of these two groups of organisms would be necessary. On the other hand, the experimental results of Markiw and Wolf (1983) created a scientific debate. Lom and Dykova (1986) were reluctant to accept the new possible life cycle of M. cerebralis

since this had not been reproduced by any other investigators and found the link between these two groups of organisms near the limits of possibility.

Recent reports from two British scientists studying the epidemiology could not confirm the presence of a developmental stage of M. cerebralis in tubificid worms culminating in actinosporidian spores, infective to fish (Hamilton and Canning, 1987). Very recently, however, El-Matbouli and Hoffman (1989) confirmed experimentally the hypothesis that the life cycle of M. cerebralis and Myxobolus cotti includes an intermediate host and a metamorphosis into actinosporea of the genus Triactinomyxon.

Although among the myxosporea M. cerebralis and C. shasta still remain the major scientific interest, two other species, members of the genus Myxidium, M. oviforme and M. giardi, have also been studied experimentally.

Walliker (1968) attempted to transmit M. oviforme, using infected tissue fed to non-infected Irish salmon (S. salar) but failed. Sadler (1979) tried to infect European eels (Anguilla anguilla) with spores of M. giardi by force feeding, but only light infection was induced. The failure to produce experimentally cysts was claimed to be the pre-mature state of the spores fed to the fish (Sadler, 1979). There was also some doubt about the absence of natural infection in the controls. Since glass eels are probably infected at sea (C. Sommerville, pers. comm.) it would be very difficult to get

Myxidiumfree controls.

In this present chapter, the life cycle of Myxidium rhodei in roach was studied by means of experimental infections introduced to both roach and carp.

Because of the difficulties researchers have encountered in experimental infections and the dispute as to the mode of transmission, it was found necessary to make an attempt to establish the best method for the experimental infection of the fish. Another important factor for the experimental infections with myxosporean species is the use of myxosporean-free experimental fish.

For the reasons explained in Chapter 1, only artificially reared fish were used in this project, which in some cases were difficult to acquire. Therefore, coi carp were also used when common carp (C. carpio) were not readily available.

6.2 MATERIALS AND METHODS

6.2.1 Examination of Mud and Tubifex Worms

Mud from Lake A Vassilios, Greece, was passed through a 56um filter to remove all benthic organisms for examination. Identification of worms, according to the Key of Janiszewska (1957) was made by taking sub-samples. Individual Tubifex spp. worms were then smeared on glass

slides and examined for the presence of actinosporea under bright field and phase contrast illumination.

6.2.2 Spore concentration and isolation technique using the Plankton Centrifuge Method

The myxosporean spores were isolated and concentrated using the methods described before in 2.5 (Chapter 2).

6.2.3 Experimental Infection of Roach and Carp

6.2.3.1 - Experiment 1: to find the best method of infection

The purpose of this experiment was to establish the best experimental method for infecting fish. For this reason, three methods were used:

1. Exposure to water seeded with spores
2. Intraperitoneal injection
3. Feeding
4. Control.

Experimental Fish

120 roach (R. rutilus) and 120 carp (C. carpio) were used in this experiment and these were obtained from Yorkshire, N England. The fish were artificially bred and held over in circular fibreglass tanks. The fish were sent alive to the Institute of Aquaculture in plastic bags filled up with water and oxygen, and after their arrival

were immediately arranged in the experimental tanks. The fish were left to acclimatise for five days before feeding started.

The fish were approximately 5cm long and weighed 30gms.

Maintenance

The fish were kept in four static, well-aerated tanks (80x70x35cm) each containing 30 roach and 30 carp. The water temperature was set at 15°C and the chlorinated tap water was filtered through a charcoal filter. The throughput water flow was set at a very low flow rate in all tanks.

The fish were left to acclimatise for five days before feeding started. The food consisted of commercial trout pellets.

Sampling

Sampling was performed on days 17, 36, 58, 116 and 140 for the methods 1, 2 and 3 as well as the Control fish. Each sample consisted of five roach and five carp. For the feeding method, the first sample of fish was taken after the training of the fish to artificial feeding, and the subsequent experimental infection. Thus the first sample was examined after 19 days, equivalent to sample 2 of the other methods. Thereafter the samples were synchronised and were taken after a month (second sample), the third sample was taken 27 days following the

second, and the fourth sample 20 days following the third sample.

Only a few mortalities occurred during the experiment. Four fish (two carp and two roach) died in the injection tank two days after the experimental infection and was probably due to handling and/or the injection technique. One carp also died in the feeding tank on day 10 of the experiment. The experiment lasted 140 days.

The fish of each sample underwent a prompt, full parasitological examination and pieces of all the organs were fixed in 10% neutral formalin for subsequent histological examination.

Preparation of Spores

The spore samples were obtained from the Yorkshire roach and were processed using the method described in Chapter 2. The spores were suspended in distilled water in which a combination of penicillin/streptomycin was added at a final concentration of 200IU/ml of suspension. The spore viability test was used to assess the viability before the spore introduction. Spore numbers were assessed using the method described in Chapter 2.

A. Method 1: Exposure

A static tank (80x70x35cm) containing 30 roach (R. rutilus) and 30 carp (C. carpio) was set up in order to try an experimental infection by simple exposure to spores (collected from roach) by simple exposure

to spores in the tank environment.

Mud collected from ponds of a myxosporean-free trout farm was placed in the bottom of the tank to a depth of approximately 5cm. The mud was screened to remove coarse material and allowed to settle for 48 hours prior to introduction of fish. A 10ml spore suspension of Myxidium rhodei containing 80,000 spores/ml was poured into the water and spread thoroughly. The water flow was cut off for 36hrs and extra aeration was used during that period. The introduction of the spores took place five days after the introduction and acclimation of the fish.

B. Method 2: Intraperitoneal Injection

The fish (roach and carp) were injected with 0.2-0.5ml of Myxidium rhodei spore concentrate containing 1,000 pores per ml intraperitoneally on the fifth day of their introduction to the tank. The fish were anaesthetised in Benzocaine and after the injection were allowed to recover in a well aerated tank. No mud was used in this tank.

C. Method 3: Feeding

After the fish (roach and carp) were introduced into the tanks, they were trained to artificial feeding with Tubifex worms for 14 days. For this purpose, worms (Lumbricidae and Tubifex spp) commercially available for angling purposes were given exclusively as food, after being killed by exposure to boiling water and chopped into small pieces. For the infection, the worms were previously injected with a

M. rhodei spore suspension containing 5,000 spores per ml and allowed to settle in a refridgerator overnight. The feeding with worms commenced on the 15th day and was continued for 20 days; thereafter the fish were fed on commercial trout pellets. No mud was used in this tank.

D. Control

The fish (roach and carp) used as Controls were kept in a tank in which a layer (5cm depth) of mud was placed. The mud was screened through a mesh screen and originated from the same source as the one used in Method 1 (Exposure). The mud was allowed to settle for 48hrs prior to introduction of fish.

6.2.3.2 - Experiment 2: to examine the possible existence of intermediate hosts in the life cylce of M. rhodei

According to the first results from the first experiment, the exposure method was found to be the most successful; therefore, a second experiment was set up in order to investigate in detail the development of the parasite in the hosts and the possible existence of intermediate hosts. For this purpose, three tanks were used. In the first tank mud from a myxosporean-free farm was sterilised and put into the tank to use mainly as a substrate for the spores which were later poured in. The second tank contained mud from the same source which was used without sterilisation in order to detect any intermediate hosts. The mud was examined and proved to contain Tubifex worms.

No mud was put into the third tank.

In all three tanks the same amount of suspension (containing the same number of spores/ml) (see spore sampling details) was poured. Finally, a Control tank was used which contained non-sterile mud from the same source as the mud from the rest of the tanks, but no spores were introduced.

Experimental Fish

The fish (C. carpio) were obtained from a Yorkshire farm. The fish were artificially reared and held over in tanks supplied with chlorinated mains water. The fish were sent live to the Institute of Aquaculture in plastic bags filled up with oxygen, and after their arrival were immediately placed into the experimental tanks. The fish were left to acclimatise for five days before any feeding was started.

Maintenance

The fish (C. carpio) were kept in four plastic, well-aerated tanks (80x70x35cm), each containing 25 carp. The water was chlorinated tap water, kept constant at 18°C by using water heaters throughout the experimental period. The throughput water flow was set at a very low rate except for the period when the spore concentrates were introduced into the tanks. During this period the aeration was cut off and the spores were left to settle for 24hrs. When mud was used, this was

allowed to settle for 48hrs before the fish were introduced. The fish were left to acclimatise for four days before the feeding started. The feeding of fish consisted of commercial trout pellets throughout the experiment.

Sampling

Sampling was performed according to the method shown in Table 42. A full parasitological examination was carried out in a Control group of 10 fish prior to the experimental infection to ensure that the fish were free of infection by myxosporeans.

TABLE 42

Sampling size and dates from Experiment 2

Days post-infection	Sample Size	Sampling date
7	4	June
10	4	June
15	3	June
20	3	June
30	2	July
40	2	July
50	2	July
60	2	July
70	2	August
80	2	20/8/86
		End of experiment

Experimental Tanks

TANK A (sterile mud, myxosporean-free)

Fresh mud from a pond of myxosporean-free trout farm was placed into the bottom of the tank after screening and sterilization.

TANK B (no mud)

This tank was filled up with dechlorinated water without mud. M. rhodei spore suspension was poured into the water and allowed to settle for two days.

TANK C (non sterile mud, myxosporean-free)

This tank was covered on the bottom with a layer of fresh non-sterile mud contaminated with M. rhodei spores. The mud was obtained from a myxosporean-free trout farm and was mixed with M. rhodei spores. The spores were allowed to settle for two days before any fish were put into the tank.

CONTROL TANK

Fresh non-sterile mud from the same source was placed on the bottom after screening through a mesh screen.

Spore Introduction

The spores of M. rhodei were obtained from heavily infected roach kidneys (R. rutilus) from Yorkshire, which were kept in holding facilities of the Institute of Aquaculture. The spores were isolated and concentrated according to the method described previously and were suspended in distilled water in which a combination of penicillin/streptomycin was put at a final concentration of 200 IU/ml. The spores were used immediately after isolation and concentration from the roach kidneys.

The spore viability test was performed before the experimental infections. The M. rhodei spore suspensions were introduced into the tanks as follows:

Tank A: A concentrate containing 150,000 spores per ml was poured into the water two days before the fish were placed into the tank. The aeration during this period was cut off.

Tank B: A suspension of M. rhodei spores (150,000 spores per ml) was poured into the water after the aeration was cut off. The spores were thus allowed to settle for two days.

Tank C: A M. rhodei spore suspension containing 150,000 spores per ml was thoroughly mixed with the mud in the tank and allowed to settle for two days, in which period the aeration was cut off.

Control Tank: No spores were put into this tank, which was used as Control.

6.2.3.3 Experiment 3: to study in detail the life cycle of M. rhodei

Experimental Fish

The 100 roach (R. rutilus) used in this experiment were obtained from Yorkshire, N England, and were artificially reared and held over in

tanks supplied with chlorinated, mains water. The 160 Koi carp were artificially reared in glass tanks. The fish were transported alive to the Institute of Aquaculture in plastics bags filled up with oxygen and water and after their arrival were immediately arranged in the experimental tanks. The fish were left to acclimatise for five days before the experimental infection.

Maintenance

The fish were put in plastic, well aerated static tanks (80x70x35cm). The water temperature was thermostatically controlled at 18°C throughout the experiment. The chlorinated tap water was filtered through a charcoal filter. Fresh chlorinated and filtered tap water was slowly poured into the tanks when necessary to maintain the water level lowered by evaporation. When mud was put into the tanks this was allowed to settle for 48hrs prior to the introduction of fish which were left to acclimatise 5 days before feeding started.

The fish were fed on commercial trout pellets.

Sampling

Sampling was performed at monthly intervals for eight months for fish in all tanks. The experiment lasted for 230 days. No mortalities occurred during the experimental period, and pieces of all organs from all the fish sampled were fixed for both light and electron microscopy

directly after sampling in order to avoid any loss of infected material during fresh examination.

A full post-mortem examination was also carried out in 10 fish of each species prior to the experimental infections to ensure that they were free of myxosporean infection.

Experimental Design

TANK A (naturally infected mud and carp)

The tank, containing 80 Koi carp 7cm long (35gms), was covered on the bottom with a layer of mud (5cm). The mud was obtained from Lake A Vassilios and had received no treatment.

TANK B (naturally infected mud and roach)

A static tank containing 50 roach (R. rutilus), 7cm long (35gms), was covered on the bottom with natural mud from Lake A Vassilios (5cm) which had received no treatment.

CONTROL TANK

A static tank containing 50 roach (R. rutilus) and 80 common carp (C. carpio) both 7cm long (35gms) was used as Control. No mud was introduced to this tank.

6.3 RESULTS

6.3.1 Examination of the Mud and Tubifex worms

Examination of the Mud for Potential Intermediate Hosts

A subsample of 10% was examined for Tubifex spp. worms. The benthic species found in the mud from Lake A Vassilios are given hereunder in Table 43.

TABLE 43

Chironomus plumosus
Chironomus thumni
Tubifex tubifex M
Tubifex oligochaetus P
Nais communis P
Nematoda spp
Tubificoides benedeni V

Examination of Tubifex spp. worms

No actinosporean species were found in the worms examined. In total, 35 worms were examined, 13 in May 1986 and 22 in December 1987.

6.3.2 Experimental Results

6.3.2.1 Experiment 1: to find the best method of infection

Full parasitological examination and blood smears were carried out in all fish killed, during the five sampling periods. Organs of fish showing signs of myxosporean infections were then taken for subsequent histological examination.

METHOD: EXPOSURE TO MUD SEEDED WITH SPORES OF M. rhodei

1st Sampling (17th day). In fresh preparations, small, trophozoites were observed in the interstitial tissue of the kidney in both roach and carp. The trophozoites meausured 15x9um, were oval in shape and very transparent with two-three vacuoles in the cytoplasm (Fig.88). The trophozoites were found in three out of the six fish examined but could not be detected in the histological sections.

2nd Sampling (36th day). No trophozoites were found in the kidneys of roach but two trophozoites were found in the interstitial tissue and in the liver of one carp. The trophozoites were identical in morphology to the ones observed during the first sampling period.

In histological sections the trophozoites were not found.

3rd Sampling (58th day). Trophozoites were found in gill smears of two roach and one carp having the same morphology and dimensions as

those found in the interstitial tissue of fish from the first and second sampling.

Histological examinations of the organs revealed early stages (pansporoblasts) of Myxidium spp. infection in the bile ducts of the liver (Fig. 89). The infection occurred in three out of six roach and two out of six carp.

4th Sampling (116th day). One cyst containing pansporoblasts of Myxidium spp. was detected in the fresh preparation of kidney tissue of one roach. The pansporoblast was disporous containing immature M. rhodei spores (Fig. 90).

Histological examination of the fish revealed one Myxidium spp. trophozoite in the bile duct of one carp. The trophozoite was large, containing developing and immature spores (Figs. 91, 92). Identification is dependent on spore size and thus, determination of the species was impossible at this stage.

5th Sampling (140th day). The fresh examination revealed a cyst containing material of indefinite origin in the kidneys of one roach. The cyst was round in shape and small in size, and appeared to have a concentric structure (Fig. 93). When the material was processed histologically, the lesions appeared to be small granulomas of undefined origin. The host reaction was characterised by the presence of a zone of epithelioid cells, and macrophages, surrounded by fibroblasts and occasionally, lymphocytes (Fig. 93A).

Furthermore, three roach out of six sampled were found to be infected with tropohozoites of Myxidium pfeifferi containing developmental stages of immature and mature spores.

