THE BIOLOGY OF TRANSMISSION OF *MYXOBOLUS NEUROBIUS* SCHUBERG AND SCHRODER, 1905 A MYXOSPOREAN PARASITE OF SALMONID FISHES

by

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The biology of transmission of Myxobolus neurobius Schberg and Schroder, 1965, a myxosporean parasite of salmonid fishes

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ABSTRACT

The morphology, development, pathogenicity, and possible mode of transmission of Myxobolus neurobius Schuberg and Schroder, 1905, a brain parasite of salmonid fishes was investigated by examining naturally infected sockeye salmon, Oncorhynchus nerka (Walbaum), and by conducting laboratory experiments on the mode of transmission.

Morphology of the spores is identical with that of the originally described material. However, the original description did not mention the existence of two nuclei in the sporoplasm of immature spores, found in the course of this work. Mature spores contain only one such nucleus. Scanning electron microscopy revealed that the spore surface is smooth; there being no ornamentation or mucus envelope. Furrows occur along each side of a prominent sutural ridge.

The vegetative stage in the form of multinucleate trophozoites is reported for the first time.

This species has a unique type of life cycle which is described for the first time. The trophozoites, up to the formation of sporonts, develop in brain capillaries. The sporonts are liberated individually into the brain tissue, leading to a diffuse infiltration, and formation of mono- and polysporous forms. Multinucleate trophozoites are distributed throughout the brain and spinal cord, but the mature spores are completely absent from the forebrain.

Fish show no pathological symptoms as a result of infection with M. neurobius. The absence of symptoms is attributed to the diffuse infiltration, absence of cyst formation and the holozoic early stage of development of the parasite.

Transmission experiments resulted in infection of some, but not all experimental fishes and can be considered a partial success.

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TABLE OF CONTENTS

Page

EXAMINING COMMITTEE APPROVAL ii

ABSTRACT iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF TABLES viii

LIST OF FIGURES x

LIST OF APPENDICES xiii

INTRODUCTION

A. PROBLEM 1

B. HISTORICAL REVIEW

1. Morphology of *Myxobolus neurobius* and its distribution 2

2. Biology of Myxosporea

(i) Survival and infectivity 5

(ii) Mode of Transmission 7

(iii) Life cycle 8

3. Pathogenicity 11

MATERIALS AND METHODS

A. SOURCES AND MAINTENANCE OF THE FISH 14

B. COLLECTION AND CLEANING THE SPORES 15

C. TECHNIQUES FOR STUDYING SPORE MORPHOLOGY 15

D. METHODS FOR STUDYING DEVELOPMENT OF *M. NEUROBIUS* 17

E. METHODS FOR STUDYING SPORE DISTRIBUTION AND PATHOGENICITY . 19

F. EXPERIMENTAL DESIGN TO DEMONSTRATE PARASITE TRANSMISSION 20

Page

RESULTS

A. MORPHOLOGY AND DEVELOPMENT OF MYXOBOLUS NEUROBIUS

1. Morphology

(i) Vegetative stages 30

(ii) Spore 34

2. Development cycle of Myxobolus neurobius 48

B. THE SPORE AS AN INFECTIVE AGENT 54

C. TRANSMISSION OF MYXOBOLUS NEUROBIUS

1. Temporal coordination between the life cycle of M. neurobius and that of its host in nature 63

2. Experimental evidence corroborating observations in natural habitat

(i) Experiment I 66

(ii) Experiment II 68

(iii) Experiment III 71

(iv) Experiment IV 71

D. DISTRIBUTION AND PATHOGENICITY

1. Parasite distribution in the brain 73

2. Pathogenicity

(i) Effects of Myxobolus neurobius infection on host histology 77

(ii) Effect of M. neurobius on its host in nature . 78

DISCUSSION

A. MORPHOLOGY AND DEVELOPMENT OF MYXOBOLUS NEUROBIUS

1. Morphology

(i) Vegetative stages 80

(ii) Spore 82

Page

2. Comparsion of developmental cycles

(i) Development of vegetative stages in the lumen of blood vessels 85

(ii) Liberation of sporonts from the trophozoite cell 87

(iii) Method of sporoblast formation 87

(iv) Time scale of spore formation 90

B. TRANSMISSION OF *MYXOBOLUS NEUROBIUS*

1. In nature (Sproat Lake) 90

2. Laboratory experiments 92

C. DISTRIBUTION IN THE BRAIN AND PATHOGENICITY

1. Distribution 94

2. Pathogenicity 97

SUMMARY 101

BIBLIOGRAPHY 104

APPENDICES 110

LIST OF TABLES

		Page
Table 1	Comparison of spore dimensions (μm) of *Myxobolus neurobius* from two published records	4
Table 2	Plan of experiment done to transmit a myxosporean parasite, *Myxobolus neurobius* into coho salmon, *Oncorhynchus kisutch*, by force feeding	24
Table 3	Plan of experiment done to transmit the fresh spores, mud-aged spores, without mud-ages spores of *Myxobolus neurobius*, into sockeye salmon, *Oncorhynchus nerka*, by exposing fish to a spore suspension	25
Table 4	Plan of experiment done to transmit the spores of *Myxobolus neurobius* into sockeye salmon, *Oncorhynchus nerka*, by force feeding	27
Table 5	Plan of experiment done to transmit the fresh and aged spores of *Myxobolus neurobius* into sockeye salmon, *Oncorhynchus nerka*	28
Table 6	The occurrence of multinucleate trophozoite of *Myxobolus neurobius* in Sproat Lake sockeye salmon, *Oncorhynchus nerka*	31
Table 7	The average dimension of fresh as well as fixed and stained spores of *Myxobolus neurobius*	46
Table 8	The disposition of various developmental stages of *Myxobolus neurobius* during the life span of its host, sockeye salmon, *Oncorhynchus nerka*	49
Table 9	Conditions of *Myxobolus neurobius* spores after being aged (without mud)	56

