THE AETIOLOGY OF WHIRLING DISEASE,

MYXOSOMA CEREBRALIS

by

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ABSTRACT

The mode of transmission of *Myxosoma cerebralis* was investigated with particular reference to the recent Wolf and Markiw hypothesis, which implicated the actinosporean genus *Triactinomyxon* as an intermediate stage in the life cycle, within *Tubifex* worms. Fish farms in the U.K. and Europe were sampled for both *M. cerebralis* and Actinosporea. *M. cerebralis* was not found in the U.K. farms sampled and there was no obvious correlation between the occurrence of *M. cerebralis* and *Triactinomyxon.*

M. cerebralis spores were added to *Tubifex* cultures in an attempt to initiate *Triactinomyxon* infections. In all cases there was no change in the prevalence of *Triactinomyxon* even after periods of 6 months. *Tubifex* worms were examined histologically and were found to take up *M. cerebralis* spores but the latter were never seen to show any signs of maturation ie. hatching, within the *Tubifex* gut.

It proved possible to infect juvenile trout in infection systems containing *Tubifex* worms and *M. cerebralis* spores, in the absence of *Triactinomyxon.* Fish exposed to *Triactinomyxon* did not develop any infection. Fish exposed to *M. cerebralis* spores alone did not develop any infection. The cross reactivity of *Triactinomyxon* spores with anti-*M. cerebralis* antiserum was confirmed, though other actinosporean species also cross reacted with this antiserum. Taken together these results shed considerable doubt on the Wolf and Markiw hypothesis.

In an attempt to aid field diagnosis of *M. cerebralis* infections, via serodiagnosis, Enzyme Linked Immunosorbent Assays (ELISA) were developed. *Aeromonas salmonicida* was used as a test organism for the antibody detection

assay and it was possible to monitor a significant change in antibody in fish inoculated with this bacterium. Using the ELISA anti-*M. cerebralis* antibodies were only detected in young fish (6 months) that had been experimentally infected while older fish (20 months) were seronegative. *M. cerebralis* antigens could not be detected in the serum of infected fish of either age group.

A combination of histological and immunohistochemical techniques were used to identify pre spore stages of the parasite in damaged cartilage. These were further investigated at the EM level.

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1.1. *Myxosoma cerebralis*

1.1.1. Description and pathogenicity

Myxosoma cerebralis was first described by Hofer (1903), in rainbow trout from Bavaria, and assigned to the genus *Myxobolus*, Butschli, 1882. A closely related genus, *Myxosoma*, was described by Thélohan (1892) and is distinguished from *Myxobolus* by the absence of an iodophilous vacuole in the former and its presence in the latter, Kudo, 1933. Most, but not all workers (Walliker, 1968; Lom, 1969a), accept the two genera and that *M. cerebralis* has no iodophilous vacuole and thus belongs to *Myxosoma*. The parasite attacks the developing cartilage of juvenile salmonids, particularly in the region around the brain and gill arches, leading to the progressive destruction of cartilage (Bogdanova, 1961; Lucký, 1970). As the area around the otoliths is destroyed, infected fish develop the characteristic symptoms of whirling disease. This is typified by tail chasing in young fish, which is seen 40 to 60 days after infection (Hoffman, Dunbar and Bradford, 1962). In heavily infected fingerling trout this often frantic whirling behaviour progressively exhausts the fish and coupled with the inability to feed effectively, leads to the high mortalities seen in some outbreaks (Hofer, 1903; Elson, 1969). Tail blackening may also be seen and this, as well as tail chasing, is more prevalent at higher water temperatures (Halliday, 1973a). In older fish which have survived the initial infection, gross morphological changes may occur, such as skeletal deformation (Hoffman, 1970a), though these symptoms may be confused with unrelated syndromes, such as dietary deficiency (Halliday, 1973b).

As ossification of cartilage proceeds, invasion by the parasite can no longer take place and various authors have observed that, once fish are older than 6-9 months, they are no longer susceptible to infection (Schäperclaus, 1954; Halliday, 1974a). The exact means by which cartilage destruction and its subsequent replacement with granulation tissue occurs, is not clear. Some workers, eg. Schäperclaus (1954) have speculated that, in addition to the direct destruction of cartilage, parasites also produce toxins which may play a part in the disruption of the otoliths.

Though the parasite is primarily localised within the cartilage of the head region, *M. cerebralis* spores have been found in other areas of the body. Halliday (1973a) observed spores within the spinal column of an infected fish, which would be responsible for the observed skeletal deformations, while Uspenskaya (1957) found spores throughout the body of infected fish.

1.1.2. Host susceptibility and dissemination

Though most of the literature deals with infections in rainbow trout, 14 salmonid species are known to be susceptible to varying degrees (Lom and Hoffman, 1971; Christensen, 1972). The disease problem is typically associated with highly intensive fish culture, and reports of infections in wild stocks are rare (Hoffman, 1970a; O'Grodnick, 1979). *M. cerebralis* has now been found throughout Europe, (Johansson, 1966; Elson, 1969), the U.S.S.R. (Bogdanova, 1969), the U.S.A. (Hoffman, 1970a) and the southern hemisphere (Van Wyk, 1968). It is generally suggested that the parasite originated in Europe and that the native brown trout, *Salmo trutta* was the original host. The latter may become infected but is considered to be resistant to the disease, unlike the rainbow trout, which has been imported from the U.S.A. and which is highly

susceptible. The spread from Europe to other continents would have occurred as a result of the transfer of fish stocks, which is a feature of the development of trout culture (Hoffman, 1970b). Schäperclaus (1954) found that the disease could not be imported into stocks via trout eggs. It is more likely that 'carrier' fish, which have developed low level infections and therefore show no obvious symptoms of the disease, are responsible for the extensive dissemination of whirling disease.

1.1.3. Diagnosis

The diagnosis of the parasite in infected fish relies principally on isolating the spore stage (Schäperclaus, 1954). Typically cartilage is removed from around the brain and gill arches and in the simplest procedure crushed and smeared for examination. Various purification regimes have been developed, designed principally to enhance the detection of spores, particularly when large numbers of fish are being processed. Enzyme digestion of skeletal elements using trypsin and pepsin, combined with acid treatments have been developed by Markiw and Wolf (1974), while O'Grodnick (1975) suggested the use of the plankton centrifuge when examining the homogenized material from whole heads. Phase separation techniques have also been used, involving polyethylene glycol and dextran, to which cartilage debris is added and then spun down (Kozel, Lott and Taylor, 1980). Spores separate out into one of the phases, with the cartilage debris in the other. In addition, histological examination of cartilage, using conventional paraffin wax embedding has been used, (Lucký, 1970; Halliday, 1973a). Histology has the advantage that it is theoretically possible to detect prespore stages. This is of particular significance in fish with low levels of infection and in those which have only recently become infected, since in neither case will spore stages be present or

easily identified. It is thought that the spore stage is only found as the cartilage is ossified in later life. Hoffman (1970a) has described trophozoites of *M. cerebralis* and Halliday (1973a) has observed a range of stages within infected cartilage. However, the detection of prespore stages has generally proved to be difficult, but this probably reflects the inefficiency of the histological techniques employed.

1.1.4. Developmental stages

It is likely that *M. cerebralis* prespore stages resemble those of other Myxosporea which have been studied (Lom and de Puytorac, 1965; Lom, 1969b). Halliday (1973a) distinguished trophozoites as large, multinucleate organisms and sporoblasts, which appeared as smaller binucleate stages each giving rise to two spores via the separation of nuclei. The prespore stages of *M. cerebralis* have not been observed at the electron microscope level, although whole spores have been examined (Lunger, Rhoads, Wolf and Markiw, 1975). The prespore stages typically reside within a mass of tissue which has been described as granulated and sometimes necrotic, connective material (Schäperclaus, 1954). Since cartilage is ossified from the exterior inwards, areas of damaged cartilage tend to become localised as the parasite is prevented from migrating to other areas.

1.1.5. Disease Control

Control of *M. cerebralis* infections has relied largely on disinfection of sites where the parasite has been found, and a number of chemical agents have been used in this context. Hoffman and Hoffman (1972) found that hydrated lime was an effective disinfectant, while Hoffman and O'Grodnick (1977)

found that chlorine did not kill all the *M. cerebralis* spores used in their tests. The application of calcium cyanamide to drained concrete ponds and raceways has been recommended (Schäperclaus, 1954). The efficacy of various agents such as ultraviolet radiation against the spores (Hoffman, 1975), has also been evaluated, though it is unlikely that this could prove commercially practicable for disease control on fish farms.

However, control methods are complicated by the apparent resistance of the parasite. It has been shown that the parasite retains its infectivity after freezing. Hoffman and Putz, (1969) found that *M. cerebralis* spores survive at -20 °C for at least 18 days. Evidence also suggests that spore viability is retained for several years in infected sites which are not disinfected; Bauer (1959) suggested that spores retained infectivity for up to 12 years.

It is possible to prevent infection by maintaining fry in concrete raceways fed by spring water, until they reach an age at which they are no longer susceptible (Schäperclaus, 1954). Should infection occur, the use of drugs to treat diseased fish is possible: Scolari (1954) found Acetarsone (Stovarsol) to be some use in suppressing the disease, while Taylor, Coli and Juncll (1973) suggested that Furazolidone might be effective. Again, as in the case of disinfectant agents such as U.V. radiation, it unlikely that drug therapy can be commercially applied.

1.1.6. Transmission

Until recently it has been thought that transmission of *M. cerebralis* is direct. However, the exact mechanisms involved are poorly understood as is also the case with other Myxosporea. Attempts to infect trout with freshly isolated

spores have not been successful (Hoffman and Putz, 1969). However, when 3-4 months before the fish were exposed the spores were added to tanks containing mud, infections were initiated (Hoffman and Putz, 1969; Hoffman and Putz, 1971). This gave rise to the suggestion that an 'ageing' period of several months duration is necessary before spores become infective. The exact nature of the process was not defined though Schäperclaus (1954) investigated, without success, the possibility that various invertebrates might serve either as carriers or intermediate hosts. The same author detected spores in the faecal material of fish-eating birds and suggested that these birds might play a part in the dissemination of the disease.

Uspenskaya (1957) reported transmission via the alimentary canal, and most workers have assumed that this is the route of infection (Schäperclaus, 1931; Noble, 1944). However, spores do not apparently discharge their polar filaments (which is presumed to be a prerequisite for hatching) in the acid condition of the stomach (Plehn, 1924). Observations that the polar filaments are discharged in alkaline conditions (Uspenskaya, 1957) has led to the hypothesis that the spores hatch in the intestine rather than the stomach. It is further presumed that the sporoplasm is released, migrates through the gut mucosa and then moves through the body via an unknown route to the developing cartilage in the head region. However, Daniels, Herman and Burke (1976) found what they described as an unidentified protozoan penetrating the skin of the head region of rainbow trout exposed to water containing *M. cerebralis*; this raised the possibility that this was an invasive stage of the parasite. These observations were not followed up.

A much more radical transmission mechanism for *M. cerebralis* was suggested by Wolf and Markiw (1981, 1984). Their results indicated that *M. cerebralis* spores pass into oligochaete worms of the genus *Tubifex* and there develop into an actinosporean intermediate stage in the life cycle, which they have described as *Triactinomyxon gyrosalmo*. *Triactinomyxon* spores are released from the worm and infect juvenile trout to complete the life cycle. In their system *M. cerebralis* spores were not directly infective to trout in the absence of *Tubifex* worms. The 'ageing' process described by previous authors could now be interpreted as the period required for the *Triactinomyxon* stage to develop. Subsequently, Wolf, Markiw and Hiltunen (1986) reported that, on addition of *M. cerebralis* spores to *Tubifex*, the prevalence of *Triactinomyxon* rose markedly after several months. This was interpreted as evidence for the involvement of *Triactinomyxon* in the life cycle of *M. cerebralis*. Markiw (1986), has given further details on the dynamics of the production of the *Triactinomyxon* stage.

1.2. Actinosporea

1.2.1. Actinosporea; definition of the group

The class Actinosporea is a parasitic group found in annelid worms, particularly oligochaetes. They have been relatively little studied, though Wolf and Markiw's work (1984, 1986) has obviously stimulated interest. Their discoverer, Stolc (1899) defined them as two layered animals or Mesozoa, and he noted their resemblence to the Myxosporea. This similarity was emphasised by other workers who suggested that the group, then termed the Actinomyxidia, had close affinities with the Cnidosporidia, that is Sporozoa characterised by the possession of polar capsules (Mrázek, 1900; Léger, 1904). This view was subsequently reinforced by Ikeda (1912) and the group was

placed in the protozoan subclass Cnidosporida, along with two other orders, the Myxosporidia and the Microsporidia, by Doflein and Reichenow (1927/8) and Hyman (1940). More recently the group has been reclassified and it is now described as a class along with the Myxosporea within the new phylum Myxozoa (Grassé, 1970; Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblick, Lom, Lynn, Merinfield, Page, Poljansky, Sprague, Vávra and Wallace, 1980).

1.2.2. Morphology

The Actinosporea are characteristically triradial with three polar capsules, each with an eversible polar filament. Within the apex of the spore is a sporoplasm containing bodies which have been variously termed gamonts or sporozoites (Hyman, 1940; Janiszewska, 1955a), surrounded by an inner endospore and an outer epispore. The remainder of the epispore is extended into a number of processes, typically three, the shape and form of which provide one of the principle diagnostic characteristics of the genera (Janiszewska, 1953; Janiszewska, 1955b; Marques and Ormieres, 1982).

1.2.3. Prevalence, localisation within the host and pathogenicity

Previous studies have shown that the prevalence of actinosporean infections in natural populations is very low; Ikeda (1912) found 1 in 400 worms were infected, while Mackinnon and Adam (1924) found a prevalence of 5 in 1250. However, Naville (1930) found a much greater prevalence in the case of *Guyenotia sphaerulosa* of between 30% and 50%.

Where infections occur they tend to be heavy (Granata, 1922a; Naville, 1930) which has led various authors to suggest that some type of autoinfection is occurring via the reinvasion of mature parasitic stages within infected worms (Mackinnon and Adam, 1924; Janiszewska, 1955a). Typically, the parasites are found either in the intestinal epithelium or its lumen, and are sometimes localised in a particular region of the worm (Janiszewska, 1953). Wolf, Markiw and Hultunen (1986) reported that the infection is visible in worms containing *Triactinomyxon gyrosalmo*, as a generalised anterior swelling and an opaque outer layer. Granata (1922b) observed *Triactinomyxon magnum* filling practically all the body cavity in a specimen of *Limnodrilus*.

Infection may cause extensive damage to the gut epithelium of the worm, even eventually causing the complete occlusion of the lumen (Granata, 1922a; Janiszewska, 1955a). There appears to be a detectable host reaction, as Janiszewska (1955b) observed host lymphocytes surrounding the earliest stages of parasite development.

1.2.4. Development

Caullery and Mesnil (1905) were the first to investigate the development of the Actinosporea while studying the new genus *Sphaeractinomyxon*, and their work was followed by the observations of Ikeda (1912), Granata (1922a, 1922b) and Mackinnon and Adam (1924), on a range of other genera. These studies were aided by the simultaneous occurrence of different developmental stages within infected worms. More recently Janiszewska (1955a) and Marques (1984) have provided detailed summaries of the literature on this subject.

Of the developmental phases studied, gametogenesis and sporogenesis have been most clearly described. Typically, gametogenesis is traced back to a single binucleate body (Léger, 1904; Granata, 1922; Mackinnon and Adam, 1924). Georgevic (1940a) traced the origin of this stage to schizogony of mononuclear cells which were themselves derived from globular elements corresponding to sporozoites. The binucleate body divides to give two somatic cells and two propagative cells; the latter in turn divide progressively to give a series of gametes termed α and β cells. At the same time the somatic cells give rise to the pansporocyst in which the gametes lie. The α and β gametes then form 8 zygotes or sporoblasts, which eventually form spores, via a complex series of steps which apparently varies in fine detail between genera (Stole, 1899; Leger, 1904; Granata, 1924; Janiszewska, 1955a). The end result is a pansporocyst containing 8 fully developed spores, each with their distinctive episporal processes. After discharge from the pansporocyst the episporal processes swell up and assume their characteristic shape (Janiszewska, 1955b; Marques and Ormières, 1982).

1.2.5. Dissemination

Janiszewska (1955a) suggested that the pansporocysts of species parasitizing the gut epithelium leave the worm along with the faeces, while those parasitizing the body cavity are released only on the death of the host. She also drew attention to the structural resemblance between the expanded processes of mature spores and the adaptations found in planktonic organisms. Ormières and Frezil (1969) and Marques and Ormières (1981), also noted the resemblance. Marques and Ormières (1982) confirmed the observations of Janiszewska (1955b) that the eight spores from a pansporocyst may link together by means of the tips of the episporal processes to form an extended

float, so that spores emitted simultaneously are kept together.

Several workers have noted the amoeboid nature of the sporoplasm within the worm host (Mackinnon and Adam, 1924; Janiszewska, 1955b), and Marques and Ormières (1982) and Marques (1984) have observed the release of the sporoplasm from free spores. These workers observed the sporoplasm changing its position in the water by vertical amoeboid movements, for up to one hour.

1.2.6. Transmission

The mode of transmission of the parasites between worms is unknown. Granata (1922b) found free spores in the intestinal lumen of worms and surmised that infection occurred by this route. Subsequently, Granata (1924) suggested that spores had to reach a state of maturity before infection could occur and that there was a possibility of a resting phase between the release of pansporocysts, and hence spores and subsequent re-infection. Janiszewska (1955a) speculated that the planktonic spore eventually sinks and then initiates infection by an undetermined route. Attempts to infect worms with actinosporean spores have been unsuccessful (Caullery and Mesnil, 1905; Granata, 1922b; Marques, 1984).

1.2.7. Genus *Triactinomyxon*

The genus *Triactinomyxon*, one of the 13 known actinosporean genera, according to the most recent review of the subject (Marques, 1984), was first described by Stole (1899), the type species being *T. ignotum.* Subsequently a range of other species were found (Mackinnon and Adam, 1924; Georgevic, 1940a,b). Marques (1984) provided a full list of the known species. There are 9 *Triactinomyxon* species out of a total of 37 identified actinosporean species.

The genus is typified by an anchor shaped epispore with three long tapering extensions, with an elongated sporoplasm containing between 2 and 100 sporozoites. Wolf and Markiw (1984) and Wolf, Markiw and Hiltunen (1986) described the actinosporean which they believed was the intermediate stage in the life cycle of *M. cerebralis* and named it *T. gyrosalmo.* Of the previously described species this most closely resembles *T. dubium* (Granata, 1924) which has 32 sporozoites in the sporoplasm. Wolf and Markiw gave the number of sporozoites in *T. gyrosalmo* as 32-50. In other respects *ie.* in size and shape, the spore of the two forms are identical. If these species are in fact identical then *T. gyrosalmo* becomes a junior synonym of *T. dubium.*

1.3. Fish immunology

1.3.1. General introduction

The ability of teleosts to mount an immune response against foreign antigens was first recognised by Metchnikoff (1892, 1901). Subsequent literature has tended to deal with attempts to induce immunity against infective agents in economically important fish species. A great deal of this work has centred on the bacterial pathogen *Aeromonas salmonicida*, the causative agent of furunculosis in farmed trout (Snieszko and Friddle, 1949; Klontz and Anderson, 1970; Paterson, 1981; Cipriano, 1982). Vaccines against this disease have been developed and this has necessitated the use of techniques to monitor the production of protective antibodies.

1.3.2. The nature of the immune response

There is also a large body of work on the mechanisms by which fish mount both cellular and humoral immune responses. It is apparent from these studies

that the immune response of teleost fish is functionally equivalent to that seen in higher vertebrates. Hence, teleosts show both primary and secondary responses (Trump and Hilderman, 1970; Desvaux and Charlemagne, 1981), appear to have cells equivalent to mammalian T and B cells (Cuchens and Clem, 1977) and can elicit mixed lymphocyte responses (Etlinger, Hodgins, Chiller, 1977). The humoral response received most of the early attention as the reviews of Corbell (1975) and Anderson (1974) indicate, though the elucidation of cell mediated responses, with particular regard to parasitic diseases, has more recently been undertaken (Hoole and Arme, 1982; Graves, Evans and Dawe, 1985). Of particular significance has been the observation that the teleost immune response is very much a temperature dependent process. Paterson and Fryer (1974) found that an antibody response in Coho salmon (*Oncorhynchus kisutch*) inoculated with *A. salmonicida* endoxin was detectable after 1 week at 17.8 °C while fish held at 6 °C took 4 weeks to mount a similar response. Such observations have led to the view that particularly in winter, when water temperatures are at their lowest, teleosts are severely restricted in their ability to mount an immune response.

1.3.3. Serodiagnosis of fish diseases

Serodiagnostic tests developed for human and veterinary medicine are also applicable to cultured fish stocks and routine use has been made of techniques, such as microfilter agglutination (Krantz and Heist, 1970; Paterson and Fryer, 1974). Most recently the highly sensitive Enzyme Linked Immunosorbent Assay (ELISA) (Voller, Bidwell & Bartlett, 1979) has proved suitable in detecting antibody responses in infected fish. Chart, Pearson and Trust (1984) used an inhibition ELISA for the detection of specific antibody against *A. salmonicida* and *Vibrio anguillarum* while Bortz, Kenny, Pauley, Garcia-Ortigoza and Anderson (1984) detected an antibody response in trout infected

with the helminth *Diplostomum spathaceum*. The latter authors stressed the advantages of the ELISA as sensitivity, reproducibility, low cost, rapid detection and the need for only small amounts of parasite antigen and fish serum.

1.3.4. Serodiagnosis of *M. cerebralis* and the occurrence of cell mediated reactions

As regards *M. cerebralis*, Lom (1969c) suggested that fish generally did not mount an immune response against metazoan parasites. Halliday (1974b), using an Indirect Fluorescence Antibody Test (IFAT), could not detect antibodies to *M. cerebralis* in rainbow trout, that were either naturally infected or inoculated with *M. cerebralis* spores, though in the latter case the experimental fish were in a poor condition. Stress of any type is known to have a depressant effect on the immune system in fish (Roales and Perlmutter, 1977), and this may explain the lack of antibody response in these fish. Pauley (1974) also could not detect an immune response against *M. cerebralis* and he suggested that the parasite antigens mimicked those of rainbow trout cartilage. It was also suggested that since the parasites were localised within cartilage, which is progressively ossified, they became effectively sealed off from the fish immune system and thus developed in an immunoprivileged site (Halliday, 1974b).

However, Griffin and Davis (1978) detected antibodies against *M. cerebralis* in infected young fish (6 months) using an IFAT. Griffin (personal communication) stressed the need for spore antigen integrity to be maintained and suggested that enzymatic treatment during extraction from the host, which some authors have used, (Pauley, 1974; Markiw and Wolf, 1980) should be avoided.

As regard cell mediated reactions, Lucký (1970) identified granulation tissue 6-12 months after infection, while Taylor and Haber (1974) identified a range of inflammatory cells in the region of damaged opercular cartilage. However, Roberts and Elson (1970) did not identify any cellular host reaction in several fish which were known to be infected.

1.3.5. Immunology of *M. cerebralis*

Halliday (1974b) showed that it was possible to raise an effective rabbit anti-*M. cerebralis* antiserum for use in an IFAT, whilst Markiw and Wolf (1978) used both indirect and direct immunofluorescence tests in studying the cross reactivity of *M. cerebralis* with other Myxosporea. Wolf and Markiw (1984) made use of this test to detect cross reactivity between *M. cerebralis* and *Triactinomyxon* spores and suggested that the shared antigens recognised by the polyclonal sera provided evidence for their interpretation of the *M. cerebralis* life cycle *ie.* that *M. cerebralis* and *T. gyrosalmo* are stages of one parasite. Both immunofluorescence and immunoperoxidase labelling have been used to study the occurrence of a range of parasites. Krotoski, Garnham, Bray, Krotoski, Killick-Kendrick, Draper, Targett and Guy (1982) used immunofluorescent labelling to detect the hypnozoite stage of *Plasmodium cynomolgi* in sections of infected tissue, and immunoperoxidase staining has been used to elucidate the exoerythrocytic stages of *Plasmodium berghei* (Hollingdal and Leland, 1982). Both techniques are applicable to the localisation of prespore stages in sections of damaged cartilage infected with *M. cerebralis.*

2. MATERIALS AND METHODS

2.1. Location of outbreaks of *M. cerebralis*

2.1.1. United Kingdom

Trout farms, in which *M. cerebralis* had previously been recognised at high prevalence, were sampled on the advice of the Ministry of Agriculture, Fisheries and Food (M.A.F.F.) inspectors and/or advisors within the Thames Water Authority. Where circumstances permitted, samples of up to 150 fish, of an appropriate age (3-6 months), were processed according to the conventional methodology for spore extraction: cartilage was removed from around the brain, particularly from the otolith region and the gill arches, and was then mechanically disrupted before filtration in Phosphate Buffered Saline (PBS), (8.0g NaCl, 0.2g KH_2PO_4, 2.4g $Na_2HPO_4.12H_2O$, 0.2g KCl/l) through a 64 um mesh (Gillespie, Evelyn, Frantsi, MacKelvie and Neufield, 1974). The macerated cartilage was then smeared on microscope slides for examination. Mechanical separation was followed by purification on Percoll gradients in the case of antigen preparation (Section 2.7.1). The enzyme treatments suggested by some authors (Markiw and Wolf, 1974), were avoided in order to maximise spore viability.