METHOD: INTRAPERITONEAL INJECTION

1st Sampling (17th day). Small trophozoites were observed in fresh preparations of peritoneum smears in two roach and one carp. The trophozoites were similar to the ones observed at the first sampling of the Exposure Method (Figs. 94, 95).

In histological sections of pancreatic tissue, small granulomas were observed in three fish (roach). The centre of the granulomas was necrotic and no obvious organisms could be seen (Fig. 96). Rodlet cells were in large numbers in this location.

2nd Sampling (36th day). Trophozoites similar to the ones observed during the first sampling were found in the liver, peritoneum and gall bladder of three roach.

3rd Sampling (58th day). Trophozoites like those in the second sampling were observed in the gills of one roach. In histological sections tropohozoites in the bile ducts were also observed in two roach and one carp. The tropohozoites contained developing pansporoblasts, immature and mature spores of M. pfeifferi. The

mature spores were always found in the centre of the trophozoite (Fig. 97).

4th Sampling (116th day). Degenerated trophozoites were found in smears of the peritoneum in one roach. In smears of kidney tissue one small round cyst was detected in the kidney of one carp containing three disporous pansporoblasts of M. rhodei (Fig. 98). The cyst, however, could not be detected in histological sections.

5th Sampling (140th day). One cyst close to melanomacrophage centre was detected in fresh preparation of kidney tissue of one roach. The cyst contained one trophozoite of M. rhodei (Fig. 99). The cyst could not be found in histological sections.

METHOD: FEEDING

1st Sampling (36th day). Two cysts were observed in the interstitial tissue of kidney of one roach. The contents of the cysts were undefined (Fig. 100).

2nd Sampling (96th day). One cyst was observed in the interstitial tissue of the kidney of one roach. The cyst contained one pansporoblast with two obvious developing M. rhodei spores and resembled in appearance a melanomacrophage centre. Next to it, an encysted calcareous deposit was also present (Fig. 101). The cysts could not be detected in histological sections.

3rd and 4th Sampling (123th and 143th day respectively). No pathological changes or parasitical evidence was found in any of the fish examined.

CONTROL

Fish in the Control tank were sampled at the same periods together with the experimental fish.

No evidence of any infection was observed and no mortalities occurred during the experimental period.

6.3.3.2 Experiment 2: to examine the possible existence of intermediate hosts in the life cycle of M. rhodei

The fish of this experiment were examined only histologically so that all the relevant tissues could be observed and lesions would not be missed.

Tank 1 (sterile mud and M. rhodei spores). Signs of myxosporean infections were obvious in this fish group only at the last sampling (80th day). Three fish were found to be infected with M. pfeifferi trophozoites in the bile ducts. The trophozoites were small and contained developing and mature spores. No lesions were observed in the liver tissue or in any other tissues of the fish.

Tank 2 (non-sterile mud and M. rhodei spores). Signs of myxosporean infections were also observed at the last sampling in three fish. Myxidium pfeifferi trophozoites were observed in the bile ducts of the liver with developing and mature spores. No lesions were observed in the other tissues of the fish.

Tank 3 (no mud and M. rhodei spores). No signs of Myxosporidian infections were observed in any of the fish in this tank throughout the experimental period.

Tank 4 (Control). Fish were infected with Ichthyophthirius multifiliis in the gills but no other infection was detected in this tank throughout the experiment.

6.3.3.3 Experiment 3: to study in detail the life cycle of M. rhodei

TANK A (infected mud and Koi carp). No parasitical evidence or pathological changes were found in the fish of this tank, until the third sampling period.

3rd Sampling (80th day). Dermocystidium spp. infection was observed in most of the internal organs of one fish. Massive diffuse infiltration of parasitic cells was observed in most organs (gills, liver, spleen and kidneys).

In the renal parenchyma the parasitic cells had replaced most of the renal tissue; in small areas containing intact tissue structures, the parasitic cells were observed within the tubular and collecting duct epithelia, within glomerular tufts and in the haematopoietic tissue (Figs. 102, 102A).

The infection was more severe in the kidneys but the liver and digestive tract were also infected. In these locations necrosis and a chronic fibrogranulomatous inflammatory reaction was seen (Figs. 103, 104, 104A).

Myxidium pfeifferi trophozoites were also observed in the bile ducts containing mature and immature spores. In histological sections small granulomas were found in the heart of one fish (Fig. 105). The lesions were small and resembled the lesions found in the naturally infected fish (Chapter 5).

4th Sampling (110th day). Dermocystidium infection was also found in the internal organs of one carp. The lesions and the pathological findings were similar to those found in the carp of the third sampling.

M. pfeifferi early developmental stages (pansporous and disporous pansporoblasts) were seen in the lumen of the bile ducts (Fig. 106) of one fish. No formed spores were seen in these ducts.

5th Sampling (140th day). M. pfeifferi trophozoites were seen in the bile ducts of one fish containing maturing and mature spores.

6th Sampling (170th day). M. pfeifferi trophozoites were observed in the bile ducts of one fish containing different developmental stages of spores.

7th Sampling (200th day). No parasitical evidence or pathological changes occurred in the fish from this sampling.

8th Sampling (230th day). In fresh preparations a mild infiltration of the capillary tuft by large granular and transparent cells was observed (Fig. 107) in the kidneys of one fish. These glomeruli were enlarged, the extra-capillary space was not existent but a central, clear area was obvious (Fig. 107).

TANK B (Infected mud and roach).

No parasitical evidence or pathological changes were observed in the fish of this tank until the second sampling.

2nd and 3rd Sampling (50th and 80th day respectively). Trophozoites were found in the bile ducts in three fish of each sample. The trophozoites were small and contained different stages of M. pfeifferi spores.

4th Sampling (110th day). M. rhodei pansporoblasts were found in kidney smears of one fish (Fig. 108). The pansporoblasts, similar

morphologically to those of M. pfeifferi, were present in small groups and contained immature M. rhodei spores.

In liver smears, early developmental stages of M. rhodei were observed within the hepatic parenchyma in two fish. These stages were dividing generative cells abundant in the smears and densely stained with Giemsa. Two to seven divisions were seen (Fig. 109) and they were considered to be most likely to be M. rhodei.

In histological sections the presence of pansporoblasts was detected next to melanomacrophage centres of the anterior kidney. The pansporoblast was inside a round cyst which was encapsulated by a thin layer of fibrous tissue. Furthermore, in the interstitial tissue small, mud parasitical stages resembling the stages found in the liver smears were detected. These stages were multinucleated and six nuclei were seen (Fig. 110). M. pfeifferi trophozoites containing immature and mature spores were also found in two fish.

5th Sampling (140th day). Eimeria rutili oocysts were found in small numbers in the melanomacrophage centres of the kidney in one fish. No host reaction was obvious and the parasites were located only in this area of the kidney.

6th Sampling (170th day). Eimeria rutili oocysts were seen in the melanomacrophage centres of the kidney without host reaction in one fish.

7th Sampling (200th day). Eimeria rutili was again seen in melanomacrophage centres of the kidney of one fish, without, however, provoking any host response.

Myxidium rhodei trophozoites were found in the kidneys of three fish (in both fresh and histological preparations). The trophozoites were located in the Bowman's space of the glomeruli (Figs. 111, 112, 112A). The changes involved hypertrophy of the renal corpuscles, enlargement of the extra-capillary space, and gradual compression of the glomeruli resulting in the splitting of the capillary tuft into two-three parts and degeneration of its structural elements.

Atrophy of the surrounding tissue was not observed at this stage.

8th Sampling (230th day). M. rhodei trophozoites were found in the glomeruli of one fish (Fig. 112B). The pathological changes observed were similar to the one observed in the fish of the previous sampling.

Cysts containing amorphous material and surrounded by a thin layer of fibrous connective tissue were also found in the interstitial tissue of the kidney and liver in one fish. The cysts were large and oval resembling the primary cysts found in the kidneys of naturally infected fish (Fig. 113). The cysts did not reveal any calcium deposits when stained with Von Kossa stain and no parasitical structures could be detected with the Giemsa stain. In one fish, however, spores of M. rhodei were detected in a small cyst in the

liver tissue (Fig. 114), together with amorphous material. Similar cysts were found also in the spleen (Fig. 115).

Control. The post mortem and histological examination of the fish in this tank revealed no evidence of parasites or pathological changes in the organs of fish.

6.4 DISCUSSION

Myxobolus spp. experimental transmissions have been tried in different fish species but only a few infections have been established experimentally.

Auerbach (1910) (cited by Mitchell, 1977), Erdmann (1912) and Shiba (1934) claimed to have produced such infections in the laboratory, but none of these workers used fish which were shown conclusively to be parasite-free. Bond (1939) used fish raised from eggs and he eventually infected three fish by feeding infusoria. According to Walliker (1968), Hoffman and Putz (1971) and Upenskaya produced infections by feeding the fish on spores of M. cerebralis aged for four months.

Hypodermal infection with Myxosoma ovalis in the muscles was claimed to be successful by Wagh (1961) in golden shiner (Notemigonus crysoleucas L).

Contaminated water with myxosporean species has been considered to be the most effective method for myxosporean experimental transmissions by many authors (Fryer and Sanders, 1970; Molnar, 1979; Wyatt and Pratt, 1963). In particular, muddy bottoms and slow flow rates have been reported to be prime sources of experimental infections in which high prevalence of infections have been reported (Hoffman and Putz, 1971; Schafer, 1968; Wyatt and Pratt, 1963).

Experimental infections with Myxidium spp. infections are very scarce in the literature; only two studies using experimental infections have been reported, M. oviforme was fed in salmon (Walliker, 1968) but the infection failed. M. giardi was also forcefed to eels and mild infections were produced (Sadler, 1979). Spores were detected from three to 16 weeks, but these were confined to low numbers in the interstitial lympoid tissue of the kidney and no formation of cysts was observed during the 154 days of his observations.

The fish were also reported to recover within 16 weeks of exposure to infection (Sadler, 1979). The latter experiments, however, cast doubts since eels may be infected with myxosporean species at very early stages of their life (C. Sommerville, pers. comm.).

Several reasons have been put forward for the negative results of Myxidium spp. experimental infections. Walliker (1968) gave as possible reasons the need for ageing of the spores, and the possible existence of an invertebrate in the Myxidium life-cycle. He also mentioned that longer exposure to the fish in his experimental conditions might have established successful results.

Sadler (1979), however, did not notice any difference in the infectivity between spores aged or non-aged for one month at 4°C.

The present results suggest that no intermediate host exists in the life-cycle of Myxidium rhodei and pfeifferi, as was demonstrated by

the examination of the Tubifex worms and the success of the experimental infections where no Tubifex worms were used.

Exposure, whether by seeding mud with collected spores or using naturally infected mud, of fish to contaminated spores has been proved, through the course of these three experiments, to be the most successful method of inducing experimental infections with Myxidium spp. in both roach and carp. This is in agreement with the findings of previous authors (Schafer, 1968). Mud is only needed as a substrate for holding the spores until these become infective to fish. This was clearly demonstrated by the exposure of fish to both sterile and non-sterile mud or mud from myxosporean-free sources. In all these cases evidence of Myxidium spp. infections was apparent. In contrast, in tanks where mud was not used, no evidence of infection could be detected (Experiment 2) presumably because the spores were easily washed out.

The Exposure Method is considered to be the most effective since most of the stages of Myxidium spp. parasites were found either in fresh or histological preparations. The Intraperitoneal Injection Method was considered to be the next most successful method since evidence of both Myxidium spp. trophozoites was also produced.

According to the results of the Exposure Method, small M. rhodei trophozoites were initially found in the renal interstitial tissue of both hosts. The parasites at this stage must be very fragile and only

in low intensity, since their presence could not be demonstrated in histological material.

Trophozoites of the same stage were also present in other organs (gills, liver and peritoneum) in lower numbers in both the Exposure and Injection Methods used, but not in the Feeding Method.

The infection thereafter was present in the bile ducts with the production of large plasmodia full of immature and mature Myxidium spp. spores (Experiments 1, 2 and 3). Early pansporoblasts were also found in this location in the carp (Experiment 3).

The existence of the infection in this location and the production of immature and mature Myxidium pfeifferi spores is very significant. M. pfeifferi infection was produced showing different developmental stages, in large numbers and relatively soon after the exposure of both hosts to M. rhodei spores only. It should be noted that in Experiment 1, previous existence of M. pfeifferi spores inside the hosts was not possible since the fish were artificially reared in. Furthermore, existence of M. pfeifferi spores in the mud must be ruled out, since the infection was also present in tanks with sterile mud (Experiment 2) and in tanks with no mud (Intraperitoneal Exposure, Experiment 1).

It is, therefore, logical to conclude that M. pfeifferi is identical with M. rhodei and most probably, is an intermediate or even final stage in its life cycle. It is interesting to note that the

experimental development in the carp stops at this stage and no further demonstration of M. rhodei trophozoites or spores could be proven with this method.

According to the results of all three experiments, the longer exposure is considered more effective than short-term exposure, since the presence of M. rhodei trophozoites in the glomeruli (which is considered the common site of this infection)(Chapter 5) was always found in the last samplings (140th day onwards). In short term experimental designs, such as Experiment 2 (three months) no trophozoites of M. rhodei in the glomeruli were found.

Interstitial cysts with amorphous material were only demonstrated in the latest samples (Experiment 3) and these may represent a late stage of degeneration. It is interesting to note here that similar cysts were found in naturally infected fish and quite often these were calcified (Chapter 5).

When comparing the experimental infections between the two hosts, it is observed that the speed of the development in the tissues of the two hosts is almost similar. The infection usually stops in carp with the appearance of M. pfeifferi in the bile ducts. The infection is present in both species in the liver. In the roach, however, pansporoblasts undergoing division were found in the liver parenchyma whereas developing pansporoblasts were found free inside the bile ducts in the carp.

Other infectious agents were also present in the non-sterile mud from Lake A Vassilios.

Carp is also very susceptible to Dermocystidium spp. infections which proved to be highly pathogenic in this species. Eimeria rutili oocysts were found in roach but these were few and concentrated in the melanomacrophage centres of the kidney.

In the Feeding Method, the cysts found in the interstitial tissue of roach in early sampling times might represent granulomas formed by the host reaction to early trophozoites located only in the interstitium of the kidney.

Similar trophozoite location and intense inflammatory reactions have also been reported by Dykova et al. (1987) in naturally infected roach. If this is the case, then it might be correct to presume that by the Feeding Method the trophozoites are rapidly established in the interstitial and renal tissue of roach, where they are well demarcated by the host response, and no further development occurs. This seems to happen only in roach hosts.

The above hypothesis, however, can not be proved by the presents results since the nature of the cysts could not be elucidated by the histological examination of the material of this method. Similar lesions, however, were observed at the final stage of the Exposure Method (Experiment 1) in roach. It is interesting to note here that recent researchers (Odening, Walter and Bokhart, 1989) have produced

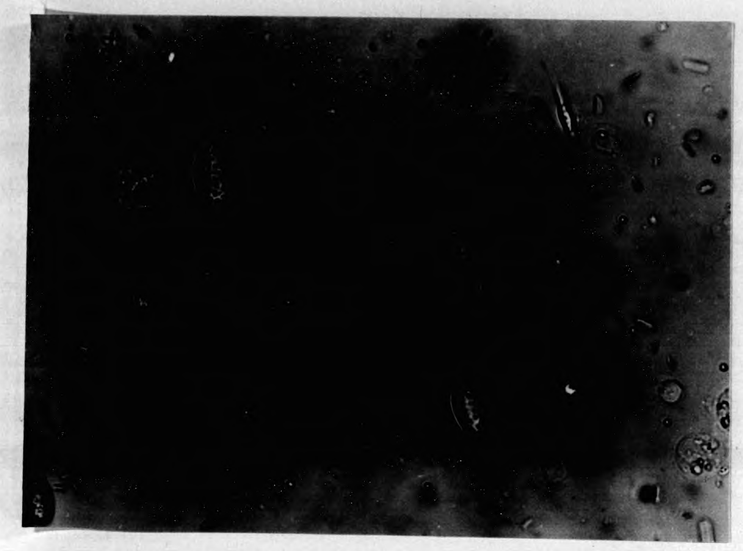
successful infections of Sphaerospora renicola to common carp by feeding kidneys in laboratory experiments.