Table 10 Conditions of Myxobolus neurobius spores after being aged in mud 57

Table 11 The distribution of three differently staining (Feulgen positive) types of spores of Myxobolus neurobius expressed as percentage occurrence 61

Table 12 Prevalence, expressed as percentage of sample examined of Myxobolus neurobius schizogony and sporogony phases in various parts of the brain of sockeye salmon, Oncorhynchus nerka 75

LIST OF FIGURES

		Page
Figure 1	Diagrammatic representation of _Oncorhynchus nerka_ showing the position of incisions in order to give access to the brain and spinal cord	18
Figure 2	Two views of multinucleate trophozoites of _Myxobolus neurobius_ within a brain capillary showing trophozoite and erythrocyte within the capillary wall	33
Figure 3	Trophozoite nuclei of _Myxobolus neurobius_ surrounded by a layer of cytoplasm and a distinct outer membrane indicating the beginning of the formation of sporonts	35
Figure 4	The locomotory ability of trophozoite of _Myxobolus neurobius_ as suggested by the two lobes of the cell and curving an erythrocyte	35
Figure 5	Scanning electron micrograph of spores of _Myxobolus neurobius_ showing the smooth surface of the shell and the prominent sutural ridge	36
Figure 6	Scanning electron micrograph of spores of _Myxobolus neurobius_ showing the straight of sutural line and the inverted lumen opening of the polar filament, situated on the suture	38
Figure 7	A squash of brain tissue showing mature spore, sporoblast, and also a pair of capsulogenic cells and of polar capsules one more advanced than the other	39

		Page
Figure 8	A spore of *Myxobolus neurobius* showing the relative position of the polar capsules in the sutural plane	40
Figure 9	An abnormal spore of *Myxobolus neurobius* containing three polar capsules	40
Figure 10	Spores of *Myxobolus neurobius* showing the affinity of polar capsules for methylene blue and Held stains	41
Figure 11	Scanning electron micrograph of *Myxobolus neurobius* spore showing the extruded polar filaments spiralled around their axes	43
Figure 12	Scanning electron micrograph of spore of *Myxobolus neurobius* showing the domed shape of valves and strongly developed sutural ridge	45
Figure 13	Fresh spores of *Myxobolus neurobius* showing variations in their shape	45
Figure 14	Various stages of the young spores of *Myxobolus neurobius*	47
Figure 15	Light micrograph showing the sporoblasts of *Myxobolus neurobius*	51
Figure 16	Polysporous sporoblast of *Myxobolus neurobius* encapsulated in sporoblast membrane	53
Figure 17	Light micrograph showing the polar filaments in the early development of polar capsules	53
Figure 18	A diagrammatic representation of three different types of *Myxobolus neurobius* classified according to distribution of Feulgen positive substances	60

Page

Figure 19 A diagrammatic representation of a probable life cycle of Myxobolus neurobius 63

Figure 20 Pansporoblast of Myxobolus neurobius containing developing spores in the brain tissue of an experimentally infected fish at 10 months post infection 69

Figure 21 Pansporoblast of Myxobolus neurobius in the brain tissue of an experimentally infected fish at 84 days post infection 72

Figure 22 Young spore of Myxobolus neurobius in the brain of an experimentally infected fish at 6 months post infection 72

Figure 23 Diagrammatic representation of distribution pattern of Myxobolus neurobius spores in the brain and spinal cord 76

Figure 24 Histological appearance of the brain tissue due to Myxobolus neurobius infection 79

Figure 25 Fusion of sporoplasm nuclei inside the spore of Myxobolus neurobius 89

Figure 26 Distribution pattern of brain capillaries of trout (Salmo irideus Gibb) (after Grodzinski, 1947) 96

Figure 27 Histological appearance of various parts of the brain of sockeye salmon 98

LIST OF APPENDICES

		Page
Appendix I	The glossary of terms used	110
Appendix II	Result of an experiment to infect the yearling coho salmon with Myxobolus neurobius in experimental tanks (Experiment I)	112
Appendix III	Result of an experiment to infect the under-yearling sockeye salmon with Myxobolus neurobius in beakers (Experiment II)	113
Appendix IV	Result of an experiment to infect the under-yearling sockeye salmon with Myxobolus neurobius in experimental tanks (Experiment III)	115
Appendix V	Result of an experiment to infect the under-yearling sockeye salmon with Myxobolus neurobius in experimental tanks (Experiment IV)	116
Appendix VI	Diagrammatic representations of sporoblasts of Myxobolus neurobius	117

INTRODUCTION

A. PROBLEM

Fish culture plays an important part in the life of the Indonesian people, for whom it provides a source of revenue (fish being treated as a cash crop by many farmers) and protein. Consequently, the government has embarked on a program of intensification of fish culture. Production in various aquaculture systems (brackish water pond, freshwater pond, cageculture, and fish-cum-rice culture) has been doubled through this program (Cholik _et al._, 1980). However, as in all other countries, aquaculture is limited by the problem of diseases and parasites. In recent years the productivity of Indonesian fish culture has been depressed in particular by losses due to parasites and diseases, and one of the pathogens involved being a species, or several species, of _Myxobolus_.