Mud samples were taken from the same habitats where possible, in order to record the presence or otherwise of Actinospora infecting *Tubifex* worms. The fish farms sampled were:

1. Cambrian, near Caerwys, Clwyd (May, 1984)
2. Hammer, Liphook, Hampshire (January, 1984)
3. Ampney, Cirencester, Gloucestershire (March, 1984)
4. Wandsford, near Great Driffield, Humberside (July, 1984)

2.1.2. Europe

In August 1984, the heads of 10 rainbow trout infected with *M. cerebralis* were sent from an outbreak of whirling disease in Brittany, France. In January 1985 I visited this infected farm and brought back a further 45 suspect fish heads along with mud samples. Serum samples were also taken from these fish for use in immunological studies. In September 1984 and March 1985, rainbow trout heads were sent from whirling disease outbreaks in Munich and Hannover, West Germany. All material of this type was simply kept cool in transit and was not chemically treated. On reaching the laboratory, heads were kept at 4 °C for periods up to 35 days before use.

In addition to the mud taken from the infected fish farms in the U.K. and France, samples were also collected from whirling disease infection tanks at the M.A.F.F. Fish Disease Laboratory, Weymouth, and from the Stratfield Saye fish farm, near Reading, Berks.

2.2. Detection of Actinosporeans

Mud containing oligochaetes was filtered through a 56 µm mesh. Worms were retained and removed from the sieve with fine forceps and placed in tap water for subsequent examination. Preliminary identification of the worms was made using subsamples of 50 worms, according to the method and key of Brinkhurst, 1971, via the use of Amman's lactophenol as a fixative (see appendix 1). To detect actinosporeans, worms were then placed individually on a microscope slide with a drop of water, and a smear was prepared by crushing the worm with another slide. Smears were observed under bright field illumination at x100 magnification. If an actinosporean species was present, some of the smear was transferred to another slide under a coverslip,

for observation at higher magnification and by phase contrast illumination. Photographs were taken as appropriate, and the species was identified with reference to the existing literature (Janiszewska, 1955a, 1957; Marques, 1984). Some methanol fixed slides were stained with Giemsa (2-10 minutes according to the species of actinosporean present) and then mounted in Canada Balsam.

2.3. *Tubifex* infection experiments

A suitable system for the culture of *Tubifex* worms was set up using *T. tubifex* originally derived from a local sewage farm. Subsamples of the population were identified (Brinkhurst, 1971) and examined for the presence of Actinosporea. Perspex tanks of 15 litres capacity were used as culture vessels. A 3:1 mixture of heat sterilised soil and sand (120 °C for 48 hours) was added to the bottom of each tank to a depth of 30 mm, and 5 litres of heat sterilised water was then poured in. Nutrient agar (Oxoid), at a concentration of 50 g/L, was introduced periodically to provide an organic input. This mud was stirred at weekly intervals during the course of the experiment. Each tank was then seeded with approximately 1000 *T. tubifex*.

Heads of infected rainbow trout, derived from the Brittany outbreak of whirling disease, were firstly washed in PBS, and then thoroughly macerated in 10 ml of PBS. The number of spores present was then estimated using a haemocytometer, and in excess of 10^6 *M. cerebralis* spores, in crude suspension, were added to each experimental culture. At no point were the spores exposed to chemical or enzymatic treatments. Cultures to which no spores had been added served as negative controls.

Initially 3 pairs of tanks, to which spores had been added, were maintained at water temperatures of 10, 15 and 20 °C respectively, and each pair had a corresponding negative control culture. The presence of Actinospora within the *Tubifex* worms was then monitored at intervals of 2, 3.5, 6, 7 and 8 months after addition of spores.

The initial experiment was extensively replicated using the same techniques; over a period of 20 months, a total of 14 *Tubifex* cultures were exposed to *M. cerebralis* spores, at 5, 10, 15 or 20 °C, with the appropriate controls. These systems were sampled at either 3 or 6 monthly intervals.

2.4.1. Fish keeping facilities

No pre-existing facilities for the culture of rainbow trout or *Tubifex* under controlled temperature regimes existed in the department. The system (shown in Fig. 1) was eventually constructed using commercially-available trout-rearing equipment and standard aquarium accessories. Each of three rectangular tanks (Purewell P105 hatching troughs) were connected by 3 inlet and 3 outlet pipes to a chiller thermocirculator (Churchill Thermocirculator, W15082). Water temperature was kept at between 10 and 12 °C during the course of parasite life cycle studies using 2-5 months old trout, and at 15 °C when older fish were used in immunological work involving *Aeromonas salmonicida*. Each trough contained 6 perspex aquarium tanks (15 l capacity), with independent charcoal filter pumps (Eheim 2007), and air supply (Whisper 600 Air Pumps). Activated charcoal (Elga B1720) in the filter pumps was initially changed at 3 weekly intervals, though subsequently the filter pumps were dispensed with. All tanks were painted black on the outside and were covered with close fitting black perspex lids. Water was first dechlorinated and then heat sterilised before being used in the tanks (Elga Spectrum SC6

Legend:

A : Churchill Thirmocirculator W15082

B : Purewell P105 hatching trough

C : 15l plastic aquarium tank

D : Eheim 2007 filter pump

1. Outlet
2. Inlet

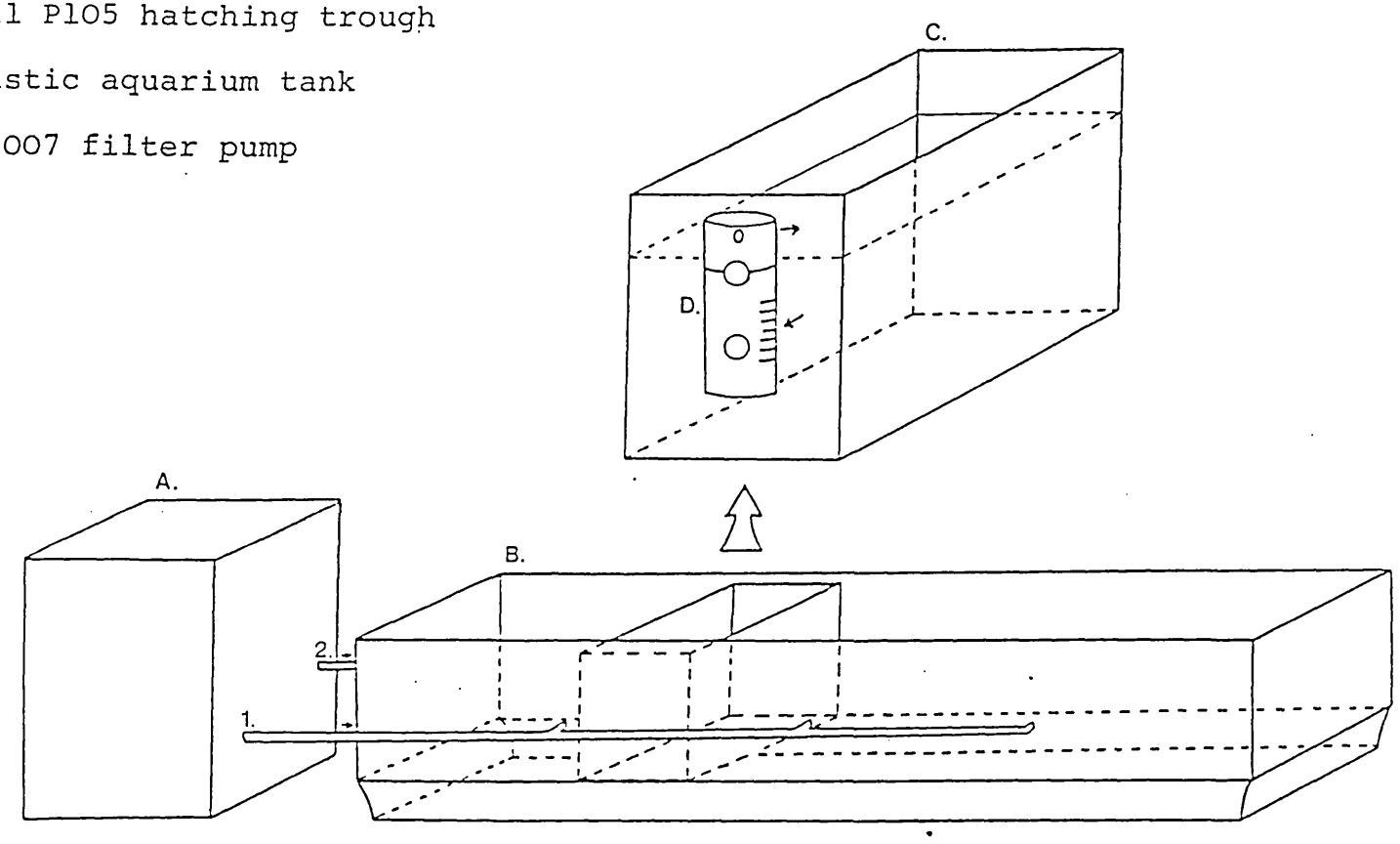


Fig.1 : Trout infection system

Dechlorination unit). All working surfaces, tanks and accessories were sterilized with Dairy Hypochlorite (FBC) at the beginning and end of any given experiment.

In addition a two tank recirculating water system was constructed using a 159l feeder tank (Sarena plastics, S.C.50) with overflow to a 55l tank (Sarena S.C.20), (Fig. 2). An Eheim filter pump 2015 was used to recirculate water with inlet from the S.C.20 tank and bifurcated outlet to the larger S.C.50 tank. Aeration was provided by Whisper 600 air pumps. All tanks and troughs were on raised platforms, 40 cm above floor surface to facilitate drainage into a disinfection trough underneath the single fitted sink in the laboratory.

2.4.2. Trout infection studies

Trout of an appropriate age, *ie.* between 1 and 3 months old, were obtained from local fish farms according to availability. Typically, 75 trout were sacrificed on day 1 of each run and were screened en masse by maceration and examination for spores, to detect any previous exposure to *M. cerebralis.* To detect any pre-spore infection some 125 of the original fish were maintained in sterile water and screened via histological examination, at monthly intervals, for *M. cerebralis.* The remaining fish were exposed to a variety of infection systems, as described below, and screened for *M. cerebralis* at appropriate intervals.

Legend:

E : Sarena Plastics S.C.50, 1591 tank

F : Sarena Plastics S.C.20, 551 tank

G : Eheim 2015 filter pump

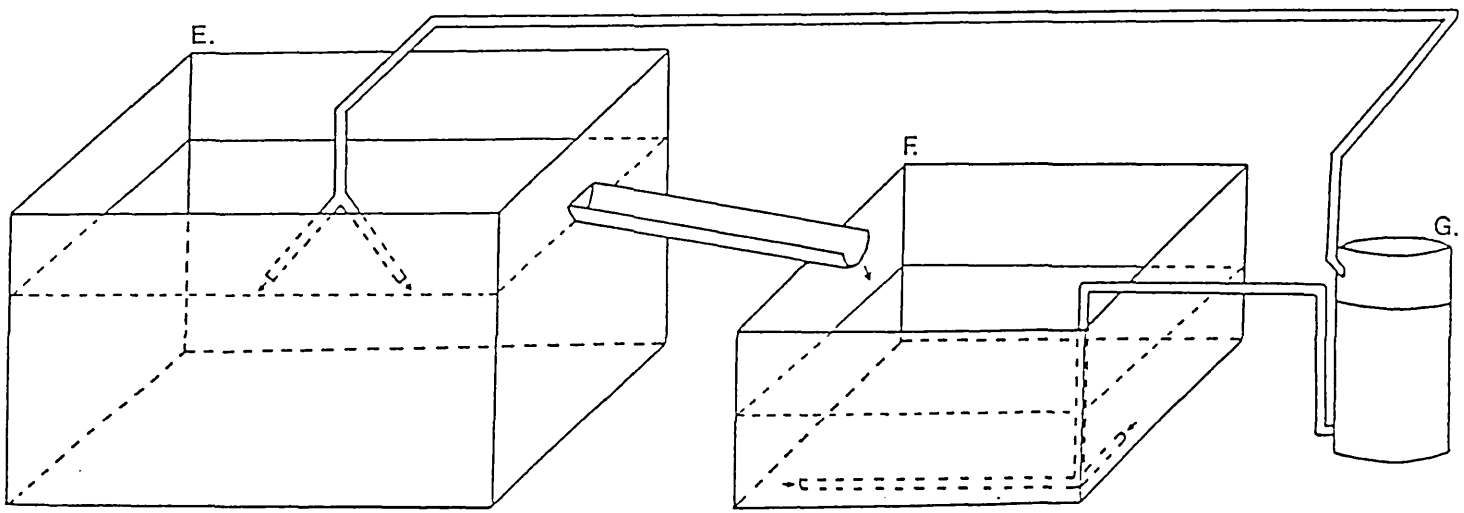


Fig.2 : Recirculating water system

Run 1: September 15th, 1984

Approximately 550 3 month old rainbow trout were collected from the Upwey fish farm, near Weymouth, Dorset. 15 fish were placed in each of the following tanks:-

a) Controls:

   (i) Heat sterilised mud and sterile water - 2 tanks

   (ii) Sterile water only - 7 tanks

b) Experimental:

   (i) Heat sterilised mud, sterile water and *M. cerebralis* spores - 3 tanks

   (ii) Sterile water and *M. cerebralis* spores - 3 tanks

   (iii) Heat sterilised mud, sterilised water, *Tubifex* worms (5×10^3/tank) and *M. cerebralis* spores - 3 tanks

M. cerebralis spores were obtained from the outbreak of whirling disease in Brittany, via simple maceration of infected heads. In excess of 5×10^5 spores were added to each of tanks b) i, ii, and iii, 4 weeks before the fish were introduced.

50 fish were placed in the 55l tank used in the recirculating system, and exposed to non sterilised mud from the Cambrian fish farm seeded with 1×10^4 *Tubifex* and 5×10^5 *M. cerebralis* spores.

Run 2: January 10th, 1985.

800 sac fry from a fish farm at Chesham, near Slough, Berkshire, were used in the same systems as detailed in run 1, but at a higher density of 30 fry per tank (all other conditions were as in run 1).

Run 3: April 25th, 1985.

Approximately 650 2-3 month old fish were obtained from the Chesham fish farm, which used spring water in its hatcheries. A density of 15 fish per tank was used and several changes were made to the type and number of tanks used:

a) Control tanks: as in runs 1 and 2

b) Experimental:

(i) Same tanks as used in runs 1 and 2

(ii) but the number of tanks was reduced

(iii) from 3 to 2

(iv) Mud from Brittany outbreak was sieved through a 50 µm mesh to remove both *Tubifex* worms and any possible *Triactinomyxon* contamination, and 5 x 10^5 spores were added - 2 tanks

(v) 3 tanks used in the *Tubifex* infection experiments (p. 29). *Tubifex* worms were screened at monthly intervals and the water column was sampled every 4 days for the presence of *Triactinomyxon.*

The use of the recirculating water system (Fig. 2), was discontinued at the beginning of this run, because of technical difficulties.

Run 4: September 31st, 1985.

Approximately 500 2-3 month old fish were obtained from Upwey fish farm, (15 fish per tank).

a) Controls and experimentals; as in run 3, but an additional group was included using *T. tubifex* derived from a sewage farm with an initial 2% infection of *T. dubium*, (400 worms sampled). The worms were first maintained in running tap water for 7 days and then added to 2 tanks containing heat sterilised mud to which agar had been added, (see p. 29),

(1 x 10^4 worms per tank). Both the water column and the *Tubifex* worms were subsequently tested for the presence of *T. dubium.*

Run 5: January 25th, 1986.

600 2 month old trout from the Chesham fish farm were used in the same infection systems as detailed in run 4.

2.5.1. Exposure of *Tubifex* worms to *M. cerebralis* spores

A sample of 80 *T. tubifex* derived from a local sewage farm were thoroughly washed in PBS and left overnight in tap water. They were then split into groups of 10, and added to 8 small plastic tubs (volume 20 ml) containing a heat sterilised mud substrate and 10 ml of tapwater. After equilibration for 48 hours in a dark box, freshly isolated *M. cerebralis* spores in a volume of 1 ml PBS, were added to 4 of the tubs, in volumes equivalent to 1 x 10^4 spores per tub. The remaining 40 worms served as controls. 10 worms, 5 from the test tubs and 5 from the controls, were sieved out at intervals of 2, 5, 7, 10, 14, 21, 35 and 42 days after the addition of spores, for subsequent histological examination.

2.5.2. Histological examination of *Tubifex* worms

Batches of worms were fixed in Carnoy's fixative for 45 minutes, then transferred to 95% ethanol, containing a few drops of Eosin, for 1 hour. After 2 changes in absolute ethanol (1 hour each), and 3 x 30 minute changes in xylene, the worms were immersed in wax (Paraplast). The wax was changed 3 times before being hardened overnight. Wax blocks were cut, using a Leitz rotary microtome, to give 7 μm sections, which were dried overnight on slides coated with egg albumin.

After removal of wax in xylene, the slides were taken down through an ethanol series and stained for 90 minutes in Giemsa stain. After differentiation with 15% Colophonium resin in acetone, slides were mounted in Green Euparol for examination.

2.6. Histological and ultrastructural examination of infected rainbow trout cartilage

2.6.1. Sectioning of material

Cartilage from rainbow trout derived from the German outbreak of *M. cerebralis*, or from fish exposed to the disease in the experimental systems, was dissected from the otolith region and fixed overnight in aqueous 3% glutaraldehyde. After dehydration, via an ethanol series, using 30 minutes for each concentration, specimens were embedded in LKB Historesin. This embedding kit consists of three components; basic resin, activator and hardener (appendix 2). Specimens were floated overnight at 4 °C in the infiltration solution (0.5 g activator and 50 ml basic resin). Once specimens had sunk, indicating complete infiltration, they were individually placed in the compartments of a Histomould and covered with the embedding medium (15 ml infiltration solution, 1 ml hardener). After polymerisation for 1 hour, the embedded specimens were freed and cut into rectangular pieces which were mounted on wooden blocks in preparation for sectioning. Glass knives for sectioning were made with a LKB 2078 Histoknifemaker and used with the Reichert Jung Supercut 2050 microtome. Sections were cut at between 0.5 and 2.0 µm thickness and floated on microscope slides for drying on a hotplate.

2.6.2. Staining of resin-embedded sections

a) A few sample slides were stained in Toluidine Blue for 30 seconds to assess the effectiveness of the sectioning.

b) For more detailed observation, slides were stained in Giemsa for 1 hour, then differentiated for 2 seconds in Colophonium in acetone. Slides were mounted in Canada Balsam.

c) To detect aldehydes, slides were washed in cold 1M HCl, then hydrolysed in 1M HCl at 60°C for 8 minutes. After another wash in cold 1M HCl, slides were stained for 30 minutes in Schiff's reagent, then washed in 3 changes of 1% sodium metabisulphite for 5 minutes, and then mounted in glycerol.

2.6.3. Examination of infected cartilage via electron microscopy

Heavily infected cartilage, derived from the German outbreaks, which had been prepared for light microscopy via fixation in 3% glutaraldehyde followed by embedding in LKB Historesin, was sectioned on an LKB ultramicrotome using glass knives cut on a LKB 7800B Knifemaker; sections were then transferred on to copper grids. Sections were cut at between 150 and 200 nm after locating areas of interest within the cartilage via examination of 1 µm Toluidine blue stained sections under the light microscope.

The copper grids were then transferred to a petri dish containing saturated alcoholic uranyl acetate (see appendix 3) and floated face down for 15 minutes. The grids were then removed and washed by rapidly dipping in boiled distilled water. Excess water was removed and the grids were dried for

a few minutes, section side uppermost. Grids were then transferred to a petri dish containing Reynold's lead citrate in a carbon dioxide free atmosphere (see appendix 4) and floated section side down for 7 minutes. Finally the grids were washed, as above, and then dried on filter paper, prior to examination using the Philips TEM 300 at voltages between 80 and 100 KV.

Cartilage taken from fish exposed to *M. cerebralis* in the experimental systems described in section 2.4.2., was processed according to more conventional electron microscopy techniques. 1 mm cubes of cartilage were taken through Karnovskys fixation schedule for vertebrate tissue (see appendix 5), and then placed in Spurr embedding media for 3 hours (hard formulation - see appendix 6). The media was then cured at 70 °C for 8 hours. Sections were then cut and transferred to copper grids prior to staining and subsequent examination using the TEM, as previously described.

2.7. Immunological Studies

2.7.1. Spore extraction and antigen preparation

Sample groups of 10 fish, aged 4-6 months and suspected of having infections of *M. cerebralis*, were processed in much the same way as detailed in section 2.1.1. Cartilage, removed from around the brain, particularly from the otolith region and the gill arches, was macerated and made up to a volume of 5 ml with PBS. The crude extract was passed through a 25 um filter and concentrated by centrifugation at 10,000 g for 5 minutes. The pellet was resuspended in 1 ml PBS. Enzymatic treatment, of the type used by Markiw and Wolf (1974; 1980), was avoided in order to maintain maximum antigenicity. In order to purify the spores further for antigen production, a phase separation technique was used. The dextran/polyethylene glycol two

phase system (Kozel, Lott and Taylor, 1980) was found to be less satisfactory than claimed by the authors. A modified technique using Percoll (Pharmacia Labs.) was more effective (Jouvenaz, 1981). A 4 phase gradient was prepared using 2 ml each of 25, 50, 75 and 100% Percoll, layered into an universal tube. 1 ml of cartilage extract was added to the top of the column and samples were centrifuged at 300 g for 10 minutes. Cleaned spores were washed in 5 ml PBS at 10,000 g for 5 minutes. The pellet was resuspended in PBS to a volume of 2 ml and filtered through a 0.8 um millipore filter (Millipore S.A., Molsheim, France). Spores were retained on the filter, which was then washed in 10 ml PBS. After centrifugation at 10,000 g for 5 minutes the filtering process was repeated twice, followed by a final centrifugation (as above). Finally, the pellet was resuspended in a volume of 1 ml PBS. Spore density was recorded using a haemocytometer and the spores were stored at 4 °C for subsequent usage as antigen.

2.7.2. Preparation of mouse anti-*M. cerebralis* antiserum

A total of 2 x 10^7 cleaned spores in 5 ml PBS were sonicated on ice for a total of 30 minutes, using a M.S.E. 150 W Sonicator with a 9 mm probe at an amplitude of 12 um peak to peak. After centrifugation at 10,000g for 5 minutes, the pellet was resuspended in 0.25 ml PBS and mixed with 0.75 ml Freund's complete adjuvant (FCA), until a water in oil emulsion was formed. 5 Balb C mice were inoculated intraperitoneally with a volume of 200 ul per mouse of the antigen preparation. A second intraperitoneal inoculation of 200 ul of the above antigen preparation in FCA was given 20 days later. After a further 7 days the mice were exsanguinated. The pooled blood was allowed to clot for 30 minutes at room temperature and at 4 °C overnight. The samples were spun down at 1000 g for 10 minutes and the serum collected. Serum was stored in aliquots at -20 °C for subsequent use.

2.8. Indirect Fluorescence Antibody Test (IFAT)

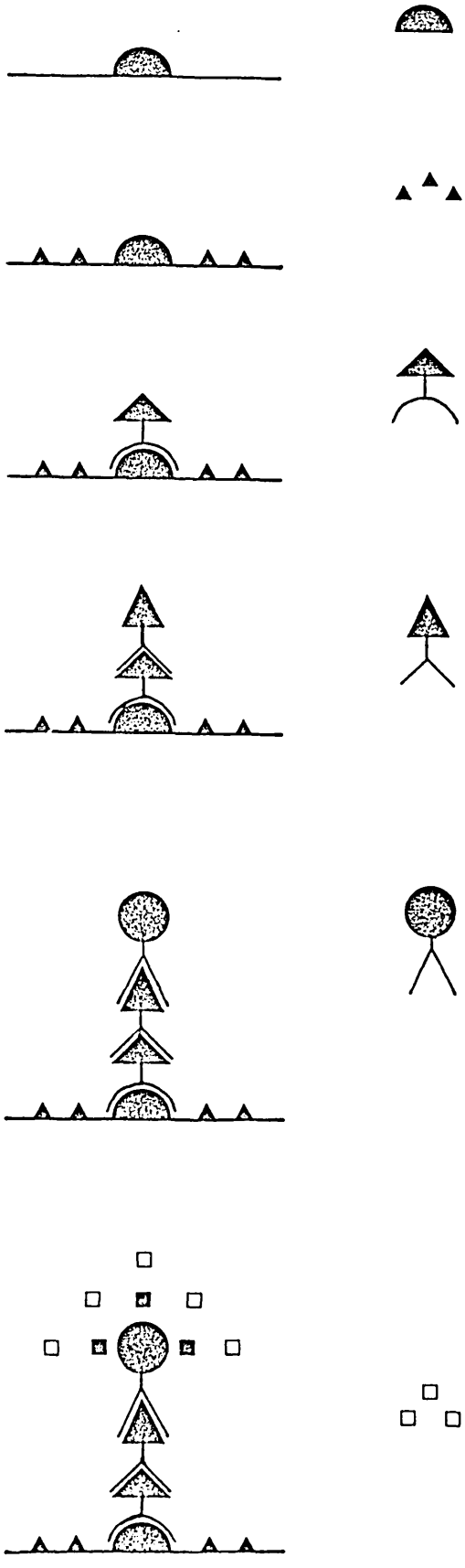
T. dubium spores were washed from a *Tubifex* smear preparation and attached to acid-cleaned slides, coated with 50% egg albumin. Slides were then dried for 15 minutes at 60 °C (Markiw and Wolf, 1978). The slides were incubated with mouse anti-*M. cerebralis* antiserum at dilutions of 1:10, 1:25, 1:40, 1:60, 1:80, 1:100, 1:120 and 1:140 for 40 minutes at 37 °C in a moist chamber. Slides were then washed three times in PBS. Fluorescein conjugated IgG swine anti-mouse antiserum, (Sw AM/FITC, Nordic labs) was added at dilutions of 1:40 or 1:80 and the slides were incubated again as above. A second series of washes with PBS was followed by a rinse with 1% Evans Blue in PBS. Finally slides were mounted in Citifluor low fluorescence mounting medium and viewed using dark ground illumination on a Zeiss microscope with U.V. illumination at 330-500 nm. Normal mouse serum was used as a negative control throughout, and spores of *M. cerebralis* were also treated as above as a positive control. The appropriate controls using PBS in place of the mouse serum or in place of the conjugate were also used. Other actinosporean species were used in place of *T. dubium* as they became available.