The pathological changes produced by Myxidium rhodei and M. pfeifferi in roach are similar to those reported in the literature for naturally infected fish (Dykova et al., 1987) and to the lesions found in naturally infected fish in this study.

The pathology of these myxosporeans in carp can not be assessed in this study, because no data on naturally infected fish is available for comparison. Through the course of this study, however, there was evidence that experimental infection with at least M. pfeifferi can be easily induced and that this stage might possibly be the final stage (and the only one?) of M. rhodei infections in this host species.

FIGURE 94. Trophozoites of Myxidium rhodei in the peritoneum
FIGURE 95. (arrow). (1st sampling, Intraperitoneal Method).
Fresh preparation

x400



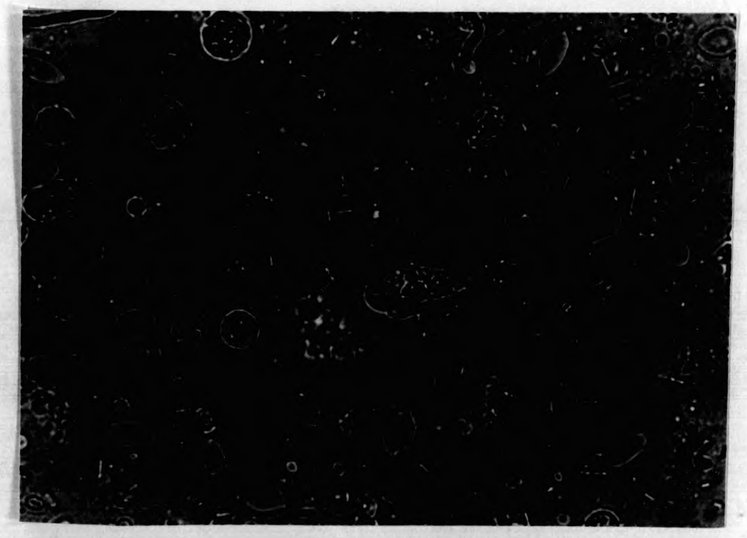


FIGURE 96. Granulomas in the pancreatic tissue (1st sampling, Intraperitoneal Injection)

x300 H&E

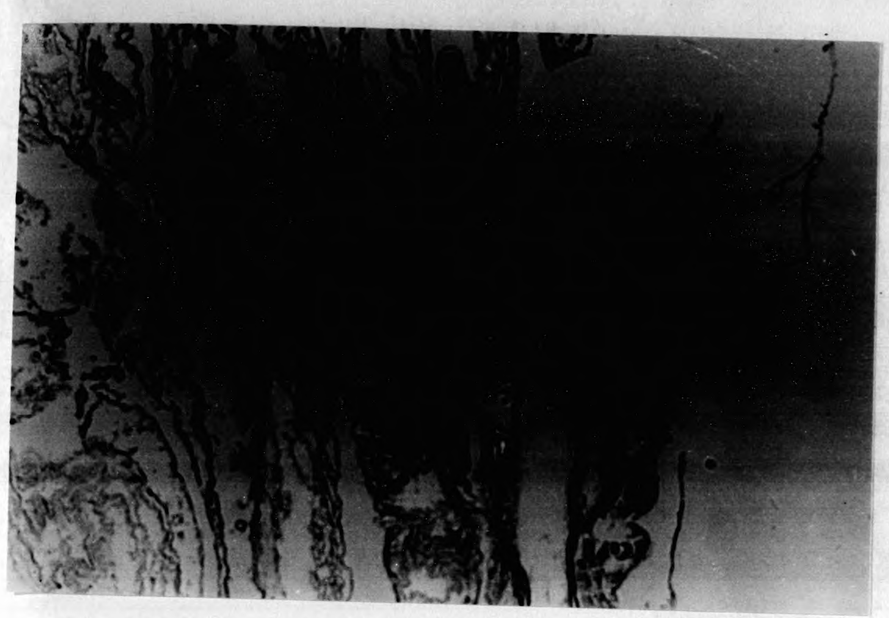


FIGURE 97. Trophozoites (arrow) of M. pfeifferi in bile ducts of roach (3rd sampling, Intraperitoneal Injection)

x300 H&E

FIGURE 98. Small, round cyst in the renal tissue of carp containing three to five disporous pansporoblasts (arrow) of M. rhodei (4th sampling, Intraperitoneal Injection). fresh preparation

x300

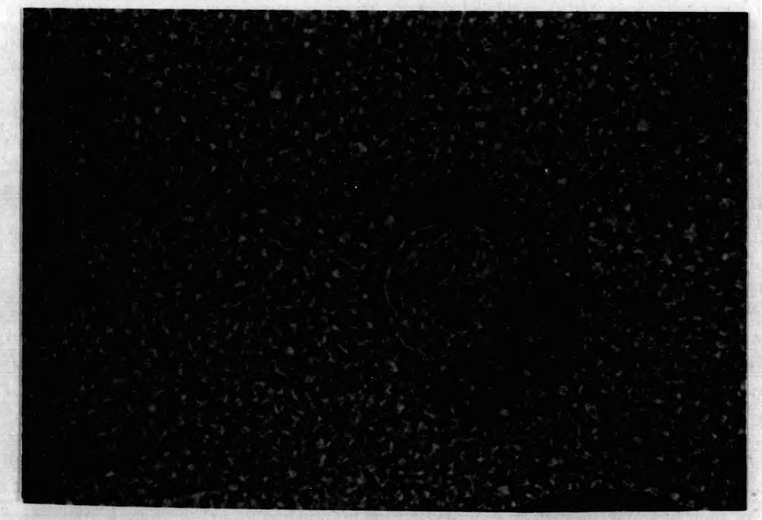


FIGURE 99. Cyst close to melanomacrophage centre containing a trophozoite of M. rhodei (arrow). Fresh preparation

x400

FIGURE 100. Cyst in the renal interstitial tissue of roach showing content of undefined origin (1st sampling, Feeding Method). Fresh preparation

x400

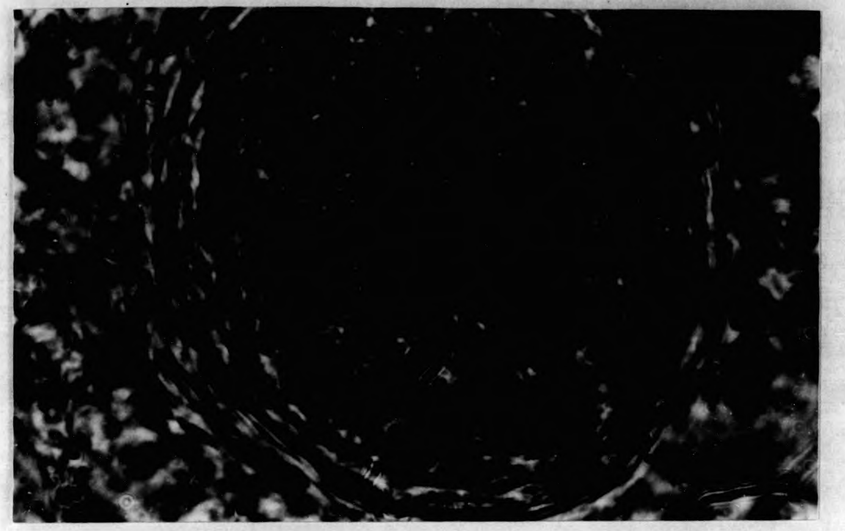


FIGURE 101. Cyst containing a disporous M. rhodei pansporoblast (arrow). The cyst resembles a melanomacrophage centre and a calcarious deposit (double arrow) is also present. (2nd sampling, Feeding Method). Fresh preparation

x100

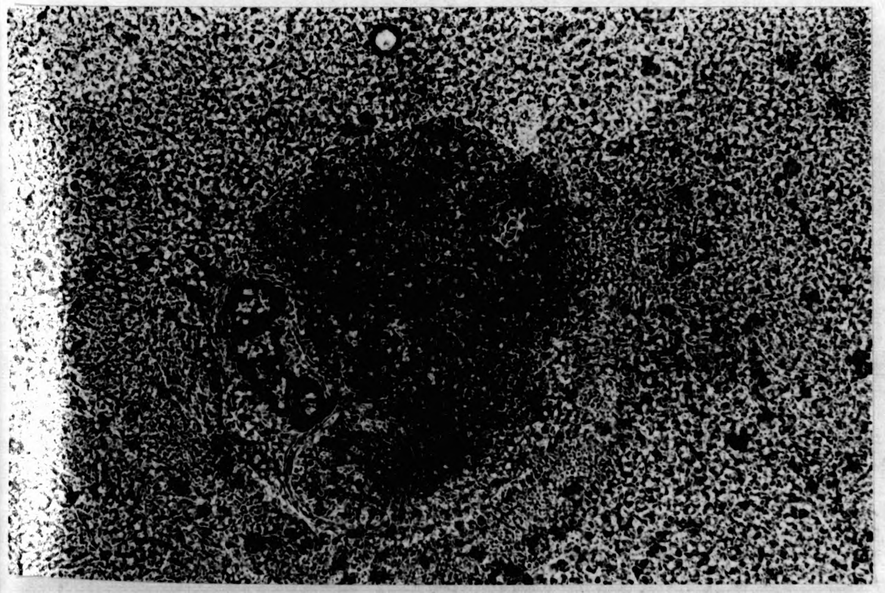


FIGURE 102. Dermocystidium spp. infection in carp kidneys. The parasitic cells have replaced most of the renal tissue

x400 H&E

FIGURE 103. Dermocystidium spp. infection in liver

x200 H&E

FIGURE 104. Dermocystidium spp. infection of digestive tract
x200 H&E

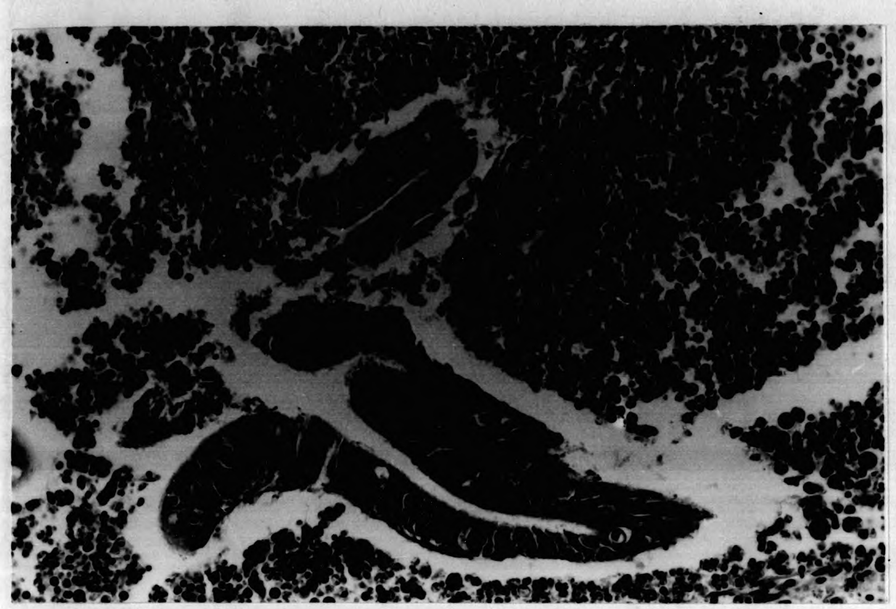


FIGURE 104A. Higher magnification showing one parasitic cell (arrow)
x400 H&E

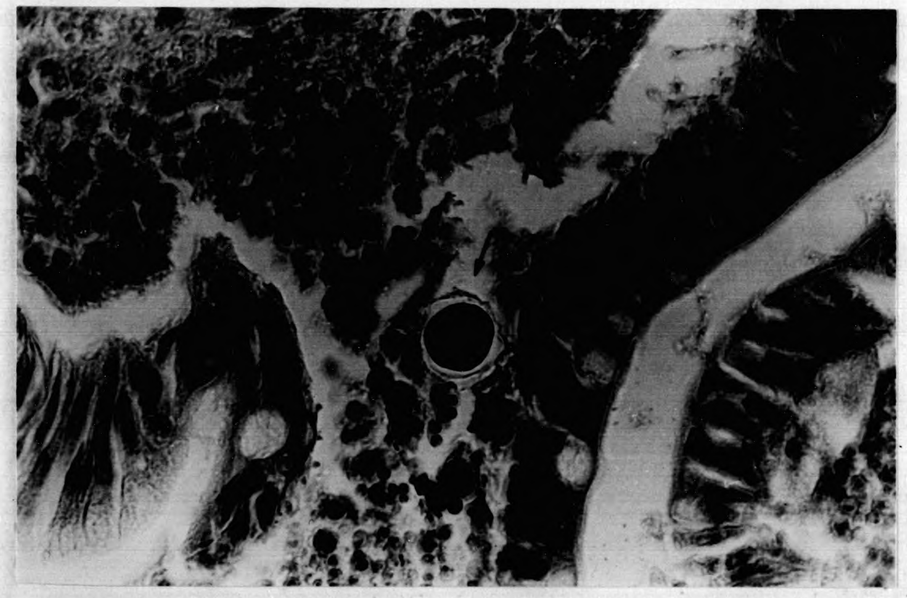


FIGURE 105. Granulomas of undefined origin in the heart musculature (3rd sampling, Tank A, 3rd experiment)
x400 H&E

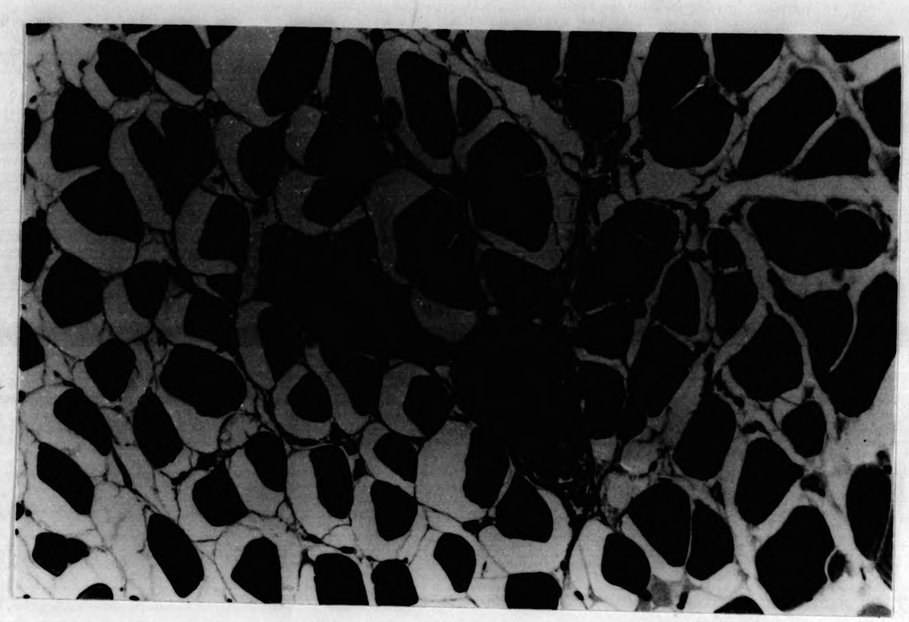


FIGURE 106. M. pfeifferi pansporoblasts in the lumen of bile *(arrows)* ducts (4th sampling, Tank A, 3rd experiment)
x400 H&E