The first outbreak of _Myxobolus_ infection in Indonesia was recorded in 1935 (Sachlan, 1952) and caused mortality in cultured stocks (_Cyprinus carpio_ and _Puntius gonionotus_). It is not known whether or not _Myxobolus_ was the sole agent responsible for fish mortality and, if not, what proportion of it could be attributed to _Myxobolus_. It is particularly difficult to assess the pathogenicity of _Myxobolus_ and to unravel its effects from those of other possible factors, because many taxonomic problems have not been resolved and the exact identity of the myxosporean has not been determined. Furthermore, the biology of _Myxobolus_ spp. is not well known, and especially that of the species in Indonesia.

It is clear, therefore, that a study of the biology of _Myxobolus_ is relevant to the general problem of aquaculture diseases. This relevance was the reason for selecting _Myxobolus_ as the subject for the IDRC sponsored research project which was subsequently to be presented in partial fulfillment of conditions required for the degree of M.Sc. The location of

the study was Nanaimo, British Columbia, and so, *Myxobolus neurobius* Schuberg and Schroder, 1905 was selected as the model parasite. Although it is not a typical member of its genus, inhabiting the brain rather than the gills of its host, and although it does not occur in Indonesia, *M. neurobius* occurs regularly in British Columbia fish stocks. Part of the information obtained from this study of *M. neurobius* biology is applicable to other species, because in spite of its unique features, it still bears similarity to other members of its genus.

The objectives of this study were as follows:

1. To determine the morphology and development of *Myxobolus neurobius*.
2. To study its distribution in the brain of its host, the possible reasons for that distribution, and the pathogenicity of the parasite.
3. To determine the infectivity of the parasite and, if possible, its mode of transmission.

It was hoped that these objectives would be accomplished by examining fresh and preserved material from naturally infected fish, and by conducting transmission experiments.

B. HISTORICAL REVIEW

1. Morphology of *Myxobolus neurobius* and its distribution in the brain

The first report of *M. neurobius* was by Schuberg and Schroder (1905) from the nervous system of *Trutta fario* L. (= *Salmo trutta*). Since that time it has been recorded in the nervous system of *Salmo trutta*, *Oncorhynchus nerka*, *O. kisutch*, *Prosopium cylindraceum*, *Thymallus arcticus*, *Salvelinus alpinus*, and *Salvelinus leucomaenis* in Russia (Pavlovskii, ed.), 1962; Konovalov, 1971), of *Salmo trutta* and *Salmo salar* in the United Kingdom

(1974), of Coregonus clupeaformis, Prosopium cylindraceum, Thymallus arcticus, Salvelinus namaycush, O. nerka, O. kisutch in Canada (Arthur, 1975; Margolis and Arthur, 1979), and of Salvelinus fontinalis in U.S.A. (O'Grodnick, 1980, personal communication). Myxobolus neurobius has a holarctic distribution in salmonid fishes (see Konovalov, 1971; Kennedy 1974).

Schuberg and Schroder (1905) described M. neurobius as occurring in cysts in almost all parts of the nervous system. The cysts were small, spherical or elongated. They reported that this parasite attained a maximum size of 0.90x 0.02 mm. The authors described thoroughly the morphology of the spore, using light microscopy techniques. In general it resembled most other species of Myxobolus. It is worth noting that the authors observed only one nucleus in the sporoplasm, and described the latter as being triangular or oval in transverse section. The polar capsules were long, extending from the anterior pole nearly to the midlength of the spore. The anterior parts of the capsules were seen as having narrow, neck-like constrictions.

In spite of its reputedly wide distribution, M. neurobius has not been studied in detail by many authors. Only two sources exist as regards the dimensions of its spores and Table 1 compares the data within these two reports. Some slight differences appear to exist in the dimension of the spores of this species though the significance of the differences is doubtful. Furthermore, Kudo (1921) pointed out, these differences can be attributed to the condition of the spores at the time of measurement. Fresh spores are usually larger than fixed ones. Fixation appears to shrink the spores of Leptotheca ohlmacheri, parasite of Rana clamitans and R. pipiens by as much as 14-22% (see Kudo, 1921).

Table 1. Comparison of spore dimensions (µm) of *Myxobolus neurobius* from two published records

Spore structure	Schuberg and Schoder (1905)	Pavlovskii (1962)
Spore length	10.0 - 12.0	10.0 - 13.0
Spore width	8.0	7.5 - 8.0
Spore thickness	6.0	5.0 - 6.5
Polar capsule length	6.0 - 7.0	6.0 - 8.0
Polar capsule width	2.0	2.0 - 3.3

It should be stressed that the early developmental stages of *M. neurobius* have not been previously described. Only fully developed spores have been recorded in the literature. The only exception to this statement is the description of pansporoblasts from sockeye salmon, *O. nerka* (cf. Konovalov, 1971). This stage contained four fully developed spores.

Schuberg and Schroder (1905) observed that the cysts of *M. neurobius* occur in almost every part of the nervous system of the fish, except the brain. On the other hand, according to Pavlovskii (1962), the spores are distributed throughout the spinal cord and the brain, between the myelin (medullary) sheath and Schwann's sheath. The affected fishes were trout (*Salmo trutta*) and a dwarf form of sockeye salmon, *Oncorhynchus nerka*. Arthur (1975) found spores in the brain and spinal cord of *Coregonus clupeaformis*, *Prosopium cylindraceum*, *Thymallus arcticus*, and *Salvelinus namaycush*.