2.9. Enzyme Linked Immunosorbent Assays (ELISA)

2.9.1. Detection of circulating antibodies produced in rainbow trout against *A. salmonicida* and *M. cerebralis*

The system used is based upon the techniques of Voller *et al.* (1979) with modifications. Fig. 3 provides a diagrammatic representation of the components involved in the *M. cerebralis* ELISA.

Fig.3 : Diagramatic representation of ELISA to detect circulating antibodies against M.cerebralis



1. Incubate soluble extract of sonicated M. cerebralis spores in carbonate buffer for 18 hours at 4°C. Wash.
2. Incubate with 3% PSA for 2 hours at 22°C. Wash.
3. Incubate host serum from trout in PBS for 2 hours at 22°C. Wash.
4. Incubate rabbit anti-trout serum in PBS for 2 hours at 22°C. Wash.
5. Incubate conjugate (Goat anti-rabbit IgG conjugated with Horseradish proxidase) for 2 hours at 22°C. Wash.
6. Add substrate O.P.D. in phosphate/citrate buffer. Reactions read after 45 mins. on Dynatech ELISA reader.

2.9.1.1. Rabbit anti-trout antiserum

Rabbit anti-trout antiserum was raised in a New Zealand White rabbit by subcutaneous inoculation with 1 ml of pooled sera from 20 adult rainbow trout (from the designated disease-free hatchery at Avon Springs, Hampshire), in an equal volume of FCA. A second inoculation in FCA was given 21 days later, and 14 days after this the animal was exsanguinated and the antiserum was prepared.

2.9.1.2. Antibody production in rainbow trout experimentally infected with *A. salmonicida*

2.9.1.2.1. Bacteria

A. salmonicida, recovered from an outbreak of furunculosis at the Stratfield Saye fish farm was used as both inoculum and antigen. The strain is uncharacterised.

Bacteria were stored on Trypticase soy agar at 4 °C for 2 months; 7 days prior to use scrapings from these cultures were transferred to new agar plates and incubated at 20 °C. The rapid development of a dark brown pigmentation within the agar indicated that culture was still viable.

2.9.1.2.2. Trout

Ten month old rainbow trout, derived from the Kingsmead fish farm, Berkshire, were maintained at 15 °C in 15l aquarium tanks. Each tank was fitted with independent charcoal filter pump and air supply. A total of 70 fish, 5 per tank, were used in the experiment.

2.9.1.2.3. Inoculation of fish and antigen preparation

A. salmonicida from agar plates were firstly suspended in PBS, then centrifuged (10,000 g for 15 minutes), and filtered (Whatman filter paper No. 2). The residue was suspended in a known volume of PBS and the bacterial concentration determined using a haemocytometer. Unanaesthetised fish were inoculated intradermally, which has been shown to initiate a sub acute form of the disease (Bucke, 1980), with doses of 10^2, 10^3, and 10^4 bacteria per individual (total volume 0.1 ml/fish, 15 fish at each dose). Control fish were inoculated with a similar volume of PBS only.

Antigen was prepared from bacteria by sonicating using a M.S.E. 150W Sonicator with a 9 mm probe at an amplitude of 12 nm peak to peak for up to 1 hour, on ice. Aliquots of this antigen suspension were stored at -20 °C for subsequent use.

2.9.1.2.4. Fish sera

Blood samples were obtained by cardiac puncture, without anaesthetic or anticoagulant. After clotting for 30 minutes at room temperature and overnight refrigeration, the samples were spun down at 1000 g for 10 minutes and the sera collected. Sera was stored at -20 °C for subsequent use.

2.9.1.2.5. ELISA

Antigen (sonicated *A. salmonicida*) was diluted in Carbonate coating buffer, pH 9.6 (1.59g Na_2CO_3, 2.93g Na NCO_3 and 0.2g NaN_3 per litre), to a volume equivalent to 5 x 10^6 bacteria/ml and applied (100 µl/well) to flat bottomed ELISA plates (Dynatech, Billinghurst, Sussex). Plates were incubated for

22 hours at 4°C, then washed three times in PBS. Porcine serum albumin (PSA, Sigma) as a 3% solution was then used as a blocking protein and as a negative antigen control (incubation for 2 hours at 20°C). After washing in PBS, fish sera at dilutions of 1:250, 1:500 and 1:750 was then added in volumes of 100 µl/well and plates were incubated at 20°C for 2 hours. After washing three times with PBS-Tween (0.5 ml/L Tween 20, Sigma), the incubation was repeated with 100 µl/well of rabbit anti-trout antiserum, at a dilution of 1:500. The final incubation (again 20°C for 2 hours) with peroxidase conjugated goat anti-rabbit antiserum (G anti-R (H + L Ig G/P), Nordic, Maidenhead, Berkshire) was followed by a further 3 washes with PBS Tween. Rinsed wells were then reacted with 100 µl enzyme substrate (orthophenylene diamine, O.P.D., Sigma) at a concentration of 0.4 mg/ml in phosphate-citrate buffer (24.3 ml 0.1M citric acid, 25.7 ml 0.2M phosphate, 66 ul H_2O_2 (30% by volume) and 50 ml water, pH 5.0). Optical densities were read after 45 minutes at 492 nm using an automatic microplate reader (Dynatech, Sussex). Background binding was assessed by wells with no fish serum added, wells with no rabbit serum added and wells with no fish or rabbit serum added. The ELISA was repeated on a further 2 occasions.

2.9.1.3. Detection of circulating antibodies produced against *M. cerebralis*

2.9.1.3.1. Fish sera

Blood samples were obtained from fish infected with *M. cerebralis*, (confirmed subsequently by examination of the cranial cartilage) as described in section 2.9.1.2.4. Samples were not pooled. Sera was then prepared, again as described in section 2.9.1.2.4. Blood samples were also taken from fish known to be free of infection with *M. cerebralis*.

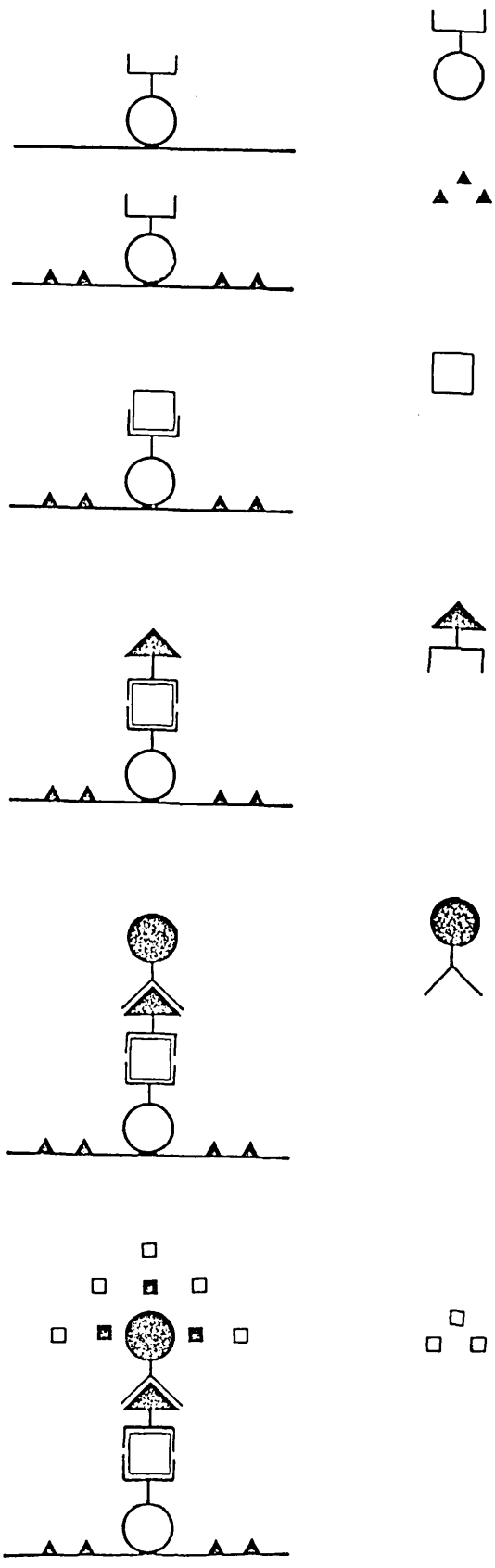
2.9.1.3.2. ELISA

Antigen, (freshly isolated, cleaned and sonicated spores of *M. cerebralis*) was diluted in carbonate coating buffer, pH 9.6, to a volume equivalent to 5 x 10^5 spores per ml and applied at 100 µl per well to 96-well rigid polystyrene ELISA plates. Plates were incubated for 18 hours at 4 °C, then washed 3 times in PBS. A 3% solution of PSA in PBS, was used to block the plates by incubating 100 µl per well for 2 hours at 22 °C. After washing in PBS, fish sera, from both infected and uninfected fish, were incubated at dilutions of 1:100, 1:250, 1:500, 1:1000 and 1:2500 at 22 °C for 2 hours. After washing 3 times with PBS-Tween, the incubation was repeated with 100 µl per well of rabbit anti-trout antiserum at dilutions of 1:250 and 1:500. The final incubation, at 22 °C for 2 hours, with peroxidase conjugated goat anti-rabbit antiserum at a dilution of 1:2000, was followed by a further 3 washes with PBS-Tween. Wells were then reacted with 100 µl enzyme substrate (orthophenylene diamine) at a concentration of 0.4 mg per ml in phosphate-citrate buffer. Optical densities were read after 40 minutes at a wavelength of 492 nm using an automatic microplate reader. Background binding was assessed in wells with no fish serum added, wells with no rabbit serum added and wells with no fish or rabbit serum added. Serum from known infection-free fish were used as negative controls. As in section 2.9.1.2.5. the ELISA was subsequently repeated.

2.9.2. Detection of *M. cerebralis* antigens using an ELISA

Figure 4 illustrates the components involved in this ELISA, which is derived from Voller *et al.* (1979), and Burkot, Zavala, Gwadz, Collins, Nussenzweig and Roberts (in press).

Fig. 4 : Diagramatic representation of ELISA to detect the presence of M.cerebralis antigen



1. Incubate Hamster anti-Myxosoma cerebralis in carbonate buffer for 18 hours at 4°C. Wash.

2. Incubate for 2 hours (at 22°C with 3% PSA) Wash

3. Incubate test serum from trout suspected of carrying M. cerebralis antigen in PBS for 2 hours at 22°C. Wash.

4. Incubate rabbit anti-M. cerebralis serum in PBS for 2 hours at 22°C. Wash.

5. Incubate conjugate (Goat anti-rabbit IgG/Po) for 2 hours at 22°C. Wash.

6. Add substrate, O.P.D., read as for Fig. 3

2.9.2.1. Mouse anti-*M. cerebralis* antiserum

See section 2.7.2.

2.9.2.2. Rabbit anti-*M. cerebralis* antiserum

A New Zealand White rabbit was inoculated subcutaneously with 2.5 x 10^7 sonicated *M. cerebralis* spores in a volume of 0.25 ml PBS and 0.75 FCA. A second inoculation was given 21 days later (as above, but using Freund's incomplete adjuvant). After a further 7 days the animal was bled from the right ear and the serum prepared. An IFAT was carried out on *M. cerebralis* spores to determine the titre of this serum.

2.9.2.3. ELISA

Mouse anti-*M. cerebralis* antiserum at a dilution of 1:250 was applied in volumes of 100 ul per well to flat bottomed ELISA plates, which were then incubated for 18 hours at 4 °C. After 3 washes in PBS, 100 µl of PSA as a 3% solution in PBS, was added and incubated for 2 hours at 22 °C. Plates were then washed in PBS and 100 µl of sera from rainbow trout infected with *M. cerebralis*, was added at dilutions of 1:10, 1:25, 1:50, 1:100 and 1:200. After incubating at 22 °C for 2 hours and washing 3 times in PBS-Tween, the plates were incubated at 22 °C for 2 hours with 100 µl per well of rabbit anti-*M. cerebralis* antiserum at a dilution of 1:250. PBS-Tween was again used to wash the plates 3 times before incubating for 2 hours at 22 °C with 100 µl per well of peroxidase conjugated goat anti-rabbit antiserum at a dilution of 1:2000. After a final wash in PBS Tween, wells were reacted with 100 µl enzyme substrate, OPD in phosphate citrate buffer (p. 45). Optical densities were read with the ELISA reader. Background binding was assessed by wells with no fish serum added, wells with no mouse or rabbit serum added and

wells with no fish, mouse or rabbit serum added; PBS was used to replace each missing component. A positive control was provided by incubation with sonicated *M. cerebralis* spores as antigen, in place of fish sera, at dilutions equivalent to 10, 10^2, 10^3 and 10^4 spores per well. Negative controls were provided by sera from fish which had not been exposed to *M. cerebralis*. Sonicated *Tubifex* were also tested as antigen along with spores of *Myxobolus* sp. at the concentrations used for *M. cerebralis*, and BSA as 1, 3, 5 and 10% solutions. As in section 2.9.1.2.5. the ELISA was subsequently repeated.

2.10. IFAT for the detection of circulating antibodies produced in rainbow trout against *M. cerebralis*.

The method is a modification of the technique used by Griffin and Davis (1978). Freshly isolated *M. cerebralis* spores were attached to acid cleaned slides (see p. 41). Slides were then incubated in a moist chamber with sera derived from known infected fish at dilutions of 1:25, 1:50 and 1:75, for 2 hours at 22 °C. The slides were then washed 3 times in PBS and incubated with rabbit anti-trout antiserum at dilutions of 1:50 and 1:100 for 2 hours at 22 °C. After washing, again 3 times in PBS, fluorescein conjugated IgG goat anti-rabbit (G anti-R/FITC) antiserum was added at dilutions of 1:50 and 1:100, and the slides were again incubated for 2 hours at 22 °C. After 3 further washes in PBS, followed by a rinse in 1% Evans Blue, slides were mounted for viewing under the U.V. microscope. Serum from a known infection-free fish served as a negative control, and the appropriate stage controls were also incubated.

2.11. Immunohistochemistry

The 2 um sections of infected cartilage prepared for Giemsa staining (p. **37**) were also suitable for immunohistochemical examination, in which absorbed mouse anti-*M. cerebralis* antiserum and fluorescein or peroxidase conjugated swine anti-mouse antiserum were used to label the parasites.

2.11.1. Absorption of mouse anti-*M. cerebralis* antiserum.

The mouse antiserum (section 2.7.2) was absorbed by incubating with macerated cartilage and bone from uninfected rainbow trout for 2 hours at 4 °C, followed by overnight incubation at 10 °C. The antiserum was then centrifuged at 10,000 g for 10 minutes and the supernatant was passed through a 0.22 um Millipore filter, previously washed with a 1% solution of PSA in PBS, to prevent antibody sticking to the filter. The antiserum was filtered a second time and stored at -20 °C for further use.

2.11.2. Immunofluorescence staining of infected cartilage sections

Cartilage sections were incubated with absorbed mouse antisera at dilutions of 1:25, 1:50 and 1:75 for 40 minutes at 37 °C. After washing 3 times in PBS, conjugate (swine anti-mouse antiserum, section 2.8.) was added at dilutions of either 1:40 or 1:80 and the slides were incubated as before. After a further 3 washes with PBS the slides were rinsed with 1% solution of Evans Blue in PBS and were finally mounted in Citifluor low fluorescence mounting medium and examined for fluorescence. Controls were used as in section 2.8.

2.11.3. Immunoperoxidase staining of infected cartilage sections

After incubation with absorbed mouse antisera (as above), slides were washed 3 times in PBS and then incubated with peroxidase conjugated swine anti-mouse antiserum (Sw anti-M(H+L IgG/P)) at dilutions of 1:500 and 1:750, for 40 minutes at 37 °C. After washing 3 times in PBS the slides were reacted with the enzyme substrate OPD in citrate-phosphate buffer, which was then rinsed off with PBS after either 30 or 90 minutes. Slides were then examined.

3. RESULTS

3.1. Transmission studies

3.1.1. Location of *M. cerebralis* outbreaks

The number and age of trout sampled from fish farms in Britain and France are given in Table 1. All British farms were negative for *M. cerebralis*; none of the fish sampled from the 4 fish farms showed any clinical symptoms eg. whirling, and *M. cerebralis* spores were not detected by smear preparations of macerated cartilage. Mud samples were taken from 3 of these fish farms for examination for tubificid worms and Actinosporea.

The Brittany trout farm was positive for whirling disease. Over 50% of the older fish (18-24 months) in the farm ponds showed skeletal deformities, particularly in the head region and the area immediately behind it, and there was some evidence of tail blackening. Large numbers of *M. cerebralis* spores were isolated from each of the 45 fish which were sampled, Table 2. Fig. 5 shows spores obtained from a cartilage smear from one infected fish. Fish sent from the German outbreaks were also heavily infected; out of a total of 24 fish all but one contained spores. One typical spore count for these fish is also shown in Table 2.

3.1.2. Occurrence of actinosporean species

Table 3 gives the prevalence of actinosporean species in tubificid worms recovered from mud from the sites from which samples were taken. Figs. 6, 7, 8, 9, 10, 11 and 12 show some of the species of Actinosporean identified in these studies. These were identified using the parameters of shape and size of

Table 2: Number of spores isolated (spores/ml) from individual infected trout

Percoll band	7 month old trout from German outbreak	18 month old trout from Brittany outbreak	5 month old trout experimentally infected (see section 3.1.4.)
x/25%	250,000	350,000	15,000
25%/50%	50,000	125,000	3,000
50%/75%	10,000	35,000	-
75%/100%	-	-	-

Table 1: Results of sampling from fish farms for presence of *M. cerebralis*

Fish Farm	Fish sampled	Presence of *M. cerebralis*	Mud sample taken
Cambrian, N. Wales	46 14-month old rainbow trout	Negative	Inlet and outlet
Hammer, Liphook, Hants.	65 6-8 month old rainbow trout	Negative	Ponds and outlet
Ampney, Cirencester, Glos.	102 3-month old rainbow trout.	Negative	-
Wandsford, Driffield, Hull.	89 4-8 month old rainbow trout	Negative	-
Lannion, Brittany.	45 18-24 month old rainbow trout	Positive	Ponds and outlet

Fig. 5 Fresh *M. cerebralis* spores from a cartilage smear suspended in PBS from an infected trout. Bright field illumination.

Figs. 13 & 14 *M. cerebralis* spores within the gut lumen of *T. tubifex* (spores arrowed).


25μm
50μm
10μm

Table 3: Prevalence of Actinosporea in tubificid worms collected at the various sites from which mud was sampled

Source		Actinosporean present	Prevalence (%) (200 worms examined unless otherwise stated)	
+Cambrian fish farm	a) Inlet:	*Echinactinomyxon radiatum*	1.0	
		Synactinomyxon tubificis	1.5	
	b) Outlet:	*S. tubificis*	2.0	
+Hammer fish farm	a) Ponds:	*Triactinomyxon ignotum*	0.5	
	b) Outlet:	*E. radiatum*	0.5	
*Lannion fish farm	a) Ponds:	None found	-	n = 107
	b) Outlet:	None found	-	n = 42
Stratfield Saye, fish farm	a) Outlet:	*E. radiatum*	0.5	n = 187
		T. ignotum	1.5	
#M.A.F.F. (Weymouth)		*E. radiatum*	9.5	
		S. tubificis	2.0	
		T. ignotum	2.0	
		T. dubium (= *T. gyrosalmo*)	0.5	
		Auractinomyxon raabiiunioris	0.5	
Sewage farm		*E. radiatum*	0.5	
		T. dubium (= *T. gyrosalmo*)	0.5	
		S. tubificis	1.0	

+ Previously diagnosed by M.A.F.F. and Waterboard inspectors as Whirling disease positive.

* Whirling disease diagnosed in the present study.

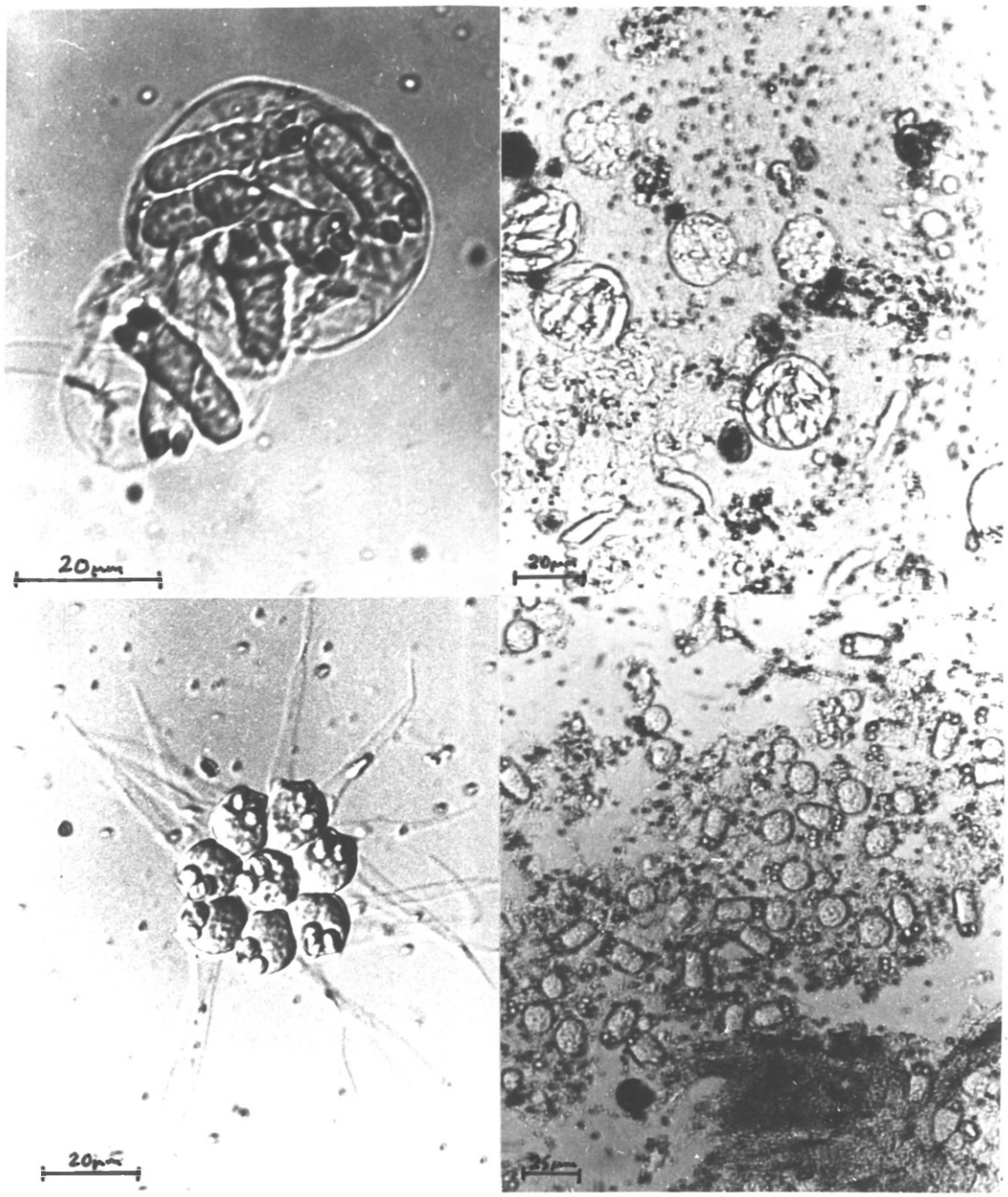
Whirling disease being cycled in experimental infections system.

Figs. 6, 7, 8 & 9 — Spores of Actinosporea derived from *Tubifex* worms.

Figs 6 & 7 — Pansporocysts of *T. dubium.*

Fig. 8 — Pansporocyst of *S. tubificis.*

Fig. 9 — Mass of *E. radiatum* spores within smear preparations of *Tubifex* gut.