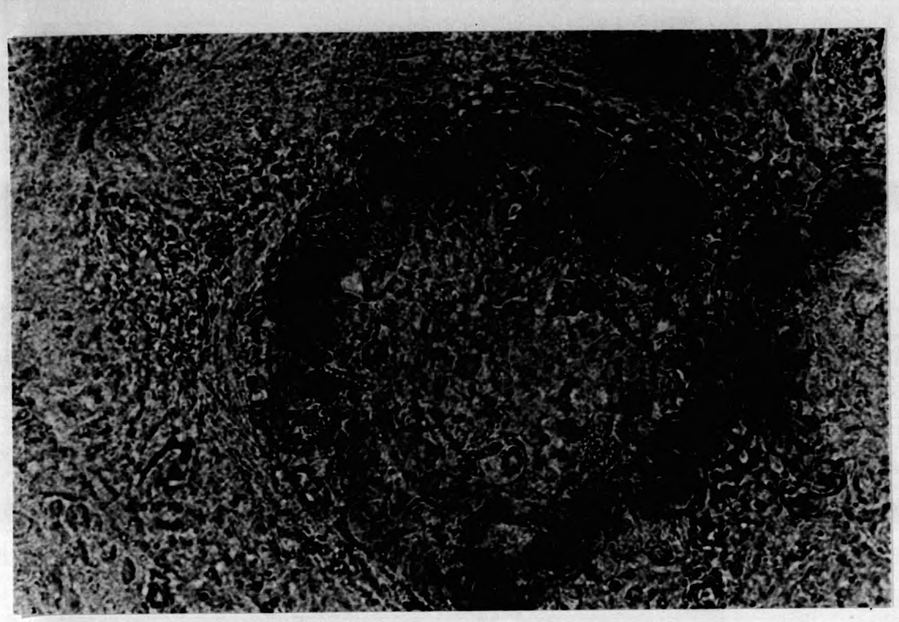


FIGURE 107. Granular, transparent cells (arrow) infiltrating the capillary tuft of the glomeruli of roach. The glomeruli are enlarged and a central clear area is apparent (8th sampling, Tank A, 3rd experiment). Fresh preparation x400

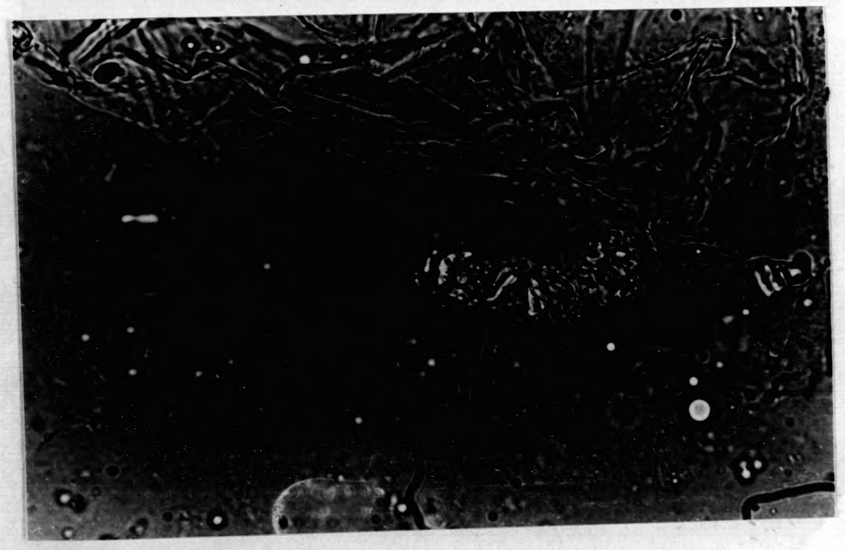


FIGURE 108. M. rhodei pansporoblasts (arrows) in kidney smears (4th sampling, Tank B, 3rd experiment). Fresh preparation x400

FIGURE 109. A-E

Developmental stages of Myxidium rhodei in liver of roach. 1-7 divisions of the generative cells can be seen.

Impression Smear, Giemsa X400

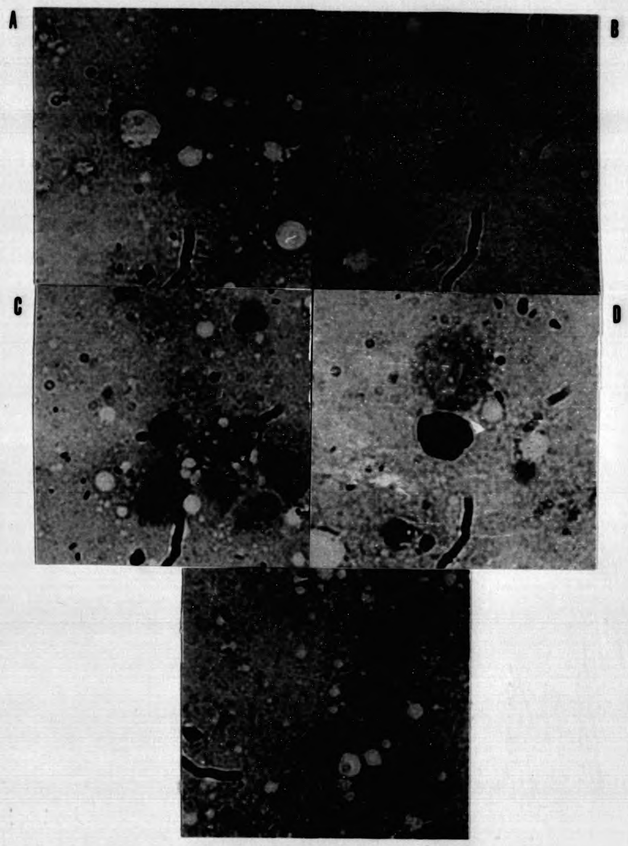


FIGURE 110. Early parasitical stages (pansporoblasts) of M. rhodei in the interstitial tissue of roach showing six deep stained nuclei

x400 Giemsa

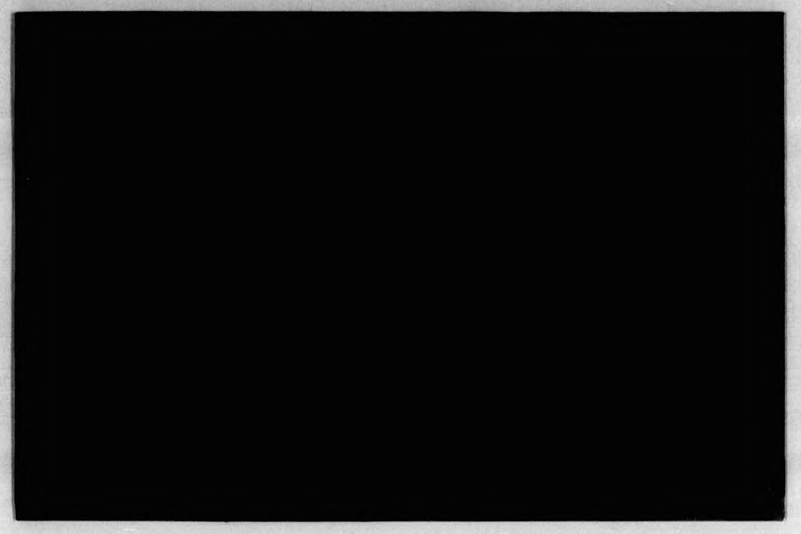


FIGURE 111. Trophozoite (arrow) of M. rhodei in glomeruli of roach (7th sampling, Tabk B, 3rd experiment)

x250 H&E

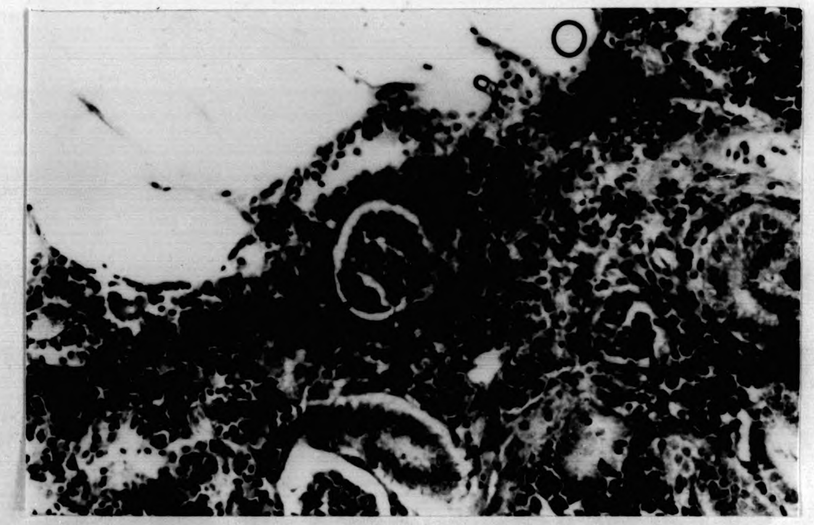


FIGURE 112. Trophozoite (arrow) of M. rhodei in glomeruli of roach (7th sampling, Tank B, 3rd experiment)
Methylene Blue x450

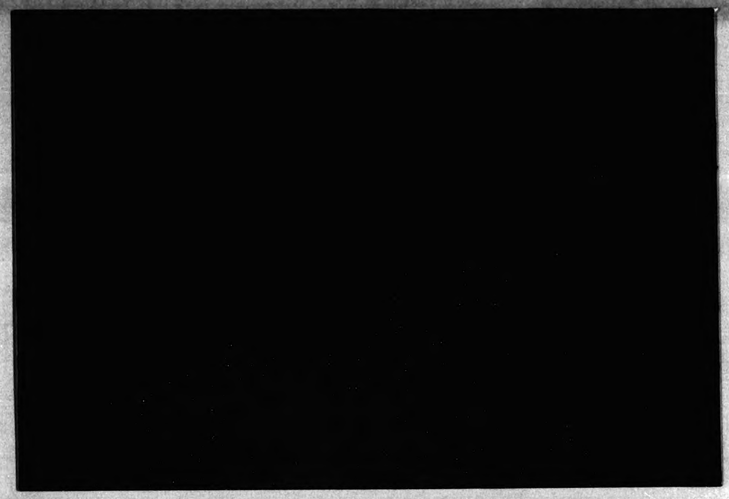


FIGURE 112A Trophozoite (arrow) of M. rhodei in glomeruli of roach (7th sampling, Tank B, 3rd experiment)
x400 H&E

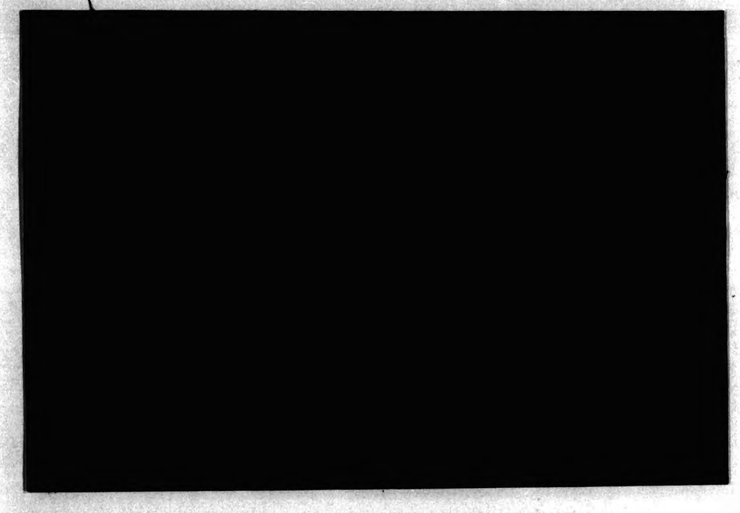


FIGURE 112B Trophozoite (arrow) of M. rhodei in glomeruli of roach (8th sampling, Tank B, 3rd experiment)
x400 H&E

FIGURE 113. Cyst containing amorphous material surrounded by a thin layer of connective tissue in the renal tissue of roach

x400 H&E

FIGURE 114. Cyst containing amorphous material in the liver parenchyma

x400 H&E

FIGURE 115. Two M. rhodei cysts in the liver parenchyma (one contains mature M. rhodei spores (arrow) and the other amorphous material (double arrow)

x250 H&E

CHAPTER 7

GENERAL DISCUSSION - CONCLUSIONS

The results of the present study showed that Myxobolus pseudodispar and Myxobolus ellipsoides are common parasites of roach. In Lake A Vassilios the infection in both species appeared throughout the year but the prevalence suggested a bimodial pattern.

Two main factors were considered to influence this bimodial pattern, the temperature and pre-spawning period of fish. Both factors may result in a suppression of the immune system of fish and temperature may also have a direct effect on the development of the parasite during the peak periods.

Myxosporeans as a group have been generally considered to be site specific, indeed, the site of infection has frequently been used as a taxonomic character. The site accorded this status, however, is generally the site in which the parasite was first recorded and reported. Subsequent, more detailed studies, may show that the parasite occcurs in several to many organs within the fish. For example, Myxidium rhodei, although a common parasite of the kidney of freshwater fish, has not been recorded from tissues such as spleen and heart. These sites of infection have been missed even in more recent studies, where it was demonstrated that the parasite could be found in liver and muscle tissue (Kerp, 1987). Where infection is

well-established, it is difficult to judge which is the primary site of infection. This question has not commonly been addressed.

A variety of criteria may be considered, for example, in higher animals, larger size would indicate the most suitable environment, i.e. a habitat which provides the most useful nutrients and permits unrestricted growth within the genetic limits of the organism.

If the ability to produce larger spores is an indication of the suitability of the site/organ for the infections, then this data suggests that the kidney may be the primary site of infection since both Myxobolus species were found to be larger in the renal tissue. Pronin and Pronina (1989) have given this problem some consideration and concluded that this "relative equilibrium" in the interactions of host-parasite systems is the situation where the frequency of parasite in the host is high, but the pathogenicity is low. In relation to this, the fact that these two Myxobolus species did not produce any pathology in this location may indicate that these are well-adapted kidney parasites which become very pathogenic if found in other organs, namely the gills (for M. ellipsoides) or the muscles (for M. pseudodispar).

The consequences of such infections in vital organs like these were considered to be serious as fish may lose their commercial value through spoilage of the flesh, as in the case of M. pseudodispar for example. On the other hand, the extensive lesions in the gills found in heavy M. ellipsoides infections may render fish less active and

prone to predation. Cyprinids are desirable edible fish species for a major part of the Greek population and in addition, some species constitute an important and cheap source of food for salmonids. Therefore, any losses of such fish due to disease would have economic consequences for the inland fishing industry in Greece. It is interesting to note here that M. cyprini, an identical species to M. pseudodispar has been reported from the muscle tissue of carp where it is believed to be very pathogenic (Dykova and Lom, 1988). Although carp from the Greek lake were not examined during the present study, it is strongly suspected that the carp population may also be infected with these Myxobolus species and this might contribute to the constant decrease of these fish catches in Lake A Vassilios. A study of the myxosporean and/or pathology of carp in this lake would be interesting and would elucidate aspects of host susceptibility and transmissibility of the myxosporean species. It might also explore other explanations for the decrease of the natural reproduction of carp in the lake, already reported by Kilikidis et al. (1984). Information conerning the decline of the catches of roach is not available for this lake, but periodic mortalities occurred during the years 1983, 1985 and 1987. The growth of roach in the last six years has been claimed by local fishermen to be retarded. The results of this study could not confirm these claims. Nevertheless, the findings here relating to the parasite pathology would indicate that there may be affects which could operate at the population level. The findings do suggest that an investigation into the health and growth performance of the populations might be useful.

One of the aims of the present project was to investigate the possible affinities of M. rhodei with other Myxidium species. The different aspects of this study, namely the seasonality, pathology, morphology and experimental transmission, pointed towards the inescapable conclusion that M. rhodei is synonymous with M. pfeifferi.