2. Biology of Myxosporea

(i) Survival and infectivity

The assessment of viability of myxosporean spores has been difficult because all attempts at reproducible tests have been unsuccessful. Transmission experiments usually failed (Schafer, 1968; Spall, 1974; Johnson, 1975; Wyatt, 1978), or produced ununiform results (Hoffman and Putz, 1969, 1971; Halliday, 1973; Uspenskaya, 1978).

In studies of Myxosporea, a commonly accepted criterion of spore viability is extrusion of the polar filament and this can be induced by a variety of treatments. The literature contains records of various methods to determine the survival of the myxosporean spores. McKinney and Bradford (1970) proposed for this purpose, measurement of spore respiration. Hoffman *et al*. (1962) used abnormalities of spore morphology

resulting from treatments as an indicator of survival of *Myxosoma cerebralis*. Uspenskaya (1978) used acridine orange and ultraviolet illumination to differentiate living (green) from dead (orange) spores.

The duration of survival of myxosporean spores outside the host has attracted attention of many scientists over years. Linton (see Bond, 1938a) observed spores of *Myxobolus lintoni*, a parasite of *Cyprinodon variegatus*, after they had been kept in sea water for 10 days. Other than some rounding of the sporoplasm, no significant changes were observed. Thelohan (see Bond, 1938a) studied spores of various species of *Myxobolus* and noted that they degenerate after 1-2 months in water. Bond (1938a) experimented with spores of various species of Myxosporea (*Myxosoma subtecalis*, *M. funduli*, *Myxidium folium*, *Myxobolus bilineatus*) and found that the species differed from each other in their ability to survive emersion in water. The survival period varied from 10 to 20 days. The sporoplasm of *Myxobolus bilineatus* rounded up after 28 days, but showed no signs of death. Bond (1938a) used several criteria as indicators of death (vacuolation of sporoplasm, pycnosis of its nuclei and inability to extrude polar filaments). It appears, however, that in addition to the specific ability to survive for a certain period outside the host, survival is determined also by the condition of the water in which the spores were stored. For example the spores of *Myxosoma cerebralis* survive storage for up to 3 years (Hoffman et al., 1968). Bauer (1959) suggested that the spores of this species survive in storage for as long as 12 years. Spores retain their infectivity after freezing at -20C for 2 months (Hoffman and Putz, 1971). Lom (1975) pointed out that the cleaner the spores are during storage the longer they survive.

The infectivity of myxosporean spores has been put to question recently.

The doubts of their infective nature arose mainly from the repeated failure of transmission experiments involving spores. So, by finding the spores of Myxosoma cerebralis in the faeces of the great blue heron (Ardea lerodias), Meyers et al. (1970) have implicated the avian vectors in the transmission of M. cerebralis. Some investigators (Hoffman and Putz, 1969, 1971; Hoffman, 1974; Halliday, 1973, 1974, 1976; Uspenskaya, 1978) considered that aging of M. cerebralis is necessary before they become infective. Others expressed the view that myxosporean infections to great extent are determined by some environmental factors (Hoffman et al., 1962; Schafer, 1968; Wyatt, 1978). Schafer (1968), for example reported that a temperature of about 10 C was required to make infection of Ceratomyxa shasta possible.

(ii) Mode of transmission

The problem of mode of transmission of myxosporean infections has two aspects. Firstly, is the question of the source of infection and secondly, is the point of entry of the host by parasite. The details of the myxosporean transmission are still unknown. It is generally accepted that the infection is transmitted by spore, which is, therefore, an infective stage. The spores of myxosporean species that live in the internal cavities or lumina (coelozoic) pass out with the excretions of the host during its life; the spores of the tissue-inhabiting species (histozoic) are released only after death and disintegration of the host (Davis, 1917).

As to the point of entry, it is generally accepted that the spores discharged from the host are suspended in water and ingested by a new host when they pass through its buccal cavity with the respiratory current. However, other modes of infection have been suggested (Pfeiffer (see Gurley, 1894); Wu et al., 1979). According to Pfeiffer (see Gurley,

1894) the infection of Barbus barbus with Myxobolus sp. might take place through its stomach, gills or wounds. Wu et al. (1979) postulated that Myxobolus dryagini enters the host through the nose, via the sensory surface cells of the olfactory bulb, olfactory nerve and to pseudolymph in the cerebral cavity. The parasite's eventual site is the central nervous system and sensory organs. This opinion (Wu et al., 1979) was based on histological observations, in which they found many mononuclear trophozoites located in the olfactory nerve between the nares and the olfactory bulb.

(iii) Life cycle

As stated above, experiments on the transmission of Myxosporea have so far no positive results. All that has been learned (Schafer, 1968; Wyatt, 1978; Yamamoto and Sanders, 1979) is that placing susceptible fish in water that contains the infective stage of the parasite produces infection. The mechanisms and paths of this process have not been elucidated.

The broad outline of the myxosporean life cycle that is presented below is based on the collective accounts of previous workers. The bases for their views are a series of histological studies or material obtained from naturally infected fish. The sequence and nature of development are arranged in a logical manner and provide the framework for interpretation. As a consequence, there are some disagreements between various investigators as regards the history and progress of the cycle (Noble, 1944). In spite of disagreements about the detail, there exists consensus as to the general features of the cycle. The spore (or spores) enters the digestive tract of a specific host (for the possibility of other entrance point see Wu et al., 1979). Extrusion of the polar filaments anchors the spore during its passage through the alimentary canal (intestine).