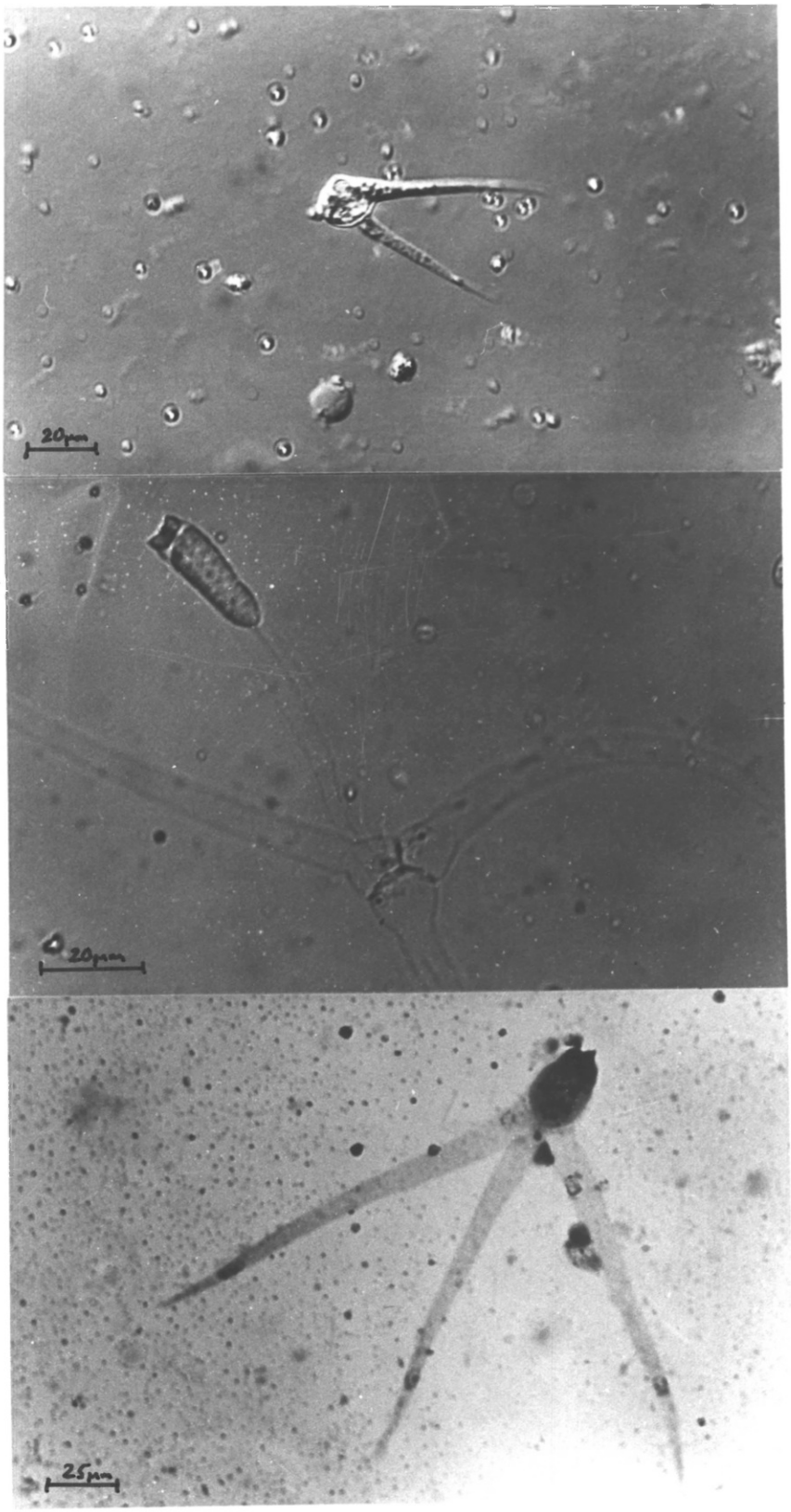

20μm
20μm
20μm
20μm

Figs. 10, 11 & 12 Spores of Actinosporea parasitising tubificid worms recovered from various mud samples.

Fig. 10 *S. tubificis*

Fig. 11 *T. dubium*

Fig. 12 *E. radiatum* (Giemsa stained)


20µm
20µm
25µm

the epispore arms at genus level and by number of sporoplasms and size of epispore arms at species level, see Table 4. Of the oligochaete worms isolated from field samples, the majority of those identified were *T. tubifex* (Table 5), except in the mud from the Brittany fish farm in which other unidentified Oligochaete worms predominated. In the other field samples the remaining worms consisted predominantly of *Limnodrilus hoffmeisteri*, with a small number of other unidentified oligochaetes. Immature worms are not included in these data. Of the worms observed from a local sewage farm and used in the *Tubifex* cultures, over 90% of those identifiable were *T. tubifex*. Since actinosporean infections were only clearly obvious after smearing the worms, which often made correct identification of the latter difficult, only limited data is available on the identity of the host species of worm involved. This data is shown in Table 6, along with a resumé of the literature relating to worm host species.

3.1.3. Attempts to infect *Tubifex* by addition of *M. cerebralis* spores isolated from infected trout.

Table 7 (a, b and c) shows the initial and subsequent prevalences of Actinosporea in tubificid worms at intervals up to 8 months from the addition of *M. cerebralis* spores to *Tubifex*, derived from a local sewage farm, held at 5°, 10°, 15° and 20°C. There were no significant changes in prevalence of any of the Actinosporea present during the period of observation. The highest prevalence recorded for *T. dubium* was 3% in one test culture held at 20°C for 3.5 months. However, a prevalence of 2.5% was recorded in one of the control cultures held at 15°C, as opposed to 0.5% and 1.5% in the corresponding test cultures held at 15°C for 3.5 months.

Table 4: Morphological characteristics on which identification of actinosporean species present in mud samples was based (with reference to Janisweska, 1955a; 1957; Marques, 1984).

Genus	Species
Echinactinomyxon	*E. radiatum*
Triradiate episporal arms emerge at 45° from bullet shaped head region. Episporal arms rigidly straight, no curvature.	Episporal arms measure between 100 and 125 µm. Between 20 and 28 sporoplasms in head region.
Synactinomyxon	*S. tubificis*
2 prominent episporal arms emerging from circular head region. Third episporal arm greatly reduced, provides contact point in pansporocyst.	Episporal arms 30 µm long, 32 sporoplasms.
Auractinomyxon	*A. raabiiunioris*
Triradiate broad leaf shaped arms, emerging from rounded head region, spore has flattened appearance.	Episporal arms between 25 and 30 µm, 16 sporoplasms.
Triactinomyxon	*T. ignotum*
Head region separated from triradiate episporal arms by elongated style.	Style between 40 and 170 µm long, arms 200 µm. 8 sporoplasms.
	T. dubium
	Episporal arms 130 - 150 µm. 32 sporoplasms.

Table 5: Species and prevalences of Oligochaetes present at the various sites from which mud samples were taken.

Source	Identifiable Oligochaetes present		Prevalence % (per 50 worms)
Cambrian fish farm	Inlet:	*T. tubifex*	82
		L. hoffmeisteri	10
		Others	8
	Outlet:	Not identified	-
Hammer fish farm	Ponds:	*T. tubifex*	74
		L. hoffmeisteri	26
	Outlet:	*T. tubifex*	80
		L. hoffmeisteri	14
		Others	6
Lannion fish farm	Ponds:	*T. tubifex*	28
		L. hoffmeisteri	20
		Others	52
	Outlet:	Not identified	-
Stratfield Saye fish farm	Outlet:	*T. tubifex*	88
		L. hoffmeisteri	12
M.A.F.F. Weymouth		*T. tubifex*	92
		L. hoffmeisteri	8
Sewage farm		*T. tubifex*	90
		Others	10

Table 6: Worm host species in which various Actinosporea were found.

Actinosporea species	Worm host identified in present study	Host records in literature
E. radiatum	3 out of 4 of infected worms were *Tubifex tubifex* in M.A.F.F. sample	*T. tubifex*; Marques, 1984
S. tubificis	2 out of 4 of infected worms were *T. tubifex* in M.A.F.F. sample	*T. tubifex*; Marques, 1984
A. raabiiunioris	-	*Limnodrilus* sp. and *Tubifex* sp; Marques, 1984
T. ignotum	-	*T. tubifex*; Leger, 1904; Mackinnon and Adam, 1924
T. dubium	4 out of 4 of infected worms in M.A.F.F. and sewage from samples were *T. tubifex*	*T. tubifex*; Janiszewska, 1959

Table 7 (a): Prevalence of Actinosporea 2, 3.5, 6, 7 and 8 months after adding *M. cerebralis* spores to *T. tubifex* cultures held at 10, 15 and 20 °C.

	T. dubium (=*T. gyrosalmo*)		*S. tubificis*		*E. radiatum*	
	CONTROL %	TEST %	CONTROL %	TEST %	CONTROL %	TEST %
(1) Initial prevalence (n = 100)						
10 °C	0.5	1.0 0	1.0	2.0 0	0.5	1.0 0.5
15 °C	0	0.5 0	0.5	0 1.0	0.5	0 0
20 °C	1.0	0.5 0.5	0.5	0.5 1.0	0	0 0
(2) 2 months (n = 200) after spore addition						
10 °C	0.5	0 0.5	0.5	0 0.5	0	0.5 0
15 °C	1.0	2.5 1.0	0.5	1.0 0.5	1.5	0.5 1.0
20 °C	0.5	1.0 0	0	1.0 0	0	0 0
(3) 3.5 months (n = 200)						
10 °C	1.0	1.0 0	0.5	3.0 1.0	1.0	1.0 1.5
15 °C	2.5	0.5 1.5	1.0	0 0.5	0.5	1.0 0
20 °C	0.5	3.0 1.5	0.5	0 0	0	1.0 1.0
(4) 6 months (n = 100)						
10 °C	0.5	0 0.5	0	0.5 0	0.5	1.5 2.0
15 °C	0	1.0 0	2.0	0 1.0	0	1.0 0.5
20 °C	1.5	1.0 1.0	1.5	1.0 0.5	0.5	0 0.5
(5) 7 months (n = 100)						
10 °C	0	0 0	2.0	1.0 0	1.0	0 1.0
15 °C	1.0	0 0	0	1.0 0	2.0	2.0 1.0
20 °C	0	1.0 0	1.0	0 2.0	1.0	0 0
(6) 8 months (n = *)						
10 °C	0 (n=61)	0 0 (n=16,-)	0	0 -	1.6	0 -
15 °C	0 (n=96)	0 0 (n=43,91)	1.0	0 1.1	1.0	0 1.1
20 °C	2.2 (n=44)	0 0 (n=57,39)	0	0 0	0	0 0

Table 7 (b): Prevalence of Actinosporea 3 and 6 months after adding *M. cerebralis* spores to *T. tubifex* cultures held at 10 and 15 °C.

	T. dubium (=*T. gyrosalmo*)		*S. ignotum*		*E. radiatum*	
	CONTROL %	TEST %	CONTROL %	TEST %	CONTROL %	TEST %
(1) Original sample (n = 200)						
10 °C	0.5	0 1.0	0	0.5 0	1.0	0 0.5
15 °C	0	0 0.5	0	0 0	0.5	0.5 1.5
(2) 3 months (n = 200)						
10 °C	0.5	0.5 0.5	1.0	0.5 0.5	2.0	0 1.0
15 °C	0.5	0 1.0	0	0 1.0	0	0.5 1.0
(3) 6 months (n = 200)						
10 °C	0	2.0 0	0.5	0 0	0.5	0 0.5
15 °C	1.0	0.5 0.5	0.5	1.0 0.5	1.0	1.5 0

Table 7 (c): Prevalence of Actinosporea 6 months after adding *M. cerebralis* spores to *T. tubifex* cultures held at 5 and 10 °C.

	T. dubium (= *T. gyrosalmo*)		*E. radiatum*		*S. tubificis*		*A. raabiiunioris*	
	CONTROL %	TEST %	CONTROL %	TEST %	CONTROL %	TEST %	CONTROL %	TEST %
(1) Original sample (n = 200)								
5 °C	1.0	0.5 0.5	0	1.0 2.0	1.0	0 0.5	0	0.5 0
10 °C	0	0 1.5	2.5	0.5 0	0.5	1.0 0	0	0 0
(2) 6 months (n = 200)								
5 °C	1.5	1.0 2.5	0.5	0.5 1.0	0.5	0.5 0	0	0 0
10 °C	1.0	0.5 0	1.0	0.5 0	0	1.0 0	0	0 0

3.1.4. Attempts to produce infections of *M. cerebralis* in juvenile rainbow trout.

None of the trout screened for prespore stages by histological examination of 1-2 µm sections of cartilage during the course of experiments 1-5, or those examined for the presence of spores at the beginning of each experiment, were positive for *M. cerebralis*.

Experiment 1: Groups of 3 month old rainbow trout making a total of 185 fish were exposed to one of the following conditions:- sterile mud + water with *M. cerebralis* spores (45 fish); sterile water with spores only (45 fish); sterile mud, *Tubifex* worms and spores (45 fish); and non-sterile mud derived from the Cambrian fish farm, seeded with spores and *Tubifex* worms (50 fish). The fish were held at water temperatures of between 10 and 12 °C, except in the latter system in which temperature was not regulated. A total of 135 fish were maintained at between 10 and 12 °C in either sterile water (105 fish) or sterile water and sterile mud (30 fish). After 50 days, 84% of the test fish and 76% of the control fish had died. Mortality was greatest in the first 14 days and was attributed to a combination of mechanical failures, as 2 of the 3 thermocirculators continually malfunctioned leading to wide temperature fluctuations, and also to disease problems, particularly *Costia*. As a result of the mortality and disease the experiment was abandoned.

Experiment 2: Groups of sac fry making a total of 320 fish were exposed to the same transmission systems as described in experiment 1 (90 fish in each of the first three infection systems described and a further 50 in the fourth system using Cambrian mud). A total of 270 fish were maintained as controls, 60 in sterile water and sterile mud and 210 in sterile water only. 92% of the experimental fish and 89% of control fish died within 65 days. None of the fish which died during the experiment nor any of the survivors were positive

for *M. cerebralis* when examined histologically on day 65 when the experiment was abandoned. Mortality was again attributed to *Costia* and to the general susceptibility of very young fish to the less than ideal conditions within the tanks.

Experiment 3: Groups of 2-3 month old fish making a total of 165 fish were exposed to the same transmision systems as described in experiments 1 and 2, (30 fish per system but the fourth system, using Cambrian mud, was abandoned) with the addition of 2 new transmission sytems as described below:-

(i) sieved mud derived from the Brittany outbreak to which *M. cerebralis* spores had been added (15 fish in each of 2 tanks); (ii) 45 fish in 3 tanks unchanged from the experiment attempting to infect *Tubifex* with *M. cerebralis* spores (page 29). These tanks contained mud, worms and *M. cerebralis* spores. A total of 135 fish were used as controls as in experiment 1. Mortality was much lower; 9% of test fish and 16% of control fish died over 90 days, spread evenly throughout the various tanks. Table 8 shows the result of exposure to the various infection systems. Transmission of *M. cerebralis* to the trout occurred only in those tanks which contained the worms derived from the original *Tubifex* infection experiments. No transmission occurred using the other combinations of conditions.

Table 9 shows the occurrence of *T. dubium* in the *Tubifex* worms within the system in which transmission of *M. cerebralis* occurred. *T. dubium* was not present either within the worms or within the water column.

Table 8: Results of experimental exposure of rainbow trout to *M. cerebralis* spores within the various infection systems.

	System	Detection of *M. cerebralis* infection	number of fish infected/examined
Experiment 3	All controls	Negative	0/114
	Heat sterilised mud, sterile water and *M. cerebralis* spores	Negative	0/23
	Sterile water and *M. cerebralis* spores	Negative	0/29
	Heat sterilised mud, sterile water, *Tubifex* worms and *M. cerebralis* spores	Negative	0/27
	Brittany derived mud	Negative	0/25
	Original *Tubifex* infection experimental tanks	Positive	25/41
Experiment 4	All controls	Negative	0/123
	Heat sterilised mud, sterile water and *M. cerebralis* spores	Negative	0/27
	Sterile water and *M. cerebralis* spores	Negative	0/27
	Heat sterilised mud, sterile water, *Tubifex* worms and *M. cerebralis* spores	Negative	0/30
	Brittany derived mud	Negative	0/28

Table 8: Results of experimental exposure of rainbow trout to *M. cerebralis* spores within the various infection systems (continued).

	System	Detection of *M. cerebralis* infection	number of fish infected/examined
	Original *Tubifex* infection experimental tanks	Positive	23/42
	Tubifex culture infected with *T. dubium*	Negative	0/28
Experiment 5	All controls	Negative	0/121
	Heat sterilised mud, sterile water and *M. cerebralis* spores	Negative	0/28
	Sterile water and *M. cerebralis* spores	Negative	0/27
	Heat sterilised mud, sterile water, *Tubifex* worms and *M. cerebralis* spores	Positive	12/28
	Brittany derived mud	Negative	0/29
	Original *Tubifex* infection experimental tanks	Positive	19/40
	Tubifex culture infected with *T. dubium*	Negative	0/26

Table 9: Occurrence of *T. dubium* in *T. tubifex* in those experimental systems in which transmission of *M. cerebralis* was achieved.

	System	Sample size of *Tubifex* worms	% infection of worms	Presence of *T. dubium* in water column
Run 3	Original *Tubifex* infection system	21,35,2	0,0,0	Negative
Run 4	Original *Tubifex* infection system	17,15,12	0,0,0	Negative
Run 5	Original *Tubifex* infection system	0,4,0	0,0,0	Negative
	Original heat sterilised mud, sterile water, *Tubifex* worms and *M. cerebralis* spores	95,121	2.1,0.8	Not tested

Experiment 4: Groups of 2-3 month old fish making a total of 195 fish were exposed to the same transmission systems as described in experiment 3, (30 fish per system, except in the system involving tanks used in the experiment attempting to infect *Tubifex* with *M. cerebralis* spores to which 45 fish were exposed) with the addition of 1 other system; 2 tanks containing 15 fish each in which there was sterile mud and Tubifex worms with an initial prevalence of 2% of *T. dubium*. A total of 135 fish were used as controls as in experiment 1.

Mortality in all the tanks was below 10% throughout the duration of this experiment. Table 8 shows the results of exposure to the various infection systems. As in run 3, all fish remained negative for *M. cerebralis* except those exposed to the original *Tubifex* infection systems. When this system was examined for the presence of *T. dubium* in the worms and the water column, none were found. Conversely, no transmission of *M. cerebralis* to trout occurred when the fish were exposed to *Tubifex* worms which were known to have infections of *T. dubium*. The water column in those tanks contained *T. dubium* when sampled on 12 out of 25 occasions.

Experiment 5: The same combinations of control and experimental systems, using the same numbers of fish, as described in experiment 4, were used in experiment 5. Mortality throughout the tanks was again less than 10%. Results for transmission of *M. cerebralis* are shown in Table 8. Transmission was only effected in the original *Tubifex* infection experimental tanks and in the tanks containing sterile mud, *Tubifex* worms and *M. cerebralis* spores. The latter were also positive for *T. dubium*, while in the former there was no evidence of *T. dubium* in the worms or in the water column, (Table 9).

3.1.5. Histological examination of *T. tubifex* worms exposed to *M. cerebralis* spores.

Figs. 13 and 14 show *M. cerebralis* spores within the gut lumen in sections of *T. tubifex*. Out of a total of 40 worms examined, 28 contained at least one *M. cerebralis* spore. The maximum number of spores seen in any one worm was 8 after 5 days of exposure. Although *T. tubifex* is clearly capable of ingesting *M. cerebralis* spores, there was no evidence, despite extensive histological examination of worms over a period of weeks, to suggest that the spores underwent any developmental process. Polar filaments were not everted, nor were there signs of hatching. Spores did not accumulate within the gut lumen with time and, thus, the worms do not apparently act to concentrate the spores for the transmission to fish.

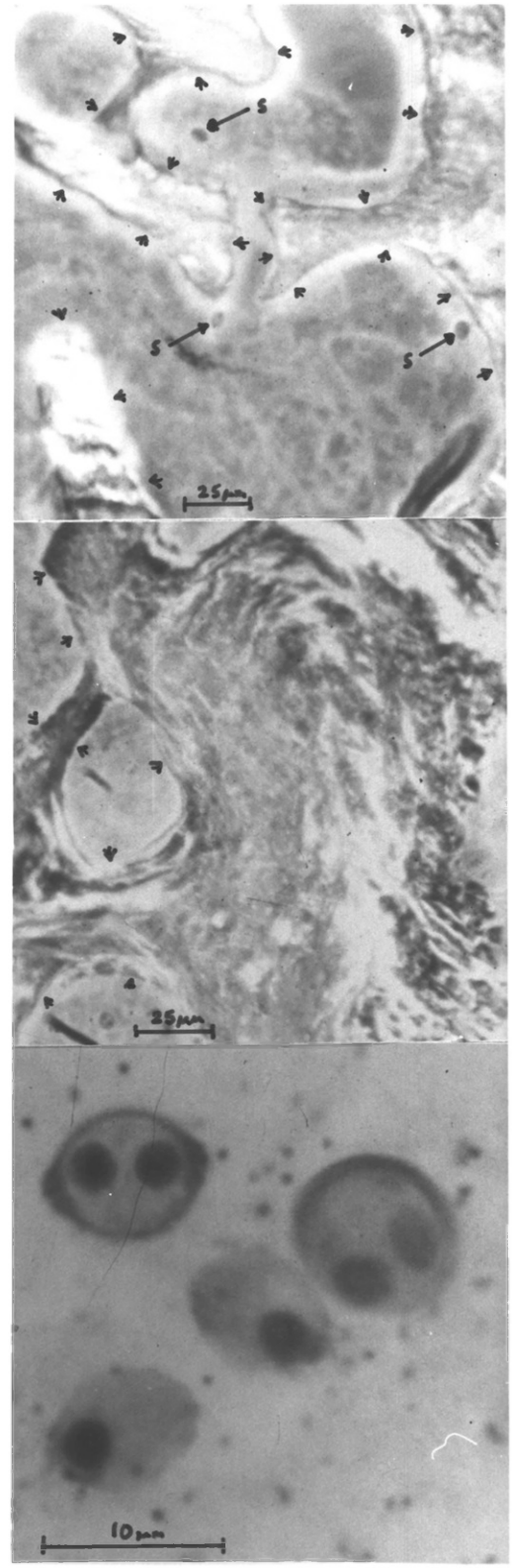
3.1.6. Histological examination of infected cartilage.

Cartilage from infected fish was examined in 0.5-2.0 µm sections after fixation in glutaraldehyde and embedding in historesin, in order to study the nature of the damage caused by the parasite and to identify possible developmental stages. In toluidine blue-stained sections of 6 month old fish derived from the German outbreaks of whirling disease, large areas of damaged cartilage were apparent (Fig. 15). Spores were easily identified at the margins of these areas, even at low power. In contrast, sections taken from fish infected in experiments 3-5 showed more limited areas of damaged cartilage, and spores were rarely seen. Using the Giemsa/Colophonium technique, an apparently fibrous network, staining light pink to purple, was observed in the damaged cartilage, (Fig. 16). Well stained spores were found only at the margin of the damaged cartilage, (Fig. 17). Presumptive pre spore stages of two types were found: a rare, apparently multinucleate stage (Fig. 19)

Fig. 15 Toluidine Blue stained section of cartilage attacked by *M. cerebralis*. Note spores (s) on margins of damaged areas, the outer edges of which are arrowed.

Fig. 16 Giemsa/Colophonium-stained thin section of cartilage attacked by *M. cerebralis*.

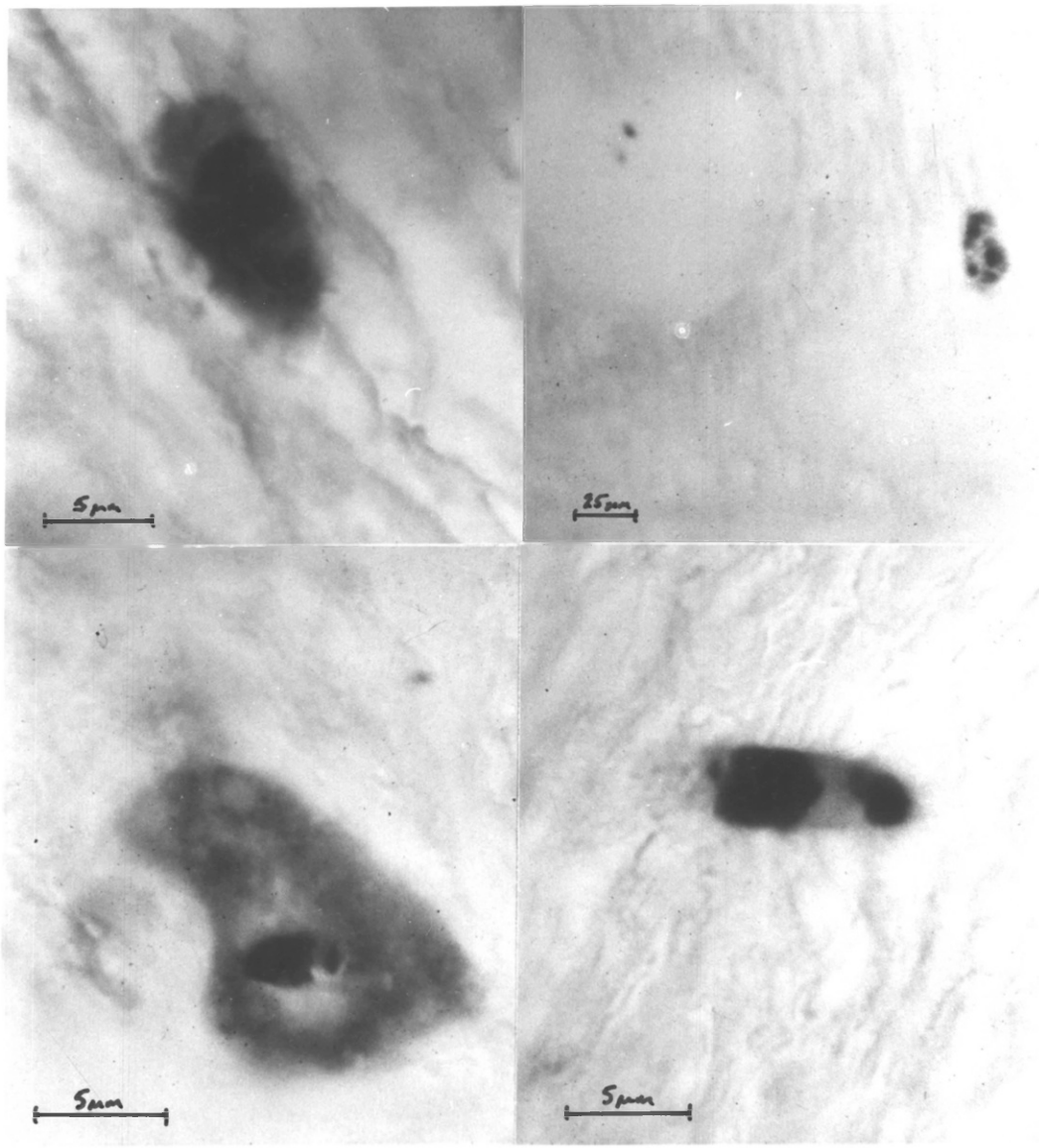
Fig. 17 *M. cerebralis* spores in thin section of infected cartilage. Giemsa/Colophonium technique.