This has already been suspected previously by other authors (Dykova et al., 1987) but further evidence on this particular subject was lacking. In response to this, the results of the present study now bring evidence from all the different approaches of the work that the two species are identical. This evidence for the synonymity is very strong.

Firstly, the results of the seasonality study of M. rhodei and M. pfeifferi showed that the trophozoite stage of the two parasites never coincided. M. rhodei trophozoites were found always in the December samples and lasted only for a short time. In contrast, M. pfeifferi trophozoites were found in Springtime only. The above findings were confirmed in all three different geographical locations where the study was carried out. This is very significant since the three lakes from which the fish originated are different, wellseparated water bodies with, in each case, different environmental and ecological conditions; and even more important, climatological conditions. This considerably reduces the possibility that the findings of this study are coincidental. However, it could be argued that the seasonal samplings in some cases (especially in the case of the British lakes) were not taken every month and, therefore, some of the information

might be misleading. This argument, although reasonable, could be overcome by the evidence provided from the results of the experimental transmission of the roach and carp with M. rhodei spores (Chapter 6).

According to these results, M. pfeifferi early developmental stages (assumed to be M. pfeifferi because of their location in the fish) appeared again in advance of trophozoites of M. rhodei in the glomeruli of the kidneys.

It must be emphasised here also that this was observed in both fish species (carp and roach) used in the experiments as well as in all the different experimental designs where modifications of the exposure method were used (Chapter 6). The same results were also obtained when the next most successful method, the intraperitoneal injection, was performed.

Furthermore, detectable M. rhodei trophozoites in the glomeruli (identified as such from the information provided by previous workers such as Dykova et al., 1987) were always found in the last samplings (140th day onwards) of the experiment which had the longest duration.

The results of the morphological study of the two Myxidium species provided further evidence that these two myxosporean species are synonymous. The morphology and dimensions of mature M. rhodei and M. pfeifferi spores, as discussed in Chapter 4, were found to be identical in both Greek and British samples and by both light and scanning electron microscopy observations.

The results were confirmed statistically for both Greek and British samples. In particular, morphological features, such as striation number and pattern, which are thought to be genetically determined (Hine, 1979), were examined in detail. As these were found to be identical in all cases these results provide strong evidence that the previously thought different Myxidium species share the same genetic pool.

Genetic studies aiming to identify biochemically these two species would be very interesting and would complement the information provided from the present project. Techniques like electrophoretic separation of proteins and subsequent staining either for proteins generally or for specific enzymic activity, is a popular technique for taxonomic purposes. Other methods in use, to detect polymorphisms are isoelectric focussing, immunological techniques and yet various methods of recombinant DNA technology are also available. In particular, immunofluorescence methods are of taxonomic importance and have been noted by Halliday (1974) and Markiw and Wolf (1978) for Myxosoma cerebralis and cytochemical methods were used by Mukherjee and Haldar (1981) in order to study the variations of Myxobolus punctatus in the different organs of fish.

Immunological, biochemical, chromosomal and DNA criteria are less likely to be influenced by environmental factors than morphology and hence are more likely to provide reliable measures of genetic differences between strains and species.

In the light of the new information so far presented, the life cycle as now suggested, may be explained as follows.

No intermediate host is required in the life cycle of M. rhodei as this was demonstrated in the experimental infections (Chapter 6) and confirmed from the examination of Tubificid worms from Lake A Vassilios where M. rhodei infections are present. (These findings are discussed in Chapter 6).

It could be presumed, therefore, that the infection starts when the mature M. rhodei spores gain entrance into the host's body through the digestive tract, gills or skin. It is generally believed that the most common method of infection is the oral route, and that the life cycle proceeds when the mature spores hatch in the intestine (Mitchell, 1977). However, Hoffman and Putz (1971) suggested that infection with Myxobolus cerebralis may take place also extraorally, through the gill openings or directly across the gill epithelium or other exposed surfaces and recently, Markiw (1989) demonstrated that portals of entry for M. cerebralis in salmonids are the skin, fins, buccal cavity and digestive tract. The suggestions of Hoffman and Putz (1971) were confirmed in the present study because M. rhodei early trophozoites were found in the gills during the early samplings in the exposure method (Chapter 6, Experiment 1).

Therefore, it must be concluded that the spores of M. rhodei enter the host via either the oral cavity or the gills. Although trophozoites were not observed directly in the skin or muscles during the

experimental infection of roach and carp, this site of infection for M. rhodei should also be considered possible.

Until quite recently, it has been supposed that after the hatching of the spores in the intestine, gills or skin, the sporoplasm becomes amoeboid (Lom and Dykova, 1987). It is then assumed that the sporoplasm penetrates the gut, gill or skin epithelium and probably migrates or is carried by the circulatory system (lymph or blood) to various organs and tissues of the host. Such transfer of developing stages could not be demonstrated in the present study but recent work of other researchers in the genus Sphaerospora has shown that there may be at least one intermediate purely proliferative (extrasporogenic) cycle without spore production in organs or tissues different from the final site of infection (Lom and Dykova, 1987). In Sphaerospora renicola, a parasite of carp, one such extrasporogenic cycle was found to take place in the circulating blood; another in the swimbladder, where the parasite causes a serious inflammation. In roach harbouring infections with different Sphaerospora species, organisms identified as mobile stages in the myxosporean life cycle were detected in the blood where they were reported to undergo a proliferative cycle (Lom, Pavlaskova and Dykova, 1985). Such extrasporogenic cycles ensure mass production of the stages which later can transform in sporogenic trophozoites and are responsible for massive infections and make autoinfection unnecessary.

When a suitable site is reached, the sporoplasm will begin growth as a trophozoite which, by repeated nuclear division and cytoplasmic growth,

becomes a multinucleated coenocyte. Its size and further development may depend on the suitability of the tissue where this coenocyte is situated.

In the exposure method of Experiment 1 (Chapter 6) of this project, trophozoites (of the Coenocyte stage) were observed at early stages in the interstitital tissue of the kidney, in the gills, liver and in the bile ducts. These were transparent, fragile and very vacuolated. In the intraperitoneal injection method (Experiment 1, Chapter 6) viable and degenerating trophozoites of this stage were also observed in the peritoneum of fish and granulomas were found in the pancreatic tissue. Although these latter granulomas were not clear whether they were due to traumatic lesions related to the injection itself or whether they were due to host response stimulated by degenerating trophozoites of M. rhodei, the latter explanation cannot be ruled out. It is interesting to note here that Clifton-Hadley, Richards and Bucke (1984) have claimed to produce successful experimental infections of Proliferative Kidney Disease (PKD) in trout using intraperitoneal method of infection. PKD is considered to be related to Myxozoa (Kent and Hedrick, 1986). A granuloma of undefined origin was also found in the renal interstitial tissue of fish during the experimental infections (Experiment 1, Exposure Method, 5th sampling). This granuloma strongly resembled the Type B lesions found in naturally infected fish (Chapter 5).

In addition to this, lesions with amorphous material were found in the heart musculature of naturally infected fish and these were thought to

be similar to Type B lesions found in the kidneys (Chapter 5). It was concluded that the Type B lesions were due to trophozoites of the same stage which were eliminated through host response.

Taking into consideration all the above findings, it must be concluded that at this stage of the life cycle, the trophozoites of M. rhodei in the gills, renal, interstitial tissue and in the heart, do not develop further and degenerate. In the renal interstitial tissue in particular, calcification of degenerate parasite lesions may occur, such as was observed in naturally infected fish (Chapter 5).

In the case of sporoplasms initially located in the skin, it is also possible that these migrate directly into the muscles where large trophozoites develop, and in later stages become surrounded by host tissue to form round, stationary histozoic cysts. These probably remain intact and viable throughout the life of the host and the spores must be shed into the environment after the death and decaying of the flesh of the fish. The above hypothesis was supported by the fact that in almost all cases, the cysts in the muscles contained mature M. rhodei spores and were surrounded by a thin layer of connective tissue without other serious host response (Chapters 4 and 5).

Degeneration of such cysts and calcification was never observed in either naturally infected or experimental fish examined in this study.

Sporoplasms which enter the digestive tract and pass directly into the gall bladder and bile ducts are considered to be the most successful.

In this location the process of development is most rapid and results in the formation of large, coelozoic trophozoites containing maturing and mature spores. This is presented as cycle 1 of the parasite.

Mature spores, thereafter, are initiated into the bile and back to the intestine, from where a second cycle of hyperinfection occurs. The spores must be transported through the blood stream directly to the glomeruli where new development starts with the production of pansporoblasts and detectable trophozoites. It is also possible that extrasporogenic stages derived from the original infection are directly carried to the glomeruli to start the second cycle of the parasite. These stages were never detected in our present study, although extensive screening of the blood smears was performed. The possibility of their existence, however, could not be ruled out in view of the scarcity and the short life of these stages in the blood stream. Such stages have only been observed in a few species of Sphaerospora despite extensive studies of numerous workers in recent years.

In contrast to the cycle occurring in the bile ducts, the development in the glomeruli is slow, and large cysts containing maturing and mature spores eventually appear, occupying the entire glomerulus (Type A cysts described in Chapter 5). These cysts often degenerate but rarely calcify in the latest stages of development. Very often 2 or 3 trophozoites invade the glomeruli and result in a subdivision of the internal space of the glomeruli, giving the appearance of 'subdivided' Type A cysts (Chapter 5).

Some of the mature spores or other stages in the bile ducts gaining access to the liver parenchyma may develop into similar cysts containing maturing and mature M. rhodei spores, as these were seen in some naturally infected fish (Chapter 5).

It is also possible that the released spores or presporogonic stages from the first cycle into the intestine are carried away by the vascular tissue in other organs such as spleen, muscles, heart, etc. where further development will depend again on the suitability of these tissues. It is believed, however, that in the heart such development is readily eliminated through host response. In the spleen tissue, the spores or other stages must be collected in the melanomacrophages where, if further development occurs this is eliminated again by demarcation and encapsulation of the entire melanomacrophage centres by host connective tissue as was observed in naturally infected fish (Chapter 5). According to Dykova (1984) melanomacrophages play an important part in the host's defence reactions. They appear capable, in some cases, of attacking small trophozoites and typically, large, mature spores which are transported to melanomacrophage centres where the parasites are isolated and gradually destroyed. The same author reported that fibroblastic encapsulation of melanomacrophage centres of spleen, kidney and hepatopancreas is often seen in myxosporean infections.

The mature spores are shed to the environment directly from the intestine or urine, most probably throughout the infection while the fish are alive. In contrast, the cysts located in the muscles and

internal organs, such as liver and spleen, may require death of the hosts before they are released to the environment.

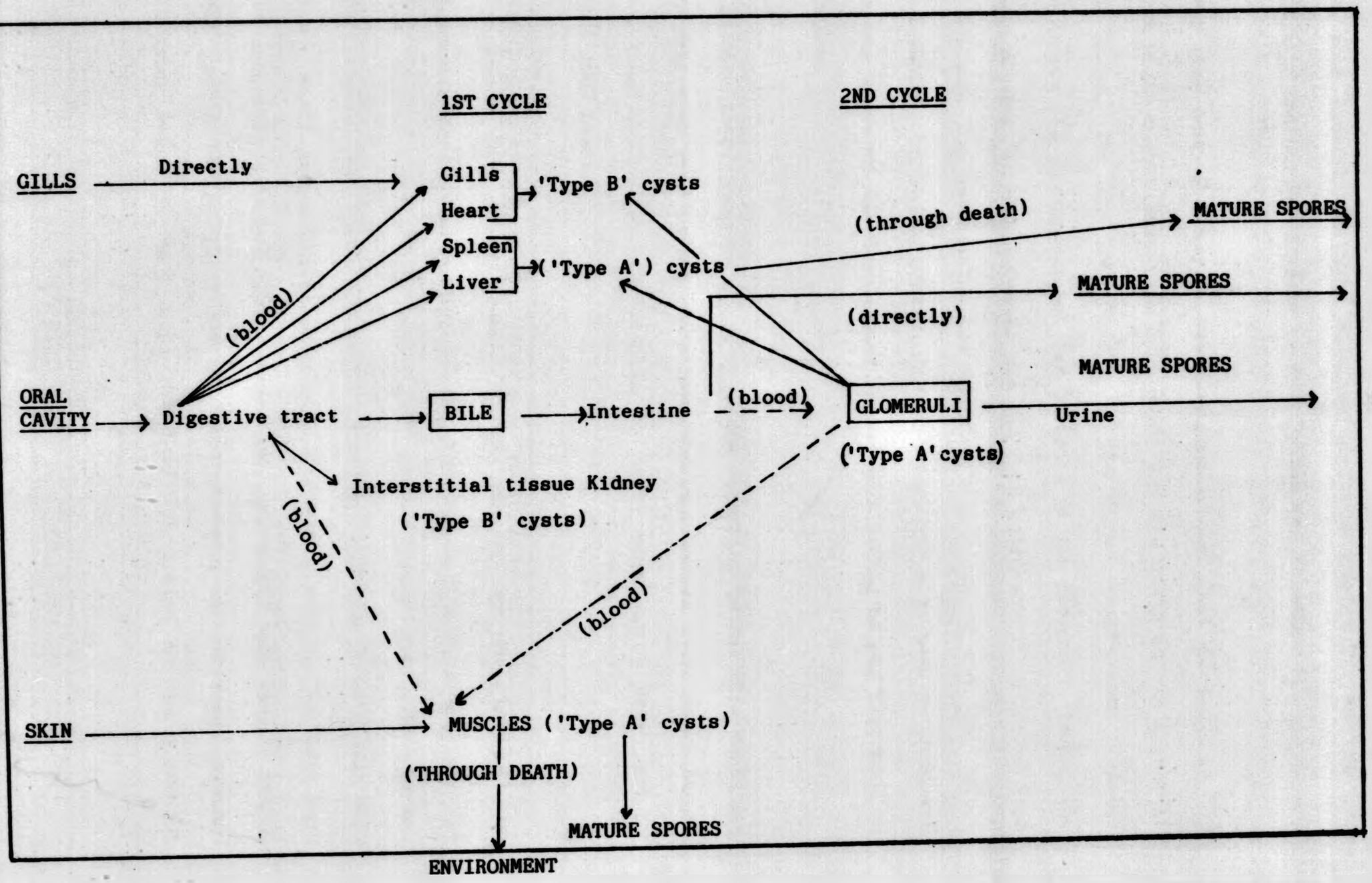
The proposed life cycle of M. rhodei is schematically represented in Fig. 116 and was based on the combined evidence of the findings of the different aspects of the present study.

It is interesting to note here that different stages of development in myxosporean life cycles are not uncommon. Molnar (1989) described two stages in the development of Myxobilatus legeri in cyprinid fishes, each of which showed distinct and specific seasonality. The intracellular trophozoites appeared in epithelial cells of tubules in Autumn whereas coelozoic plasmodia appeared in the lumen of tubes, ureters and urinary bladder in Spring time.

It is therefore possible that M. rhodei shows distinct seasonal patterns each occurring in different locations in the host, as this is suggested by the results of this project.

In summary, the existence of two separate cycles of reproduction of M. rhodei was demonstrated by the results of the experimental infections where trophozoites and spores in different stages of development were found initially located in the bile ducts and gall bladder. These never coincided with trophozoites in the glomeruli. These results were obtained in both carp and roach and confirmed in three separate experiments.

FIGURE 116: Proposed life cycle of M. rhodei



The same observations were obtained when the seasonality of the two Myxidium species was studied in both Greece and Britain.

From this part of study it was also demonstrated that the cycle in the bile ducts occurs mainly in Springtime and the cycle in the glomeruli in Winter. The low temperatures of the Winter months most probably influence the development of the parasite in the glomeruli, hence, the slow process in this location and the rare existence of calcified Type A lesions. The prespawning and spawning activities of the fish before and during the Spring period, respectively, may also have an influence on the speed of the development in the bile ducts, as was discussed in Chapter 3.