The valves open and the sporoplasm emerges into the intestine. The reason for postulating this course of events is based on the observations that digestive secretions cause the spores of most myxosporean species to discharge their filaments, open the valves and liberate the sporoplasm. For example, Erdman (1917) was able to demonstrate it in *Chloromyxum leydigi*; Kudo (1922) in *Leptotheca ohlmacheri*; Davis (1923) in *Lentospora* (= *Myxosoma*) *ovalis*; and Noble (1943) in *Myxidium gasterostei*. Having been released, the sporoplasm penetrates between the intestinal cells and migrates with the blood stream, lymph or coelomic fluid until it finds an appropriate site. Once established, the sporoplasm, now known as the trophozoite, enlarges by nuclear division and cytoplasmic growth. The nuclei may be differentiated into vegetative (controlling nutrition, growth, and other functions) and generative (participating in spore formation) forms. Multinuclear division, frequently supplemented by division of the cytoplasm into plasmodia by means of simple fission or budding, external or internal, results in a new trophozoite, spreading infection to a new site in the host. This has been described by Noble (1941) for *Ceratomyxa blennius*.

The factors governing the growth of the trophozoite are unknown but at some stage the process of spore formation begins. Some nuclei become surrounded by cytoplasm and become the sporonts. These sporonts then grow and their nuclei divide several times, forming six to eighteen nuclei, each with a small mass of cytoplasm and each giving rise to a spore. At least six nuclei are needed to produce a spore. The number of the nuclei produced however, depends on the structure of the mature spore, species, and also on the number of spores that each sporont will eventually develop. When the sporont develops into a single spore, it is called a monosporoblastic sporont and if two spores are formed within

it, it is known as disporoblastic, or a pansporoblast. According to Noble (1944), the nucleus within a pansporoblast divides twice to form four nuclei. Two of them discontinue division and become residual bodies of the pansporoblast. Within each sporoblast the nucleus divides twice, after which two of the nuclei cease dividing while the other two divide once more (this division is apparently reductive in one of the nuclei). Of the six sporoblast nuclei thus formed the first pair, together with the surrounding cytoplasm, give rise to the valves of the spore. The second pair gives rise to the polar capsules and another pair become the embryonic nuclei.

There is substantial amount of information on the time scale of development in some species of Myxosporea, although that describing its initial stages is limited. Schafer (1968) found that a 15 minute exposure of trout to water containing the infective stage of Ceratomyxa shasta produced an infection, which was detectable 10-20 days later, depending on temperature. Yamamoto and Sanders (1979) exposed 6 month old chinook salmon for 48 hours to water containing the infective stage of C. shasta and maintained them in well water at 17.8 C. The infection was detectable after 7 days. A large number of pansporoblasts were detected after 14 days in varying stages of development and spores appeared in 21 days.

Hoffman and Putz (1969) reported that trout fry become infected with Myxosoma cerebralis as early as 2 days after hatching, when placed in water with spores that have been aged for 4 months. They observed multinucleate amoeboid trophozoite 40 days postinfection. Spores were found in the cartilage of fish infected with M. cerebralis 4-6 months after infection (Hoffman and Putz, 1969; Putz, 1970; Tidd and Tubb, 1970). Wyatt (1978) reported that the infection with Myxobolus insidiosus was

in fish 22 days after its exposure to contaminated water. He found morphologically distinct spores 51 days after the infection. Wu *et al.* (1979), who studied the development of *Myxobolus dryagini* in silver carp, *Hypophthalmichthys molitrix*, reported that the fish larvae hatched and were already infected one month later. The first developmental stage to be found in the host was the trophozoites in the nervous system and pseudolymph. The trophozoites were found in increasing numbers after 2-3 months. The spores were first observed in the pseudolymph after 4 months.

3. Pathogenicity

Myxosporea are cosmopolitan parasites that infect both marine and freshwater fishes and occur in almost every organ and tissue of their hosts. In common with other parasitic organisms, Myxosporea exert a pathogenic influence on their hosts. These effects depend on the species of the parasite and host, age and condition of the host and environmental factors (Shulman, 1966). They range from the apparent absence of harmful effects through local tissue damage and some degree of host debility to lethal effects.

Relatively few Myxosporea species have been found in the brain or nervous tissues of fishes. Pfeiffer (see Schuberg and Schroder, 1905) found *Myxobolus* in the brain and spinal column of greyling (*Thymallus thymallus*). This parasite was later described as *Myxobolus mulleri* by Thelohan (see Schuberg and Schroder, 1905). Schuberg and Schroder (1905) found a species of *Myxobolus* in the nervous tissue of *Trutta fario* (= *Salmo trutta*) and named it *Myxobolus neurobius*. This species differs from *M. mulleri* in the morphology of the spores and the host affinity.

Bond (1938b) reported *Myxobolus bilineatus*, parasitic in various

organs of Fundulus heteroclitus, including the brain, particularly the ventricles and subthecal space of the brain. Yasutake and Wood (1957) described Myxobolus kisutchi from the spinal cord of coho salmon (Oncorhynchus kisutch). This species, however, is morphologically identical with M. neurobius. Konovalov (1971) considered M. kisutchi as a junior synonym of M. neurobius. Arthur (1975) supported this view. Myxobolus dryagini, was reported from the nervous system of silver carp (Hypophthalmichthys molitrix) in China (Wu et al., 1979).

Members of other myxosporean genera are also known to live in the brain of their hosts. Paperna and Zwerner (1974) described Kudoa cerebralis from the brain and spinal cord of Morone saxatilis. Narasimhamurti and Kalavati (1979) found K. tetraspora in the brain of Mugil cephalus. The occurrence of an organism resembling a myxosporean was reported by Marquet and Sobel (1970) from the lung fish, Polypterus enlicheri.