S
S
S
25μm
25μm
10μm

Figs. 18, 19, 20 & 21 — Presumptive pre spore stages of *M. cerebralis* in thin sections of damaged cartilage stained by Giemsa/Colophonium.

Figs. 18, 20 & 21 — Small pre spore stages containing 1-3 darkly staining bodies.

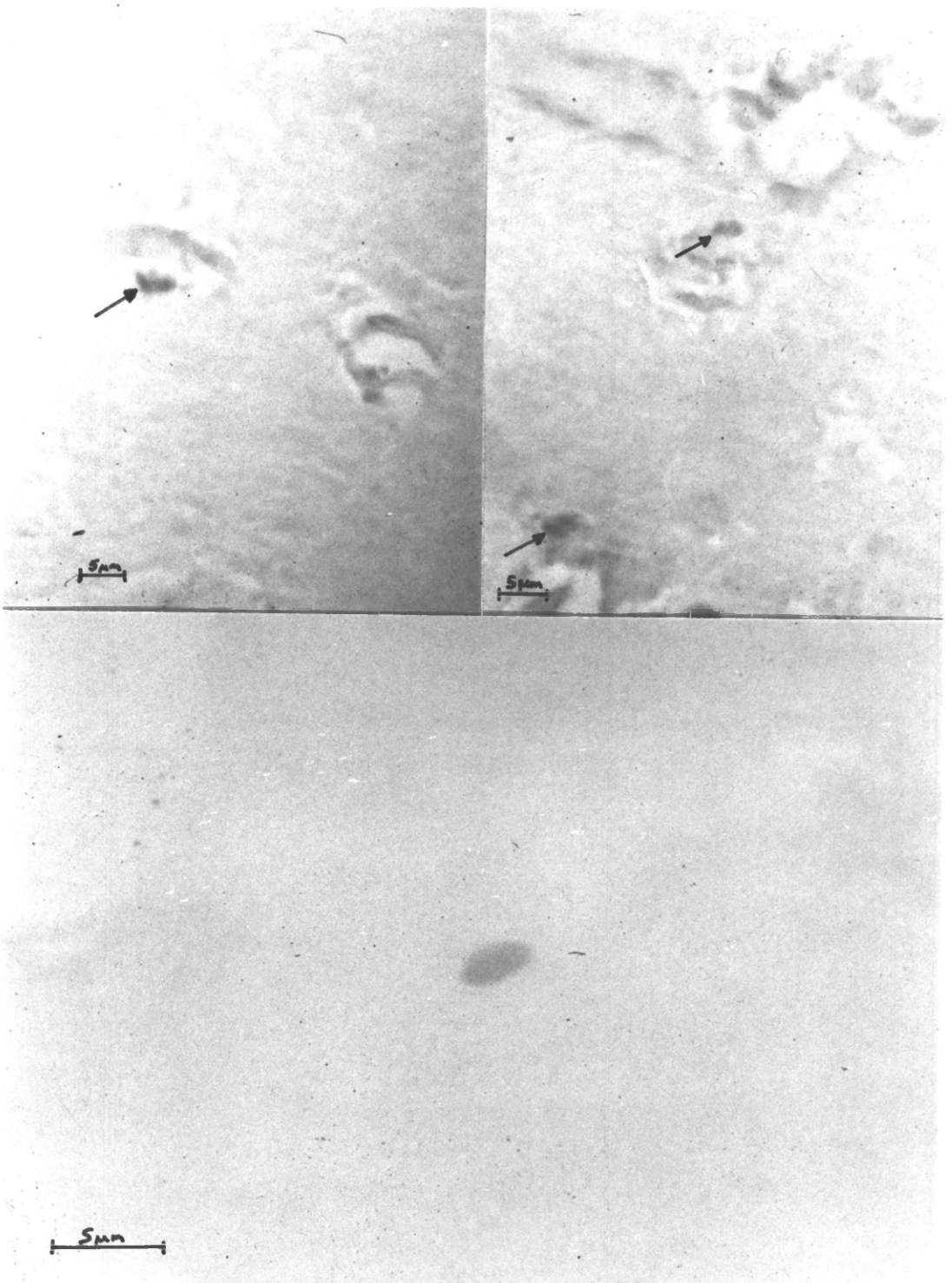
Fig. 19 — Rare apparently multinucleate stage.


5μm
25μm
5μm
5μm

Figs. 22, 23 & 24 Staining of spore and pre spore stages of *M. cerebralis* with Schiff's reagent.

Figs. 22 & 23 Binucleate pre spore stages (arrowed) within damaged cartilage

Fig. 24 Nucleus of spore stage on margin of damaged cartilage


5μm
5μm
5μm

and a commoner small stage containing 1-3 darkly staining bodies, (Figs. 18, 20 and 21). In the experimentally infected fish exposed to cultures of *T. tubifex* worms, *M. cerebralis* spores and mud (Experiments 3, 4 and 5), pre spore stages predominated, while in the sections taken from fish from the German outbreak pre spore stages were comparatively rare. Staining with Schiff's reagent showed faint pink areas of DNA within spores and in the presumptive pre spore stages, demonstrating that the areas which stained darkly with Giemsa were nuclei. Some of the pre spore stages were binucleate, (Figs. 22, 23 and 24).

3.1.7. Examination of infected cartilage via electron microscopy

It proved very difficult to cut the Historesin embedded material to sections approaching the optimum of 90 nm for electron microscopy, and unfortunately this material included the most heavily infected cartilage. As a result sections were cut at between 150 and 200 nm which greatly reduced contrast and necessitated the use of higher beam voltages (80-100 KV), which in turn often caused distortion of both the material and the Historesin. As a result it was not possible to observe definitive pre spore stages, even after their initial localisation using 1 µm sections at the light microscopy level. However, spores were identified (Fig. 25) though little detail, apart from the prominent sutral ridge, could be discerned. In addition, very small (1 µm) structures were identified (Fig. 27), which appeared to possess an outer unit membrane; their possible relationship to *M. cerebralis* is referred to in section 4.4.

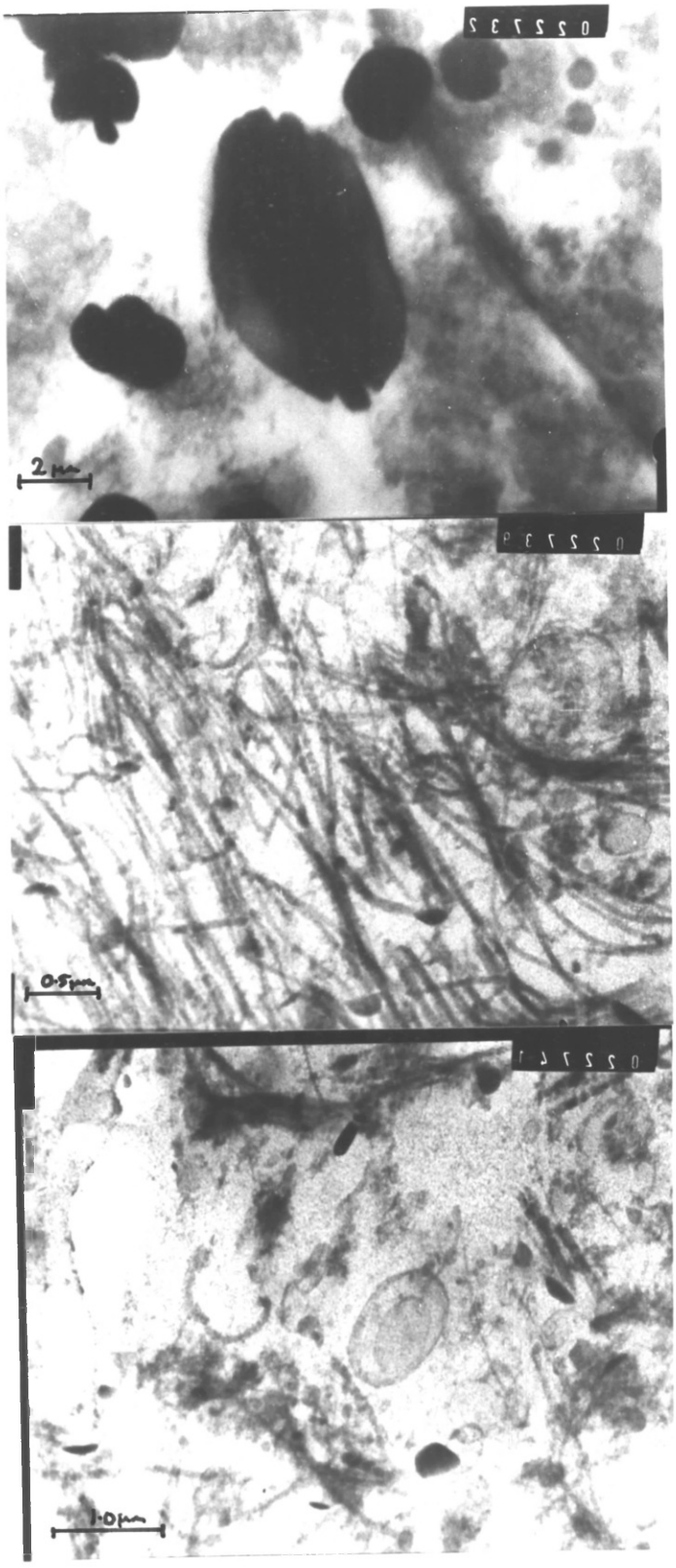
The material embedded in Spurr resin proved much easier to section down to 90 nm. However, since this came from the fish which had been experimentally infected, there were fewer parasitic stages and none were located under the electron microscope. It was possible to study the nature of the fibrous matrix

Figs 25, 26 & 27 Examination of infected cartilage via electron microscopy

Fig. 25 *M. cerebralis* spore (sutral ridge arrowed)

Fig. 26 Banded fibrils comprising the matrix of damaged cartilage.

Fig. 27 Unidentified small (1 μm) structure within damaged cartilage.


2 μm
0.5 μm
1.0 μm

of damaged cartilage using this material; it appeared to be comprised of a network of irregularly arranged, banded fibrils, approximately 50 nm in diameter, (Fig. 26).

3.2.1. Percoll separation of *M. cerebralis* spores

Preparations of *M. cerebralis* spores contaminated with only small amounts of cartilage were obtained by phase separation of triturated infected cartilage on a discontinous Percoll gradient. The results of the separation are illustrated in Fig. 28. Clean spores were found at the x/25% interface. Spores were also present at the 25%/50% and at the 50%/75% interfaces, but with a higher level of contamination. The latter were effectively purified after reprocessing through the system. Bacteria were largely confined to the band containing the original extract at the top of the column. Table 2 shows numbers of spores isolated from given bands on the gradient for individual fish.

3.2.2.1. IFAT using mouse and rabbit anti-*M. cerebralis* antisera on *M. cerebralis*, *T. dubium* and other actinosporean spores.

When 8 x 10^6 sonicated *M. cerebralis* spores in Freunds complete adjuvant were inoculated intraperitoneally into each of 5 Balb C mice in two separate doses of 4 x 10^6 spores at an interval of 20 days, antibodies were produced which were reactive in an IFAT against freshly isolated *M. cerebralis* spores. Using fluorescein conjugated IgG swine anti-mouse antiserum at 1:40 the titre of the pooled mouse antiserum was 1:120 (Table 10). The pooled mouse antiserum also reacted when *T. dubium* spores were used as antigen but the titre for this system was 1:60. Fig. 29 shows a fluorescing spore of *M. cerebralis* and Fig. 30 illustrates the cross reactivity of the mouse antiserum with a spore of *T. dubium*.

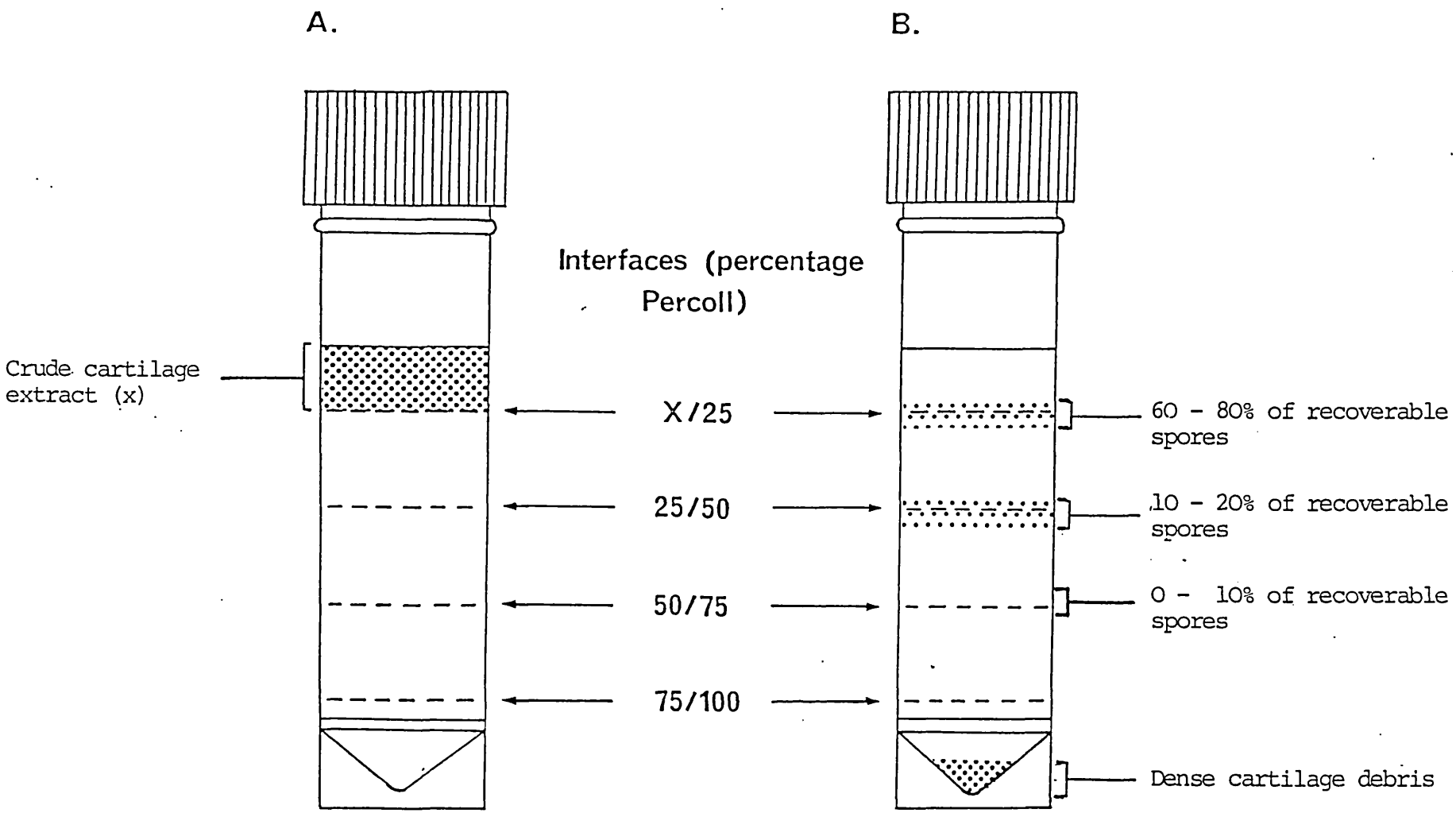


Fig.28 : Percoll separation technique for the purification of M.cerebralis spores; A, before centrifugation, B, after.

Table 10: Results of the IFAT on *M. cerebralis* and *T. dubium* spores using mouse and rabbit anti-*M. cerebralis* antiserum.

1. Mouse anti-*M. cerebralis* antiserum (conjugate 1:40 throughout)

a) reacted with *M. cerebralis* spores								
Antiserum titre	1:10	1:20	1:40	1:60	1:80	1:100	1:120	1:140
Fluorescence	+++	+++	+++	++	++	++	+	-
b) reacted with *T. dubium* spores	+++	+++	++	+	-	-	-	-

2. Normal mouse serum

a) reacted with *M. cerebralis* spores	+	-	-	-	-	-	-	-
b) reacted with *T. dubium* spores	-	-	-	-	-	-	-	-

3. Rabbit anti-*M. cerebralis* antiserum

a) reacted with *M. cerebralis* spores	+++	+++	++	++	+	+	-	-

4. Normal rabbit serum

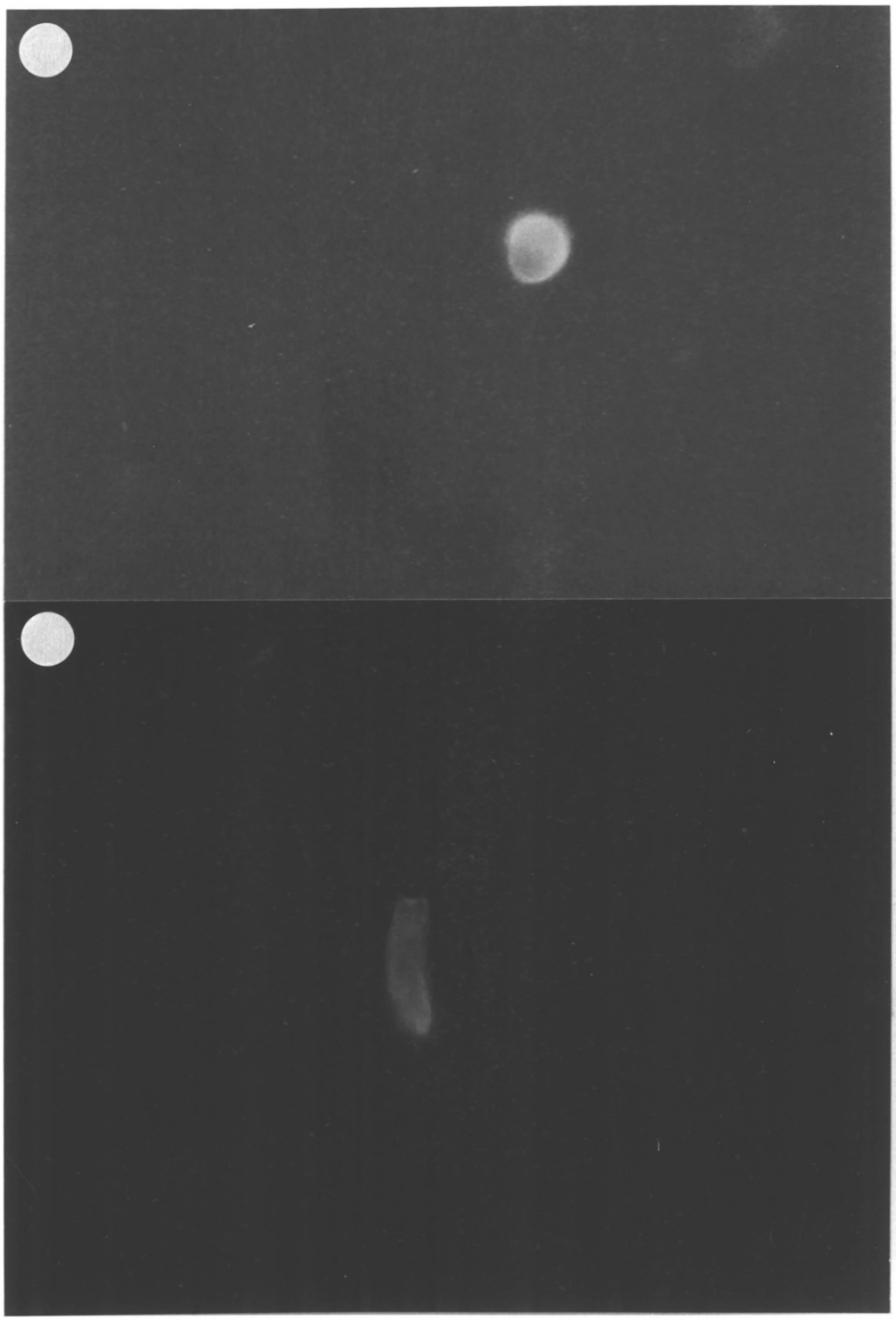
a) reacted with *M. cerebralis* spores	+	+	-	-	-	-	-	-

Fluorescence Key:

+++ : strong positive
++ : moderate positive
+ : weak positive
- : negative

Fig. 29 *M. cerebralis* spores fluorescing in an IFAT using mouse anti-*M. cerebralis* antiserum.

Fig. 30 Cross reaction of *T. dubium* when incubated with anti-*M. cerebralis* antiserum in a similar IFAT.



The polar capsule region of *M. cerebralis* was less reactive than the remainder of the spore wall, which appeared as a pronounced fluorescent halo. With further dilution (to above 1:120), the fluorescence pattern became indistinguishable from the background; firstly the fluorescence around the polar capsules disappeared, followed by the fluorescence from the spore wall itself.

Only the head region of *T. dubium* spores were positive in the IFAT; the remainder of the epispore, including the triradiate arms, showed litte or no fluorescence. As with *M. cerebralis*, the polar capsules of *T. dubium* were less reactive in the IFAT than the remainder of the head region.

Both *M. cerebralis* and *T. dubium* spores incubated with normal mouse serum showed almost undetectable fluorescence, as did spores incubated with PBS in place of either the antiserum or the conjugate.

Two other species of Actinosporean were cross reacted with mouse anti-*M. cerebralis* antisera combined with the fluorescein labelled conjugate. These were *S. tubificis* and *E. radiatum*. Both species fluoresced significantly around the head region of the spore down to a mouse antiserum titre of 1:60, as in the case of *T. dubium*.

3.2.2.2. Rabbit anti-*M. cerebralis* antiserum.

When 2.5 x 10^7 sonicated *M. cerebralis* spores in Freunds complete adjuvant were inoculated subcutaneously into a New Zealand White rabbit in two separate doses at an interval of 21 days, antibodies were produced which were reactive in an IFAT against freshly isolated *M. cerebralis* spores. Using fluorescein conjugated IgG goat anti-rabbit antiserum at 1:40 the titre of the

rabbit antiserum was 1:100 (see Table 10). The fluorescence pattern was the same as that described using mouse anti-*M. cerebralis* antiserum. The normal mouse serum and the normal rabbit serum used as negative controls gave a weak fluorescence reaction at dilutions of 1:10 and 1:20 respectively when reacted with *M. cerebralis* spores. Neither normal serum gave any reaction with *T. dubium.*

3.2.3. ELISA for the detection of antibodies produced in rainbow trout experimentally infected with *A. salmonicida.*

When a total of 45 10-month old rainbow trout, split into 3 groups of 15, were inoculated with viable *A. salmonicida* at doses of 10^2, 10^3, or 10^4 bacteria per individual, it was possible to detect a significant change in antibody titre against *A. salmonicida* over a period of 28 days (Fig. 31). To produce the approximate conversion between optical density and serum titre, sera from 5 of the sample fish giving widely different optical density values were titred out to establish an equivalent serum titre, which are shown in Fig. 32. (The relationship between optical density and antibody titre is linear between values of 0.1 and 2.0). The remaining serum titre values were then extrapolated from this Fig. (for full conversion see appendix 7). During the experiment, 4 of the fish inoculated with 10^2 bacteria died, along with 7 of the fish inoculated with 10^3 bacteria, 8 of the fish inoculated with 10^4 bacteria and 2 of the control fish. Sera was not taken from these fish since most of them died during the night. The dilution of the rabbit anti-trout antiserum in this ELISA was 1:500 and the conjugate dilution was 1:2000; in the subsequent ELISA (section 3.2.4.) the trout serum dilutions were 1:250 and 1:500.