The more rapid progress of development occurring in the bile ducts was demonstrated also in the three separate experiments where early stages and trophozoites were detectable in early samplings (days) and in both fish hosts.

The slower rate of development of the parasite in the glomeruli was demonstrated in the finding of the parasite in the glomeruli only in the final samples of experiment which lasted for six months. Furthermore, the coenocytic stage of these trophozoites must last only for a short period since, despite the extensive and regular samplings of naturally infected fish, these were detectable only rarely during the period of the study and at specific times of the year (Winter: December/January). Very early developmental stages in the glomeruli

are obviously very difficult to detect because of their size and location.

In relation to these findings, it is surprising to note here that Dykova et al. (1987) found trophozoites of M. rhodei in the glomeruli and within renal interstitial tissue of roach from Bulgaria and Czechoslovak localities in abundance. Although information on their seasonal appearance was not noted, it seems most likely that their material must have been collected at specific times of the year when the infection in the glomeruli was in peak. This remains to be confirmed.

The slower development of the parasite in the glomeruli might explain the fact that the spore filled cysts (Type A) found in the kidneys of naturally infected fish were always larger in this tissue and in the older fish (Chapter 4). Whereas such large cysts were never found in small fish, it is also important to note here that the lesions due to degenerating trophozoites found in the interestitial tissue of roach were more often observed in younger fish (Chapter 5).

These lesions were thought to be due to trophozoites initially located in the interstitial tissue which then became encapsulated by the host response so that no further development could occur, resulting in death and degeneration. According to the above proposed life cycle, these lesions appear around the same time as the development in the bile ducts soon after the initial transfer of amoebas in the different tissues. Juveniles born in March, for example, may become rapidly

infected and show Type B cysts of M. rhodei due to the progress of the first cycle of infection. As the fish get older, the second cycle results in the appearance during the Winter of the Type A M. rhodei lesions and cysts which is due to the cycle in the glomeruli. Some calcified lesions due to degenerating trophozoites as well as mature spores in the gall bladder, may still remain, however, throughout the year.

The pathology of M. rhodei in freshwater fish has until now been thought to be limited in the renal tissue. Even the more recent work of Dykova et al. (1987) described the histological changes observed in the roach kidney without any reference to other tissues. Kerp (1987) in a short publication reports the incidence of M. rhodei cysts in the liver and muscles, but no other information on the pathology has so far been available.

The results of the present study, therefore, have brought to light the wider aspects of the pathology of this species occurring in other tissues, which can be as serious as the lesions in the renal tissue during heavy infections and, indeed, is in many instances more severe than that in the kidney. However, the renal tissue was here seen to be the most commonly and seriously infected organ. The spore filled cysts in particular, were considered to be quite serious for two reasons. Firstly, as these lesions represent trophozoites which eventually destroy the entire glomerulus, the function of these essential elements of kidney are destroyed in large numbers and thus, the metabolism of the fish must be seriously affected. The corruption

of the kidney tissue was well demonstrated using the digital computer analysis method.

Secondly, affected glomeruli were usually very enlarged - as much as seven times of the size of the normal glomeruli in the case of Greek fish and four times in the case of British fish. In addition to the disturbances of the glomerulus function they also, by occupying an extensive area of the interstitial tissue, interefere with the normal function of this tissue so that haemopoiesis must surely be diminished.

The pathological changes due to the degenerating trophozoites and the granulomas found in the interstitial tissue must also be considered when studying the pathology of M. rhodei in this tissue since they too occupy, and presumably interrupt, interstitial tissue function.

The significance of these lesions in the kidney must also be assessed in conjunction with the other pathological conditions that are coincident with them. When calcarious deposits in the tubules, for example, which were probably due to environmental conditions, are found in kidneys already infected extensively with M. rhodei, then the functional ability of the renal tissue must be seriously disturbed. In the Greek lake, not only such lesions existed alongside M. rhodei, but other pathological conditions and parasites have been found in the roach (Athanassopoulou, 1981, 1985; Athanassopoulou and Vlemmas, 1986). These conditions must further affect the health of fish in this lake.

As far as the pathological effect of M. rhodei on other organs is concerned, the results of the present study showed that the bile ducts and liver can be seriously and extensively affected, which may lead to additional metabolic dysfunction.

The cysts in the muscles, although they do not provoke serious host reaction, when found in large numbers may render the fish undesirable for consumption and/or make fish more susceptible to predation.

The significance of the infection with M. rhodei in roach, therefore, has been shown to be much more serious than it was first perceived. In Greece, where cyprinids are an essential part of the fishing industry and where the climatic conditions favour the fish farming of such species, infections with such pathogenic species as M. rhodei have serious consequences in terms of both economy and fish health monitoring.

CONCLUSIONS

- The seasonality of the two Myxobolus and two Myxidium species investigated in the present study showed that in each of the different habitats all the parasites had at least one peak in prevalence in Springtime. In addition to this, the two Myxobolid species found in the Greek lake as well as Myxidium rhodei in all three locations, show another peak during the Winter months (December to February). Myxidium pfeifferi showed only one peak in Spring (April to May) in all three lakes.

- The two Myxobolus species studied in the present project were considered well adapted kidney parasites provoking no host response to this tissue. In contrast, M. pseudodispar in the muscles and M. ellipsoides in the gills were found to be very pathogenic.

- The spore morphology and variability study of these two species showed that M. pseudodispar has a distinct variability in the spore dimensions between the organs of fish, although M. ellipsoides showed only slight differences.

- From the study of the two Myxidium species, evidence was produced that M. rhodei is synonymous with M. pfeifferi. This evidence was produced from the morphological investigation and the experimental infections carried out in two fish hosts and was supported by the seasonal data.

- Through three different experimental infections in carp and roach, the life cycle of M. rhodei and its development in the hosts was proposed. No intermediate hosts could be detected in the life cycle of the parasite and the Exposure Method was considered the most effective, and the Intraperitoneal Injection Method the second most successful one.

- Two cycles of development of M. rhodei infection were detected in roach. The first, occurring in the bile ducts, had a fast process resulting in the production of mature and maturing spores. The second started with the infection of glomeruli and had a slow progress with the final production of cysts containing mature spores.

Lesions due to degenerating trophozoites were also detected in the interstitial tissue of the kidney, where they were usually calcified, and in the heart. Large mature cysts were also found in the muscles of the fish.

In the liver, disturbance of the metabolism of the body was assessed from the extent of the lesions in heavy infections with M. rhodei

- The pathogenicity of the parasite was thought to be serious in both renal tissue and liver/bile ducts. In the kidneys, the pathology concerned with the glomeruli and interstitial tissue. The pathology was assessed for the first time in terms of area of lesions and volume of affected tissues as well as in relation to the environmental conditions of the habitats.

- Other concurrent infections and conditions in fish with M. rhodei were considered to enhance the pathology of M. rhodei in the hosts.

- Finally, the infection of M. rhodei was considered important in terms of both Greek economy and fish health monitoring.

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APPENDIX I

ENVIRONMENTAL PARAMETERS OF THE LAKE AGIOS VASSILIOS, NORTHERN GREECE, AND LOCH FAD, SCOTLAND

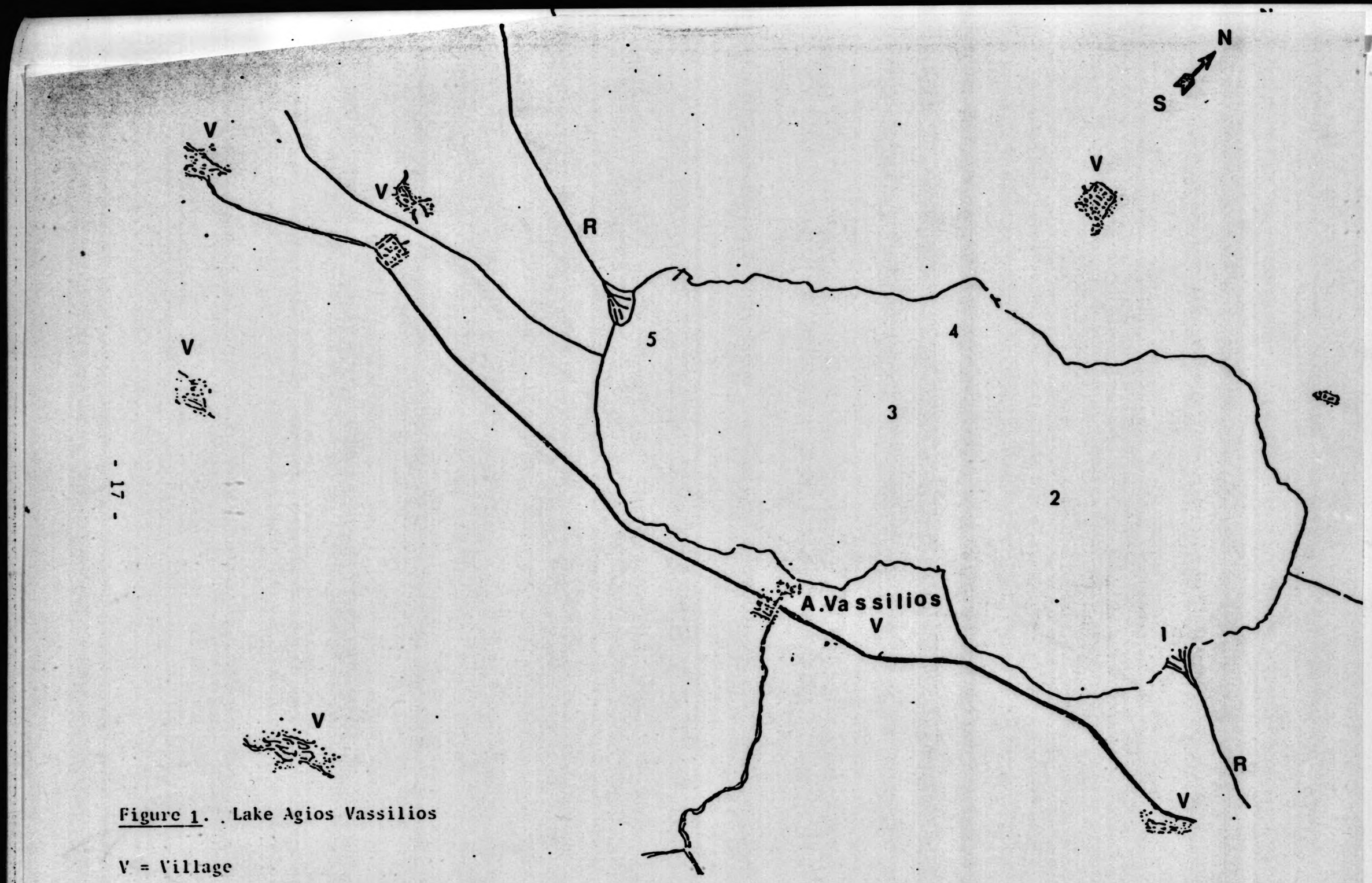


Figure 1. . Lake Agios Vassilios

V = Village

R = River

1,2,3,4,5 = Sampling stations

Scale: 1 : 73,000

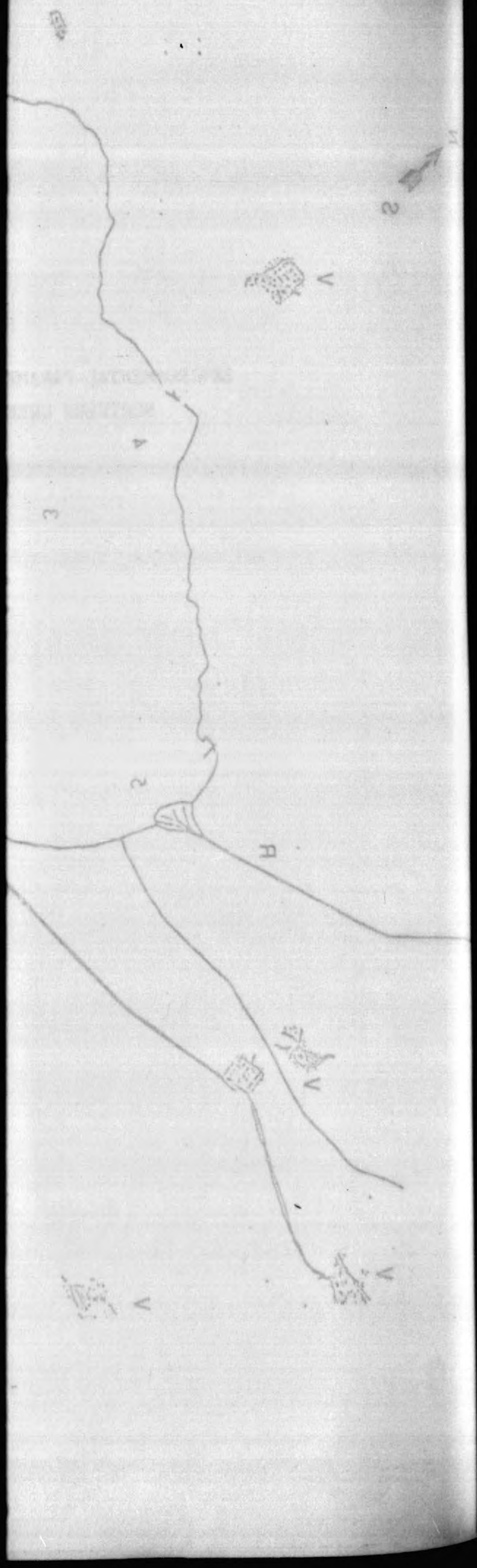


TABLE 1

Different environmental parameters of the Lake Agios Vassilios, Greece

(Kilikidis et al., 1984)

Temperature:	Summer (22° - 24°C) (max) Winter (6° - 7°C) (max)		
Depth:	4m	**Area**:	$42Km^2$
L_1:	11.4Km	L_2:	5.1Km
	Eutrophic; polluted		

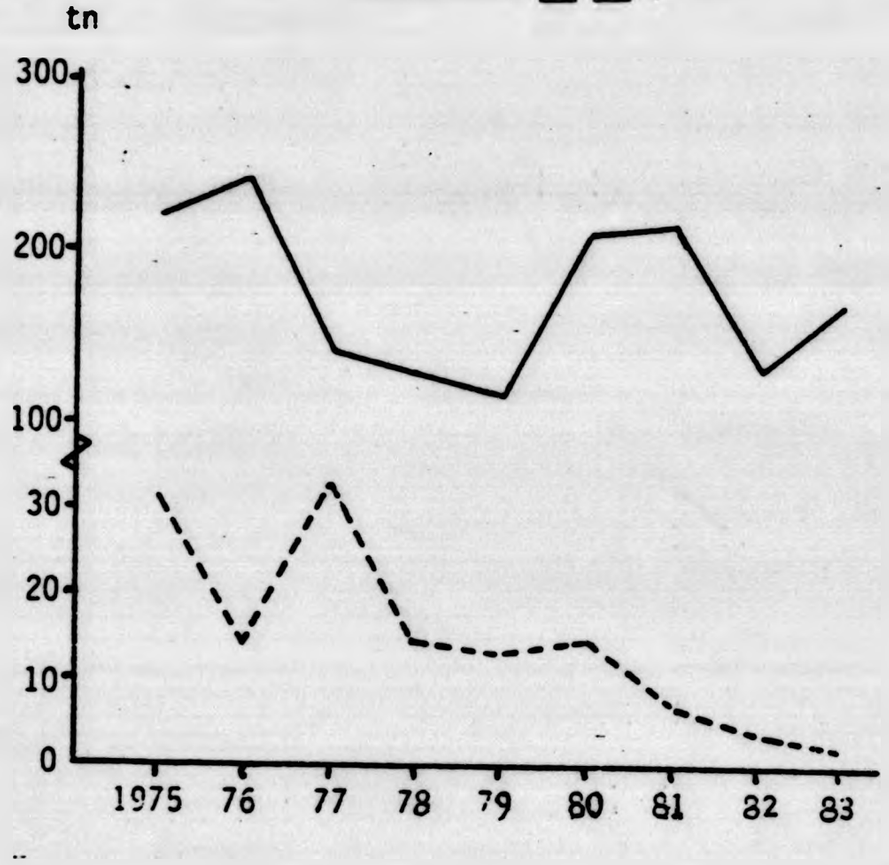
L_1 = maximum length of the lake

L_2 = maximum width of the lake

TABLE 2

Productivity of Lake A. Vassilios during the period 1975-1983

(Kilikidis et al., 1984)



——— Total fish production

- - - - Production of carp

TABLE 3

Phytoplankton species of Lake A. Vassilios, N. Greece
(Kilikidis et al., 1984)

A. CYANOPHYTES

Microcystis flos-aquae
Microcystis aeroginosa
Anabaena scheremetieri v. recta
Anabaena flos-aquae v. indermedia
Anabaena f. spiroides
Aphanizomenon flos-aquae
Aphanocapsa spp.
Gomphosphaeria aponina v. delicatula
Tetrapedia trigona
Chroococcus dispersus
Chroococcus limneticus

B. CHLOROPHYTA

Pediastrum boryanum
" tetras v. tetraodon
" simplex
" spp.
Coelastrum microporum
Scenedesmus falsatus
" quadricauda
obliguus
flexuosus
Tetraedron minimum
Tetraedron trigonum
Staurastrum leptocladum
Cosmarium spp.
Elakatothrix gelatinosa
Crucigenia rectanguloris
Oocystis spp.