It might be expected that parasites inhabiting a major organ or tissue would have a correspondingly great effect on it and on the host. However, little is known about the influence of the brain-inhabiting Myxosporea. Pfeiffer (see Schuberg and Schroder, 1905) was inclined to attribute mass mortality of the infected greyling in the river Ilm to M. mulleri. The disease that he believed was caused by the myxosporean was named by him "polyneuritis parasitica". Pfeiffer's views were not, however, supported by concrete evidence. There are grounds to believe that the die off might have been due to pollution of the river.

Schuberg and Schroder (1905) were also inclined to attribute a pathogenic effect to the species, M. neurobius. However, apart from slight mechanical damage to the nerve tissue no pathogenic changes were to be seen. Bond (1938b) was another worker who failed to find any pathological effects

of *M. bilineatum* on the brain of its host, *F. heteroclitus*. However, Marquet and Sobel (1970) found large wandering glial cells in the spinal cord of *P. enlicheri* and they attributed this phenomenon to the infection even though the fish showed no morphological changes and its behaviour remained normal. Wu *et al.* (1979), on the other hand, believed that *M. dryagini* was definitely pathogenic, causing the so-called whirling disease. Unfortunately, they produced no description of the pathological signs of this disease.

MATERIALS AND METHODS

A. SOURCES AND MAINTENANCE OF THE FISH

Throughout the study period, June 1980 to January 1982, naturally infected sockeye salmon, Oncorhynchus nerka (Walbaum), were provided by the staff of the Pacific Biological Station, Department of Fisheries and Oceans, Nanaimo, B.C. They were obtained by seining and trawling. The smolt, underyearling and adult sockeye salmon were collected mainly from Sproat Lake, Henderson Lake and its river. Some fish, however, were obtained from Kennedy Lake and Hobiton Lake. All these locations are situated on Vancouver Island, B.C. The sockeye populations of these lakes are known to be naturally infected with Myxobolus neurobius Schuberg and Schroder, 1905 (T. McDonald, personal communication.)

Most of the fish were brought to the Pacific Biological Station on ice and stored in a cool chamber until examination. The fish were usually necropsied within 24 hours after capture. Some specimens, however, were preserved in 10% formalin before examination. Storage periods could extend up to a month.

On one occasion the underyearling fish were brought to the laboratory alive for study of the duration of the developmental cycle of M. neurobius. They were held in 34 l cylindrical fibreglass tanks supplied with a continuous flow of water (2.8-3.0 l/min), at temperature with seasonal fluctuations 5-15 C.

Uninfected fish were obtained from Rosewall Creek hatchery on Vancouver Island, about 25 miles north of Nanaimo and were used in transmission experiments. This hatchery is free of M. neurobius. These fishes were produced by artificial spawning in which hatching and rearing were conducted in containers supplied with well water. The fish included 11 month old

coho salmon (O. kisutch Walbaum) and 2 month old sockeye salmon fry. They were maintained in 1,000 l oval fibreglass tank supplied with a continuous flow (38 l/min) of dechlorinated domestic water at a seasonally fluctuating temperature 5-20 C. The two species were resident in the same tank but at different times. All fish were fed with an Oregon Moist Pellet (OMP) diet ad libitum.

B. COLLECTION AND CLEANING OF SPORES

The top of the cranium was opened by a horizontal incision and the brain removed. The brains were then grounded in a mortar, one or two brains at a time. Distilled water (10 ml) was added to each sample of ground brains and the suspension collected in a 250 ml erlenmeyer.

The 250 ml liquid sample was filtered through a triple thickness of cheesecloth to remove coarse particles and the resulting filtrate was re-filtered through a filter funnel. Clean distilled water was added to the funnel. The resulting filtrate had a volume of about 400 ml and this filtrate was then transferred into 15 ml plastic centrifuge tubes and centrifuged in an International Clinical Centrifuge at 3393 rev/min for 5 min. The supernatant fluid was drawn off and the pellet was left in 0.5 ml of suspension. All pellets were collected in a 25 ml volumetric glass. Clean distilled water was added to make 25 ml volume. This stock of spores was stored in a refrigerator at 4 C.

C. TECHNIQUES FOR STUDYING SPORE MORPHOLOGY

The spores were studied under (a) phase contrast, (b) bright field, (c) fluorescence illumination, and with the aid of a (d) scanning electron microscope.

(a) Wet mounts of spores were studied, after sealing the coverslip with paraffin wax, using phase contrast microscopy. The variations in the morphology of the spores were recorded by photomicrography, as recommended by Lom (1969)

(b) To reveal the presence of mucous envelopes, the India ink technique was used (Lom and Vavra, 1963). Polyvinyl lactophenol iodine was used to detect iodinophilic vacuole (Davies, 1968). For other spore details, thin smears of spore suspension were air dried, fixed in absolute methanol and stained with Giemsa's stain (see Amlacher, 1970). Feulgen's technique was also employed to stain these smears after having been fixed in formol saline.

(c) The acridine orange fluorescent technique was used to differentiate live from dead spores, using fluorescent microscopy (see Uspenskaya, 1978).

The above staining procedures are commonly used for studying Myxosporea.

(d) For scanning electron microscopy, spores of M. neurobius were prepared as follows. After fixation in 2.5% buffered phosphate glutaraldehyde (pH 7.3) overnight, the spores were collected on millipore filters in a plastic chamber by using a plastic syringe, washed in phosphate buffer (pH 7.3) and dehydrated in ethyl alcohol. Drying was accomplished with the aid of a critical point drier Bomar SPC 900 with freon 13, after passing through an ascending series of Genesolv-D. The millipore filters were attached to the supporting specimen stubs by transparent, double-coated tape. After a thorough gold-palladium coating in a Technics Hummer V sputter coater, the stubs were examined with an ETEC Autoscan at 20 kv. Some spores were treated with 1.5-2.0% KOH for extrusion of polar filament prior to fixation.