Fig. 31 Antibody response of rainbow trout inoculated with A.salmonicida

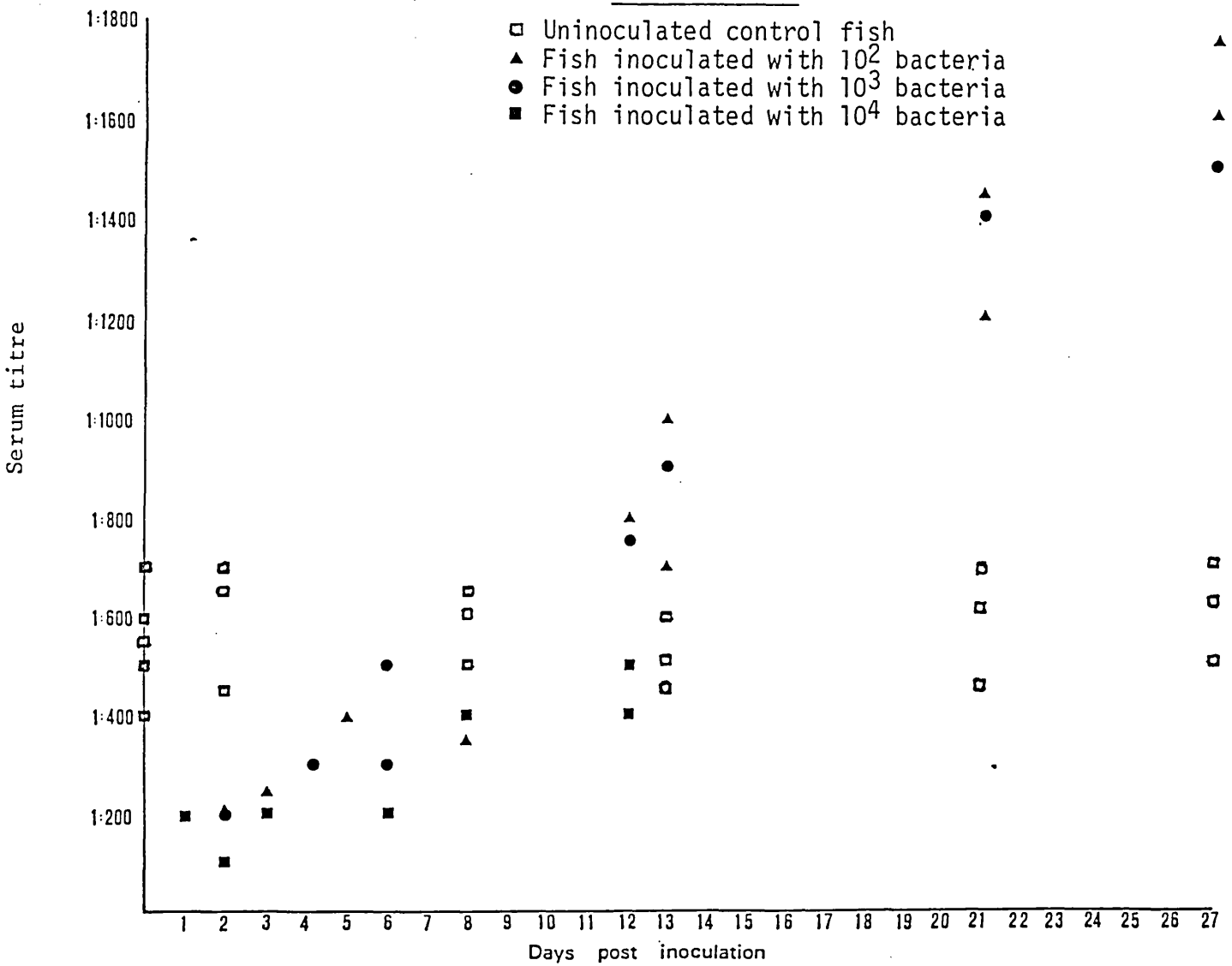
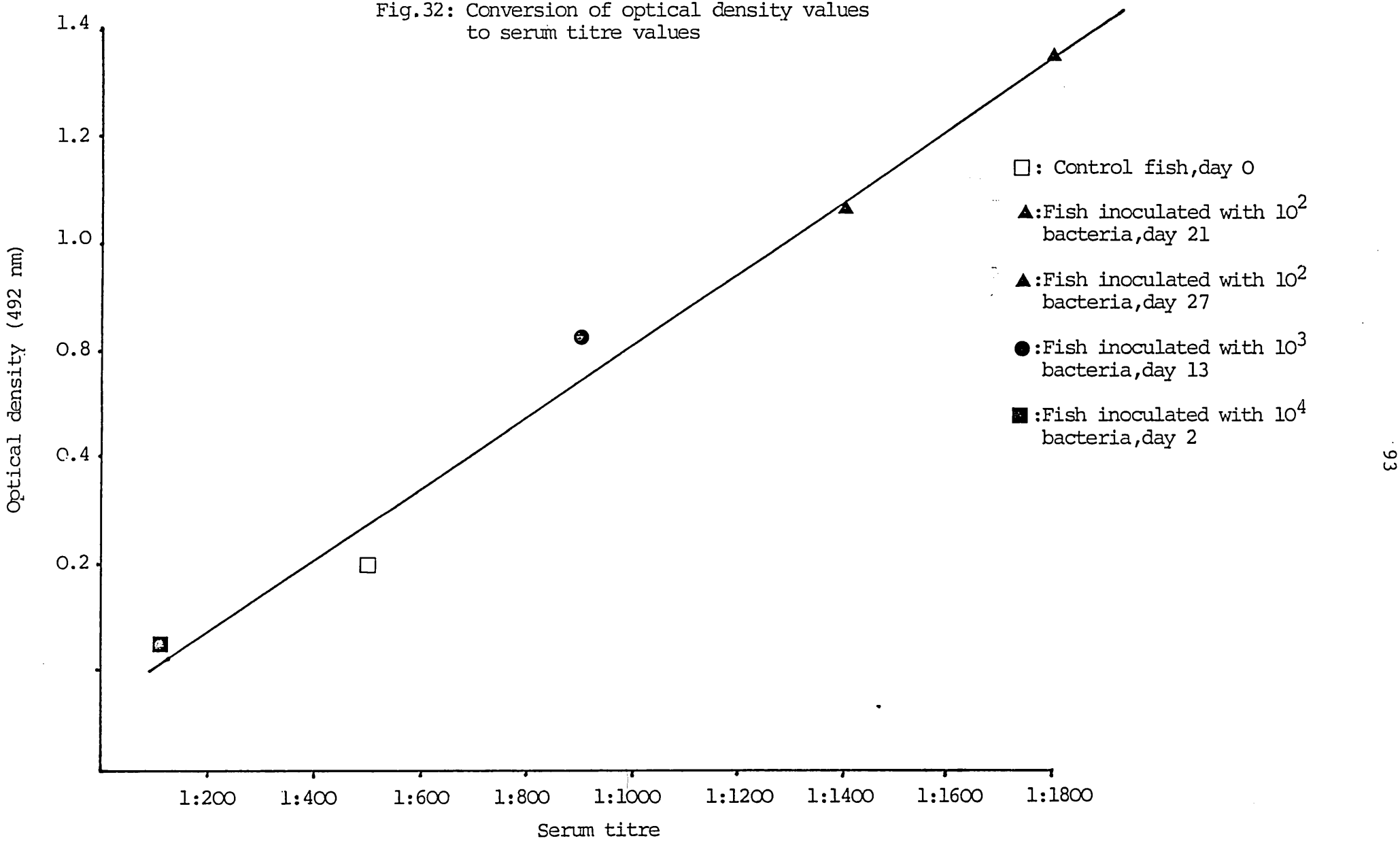


Fig.32: Conversion of optical density values to serum titre values



3.2.4. Detection of circulating antibodies produced in rainbow trout against *M. cerebralis* using an ELISA.

Figures 33 and 34 illustrate the results of this ELISA. Background level readings, *i.e.* those taken from wells containing no trout serum or no rabbit and trout sera have been subtracted from the optical density values shown (all results are included in appendix 8). Using 18-24 month old trout derived from the Brittany outbreak, the mean antibody levels aginst *M. cerebralis* spores in the ELISA (expressed as optical densities) were not significantly different from those of uninfected fish of the same age, (Fig. 33). Using 5-6 month old fish experimentally infected with *M. cerebralis* there was a significant difference in antibody level compared with unexposed fish, at titres of 1:100 and, in all except one fish, also at 1:250, (Fig. 34). Antigen concentration was critical in the detection of an immune response against *M. cerebralis.* Significant levels of anti-*M. cerebralis* antibodies were not detectable when the equivalent of 5×10^3 - 1.0×10^4 spores per well were used as antigen. Antigen concentrations of 5×10^4 spores per well were necessary to obtain the results shown in Figs. 33 and 34.

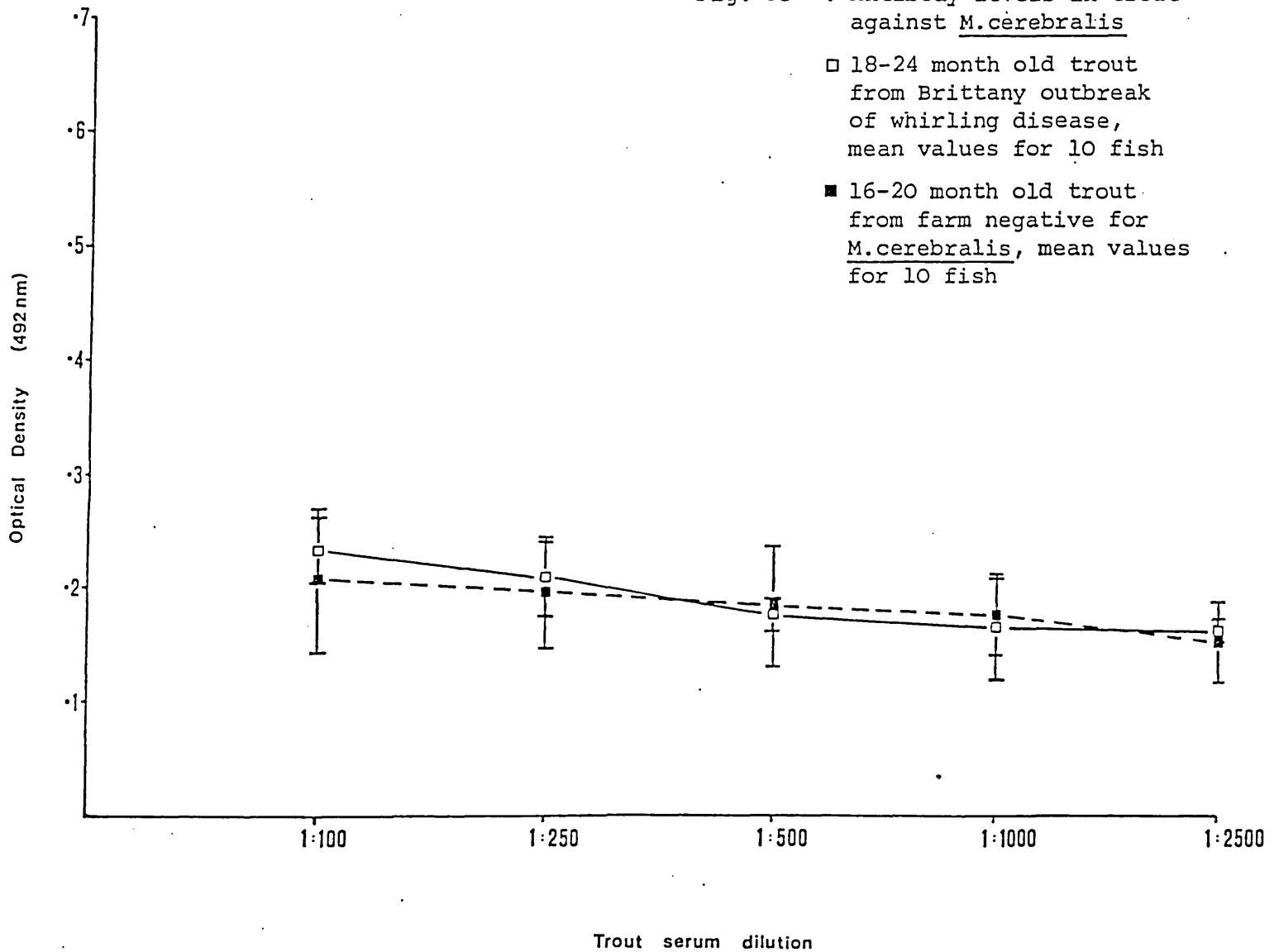
3.2.5. IFAT on *M. cerebralis* spores using immune trout sera.

When freshly isolated *M. cerebralis* spores were firstly incubated with serum at dilutions of 1:25, 1:50 and 1:75, from the same batch of 5-6 month old trout which had given a positive reaction in the ELISA for anti-*M. cerebralis* antibody, then rabbit anti-trout antiserum at a dilution of 1:100, followed by goat anti rabbit (FITC) at 1:100, there was a strong fluorescence, (Fig. 35). This fluorescence pattern was similar to that seen in section 3.2.2.1. In contrast, when the spores were incubated with normal trout sera even at dilutions of 1:25 and 1:50, there was little or no fluorescence, (Fig. 36). Similarly, sera from 18-20 month old trout from the Brittany outbreak did not

Fig. 33 : Antibody levels in trout against M.cerebralis

□ 18-24 month old trout from Brittany outbreak of whirling disease, mean values for 10 fish

■ 16-20 month old trout from farm negative for M.cerebralis, mean values for 10 fish



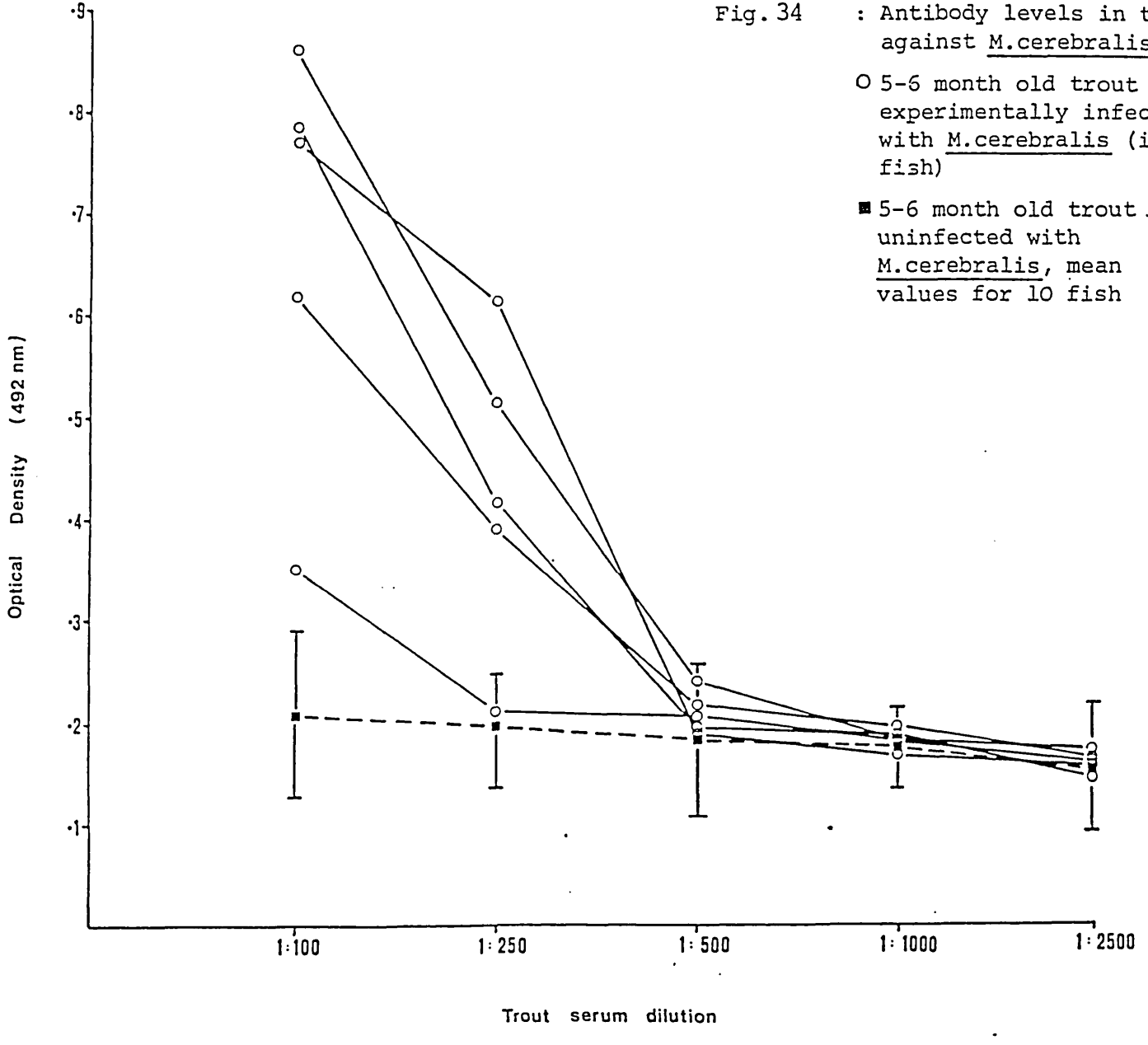


Fig. 34 : Antibody levels in trout against M.cerebralis

O 5-6 month old trout experimentally infected with M.cerebralis (individual fish)

■ 5-6 month old trout uninfected with M.cerebralis, mean values for 10 fish

Figs. 35 & 36 Indirect Fluorescence Antibody Test on *M. cerebralis* spores using immune and non immune trout serum.

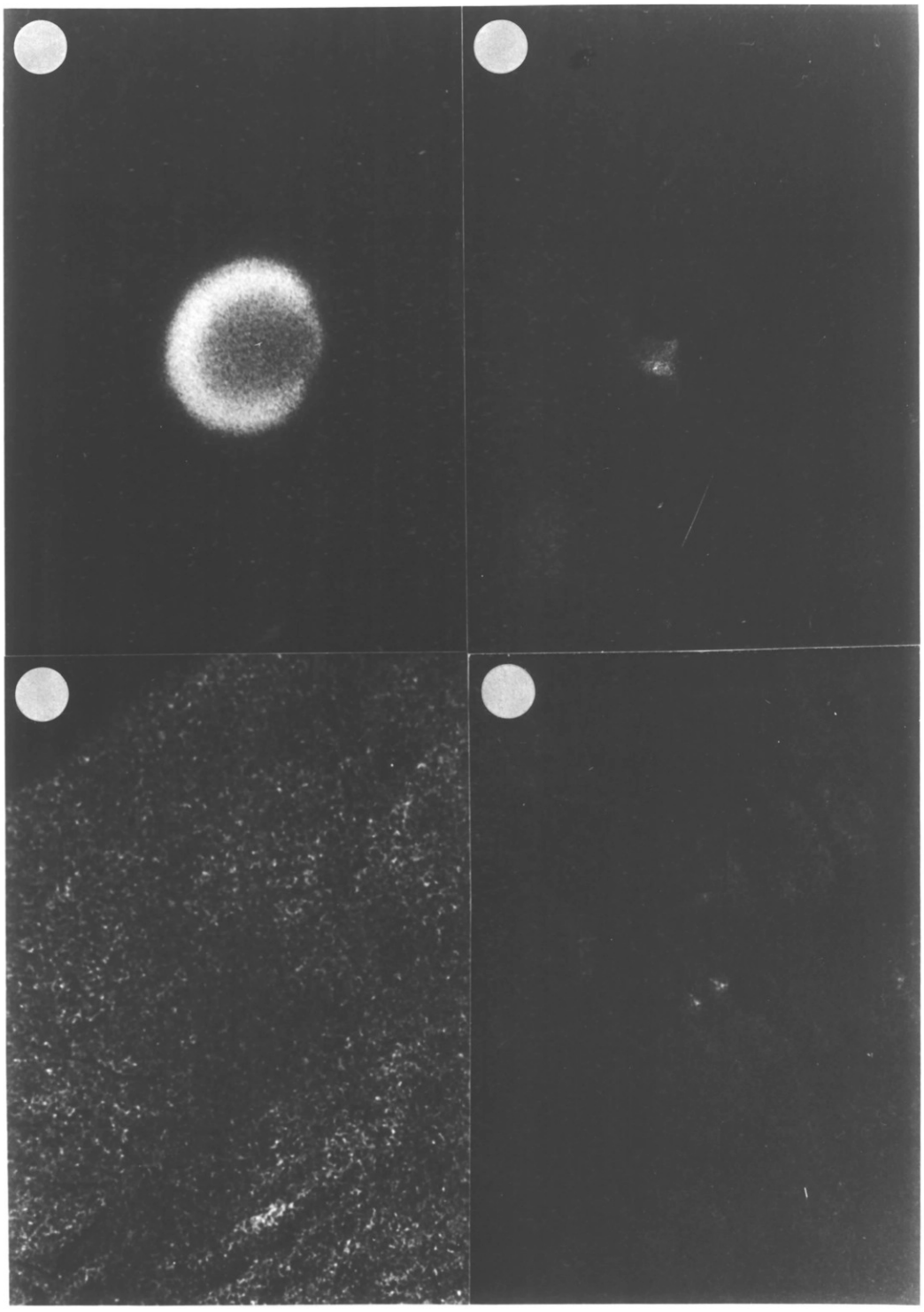
Fig. 35 Freshly isolated *M. cerebralis* spore incubated with immune serum from 5 month old trout infected with *M. cerebralis.*

Fig. 36 *M. cerebralis* spore incubated with non immune serum from an uninfected trout of the same age.

Figs. 43 & 44 Indirect Fluorescence Antibody Tests on sections of cartilage infected with *M. cerebralis* incubated with mouse anti-*M. cerebralis* antiserum.

Fig. 43 1 μm section from an area of damaged cartilage incubated with mouse anti-*M. cerebralis* antiserum showing generalised fluorescence.

Fig. 44 1 μm section of damaged cartilage incubated with normal mouse serum.



produce a positive IFAT even at dilutions of 1:25. This corroborated the results of the ELISA described in section 3.2.4.

3.2.6. ELISA for the detection of *M. cerebralis* antigen in rainbow trout infected with *M. cerebralis*.

Figure 37 illustrates the ability of the ELISA to detect *M. cerebralis* antigen in the form of spore sonicate. When plates were coated with 100 ul of pooled mouse anti-*M. cerebralis* antiserum at a dilution of 1:250, then incubated with 200 ul of antigen suspension equivalent to 10, 10^2, 10^3 or 10^4 sonicated spores of *M. cerebralis*, and then with rabbit anti-*M. cerebralis* antiserum at a dilution of 1:250, followed by peroxidase conjugated goat anti rabbit antiserum.at a dilution of 1:200 and O.P.D substrate, significant differences in O.D. were observed with spore concentrations of 10^3 and 10^4 when compared with controls using sonicated *Tubifex* or BSA in place of *M. cerebralis* antigen. When sonicated *Myxobolus* sp. spores were used as antigen under the same conditions, similar results were obtained as for *M. cerebralis*.

However, when this ELISA was used for the detection of antigen in trout serum from 5-6 month old fish from the same batch, which had been positive at titres of 1:250 in the ELISA for antibody detection (section 3.2.4.) there were no differences in optical density values between uninifected and infected fish even when the trout serum was used at a dilution of 1:10 (Fig. 38). Sera taken from fish infected in the Brittany outbreak also showed no difference in O.D. values compared with sera from uninfected trout, (full results in appendix 9).

Fig.37: Detection of M.cerebralis spore sonicate by ELISA

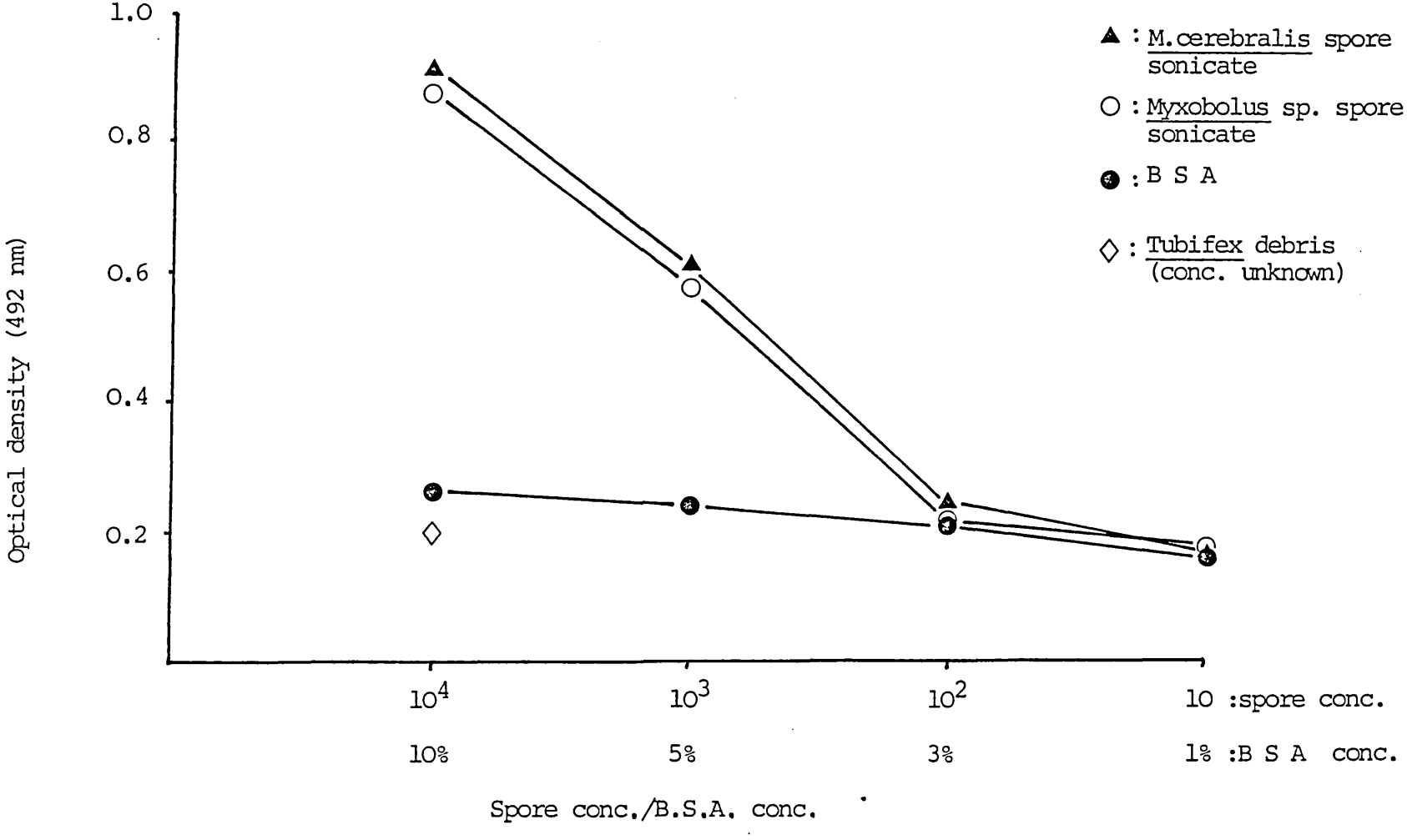
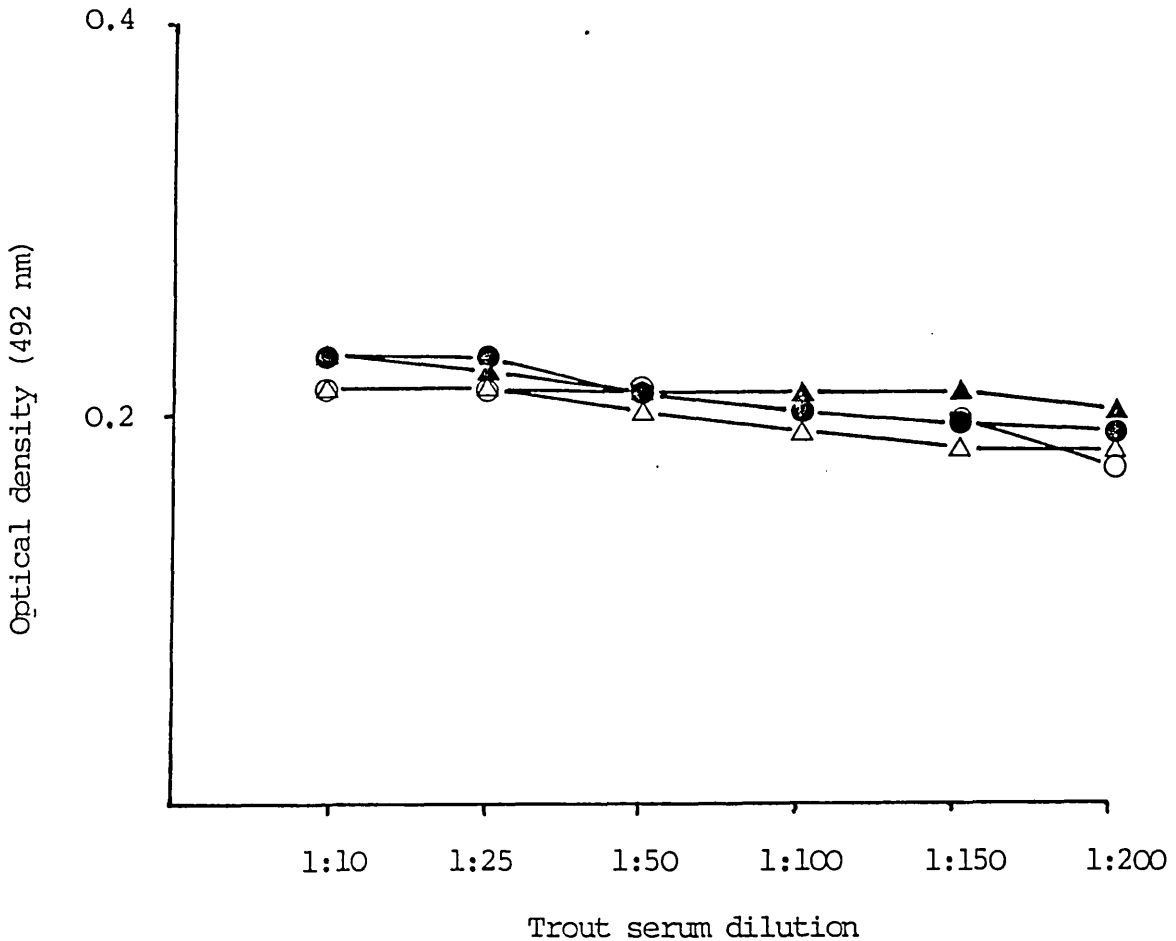