C. DIATOMA

Cyclotella spp.
Melosira granulata v. valida
Cocconeis spp.
Gyrosigma spp.
Fragilaria crotonensis
Amphora spp.
Nitzschia spp.

D. DINOPHYTA

Ceratium hirundinella
Peridinium spp.

E. CRYPTOPHYTA

Cryptomonas erosa

E.

Euglena acus
Phacus pleuronectes

TABLE 4

Seasonal variation of phytoplankton in Lake A. Vassilios

(Kilikidis et al., 1984)

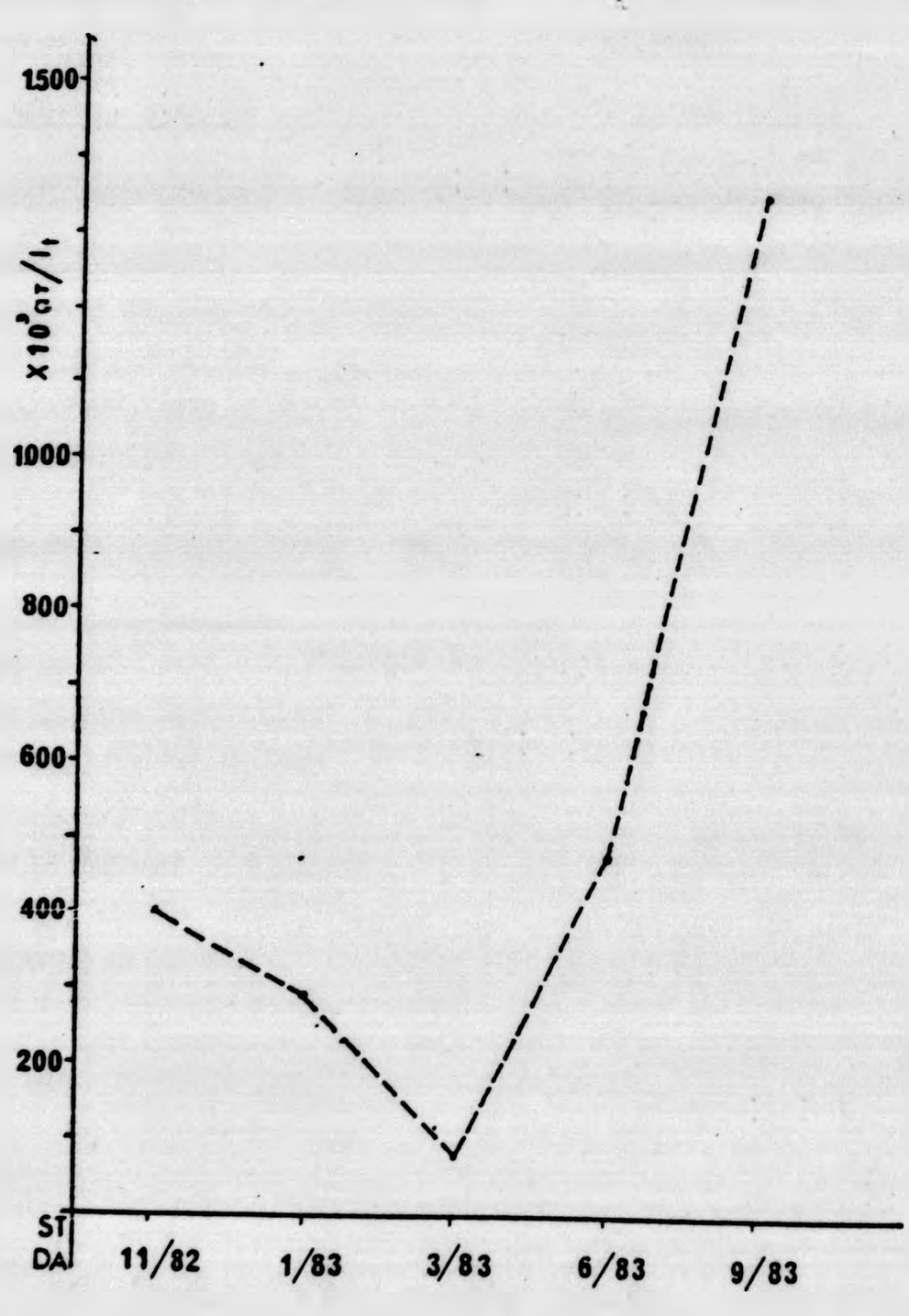


TABLE 5

Different zooplankton organisms found in
Lake A. Vassilios
(Kilikidis et al., 1984)

A. TROCHOPHORA

Trichocerca pusilla
Trichocerca birostris
Ketarella cochlearis f. tecta
Ketarella tropica
Polyarthra trigla
Pompholyx complanata
Anuraeopsis fissa
Hexarthra mira

B. CLADOCERCA

Bosmina longirostris
Bosmina sp.
Ceriodaphnia pulchella
Diaphanosoma brachyurum
Daphnia cucculata
Daphnia hyalina
Chydorus ovalis
Leptodora kindtii

C. COPEPODA

Cyclops vicinus
Acanthocyclops robustus
Arctodiaptonus bacillifer
Macrocyclops albidus

D. CILIATES

Tintinnidium fluviatile

TABLE 6

Benthic organisms of Lake A. Vassilios
(Kilikidis et al., 1984)

Chironomus plumosus	Limnodrilus hoffneisteri
Chironomus thummi	Nais obtusa
Chaoborus crystallinus	Nais communis
Tubilex tubilex	Nematoda
Tubilex oligocetaceus	

TABLE 7

Seasonal variations of benthic organisms in Lake A. Vassilios

(Kilikidis et al., 1984)

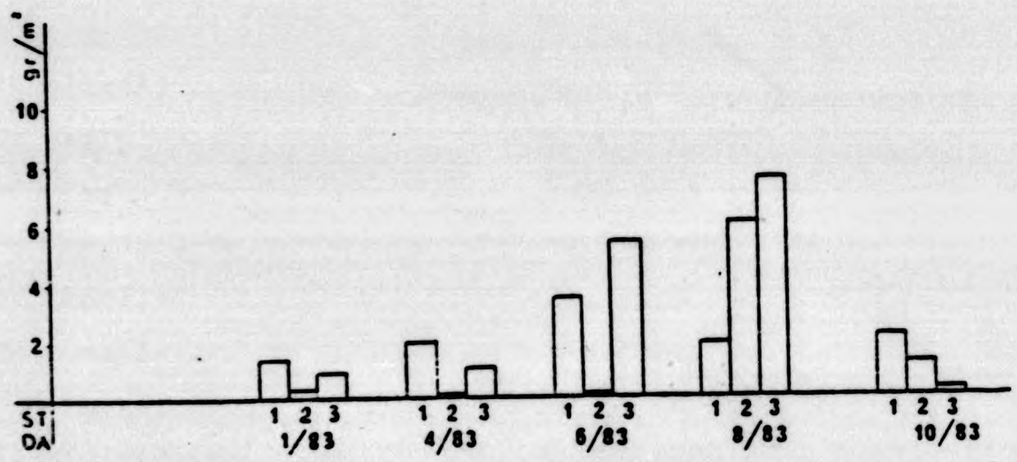
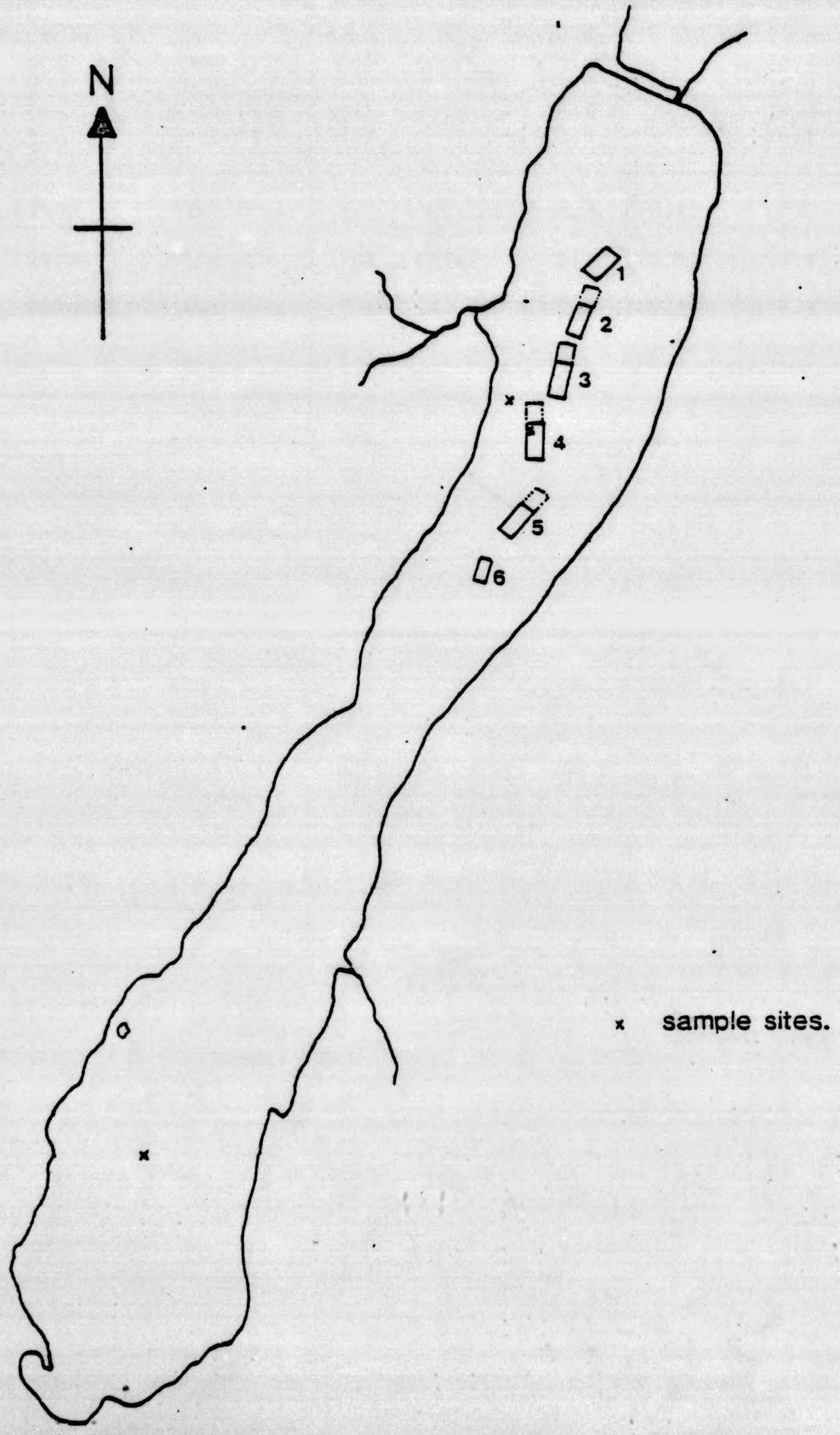


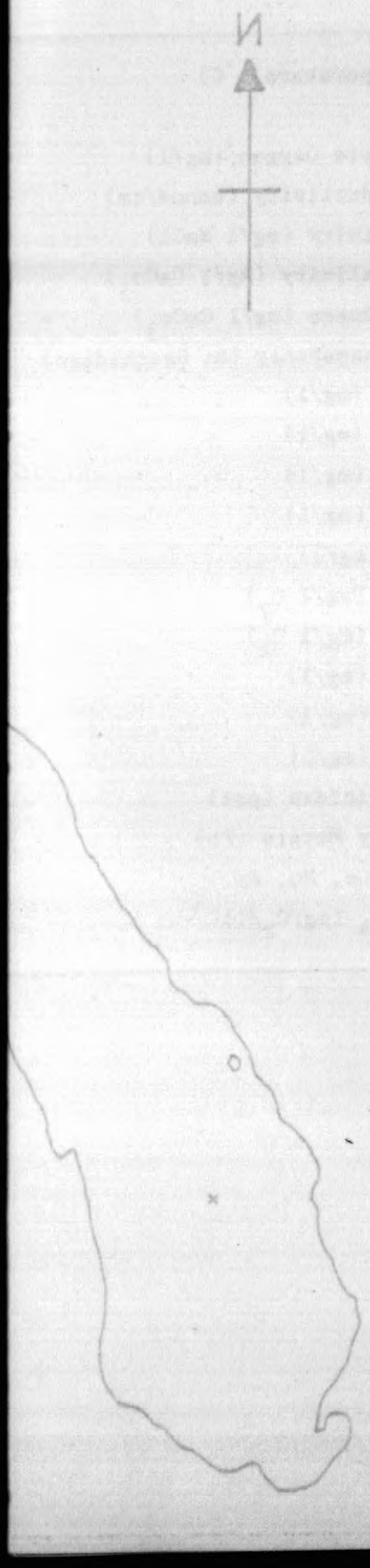
TABLE 8

Water parameters from Lake A. Vassilios
(Kilkilidis et al., 1984)

Temperature (°C)	6 - 24
pH	6.5 - 7.5
Dilute Oxygen (mg/l)	6.8 - 12
Conductivity (umhos/cm)	1150 - 1700
Salinity (mg/l NaCl)	550 - 825
Alkalinity (mg/l $CaCo_3$)	320 - 440
Hardness (mg/l $CaCo_3$)	60 - 180
Transparency (m, Secchidisc)	0.20 - 0.60
CO_2 (mg/l)	0 - 20
NO_3 (mg/l)	0 - 26.4
PO_4 (mg/l)	0 - 2.36
SO_4 (mg/l)	50 - 85
Si (mg/l)	1.65 - 6.50
BOD (mg/l O_2)	1.0 - 5.5
COD (mg/l O_2)	49.6 - 280
NO_2 (mg/l)	0 - 0.092
NH_3 (mg/l)	0 - 0.268
H_2S (mg/l)	Ø
Pesticides (ppt)	64.8 - 118.6
Heavy Metals (Pb)	0.001 - 0.002
Cu, Co, Mu, As	0.1
KM O_4 ($mg/O_2/l$)	0.57 - 2.40

FIGURE: 2 **Loch Fad.**





Descriptions of environmental conditions for Loch Fad, Scotland

Table 9 Water Quality in Loch Fad. Summer data 1982-1984

(A Stewart, 1985)

			1982	1983	1984
Temperature °C	May		12.3	11.6	14.0
	June		16.3	15.1	16.4
	July		18.3	19.0	18.2
	August		16.9	19.0	19.0
	Average		16.0	16.2	16.1
Oxygen mg/l (at 2 m depth)	June - Sept.	Loch ave.	9.0 (↓*0.7)	8.3 (↓0.6)	7.7
		Cage ave.	7.5 (↓1.1)	6.4 (↓0.5)	5.9
	August	Cage ave.	7.3 (↓0.5)	6.8 (↓0.5)	6.3
	Minimum 24 hr	Cage level	5.8 (↓1.6)	4.2 (↓0.5)	3.7
Chlorophyll 'a' ug/l surface	June-Sept.		62 (↑46)	108 (↑32)	76
Nutrient levels ug/l (surface samples)	June-Sept.				
Ammonia		ave.	216 (↑84)	300 (↑130)	430
		peak	400	700	800
Nitrite		ave.	12.3 (↑23)	35.3 (↑9.0)	44.3
		peak	140	310	150
Phosphate		ave.	29.3 (↑5.3)	34.6 (↑4.1)	38.7
Secchi disc (m)		ave.	0.93	0.77	0.57
		weeks below			
June-Sept.		0.5 m	0	10	10
Food input (tonnes dry wt.)					
	1st April - 31st August		108 (↑59%)	172 (↑101%)	346
	1st Jan. - 31st Dec.		254 (↑47%)	374 (↑55%)	508
Rainfall	August	mm	152	35	66
		% of ave.	128	30	55

* ↑ = increase, ↓ = decrease from one year to the next

Table 10 Temperature, dissolved oxygen and turbidity (Secchi disc depth) in Loch Fad on the 20th December 1988.