Measurements of both fresh and fixed spores were made with the aid of an ocular micrometer. Drawings were made by tracing the picture on the scanner screen through microfiches, as well as by drawing freehand, with the aid of an eye-piece graticule.

D. METHODS FOR STUDYING DEVELOPMENT OF M. NEUROBIUS

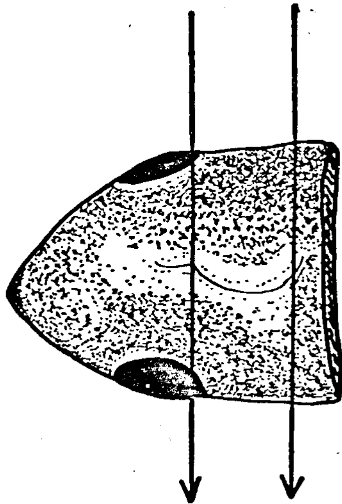
A series of successive monthly samples of underyearling sockeye salmon were taken from Sproat Lake during the period August-December 1981. The August sample was fixed in 10% formalin immediately after catching, and brought to the Pacific Biological Station. The September sample was transported to the Station on ice and fixed in Bouin's solution. The October sample was maintained alive in a 34 l fibreglass tank supplied with a continuous flow (2.8-3.0 l/min) of dechlorinated water at a seasonal fluctuation of temperature (5-12.5 C). This sample was used in three portions: one was examined during October and constituted the October sample proper, the second and third were used as November and December samples respectively.

The entire fish were fixed in 10% formalin or Bouin's solution for a minimum of 24 hours before processing. The fish were decapitated by cutting between the operculum and pectoral fin. Each head was then further divided by a sagital or a coronal incision. Two coronal incisions were made in the translucent cranium that was not sagitally incised, one just behind the eye and another in a position between the optic tectum and the cerebellum, so that the brain was cut into its fore- mid- and hind-brain sections (Fig. 1a). All parts of the head were dehydrated through an alcohol series, cleared in xylene and infiltrated with paraffin wax (56 C) in an automatic tissue processor (Autotechnicon Ultra). The tissues

Fig 1. Diagrammatic representation of *Oncorhynchus nerka* showing the position of incisions in order to give access to the brain and spinal cord.

a. Two coronal incisions dividing the brain into its fore- mid- and hind regions.

b. Three incisions in the spinal cord.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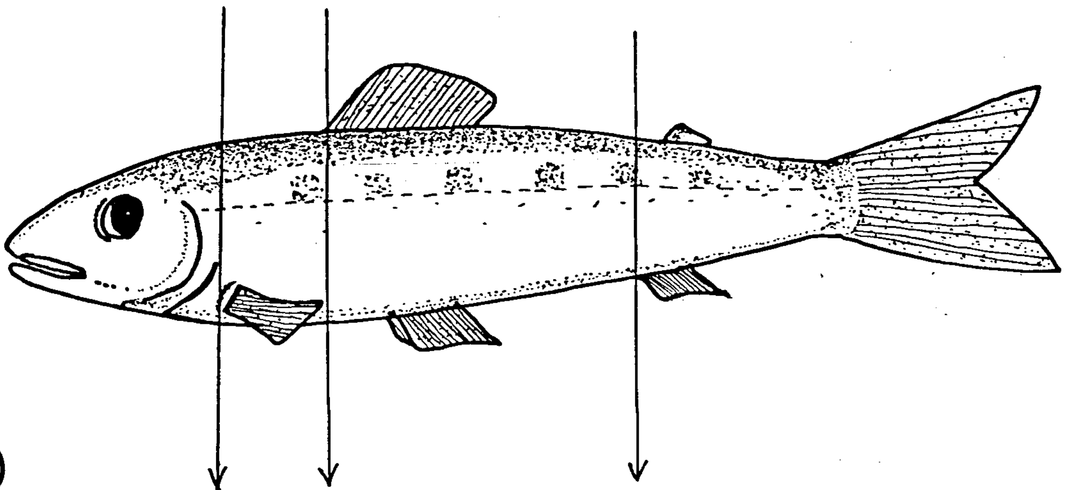
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were immersed in molten paraffin wax under negative pressure (- 11.55 kg) for 30 min and embedded in fresh paraffin wax of the same melting point. The paraffin-embedded tissues were sectioned on a rotary microtome at 8 um. Sections were floated in a water bath containing gelatin, mounted on frost-end glass slides and coded. The slides were stained with haematoxylin-eosin after hydration, dehydrated and mounted with Permount. Some slides were stained with methylene blue, or methylene blue with eosin in alcohol as counterstain.

Slides were examined under bright field illumination of a Reichert or Leitz Dialux 20 microscope. Photomicrographs were taken on Kodak Ectachrome 64 slide film, or Kodachrom 40 slide film. Freehand drawings were made with the aid of eye-piece graticule, at magnification x 1,000. Measurements were made with the aid of an ocular micrometer.

In addition, examinations were made of sockeye salmon smolts caught during their seaward migration. The processing of these fish was similar to that described above. Squash samples of the brain of these were also studied and photographed. Thin squashes of brains were fixed in methanol and stained with Giemsa's stain, or methylene blue and eosin. A few samples were fixed in Held fixative and stained with Held test (Gray, 1960). This stain was commonly used for microglia. Photomicrographs were taken of smear preparations.