Fig.38: Detection of M.cerebralis antigen in infected trout



▲ : 5-6 month old trout expermentally infected with M.cerebralis

△ : 4-6 month old trout negative for M.cerebralis

● : 18-24 month old trout from Brittany outbreak

○ : 16-20 month old trout negative for M.cerebralis

(mean values for 10 fish in each case;error bars not shown)

3.2.7. Immunofluorescent staining of cartilage sections from fish infected with *M. cerebralis* .

When mouse anti-*M. cerebralis* antiserum was first absorbed with macerated cartilage and bone from uninfected fish and then incubated with 1-2 µm sections of infected cartilage, at dilution of 1:25, 1:50 and 1:75, followed by incubation with swine anti-mouse antiserum, FITC, at dilutions of either 1:40 or 1:80, it was possible to detect a range of parasitic stages both within the matrix of the damaged cartilage and in the area around it. Figs. 39 and 41 show fluorescent *M. cerebralis* spores; fine detail such as the polar capsules and sutral ridge are evident. The area of damaged cartilage fluoresced strongly compared with the surrounding normal cartilage (Figs. 42 and 43). Presumptive prespore stages could occasionally be discerned within this area, though with little detail, (Fig. 42).

The fluorescent pattern was clearest at a mouse anti-*M. cerebralis* antiserum titre of 1:50, combined wth swine anti-mouse antiserum FITC at 1:40. Backgound levels of fluorescence, as assessed by the use of normal mouse sera and PBS in place of the fluorescent conjugate, were significantly lower, though areas of damaged cartilage did show some reaction. (Figs. 40 and 44).

3.2.8. Immunoperoxidase labelling of thin sections of damaged cartilage.

After incubation of cartilage sections with mouse anti-*M. cerebralis* antiserum (as per section 3.2.7.), followed by incubation with peroxidase conjugated swine anti-mouse antiserum at dilutions of 1:500 and 1:750, and subsequent reaction with O.P.D. substrate, the pattern of immunoperoxidase staining was similar to that seen with immunofluorescence, though background binding appeared to be higher. Figs. 45, 46 and 47 show typical results; the damaged

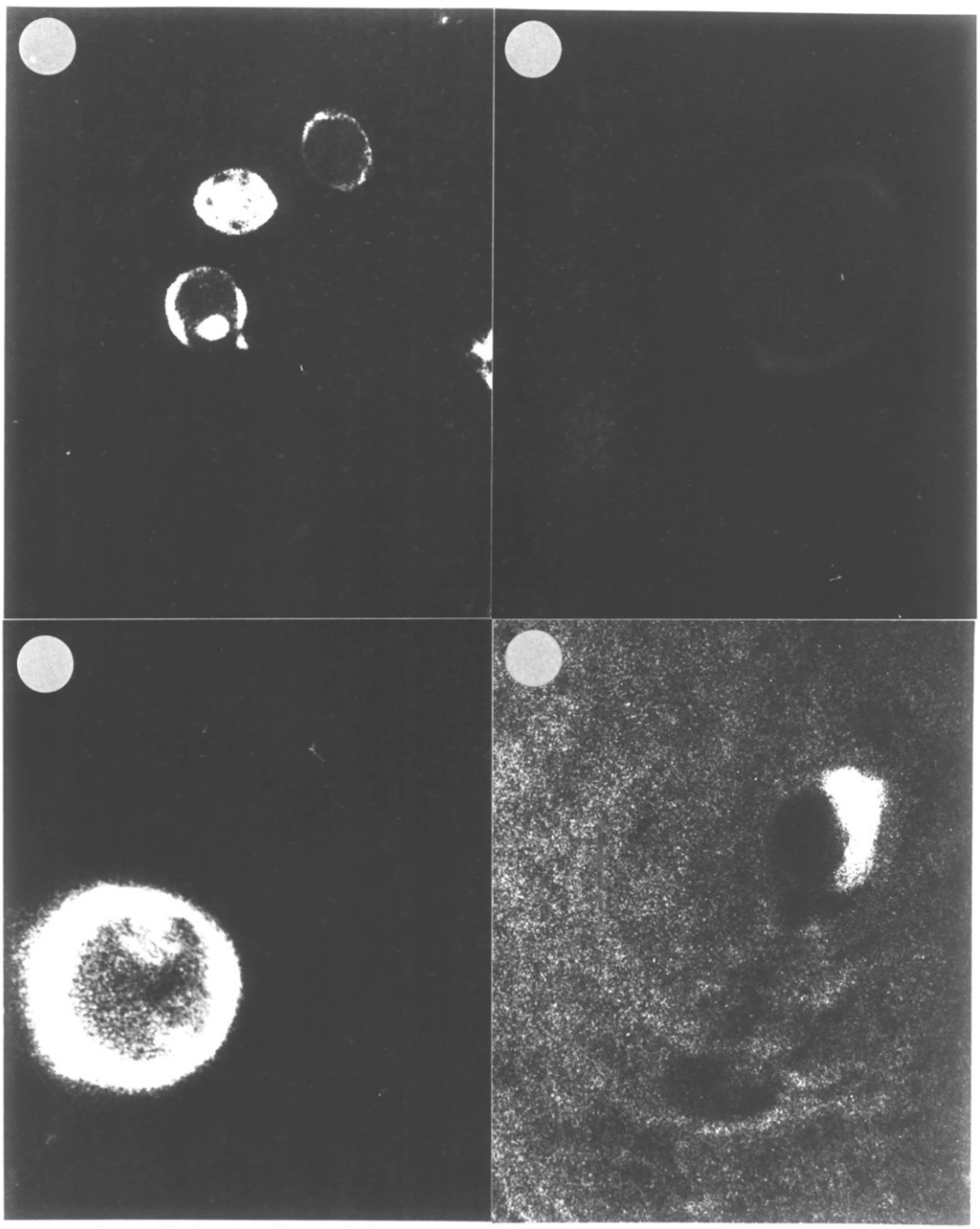
Figs. 39, 40, 41 & 42 — Indirect Fluorescence Antibody Tests on sections of cartilage infected with *M. cerebralis* incubated with mouse anti-*M. cerebralis* antisera and normal mouse serum.

Fig. 39 — Fluorescent *M. cerebralis* spores in 1 µm sections of infected cartilage incubated with mouse anti-*M. cerebralis* antiserum.

Fig. 40 — *M. cerebralis* spore in 1 µm section incubated with normal mouse serum.

Fig. 41 — *M. cerebralis* spore in 1 µm section incubated with mouse anti-*M. cerebralis* antiserum showing fluorescence of polar capsules.

Fig. 42 — 1 µm section of infected cartilage incubated with mouse anti-*M. cerebralis* antiserum showing generalised fluorescence and a presumptive pre spore stage.



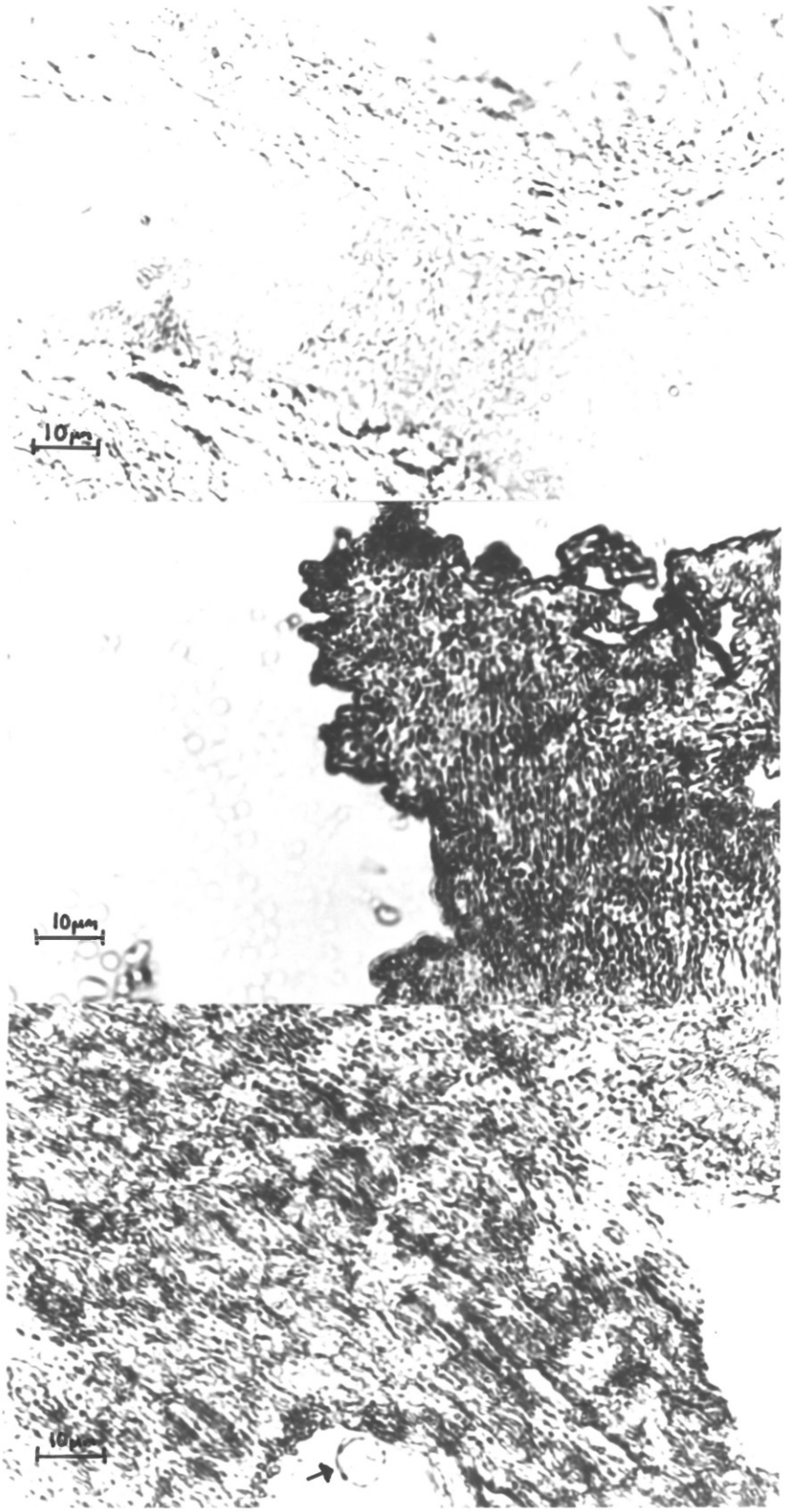
area of cartilage showed significantly more reaction product than controls at mouse anti-*M. cerebralis* antiserum titre of 1:25 and 1:50.

Figs. 45, 46 & 47 — Immunoperoxidase staining of damaged cartilage.

Fig. 45 — Damaged cartilage incubated with normal mouse serum as a control (outer margin of areas of damaged cartilage arrowed).

Fig. 46 — Incubation of damaged cartilage with mouse anti-*M. cerebralis* antiserum. Note darkly stained damaged area (arrowed).

Fig. 47 — As for Fig. 45 with stained *M. cerebralis* spore (arrowed).


10μm
10μm
10μm

4. DISCUSSION

4.1.1. Location of outbreaks of *M. cerebralis*

The inability to detect *M. cerebralis* spores in samples from British trout farms, which had been previously designated as positive for this pathogen by Ministry of Agriculture, Food and Fisheries inspectors *ie.* Cambrian, Hammer, Ampney and Wandsford, could have been due either to deficiencies in the spore detection methods, or to the disappearance of the disease. Spores were detected by otolith/cranial cartilage extraction followed by maceration, filtration and Percoll separation, techniques which may not have picked up low level pre spore infections at these sites. The intervals between the sites being sampled by MAFF inspectors, and by the author, were 12-24 months and there was no evidence that corrective disinfection methods had been applied by the various fish farmers at the British sites tested. Stratfield Saye was a site that Thames Water Board officials found negative for *M. cerebralis.* The absence of disease may be due to this farm never having been exposed to the pathogen, though the fact that brown trout are produced on this farm, which are thought to be resistant to *M. cerebralis*, may account for the absence of the disease.

In marked contast was the situation on the three European sites from which samples were obtained, where not only were large numbers of spores recoverable but where classic external symptoms of the disease, such as skeletal deformities and tail blackening, were in evidence. Such gross morphological symptoms have been described in outbreaks within the U.K. (Elson, 1969) but have tended to be more representative of the disease in Continental Europe (Schäperclaus, 1954; Sweeting, personal communication). The epizooticology of whirling disease is poorly understood, though certain

physical and ecological factors play a part in determining the intensity and persistence of outbreaks. Temperature is one such factor (Halliday, 1973a) and this may have determined the differences in outbreak intensity between British and Continental farms. Other factors on fish farms, which may play a part, are related to poor farm management, and are illustrated by the heavy and persistent outbreak at the Lannion fish farm in Brittany. These are:

(i) failure to maintain young fish in spring water, which is free of pathogens, including *M. cerebralis.*

(ii) the predominance of mud rather than concrete lined raceways and ponds. The link between whirling disease transmission and mud substrates has long been suspected and various authors have stressed the advantages of concrete lining as a preventative measure (Schäperclaus, 1954).

(iii) non removal of older carrier fish, even after it is clear that such individuals are infected. If such fish are allowed to die and sink to the bottom of mud ponds the disease may be constantly cycled.

4.1.2. Distribution of Actinosporea in *Tubifex* from mud samples.

The high levels of organic matter in sewage farms and intensive trout farm facilities explains the predominance of *T. tubifex* in the mud samples examined. *L. hoffmeisteri*, the other tubificid present in appreciable numbers, also favours such conditions (Brinkhurst, 1971). Samples of these tubificids, collected from a range of environments, show that the Actinosporea are of widespread occurrence, with species such as *S. tubificis* and *E. radiatum* present in many types of environment, from trout farms in different areas of the United Kingdom, to sewage works. Actinosporea were present in *T. tubifex* from all localities from which a sufficiently large sample of worms was

collected. The low prevalences of Actinosporea found in all field sites examined is in accordance with the observations of many previous workers. Thus Mackinnon and Adam (1924) found *Triactinomyxon* sp. in only 5 of 1250 *Tubifex* worms examined, while Ikeda (1912) observed an even lower prevalence of 1 in 400 in the case of *Tetractinomyxon intermedium* infesting sipunculoid worms.

The highest prevalence of an actinosporean species recorded in the present survey was in the case of *E. radiatum*; 9.5% of *T. tubifex* collected from the experimental tanks at the MAFF laboratories, Weymouth, where trout were being exposed to infection with *M. cerebralis*, were infected with this species. High prevalences have occasionally been recorded, as by Naville (1930) who found *Guyenotia sphaerulosa* in between 30 and 50% of *T. tubifex*, taken from an open water course rather than a closed tank system. Wolf and Markiw (1984) and Wolf, Markiw and Hiltunen (1986) make no references to the occurrence of actinosporean genera other than *Triactinomyxon* in their sample worm populations, in marked contrast to the present study in which several species belonging to different genera were found in all populations which had been effectively sampled.

Of particular note from these field studies was the absence of *T. dubium* from 3 out of the 4 sites in which whirling disease had been diagnosed previously, *ie.* the Cambrian, Hammer and Lannion fish farms, though in the latter case the dominance of oligochaete species other than *T. tubifex* meant that effective sampling of *T. tubifex* was difficult. This observation is in itself of interest since Wolf and Markiw (1984) stated that *T. tubifex* is the single host for the intermediate stage in the hypothesised life cycle. However, at the very heavily infected Lannion fish farm in Brittany visited by the present author,

T. tubifex comprised fewer than 30% of the total worm population, and *T. dubium* was not found in those worms examined. At the only site in which *T. dubium* was found in the presence of *M. cerebralis*, namely at the MAFF laboratories, Weymouth, 4 other actinosporean species were also found, 3 of which, *E. radiatum, S. tubificis* and *T. ignotum*, occurred at higher prevalences. If Wolf and Markiw (1984) are correct in their views of the interrelationship between the Myxosporea and the Actinosporea, then *E. radiatum*, and the other actinosporean species present, should represent the intermediate stage of another myxosporean species. However, the MAFF workers had not observed any Myxosporea other than *M. cerebralis* within the experimenal trout. The mud used in the MAFF system had been derived from the Cambrian fish farm some 12 months previous to the worms being sampled and so it is possible that other fish species were present at this site, which could have been infected with myxosporean species other than *M. cerebralis*. However, it is difficult to envisage how actinosporean infections arising from this source could have been maintained within the Weymouth system over such a period of time, in the absence of the necessary fish species and their attendant Myxosporean parasites.

Conversely, the occurrence of *T. dubium* in *Tubifex* populations taken from sewage farms, an environment from which trout are obviously excluded, is not immediately explicable in terms of the Wolf and Markiw hypothesis. Other fish species would also be excluded, making the occurrence of other Actinosporea such as *S. tubificis* and *E. radiatum*, in sewage farm samples, a similarly difficult phenomenon to explain in terms of their hypothesis.

The field observations have thus shown that Acinosporea may be present in tubificid populations from sites where fish may be absent, or, if present, are

not infected with Myxosporea which could represent the other part of their life cycle. Thus no correlation has been found between the occurrence of *M. cerebralis* and *T. dubium.*

4.2. *Tubifex* infection experiments

In contrast to the observations of Wolf, Markiw and Hiltunen (1986) who found an increase in the prevalence of *Triactinomyxon* in *Tubifex* cultures after the addition of *M. cerebralis* spores, in the present attempts to reproduce these experiments there were no significant increases in the prevalences of *T. dubium* in *Tubifex* cultures, even after periods of six months or more. Since great care was taken in the extraction of the *M. cerebralis* spores used in these studies to avoid treatments which would in any way affect their viability, there is no reason to suspect that they would have lost their infectivity.

The other actinosporean species present within the *Tubifex* cultures followed a similar pattern in prevalence to that shown by *T. dubium*, and the fact that these actinosporean species were still present six months after the experiments began suggest either that the worms and their actinosporean infections are long lived or that the parasites are transmitted directly from worm to worm. However, actinosporean transmission is itself problematical, though few studies have been conducted. Both Granata (1922b) and Marques (1984) failed to obtain a significant increase in prevalence on exposure of *Tubifex* to actinosporean species, though the latter has stressed (personal communication) the great difficulty encountered in finding *Tubifex* populations which are free of preexisting actinosporean infections, and which are a prerequisite, in statistical terms, for such experiments.

4.3. Exposure of *Tubifex* worms to *M. cerebralis* spores

Spores of *M. cerebralis* were found in the lumen of the gut of *T. tubifex*, although the number of spores present at any one time was not large in relation to the number of spores added to the cultures. Despite extensive histological examination of the worms over a period of weeks, no morphological changes were observed in the *M. cerebralis* spores ingested by the worms: the polar filaments were not everted and spore valves did not open. Also, spores were not concentrated in the worms' gut by accumulation with time. As tubificids are known to ingest particulate matter as part of their feeding process, (Wavre and Brinkhurst, 1971), it seems likely that intake of *M. cerebralis* spores would be part of the normal feeding process and that they would pass through the gut unchanged.

4.4. Histological and ultrastructural examination of cartilage infected with *M. cerebralis*

The methods for extraction of *M. cerebralis* spores from cartilage used both in this study and by previous workers, with various modifications, (Markiw and Wolf, (1974) - acid treatments; O'Grodnick (1975) - plankton centrifuge; Kozel, Lott and Taylor (1980) - polyethylene glycol and dextran phase separation), are unsatisfactory for the diagnosis of low level or early infections, in which pre spore stages predominate. Early stages can be detected by histological methods, (Halliday, 1973a) and Giemsa staining of paraffin wax embedded sections has been used routinely at the MAFF laboratories at Weymouth, (Alderman, personal communication). However, water soluble embedding resins, such as Historesin, have a number of advantages over wax embedding when studying cartilage infected with *M. cerebralis*:

(i) it is effective with hard specimens which are difficult to cut. Thus the use of decalcification techniques when processing the highly ossified cranial cartilage of older fish (12 months and older), which might disrupt or destroy pre spore stages, is unnecessary.

(ii) thin sections (0.5 - 2 μm) are easily cut and, as a result, resolution is much higher.

(iii) it is unnecessary to remove the resin prior to staining, as in the case of paraffin wax embedding.

These advantages suggest that such resins should be selected for studying cartilage infected with prespore stages of *M. cerebralis*, and for that matter, other myxosporean species which attack bony structures, such as *Myxosoma cartilaginis*. Such resins are likely to replace paraffin wax as embedding media in routine diagnostic procedures.

The pre spore stages identified in the Historesin sections after staining with Giemsa or Schiffs' reagent are similar to those observed by Halliday (1973a). There were large multinucleate trophozoites, which may represent the multiplicative stage in the development of the parasite, and smaller binucleate sporoblasts, which presumably give rise to spores. These pre spore stages were found exclusively within a matrix of fibrous tissue representing the remnants of damaged cartilage. There was no evidence of a network of membranes around the sporoblasts as described by Halliday (1973a), and fully developed spores were found only at the margins of damaged areas, and not within the confines of what he described as a 'tissue network'.

It has been suggested (Schäperclaus, 1954) that as ossification proceeds, preventing the further spread and subsequent multiplication by the parasite,

the trophozoite and sporoblast stages would become rarer and be replaced by fully developed spores. This was supported by the present study. Sections taken from older fish (6-9 months) from the German outbreak of whirling disease, contained more spore stages and fewer pre spore stages than those taken from younger fish (4-6 months) that were experimentally infected. Although some of the sections taken from the German fish contained large numbers of spores, along with the rarer prespore stages, most of the sections contained very few parasitic stages of any type, though there was still massive host tissue disorganisation. This would suggest that even small numbers of parasite can cause significant damage to areas of cartilage, though the means by which this is accomplished is unclear.

Prespore stages were not recognised in 150-200 nm sections of infected cartilage embedded in Historesin, However, as well as spores, small structures, measuring 1 um diameter, which are apparently surrounded by a unit membrane, were present within the damaged area of cartilage. Whether or not those were stages of *M. cerebralis* is uncertain. Apart from the outer unit membrane there was no clear evidence of intracellular organisatin such as mitochondria or endoplamsic reticulum. In addition the small size of those structures does not equate with the much larger structures (10-20 um) identified as prespore stages via light microscopy.

The fibrous network, in which pre spore stages were identified at the light microscopy level, actually consisted of a mass of disorganised collagen fibrils, with the typical banded, thin (50 nm), cable-like appearance of this fibrous protein. This collagen matrix presumably represents a cellular repair response to the damage caused by the parasite ; fibroblasts would have invaded damaged areas and deposited collagen.