TABLE 11 Water quality in Loch Fad during 1988

(Philips et al., 1989)

Depth (m)	T (°C)	DO (mg/l)	T (°C)	DO (mg/L)	T (°C)	DO (mg/l)	T (°C)	DO (mg/l)
0	6.5	10.6	6.4	10.1	6.6	10.3	6.6	10.2
1	6.4	10.6	6.4	10.1	6.6	10.2	6.6	10.2
2	6.4	10.6	6.4	10.1	6.5	10.2	6.6	10.2
3	6.4	10.6	6.4	10.1	6.5	10.2	6.5	10.2
4	6.3	10.6	6.4	10.1	6.5	10.2	6.5	10.2
5	6.3	10.6	6.4	10.1	6.5	10.2	6.5	10.2
6	6.3	10.6	6.4	10.1	6.5	10.2	6.5	10.2
7	6.3	10.6	6.4	10.1	6.5	10.2	6.4	10.2
8	6.3	10.6	6.4	10.1	6.5	10.2	6.4	10.2
9	6.3	10.6	6.4	10.1	6.5	10.2		
10	6.3	10.6	6.4	10.1	6.5	10.2		
Secchi disc depth (m)	2.5		2.5		2.3		2.3	

TABLE 11 : Water quality in Loch Fad on the 20th December 1988.
(Philips et al., 1989)

Site	1			2			3			4		
depth (m)	0	5	10	0	5	10	0	5	10	0	4	8
pH	7.22	7.14	7.28	7.19	7.26	7.19	7.22	7.14	7.18	7.20	7.22	7.18
Alkalinity (meq/l)	0.86	0.86	0.84	0.86	0.87	0.85	0.84	0.87	0.85	0.86	0.86	0.85
Conductivity (µs/cm)	160	160	165	162	165	165	160	164	160	165	165	160
Ammoniacal nitrogen (µg/l as N)	-	737	1051	768	777	748	801	803	829	808	801	794
Nitrite nitrogen (µg/l as N)	7	6	7	6	6	5	5	6	5	5	6	5
Nitrate nitrogen (µg/l as N)	472	507	551	395	472	468	442	374	461	465	411	449
Dissolved reactive phosphorus(µg/l as P)	141	142	141	147	148	145	154	154	157	169	152	148
Total dissolved phosphorus(µg/l as P)	167	156	169	175	163	178	177	184	184	175	189	179
Total phosphorus(µg/l as P)	198	197	201	203	213	206	208	221	225	207	211	207

TABLE 12

Chemical analysis of a sample of water from Sandall Park Boating Lake taken on 22 February 1973

(Yorkshire River Authority, 1974)

Chemical parameter	Result in milligrammes per litre
Suspended solids	10.0
Chlorides as Cl	76.0
Ammoniacal Nitrogen	0.4
Nitrous Nitrogen	0.05
Nitric Nitrogen	Not detectable
Permanganate value 4 hours (total)	4.1
Alkalinity as Ca CO_3 to M.O.	240.0
Total hardness as Ca CO_3	333.0
Ortophosphates as P	0.02
pH	8.1

APPENDIX II

HISTOPATHOLOGICAL TECHNIQUES

Regime for the wax embedding of formalin fixed tissue samples for light microscopy using the Reichert-Jung, Histokinette 2000 automatic processor

1. 50% Meths for 1 hour
2. 80% Meths for 2 hours
3. 100% Meths for 2 hours
4. 100% Meths for 2 hours
5. 100% Meths for 2 hours
6. 100% Alcohol for 2 hours
7. 100% alcohol for 2 hours
8. Chloroform for 2 hours
9. Chloroform for 1 hour
10. Wax for 2 hours
11. Wax for 2 hours

MALLORY HEIDENHAIN TRICHROME STAIN

1.	Xylene	5 minutes
2.	Absolute Alcohol	2 minutes
3.	Meths	1 minute
WASH		
4.	Mallory Heidenhain Casson stain	15 minutes
5.	Running tap water	1 minute
6.	Distilled water	30 seconds
7.	Meths	30 seconds
8.	Absolute Alcohol I	2 minutes
9.	Absolute Alcohol II	1 minute
10.	Xylene	5 minutes

Staining Schedule for the Periodic Acid-Schiff (PAS) Reaction
from Drury and Wallington (1980)

1. Sections to water
2. Oxidize in 1% aqueous periodic acid for 5 mins
3. Wash sections in tap water (5 mins+)
4. Rinse in distilled water
5. Schiff's reagent for 10–20 mins
6. Wash thoroughly in running tap water for 10 mins
7. Stain sections in Haematoxylin (= 4 mins)
8. Wash in tap water
9. Differentiate in 0.5% acid-alcohol for a few seconds
10. Wash in tap water
11. 'Blue' in Scott's tap water substitute for 1 min
12. Wash in tap water
13. Meths for few seconds
14. Counterstain with 0.3% saturated solution of tartrazine in cellosolve for 5–10 mins
15. 70% alcohol for 1–2 mins
16. Absolute alcohol I for 1–2 mins
17. Absolute alcohol II for 1–2 mins
18. Xylene for 5 mins
19. Mount

Schiff's Reaction (Lillie, 1951)

Basic fuchsin	1.0g
Sodium metabisulphite	1.9g
N hydrochloric acid	15.0ml
Distilled water	85.0ml

Activated charcoal	0.5g

Results: Nuclei blue

PAS positive substances, including glycogen, red cytoplasm, connective tissue and muscle yellow

PERIODIC ACID SCHIFF (PAS) STAIN

1.	Xylene	5 minutes
2.	Absolute Alchol	2 minutes
3.	Meths	1 minute
WASH		
4.	1% Periodic Acid	10 minutes
WASH		
5.	Schiff's reagent	20 minutes
WASH		
6.	Haematoxylin	5 minutes
7.	1% Acid Alcohol	1-3 dips
8.	Scott's Tap water Substitute	Until blue
WASH		
9.	Meths	30 seconds
10.	Absolute Alcohol I	1 minute
11.	0.3% Tartrazine in cellosolve	3 minutes
12.	Absolute Alcohol II	1 minute
13.	Xylene	

VON KOSSA STAIN

1.	Xylene	5 minutes
2.	Absolute Alcohol	2 minutes
3.	Meths	1 minute
	WASH	
	DISTILLED WATER	
4.	1.5% Silver Nitrate	10–20 minutes (in dark)
	WASH IN DISTILLED WATER	
5.	0.5% Hydroquinone	5 minutes
	RINSE IN DISTILLED WATER	
6.	2.5% Hypo	5 minutes
	WASH	
7.	Neutral Red	30 seconds
8.	Meths	2 minutes
9.	Absolute Alcohol I	2 minutes
10.	Absolute Alcohol II	1 minute
11.	Xylene	5 minutes

GIEMSA STAINING FOR SECTIONS

from Drury and Wallington (1980)

1. Sections to water
2. Stain in dilute Giemsa solution (1 part Giemsa stock solution to 9 parts water) for 20 mins
3. Rinse rapidly in distilled water
4. Dehydrate very rapidly in absolute alcohol
5. Differentiate with a 0.2–0.5% colophonium solution in absolute alcohol for 10–20 secs
6. Absolute alcohol for about 5 secs to eliminate any traces of colophonium
7. Xylene for 5 mins
8. Mount

Results: Nuclei dark red

Red blood cells pink

Polar capsules dark blue

ZIEHL-NEELSON STAIN

1.	Xylene	5 minutes
2.	Absolute Alcohol	2 minutes
3.	Meths	1 minute
WASH		
4.	Carbon Fuschin (flood)	
5.	Warm slide gently until steam rises	5 minutes
WASH		
6.	3% Acid Alcohol	1-3 dips
WASH		
7.	Haematoxylin	5-10 minutes
8.	1% Acid Alcohol	1-3 dips
9.	Scott's Tap Water Substitute	Until blue
10.	Meths	30 seconds
11.	Absolute Alcohol I	2 minutes
12.	Absolute Alcohol II	1 minute
13.	Xylene	5 minutes

HAEMATOXYLIN AND EOSIN STAINING SCHEDULE

from Drury and Wallington (1960)

1. Section to water
2. Haematoxylin for 2-20 mins depending on stain type and strength. Mayer's haematoxylin was used here for 5 mins.
3. Wash in running water for 2 mins
4. Differentiate in 0.5% acid-alcohol for a few seconds. Check differentiation by examination under a microscope.
5. If nuclei are sufficiently stained, 'blue' in Scott's tap water substitute for 5 mins
6. Wash in water
7. Stain in 1% aqueous Eosin for 3-5 mins
8. Remove excess Eosin by rinsing in methos
9. Absolute alcohol I for 1-2 mins
10. Absolute alcohol II for 1-2 mins
11. Clear in Xylene for 5 mins
12. Mount

HAEMATOXYLIN - MAYER'S (1903) HAEMALUM

- cited from Lendrum and McFarlane (1940)

Haematoxylin	1.0g
Sodium Iodate	0.2g
Potassium alum	50.0g
Citric acid	1.0g
Chloral hydrate	50.0g
Distilled Water	1000.0ml

Acid-alcohol

0.5% Hydrochloric acid in 70% alcohol

Scott's Tap Water Substitute (Scott 1912)

Sodium bicarbonate	3.5gm
Magnesium sulphate	20.0gm
Distilled water	1000.0ml

Add a few crystals of thymol to prevent mould growth.

1% Aqueous Eosin

Eosin	1.0g
1 crystal thymol	100.0ml

Results: Nuclei blue

Cytoplasm, connective tissue, red blood cells and muscle pink/red

Giemsa Staining Schedule for Impression Smears
from Disbrey and Rack (1970)

1. Air dry
2. Methanol 2 mins
3. Stain in dilute Giemsa solution [1 part Giemsa stock solution to 9 parts buffered (pH 6.8) distilled water] for 1 hour
4. Methanol rinse for approximately 4 seconds
5. Differentiate in distilled water for 2-5 mins (pH 6.8)
6. Methanol for a few seconds
7. 70% alcohol for 1-2 mins
8. Absolute alcohol I for 1-2 mins
9. Absolute alcohol II for 1-2 mins
10. Xylene for 2 mins
11. Mount

Results: Nuclei dark red

Red blood cells pink

Polar capsules dark blue

PREPARATION OF SAMPLES FOR ELECTRON MICROSCOPY

from Bullock (1978) and Glauert (1975)

1. Tissue samples (= $1mm^3$) from freshly killed fish are placed in *2.5% glutaraldehyde in phosphate buffer for 1½ hours at 4°C. The tissue is cut into sufficiently small pieces by placing it onto a sheet of dental wax in a Petri dish and adding enough fixative to keep the tissue moist. It is then cut with a new scalpel, great care being taken to ensure a clean cut with little tissue damage. It is then transferred to fixative in a bottle.

2. Rinse several times in quick succession in +Millonig's buffer at pH 7.2. Three times over 2 hours is sufficient. Samples can be stored in buffer in the cold (= 4°C) at this stage for long periods before post-fixation.

3. Post-fixation: fix for 1 hour in *1% osmium tetroxide in Millonig's buffer

4. Wash in buffer, 2 times 30-60 mins

5. Dehydrate in alcohol as follows: 70% 2 x 10 mins
   200% 2 x 20 mins
   If required samples can be stored for a while in 70% alcohol

6. 50/50 mixture of propylene oxide and absolute alcohol for 15 mins

7. 100% propylene oxide for 15 mins twice

8. Mix up batch of resin with hardener and activator. Soak tissues in a 50/50 mixture of propylene oxide and resin and leave overnight (a minimum of 6 hrs)

9. 75%:25% propylene oxide and resin for 1 hour

10. 100% resin for 6 hrs at 40°C

11. Fill polythene Emcaps with resin and position the tissue within them. Polymerize overnight at 60°C

***Glutaraldehyde in phosphate buffer** (Bullock 1978)

Buffer:	0.067M KH_2PO_4	1 part (9.118 g/l)
	0.067M Na_2HPO_4	3 parts (9.512 g/l)

A percentage of glutaraldehyde is added to this buffer. 10ml of a 25% glutaraldehyde solution with 90ml of buffer gives 100ml of 2.5% solution.

†Millonig's phosphate buffer (Bullock, 1978)

Stock acid solution: monosodium phosphate 2.26% (2.6gm in 100ml water).

Stock alkali solution: sodium hydroxide 2.52% (2.52gm in 100ml water).

Buffer:	NaH_2PO_4 soln.	83.0ml
	NaOH soln.	variable, adjust to give correct pH, 17ml
	Distilled H_2O	10.0ml
	Sucrose	0.54gm

pH 7.2 - 7.4

Glucose may be used instead of sucrose.

***% osmium textroxide**

Osmium tetroxide is supplied as a 4% aqueous solution. To produce a 1% solution this is diluted with Millonig's buffer. 1ml of 4% osmium tetroxide solution plus 3ml of buffer gives 4ml of 1% solution.

STAINING SCHEDULE FOR ULTRA-THIN SECTIONS

from Bullock (1978)

Lead Citrate Stain:

Lead nitrate $Pb(NO_2)_2$	1.33gm
Sodium citrate $Na_3C_6H_5O_72H_2O$	1.76gm
Distilled water	30.0ml

Uranyl Acetate Stain:

Uranyl acetate (5% solution)	13.0ml
0.1N Hydrochloric acid	7.0ml
Michaelis buffer	5.0ml

Michaelis Buffer:

Sodium veronal (barbital sodium)	14.7g
Sodium acetate	9.7g
Distilled water	500.0ml

Method

1. Place the grids, section downwards, onto a blob of uranyl acetate for 20 mins
2. Wash the grids in distilled water. Blot dry
3. Place the grids, section downwards, onto blobs of lead citrate for 10 mins
4. Wash the grids in (a) 0.02N sodium hydroxide, and (b) distilled water (twice). Blot dry

Stain blobs are placed onto pieces of dental wax inside Petri dishes, and for Stages 3 and 4 pellets of sodium hydroxide are placed in the Petri dishes alongside the samples, in order to absorb carbon dioxide which could otherwise cause precipitation of the lead in the sections as lead carbonate.