4.5. Experimental infection of rainbow trout with *M. cerebralis*

The fact that transmission of *M. cerebralis* occurred in an experimental system containing *M. cerebralis* spores aged for at least 6 months in mud containing *Tubifex* that did not contain *Triactinomyxon* strongly suggests that the latter is not necessary for the transmission of whirling disease. This view is supported by the observation that fish exposed to *Triactinomyxon* did not develop any *M. cerebralis* infection, as would be predicted from the Wolf and Markiw hypothesis. However, *M. cerebralis* spores in combinations of sterile mud and/or water without *Tubifex* worms also did not produce infections. This suggests that the *Tubifex* may have a very subtle role to play in transmission (see resumé). This view is strengthened by the fact that the mud derived from the Brittany outbreak, from which tubificids had been removed, and to which *M. cerebralis* spores were added, also produced no infection. However, one factor, which is discussed in some detail in the resumé, which may also have played a significant role in determining spore infectivity, is temperature. Although transmission experiments were all carried out at 12°C, the *M. cerebralis* spores used to seed the experimental systems were kept for periods of up to 35 days at 4°C prior to use. Prihoda (1983) found that maintaining spores at low temperature was important in producing infectivity, and so variations in the time from which spores were kept at 4°C may explain why certain experimental systems, such as the Brittany-derived mud, with added spores, remained negative.

4.6. Percoll separation of *M. cerebralis* spores and cross reactivity of *M. cerebralis* and *T. dubium* in the IFAT's using mouse and rabbit anti-*M. cerebralis* antisera.

As a result of the differences in the intensity of *M. cerebralis* infection in the particular fish used by previous authors, it is difficult to estimate the relative

effectiveness of the methods which have been used to isolate spores. Hence, O'Grodnick (1975) recovered an average of 91,000 spores per fish using his plankton centrifuge method, while Tidd, Tubb and Wright (1973) recovered between 15,300 and 2,885,000 spores per fish from the head region alone, using their wash/filter/centrifuge technique. The spore counts obtained using Percoll, of between 16,000 spores per fish in young experimentally infected fish and 510,000 spores per fish (head only) in older naturally infected fish, compare reasonably well with these previous studies. However, the principal advantage of this method is that it avoids the use of acid or enzyme treatments, as has been suggested by some authors (Markiw and Wolf, 1974), which could substantially alter the antigenicity of the spores when used in immunological studies.

The cross reactivity of polyclonal rabbit anti-*M. cerebralis* antiserum with *T. dubium* is in agreement with the observations of Wolf and Markiw (1984). These authors argued that the sharing of antigens indicates that the two spore types are part of the same life cycle. However, the sharing of antigenic determinants recognised by polyclonal sera could simply be indicative of a close taxonomic relationship between organisms, rather than of taxonomic identity. This view is supported by the observation of the cross reactivity of rabbit anti-*M. cerebralis* antiserum with actinosporean species other than *T. dubium*, such as *S. tubificis*, which is rather indicative of a general cross reactivity between the two groups of Myxozoa.

4.7. ELISA for the detection of antibodies produced in rainbow trout experimentally infected with *A. salmonicida*

A. salmonicida was used to assess the ability of the triple layer ELISA to monitor the humoral response in teleosts and has served to illustrate some of

the features of the fish immune system, such as its temperature dependence, which are involved in the response to infection with *M. cerebralis*. The antibody titre curve recorded over a period of 28 days is characteristic of the secondary immune response seen in mammals, with a rapidly rising antibody titre, which does not, however, peak within the time course of the experiment. The high background level of antibody against *A. salmonicida* (titres = 1:400-1:700), in fish derived from wild/farm conditions has been described by previous workers (Paterson and Fryer, 1974). As the trout were obtained in October shortly after the peak water temperatures, which are known to enhance pathogenicity, and hence lead to the dissemination of *A. salmonicida*, there are several possible explanations for the presence of these antibodies. The fish may have been partially or fully immune as a result of recent exposure and recovery. There may have been active infections of *A. salmonicida* at a sub clinical level, stimulating a low to moderate antibody response. The antibodies may have been raised against antigen other than *A. salmonicida* but which cross reacted with *A. salmonicida* and gave a positive reaction in the ELISA.

The initial fall in antibody titres in the first three days after immunisation can be explained by the binding of antibody to antigen so that the antibodies are no longer available to react in the ELISA. This was particularly evident when the higher doses of antigen were given. However, it is also known that the toxins produced by *A. salmonicida* are capable of destroying blood proteins, which may have played some part in the disappearance of antibodies in the first few days after inoculation.

The fall in antibody titre was followed by a rapid rise, which was probably accelerated by the elevated water temperature in the experimental system

(15 °C). Thus between days 5 and 28 antibody titres increased from around 1:400 to around 1:1600. These results are in general agreement with the findings of Paterson and Fryer (1924), who detected an immune response after 7 days, at a higher water temperature of 17.5 °C, using microtiter agglutination and gel precipitin.

4.8. Detection of circulating antibodies produced in rainbow trout against *M. cerebralis* using an ELISA

Although it proved possible to detect an immune response in young fish experimentally infected with *M. cerebralis* using the ELISA, the test is not routinely applicable to the diagnosis of the disease, because of the need to use large numbers of spores to obtain a significant result. Antigen concentration is known to play a key role in the effectiveness of the ELISA (Voller *et al.*, 1979). At a concentration of 5×10^4 spores/well there were numerous available binding sites for the trout antibodies, but there appeared to be insufficient binding sites provided by lower concentrations of spores to obtain a reaction.

The problem of antigen procurement might be surmounted by idcotyping. If a monoclonal antibody (Mab) was raised against *M. cerebralis* spores and then a second Mab was raised against the first Mab, the second would be an effective replica, in terms of antigen binding sites, of the *M. cerebralis* spore. The second Mab would then be used as the antigen to coat the plates.

Two factors may play a part in the observations that mean antibody titre in fish aged 18-24 months, derived from the Brittany outbreak, were no different from the mean antibody titres recorded from control fish. Firstly, as suggested by Halliday (1974b), that *M. cerebralis* develops within a so called

immunoprivileged site: as the infected cartilage is progressively ossified after the first 5-6 months of life, the parasites are effectively sealed off from the fish immune system and so do not elicit an immune response. Secondly, the samples of sera were taken from these fish in early January, when water temperatures were approaching the lowest annual levels (2-5 °C). The fish immune system is known to be highly temperature dependent, (Avtalion, Wojdani, Malik, Shrahrabani and Dùczyminer, 1973) and would have been practically inactive at this time of the year. Antibodies produced earlier in the year would have declined during the inactive period. This means that the role of the postulated immunoprivileged site in older fish could only be determined by maintaining infected fish, over a long time period, at water temperatures at which the immune system is fully functional (*ie.* 10-15 °C), so that serum samples could be taken at intervals.

Notwithstanding the possible use of ideotyping to generate 'artificial' antigen, the detection of a humoral immune response against *M. cerebralis*, and to a certain extent against other pathogens, such as *A. salmonicida*, as a means of diagnosis, is handicapped by a number of factors:

(i) the temperature dependent nature of the process; carrier fish would appear seronegative during the winter months.

(ii) age related factors; older fish may appear seronegative if the parasite resides in an immunoprivileged site.

(iii) problems of cross reactivity; other Myxosporea, which are known to infect salmonids, such as *Myxidium truttae*, may also initiate an antibody response which could cross react with *M. cerebralis* and give false positives. This would need to be tested.

In general terms fish immunodiagnostics would be effective if techniques such as ELISA, coupled with the production of Mab's were geared towards the detection of pathogen antigens rather than to the antibodies produced in response to the disease (see Section 4.10). Such methods would not only overcome the problems caused by cross reactivity and temperature dependence, but would also overcome the problem of false positives arising from seropositive fish which have recently been infected with a pathogen, but which have now recovered fully from the disease and are thus no longer carriers.

4.9. IFAT on *M. cerebralis* spores using immune trout sera.

As an immunodiagnostic tool the IFAT has the same drawbacks as those listed for the ELISA, and in addition it is highly a subjective test. However, it is relatively simple and uses very little antigen (spores) and it may be of some use in studying the immune response against *M. cerebralis* in experimental systems rather than in field diagnosis.

The fluorescence pattern observed was identical to that described by previous workers (Griffin and Davis, 1978), and the pattern of the immune response between the different fish sera used is the same as shown by the ELISA. Of particular note is the pronounced fluorescent corona around the *M. cerebralis* spores when incubated with immune trout sera or with rabbit or mouse anti-*M. cerebralis* antisera. Griffin (personal communication) noted that if the IFAT was performed either on spores that had been enzymatically treated during isolation, or on those that had been aged after removal, the corona effect was greatly reduced in the IFAT. *M. cerebralis* spores have a surface coat, particularly around the posterior half (Lom and Hoffman, 1971). If this coat is protein or glycoprotein and is antigenic, its loss by ageing or removal

by enzymes would greatly reduce the fluorescence. It is therefore essential that only freshly isolated spores be used in any attempts at immunodiagnosis.

4.10. ELISA for the detection of *M. cerebralis* antigen in infected rainbow trout.

The reliability of the ELISA for the detection of antigen in the form of *M. cerebralis* spore sonicate was recognised at concentrations equivalent to 10^3-10^4 spores/well. However, no *M. cerebralis* antigen was detected in sera from infected fish. Whilst the specificity of this ELISA would be greatly improved by the use of monoclonal rather than polyclonal antibodies, it seems unlikely that there would be large amounts of circulating antigen to detect since the parasites are essentially localised in the cranial cartilage. There was a high degree of cross reactivity of the anti-*M. cerebralis* sera with spores of *Myxobolus* sp. Both Walliker (1968) and Lom (1969a) have suggested that the genus *Myxosoma* is indistinguishable from the genus *Myxobolus* and the observed cross reactivity between the two appears to emphasise their close relationship. An extensive series of tests with the ELISA using antisera to different species of Myxosporea, in tests for cross reactivity between genera, along the lines suggested by Markiw and Wolf (1978), would be useful in assessing taxonomic relationships within the group.

4.11. Immunolabelling of infected cartilage sections

Antisera raised against the spores of *M. cerebralis* reacted in the immunofluoresence test with pre spore stages in sections of cartilage. This provided useful confirmation of the identity of the multinucleate stages as pre spore stages of *M. cerebralis*.

The generalised labelling of the area of damaged cartilage, in which the pre spore stages were found, suggests that parasite antigens are released into this area. Those antigens may represent enzymes released by the parasites and which may be responsible for the destruction of the cartilage. This might explain the contrast between the large areas of damaged cartilage and the relative paucity of parasite stages seen in most sections (see Section 4.4.).

4.12. Resumé of studies on the Wolf and Markiw hypothesis for the transmission of whirling disease.

To date there has been no independent confirmation of the Wolf and Markiw hypothesis (Markiw and Wolf, 1983; Wolf and Markiw, 1984; Wolf, Markiw and Hiltunen, 1986; Markiw, 1986) on the involvement of *Triactinomyxon* in the transmission of *M. cerebralis*. Apart from the work of Uspenskaya (1957; 1978) the most recent account of the direct transmission of the disease is contained within the abstract of Prihoda (1983). Lom (personal communication) kindly provided unpublished data on the means of by which Prihoda transmitted the disease. These were by:

(i) adding mud from the benthos of a pond heavily infected with *M. cerebralis* to an aquarium with small rainbow trout fry which were just beginning to feed. The mud had previously been kept in polythene bags at +4 °C for 15 months. It is highly unlikely that tubificids could have survived in such conditions and indeed Prihoda had not seen them.

(ii) by adding spore suspension, without any possibility of tubificid or *Triactinomyxon* contamination, to juvenile trout. The spores had been obtained from the cleaned heads of infected trout, and had been kept for 24 months at +4 °C.

In both instances, clinical symptoms appeared 69 days after exposure. Prihoda (1983) suggested that temperature might play a significant role in bringing the spores to infectivity, a factor which will be discussed subsequently.

The evidence presented in the present work which also questions the Wolf and Markiw hypothesis is as follows:

(i) addition of *M. cerebralis* spores to *Tubifex* cultures did not generate infections of *Triactinomyxon* in the worms.

(ii) field observations on the occurrence of *Triactinomyxon*, and of the Actinosporea in general, do not support the view of a direct link with *M. cerebralis* or other Myxosporea.

(iii) *M. cerebralis* spores ingested by Tubifex showed no signs of development within the worms.

(iv) transmission of *M. cerebralis* has been possible in the absence of *Triactinomyxon*, using aged *M. cerebralis* spores included in a *Tubifex* culture system.

(v) immunological studies using anti-*M. cerebralis* antiserum to test the cross reactivity with *Triactinomyxon* and other Actinosporea do not support the contention that shared antigens between the 2 spore types involved in the Wolf and Markiw hypothesis is clear evidence for their interrelationship.

Indirect support of these observations has come in a number of personal communications. Hoffman (personal communication) states that recent work on *Ceratomyxa shasta*, which attacks a range of salmonid species, and which is a particular problem on the West coast of the U.S.A. has shown no indication of the involvement of an intermediate host. Similarly, Moser (personal

communication) has quoted recent unpublished work by Kabata which shows that no intermediate stage is necessary, though no further detail is available yet.

Although Wolf and Markiw's experimental work may appear to be definitive on closer examination there are a number of practical and theoretical considerations which should be studied carefully. Firstly, their technique for diagnosis of whirling disease relied solely on the detection of spores. This method is not reliable when low level or early phase infections are present. Early infections need to be demonstrated histologically as described in sections 2.6. and 3.1.6. Secondly, the screening meshes used by Wolf and Markiw to isolate the infective agent separated intact actinosporean spores but would not have prevented the sporoplasms they contain, measuring about 2 µm, from passing through. They stated that the major peak of infectivity was trapped on a 50 µm screen while filtrate from a 25 µm screen produced no infectivity. Marques and Ormières (1982), and Marques (1984), observed that sporoplasms are regularly released from Actinosporean spores and are then seen to move in the water column. The sporoplasms might be infective after release from the spores and would have passed easily through the 25 µm mesh to initiate an infection. Their failure to detect infections in fish exposed to the 25 µm mesh filtrate could, of course, have been due to low levels of infection remaining undetected, which is a shortcoming of their detection method. Thirdly, while the number of described myxosporean species is in the hundreds there are only 33 known actinosporean species (Marques, 1984). The Wolf and Markiw hypothesis would require that for every myxosporean species there is a corresponding actinosporean species. Even given the relatively limited amount of research on the Actinosporea there is obviously an enormous disparity in numbers between the 2 groups. The paucity of actinosporean species has been

confirmed in the present work which has revealed only 5 species in the various habitats sampled in the U.K. Lastly, there are profound morphological differences between the 2 spore types, centred around the possession of 3 polar capsules in *Triactinomyxon* and 2 in *M. cerebralis*. In the case of *M. cerebralis* and *T. dubium* there is also a great disparity in size.

Although the present results strongly suggest that *Triactinomyxon* is not involved in the transmission of *M. cerebralis* between fish, it was nevertheless impossible to infect juvenile trout with *M. cerebralis* spores which had been aged on sterile mud alone. Transmission occurred only in those experimental tanks containing *Tubifex*, hinting at the possibility that the worms may have a more subtle role to play than that described by Wolf and Markiw. The worms have been shown to take up *M. cerebralis* spores and it is possible that passage through the gut of the worm may be necessary for spores to become infective. However, Prihoda's results suggest that there is no role for *Tubifex*, though he states that the oxygen concentration within the mud substrate is a significant factor in defining spore infectivity, a factor which is of course influenced by the activity of the mud macroflora.

Before Wolf and Markiw proposed the involvement of tubificids and Actinosporea, a 3 to 4 month ageing process was thought to be necessary during which *M. cerebralis* spores became infective under the influence of various physio-chemical/biological interactions (Schäperclaus, 1954; Hoffman and Putz, 1969). In this context *M. cerebralis* transmission should be examined in 2 distinct environments: in the artificial, high density fish farms, in which rainbow trout are produced and in which mortality as a result of *M. cerebralis* may be extremely high; and in the environment from which *M. cerebralis* originated, *ie.* as a parasite of brown trout in natural river and lake systems.

In rainbow trout farms it may be envisaged that for the 6 months following spawning, which usually occurs between December and March, the young trout are susceptible to attack by *M. cerebralis*. During this period a number of heavily infected individuals will die, spores will be released back into the system and surviving fish will become carriers. By the time spores are available for transmission, most of the available host fish will be approaching the age at which ossification has begun or has occurred, so that infection is made difficult or impossible. This should occur by late summer. The next spawning, and the period immediately after, when trout are most susceptible to infection, does not occur for another 3-5 months. In order to maximise disease transmission there is therefore theoretical justification for an 'ageing' period. This model applies equally well to transmission hypotheses involving either physio-chemical factors acting on the spore to induce infectivity or intermediate hosts as suggested by Wolf and Markiw.

A more complex picture emerges for transmission between wild brown trout, which most authors agree were the original hosts of *M. cerebralis*. The habitats of these fish are clear water streams, rivers and lakes, environments in which *T. tubifex*, which strongly favours organically polluted waters, are very uncommon. Wild trout occur at much lower density than in fish farms, so that transmission between individuals is more difficult. Given such a situation, Wolf's observation (personal communication) that *Triactinomyxon* spores are only viable for a few days after release from *Tubifex* would further reduce the possibility of transmission.

Individual wild brown trout are unlikely to be exposed to large numbers of infective stages and consequently *M. cerebralis*-induced mortality would be rare. Most, if not all infected brown trout will become asymptomatic carriers

and will die of other causes throughout the year. Even allowing for the 3-5 month 'ageing' process, infective spores would be available throughout the year, so spore infectivity is not synchronised with the period when susceptible juveniles are available.

A factor that can induce synchronicity is temperature. Prihoda obtained infections by keeping *M. cerebralis* spores at low temperature (4°C) for varying time periods, though he also stated that keeping spores for 20 days at 4°C does not guarantee that spores will become infective. These observations suggest that cooling is necessary over a reasonable length of time to induce infectivity of spores. Under natural conditions this occurs in winter which coincides with the onset of the breeding season. Thus, irrespective of when spores are released upon the death of the carrier fish, the chilling necessary to bring them to infectivity will only occur at the time when susceptible hosts are once more available.

Future studies on the transmission of *M. cerebralis* should, therefore, take account of the possible effects of temperature on inducing spore infectivity. The Wolf and Markiw hypothesis should be regarded with caution and should in no way be considered as proven until further independent data on the possibility of indirect transmission is produced. Much more detailed work on the transmission and general biology of the Myxosporea as a whole is necessary before a full understanding of the life history of this important group of fish parasites can be reached.

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APPENDIX 1: Constituents of Amman's lactophenol for fixation of Tubificid worms.

Carbolic acid	400 g
Lactic acid	400 ml
Glycerol	800 ml
Water	400 ml

APPENDIX 2: Constituents of LKB Historesin and embedding procedure.

Historesin constituents:	Basic resin	-	Glycolmethacrylate
			Hydroquinone
			PEG 400
			Water
	Activator	-	Benzoyl peroxide plasticizer
			Hardener - Barbituric acid (derivative)
			Dimethyl sulfoxide

Activator (0.5 g) + basic resin (50 ml) produces infiltration solution. Overnight infiltration at 4 °C of specimen, which sinks when completely infiltrated.

For embedding medium: 15 ml infiltration solution + 1 ml hardener, mixed for 1 minute.

Lower depression of Histomould filled with embedding medium, into which specimen placed. Polymerised for 1 hour and then removed.

APPENDIX 3: Preparation of alcoholic uranyl acetate.

Make saturated solution (> 8%) of uranyl acetate in 50% ethanol. Prepare fresh solution prior to use.

APPENDIX 4: Preparation of Reynold's lead citrate.

Lead nitrate	1.33 g
Sodium citrate	1.76 g
Boiled distilled water	30 ml

Shake for 30 minutes, to produce milky suspension. Add 8 ml 1N NaOH and dilute to 50 ml, solution then clears. Do not use if cloudy.

APPENDIX 5: Karnovskys fixation schedule (vertebrate tissue)

1. SOLUTIONS

(A) 1° FIXATIVE. 2 gr Paraformaldehyde + 25 mgm $CaCl_2$ + 20 ml dist H_2O

Heat to 65°C; add 2 drops 1M NaOH. Stir until clear.
Cool and add 10 ml 25% Glutaraldehyde.
Make up to 50 ml with 0.2M cacadylate buffer pH 7.4.
Store at 4°C.

(B) 2° FIXATIVE. Equal vols. 5% osmium tetroxide and 0.2M cacadylate pH 7.4.
Store at 4°C.

(C) WASH BUFFER. 3 volumes 0.2M cacadylate + volumed dist H_2O.
Store at 4°C.

(D) CACODYLATE BUFFER. 42.8 gr Na cacadylate in 1 litre dist H_2O. To 50 mls of this add 2.7 ml 0.2M HCl.

2. FIXATION

(1) Cut tissue into 1mm cubes in fixative at room temp.
Change fixative after 10 mins and fix for 1 hour at 4°C.

(2) Wash twice in 0.12M cacodylate. 15 mins each at 4°C.

(3) Fix in 2.5% OsO4 in 0.1M cacodylate for 1 hour at 4°C on rotator.

(4) Wash twice in 0.1M Na acetate. 10 mins each at 4°C on rotator.

(5) Stain in 0.25% aqueous uranyl acetate for 1 hour at 4°C.

(6) Wash twice in 0.1M Na acetate. 10 mins each at 4°C on rotator.

(7) 35% acetone. 5 mins at 4°C.

(8) 50% acetone. " "

(9) 70% acetone + 1% PTA + 1% uranyl acetate overnight at 4°C.

(10) 90% acetone. 10 mins at room temp.

(11) Wash three times in absolute acetone for 15 mins each at room temp.

(12) Transfer to Spurrs resin.

APPENDIX 6: Spurrs hard formula

Vinylcyclohexene dioxide (VCD)	10 ml
DER 736 Flexibilizer	4 ml
Nonenyl succinic anhydride	26 ml
Dimethylaminoethanol Accelerator (SI)	0.4 ml
Cure 70 °C	8 hours

Appendix 7 Conversion of potical density values to serum titre values for trout experimentally infected with A. salmonicida (Fig. 32)

O.D. = optical density

s.t. = serum titre

(values marked with * are those used in Fig. 32)

i) Fish inoculated with 10^4 bacteria

O.D.	s.t.
0.28	1:200
0.25	1:100*
0.29	1:200
0.28	1:200
0.40	1:400
0.42	1:400
0.47	1:500

ii) Fish inoculated with 10^3 bacteria

O.D.	s.t.
0.80	1:900*
0.28	1:200
0.35	1:300
0.37	1:300
0.48	1:500
1.07	1:1400
1.13	1:1500

iii) Fish inoculated with 10^2 bacteria

O.D.	s.t.
1.35	1:1800*
1.05	1:1400*
0.28	1:200
0.33	1:250
0.41	1:400
0.37	1:350
0.68	1:800
0.60	1:700
0.80	1:1000
0.94	1:1200
1.20	1:1600

iv) Control fish

O.D.	s.t.
0.40	1:500*
0.40	1:400
0.50	1:550
0.55	1:600
0.60	1:700
0.62	1:700
0.57	1:650
0.44	1:450
0.59	1:650
0.54	1:600
0.47	1:500
0.53	1:600
0.48	1:500
0.44	1:450
0.60	1:700
0.53	1:600
0.43	1:450
0.61	1:700
0.52	1:600
0.47	1:500

Appendix 8 Antibody levels in trout against M. cerebralis

i) 5 - 6 month old trout experimientally infected with M. cerebralis
S.D. = serum dilution
O.D. = optical density
All values shown have had maximum background reading of 0.85 subtracted from them.

Fish	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1	1:000	0.35	1:250	0.21	1:500	0.21	1:1000	0.20	1.2500	0.18
2	"	0.78	"	0.43	"	0.20	"	0.20	"	0.17
3	"	0.62	"	0.40	"	0.22	"	0.21	"	0.20
4	"	0.79	"	0.63	"	0.20	"	0.19	"	0.19
5	"	0.86	"	0.53	"	0.26	"	0.20	"	0.20

ii) 5 - 6 month old trout uninfected with M. cerebralis (mean values for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:100	0.21	1:250	0.2	1:500	0.19	1:1000	0.18	1:2500	0.17

iii) 18 - 24 month old trout from Brittany outbreak of whirling disease (mean value for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:100	0.23	1:250	0.21	1:500	0.19	1:1000	0.18	1:2500	0.18

iv) 16 - 20 month old trout from farm negative for M. cerebralis (mean values for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:100	0.21	1:250	0.20	1:500	0.19	1.1000	0.18	1.2500	0.17

Appendix 9 (1) Detection of *M. cerebralis* spores sonicated by ELISA

O.D. = optical density

S.C. = spore concentration

Conc.= concentration

All values shown have maximum brackground reading of 0.62 subtracted from them.

(a) *M. cerebralis*

S.C.	O.D.
10^4	0.92
10^3	0.61
10^2	0.25
10	0.16

(b) *Myxobolus* sp.

S.C.	O.D.
10^4	0.89
10^3	0.59
10^2	0.23
10	0.17

(c) B.S.A.

Conc.	O.D.
10%	0.24
5%	0.22
3%	0.22
1%	0.16

(d) *Tubifex* debris*

O.D.
0.2

* amount of protein not quantified

Appendix 9 (ii) Detection of *M. cerebralis* spore antigen in infected trout

(a) 5 - 6 month old trout experimentally infected with *M. cerebralis*

O.D. = optical density

S.D. = serum dilution (mean values for 10 fish)

All values shown have maximum background reading of 0.5 subtracted from them

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:10	0.23	1:25	0.22	1:50	0.21	1:100	0.21	1:150	0.21	1:200	0.20

(b) 4 - 6 month old trout negative for *M. cerebralis* (mean values for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:10	0.21	1:25	0.21	1:50	0.20	1:100	0.19	1:150	0.18	1:200	0.18

(c) 18 - 24 month old trout from Brittany outbreak of whirling disease (mean values for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:10	0.23	1:25	0.23	1:50	0.21	1:100	0.20	1:150	0.19	1:200	0.19

(d) 16 - 20 month old trout negative for *M. cerebralis* (mean values for 10 fish)

S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.	S.D.	O.D.
1:10	0.21	1:25	0.21	1:50	0.21	1:100	0.20	1:150	0.19	1:200	0.17