E. METHODS FOR STUDYING SPORE DISTRIBUTION AND PATHOGENICITY

Distribution of spores in the brain was studied in smolt, underyearling, and adult sockeye salmon. Longitudinal and transverse sections of the brain tissue, processed as described above were examined. For the adult fish the brains, rather than whole heads were processed, because of the

volume of tissue.

Serial sections of the brain tissue were stained with methylene blue and eosin in alcohol as counterstain. This stain proved to be excellent for the distribution study, because the spores took on the blue colour and so were readily distinguishable from the red brain tissue, even under low magnification. The nuclei of the brain cells were also blue, but their shape was clearly distinct from that of the spores, and so minimizing confusion.

The staining method was the same as that mentioned in the section above. However, the slides were first immersed in eosin alcohol (5 ml eosin + 100 ml alcohol) before continuing dehydration and clearing.

Spore distribution along the length of the spinal cord was studied in transverse sections taken from various parts of the cord. The body was cut at three points, as shown in Fig. 1b. The tissues were processed in the same manner as the brain samples and spores located in the stained spinal cord tissue.

Semidiagrammatic maps of spore distribution in the brain and spinal cord were made after examination under a dissecting scope at low magnification (x 12) or at 40, especially for the spinal cord. The locations of the spores in the brain were also recorded photomicrographically using Kodak Ectrachrome 64 slide or black and white slide film.

All processed tissues were examined for possible pathogenic influence of _M. neurobius_. Any histological changes in the brain were noted and photographed.

F. EXPERIMENTAL DESIGN TO DEMONSTRATE PARASITE TRANSMISSION

Both fresh and aged spores were used as infective material in experiments

aimed at transmitting M. neurobius from one fish to another. Ageing of spores was attempted by placing infected brains from adult fish in four 100 ml beakers, four brains in each. The beakers were covered individually with nylon netting. Each beaker was placed individually in a 1.0 l beaker. These beakers were then separately placed in 34 l fibreglass tanks supplied with dechlorinated domestic water with continuous flow (1 l/min) at seasonal fluctuating temperatures(5-12 C).

Another series of aged spores was prepared by putting ground infected brains in glass jars containing mud collected in the yard of the fish culture building. Each inoculum contained 12 ground, infected brains. The open jars were individually kept in separate 34 l fibreglass tanks, with the same water regime as that of the preceding series.

The morphology of the spores in each monthly sample from both series was examined. Spores were cleaned for this purpose by sieving through a triple thickness of cheesecloth, re-filtering through a filter funnel, and centrifuging as that previously described of spore cleaning. Smears of spore suspension were made and stained with Feulgen's stain after being fixed in 10% formol saline. The filament extrusion ability of the spores was tested by treating with 1.5-2.0% KOH. The acridine orange technique (see Uspenskaya, 1978) was also employed. The spores were centrifuged in an International Clinical Centrifuged at 3393 rev/min for 5 min. After the supernatant was drawn off, acridine orange solution at dilution 1:20,000 was added to the pellet. After 10 min the spores were examined with the aid of a Zeiss fluorescence microscope.

Spore counts for the transmission experiments were made by using an AO Spencer bright line haemacytometer well 0.1 mm deep. The count was based on the number of spores in five groups of 16 small squares

with 20 replicates, observed under 40 x 15 magnification. The number of spores in 1 ml of solution was calculated according to the following formula:

$$N = \frac{\overline{p} \times 4,000 \times 10^3}{q}$$

where, $\overline{p}$ = the average number of spores in 20 counts

q = the number of small squares counted

N = the number of spores in 1 ml of solution

Four transmission experiments were done. Spores were introduced into fish by force-feeding or by placing the fish in spore suspensions. Since no data are available on the number of spores that should be injected into the fish, an arbitrary amount of spores was administered to the fish in these experiments.

In the first experiment (August 7, 1980) fresh spores from an adult fish (Oncorhynchus nerka Walbaum) were administered by force-feeding the experimental fish, Oncorhynchus kisutch (Walbaum). The fish were force-fed 0.1 ml doses of spore suspension (28,000 and 70,000 spores respectively). The first dose constituted the group of Treatment 2, and the second was that of Treatment 3. The administration was carried out with the aid of a 1 ml disposable plastic syringe, equipped with a technicon 020-116-0536-05 rubber tube. The tube was gently inserted into the oesophagus of the fish and the spores were carefully injected, to avoid their expulsion by the fish. The fish were then placed in 34 l fibreglass tanks supplied with continuous water flow at 2.8-3.0 l/min. The water was supplied from the domestic, dechlorinated source at temperature 15 C. In addition, a batch of 10 fish, treated with distilled water

in place of spore suspension was used as a control. A replicate was produced for each of these three batches. The control fish, like those in the treated batches were in the 43-93 mm size range. No anaesthetics were used when administering the spores. The details of treatment are summarized on Table 2.

The second experiment (March 3, 1981) consisted of putting the experimental fish into suspensions of fresh or aged spores (Table 3). One batch of 30 fish was exposed to fresh spores (T.2), one to spores aged in mud for 109 days (T.3), and the third to spores aged for 131 days without mud (T.4). This experiment was conducted in 3 l beakers containing 1 l of dechlorinated water and equipped with air pumps. Experimental fish were 3 month old sockeye salmon, 35-37 mm long. On the first day of the experiment 5 ml of fresh spores (160 x 10^3 specimens/ml), mud-aged spores (145 x 10^3 specimens/ml), and spores aged without mud (180 x 10^3 specimens/ml), were added to each of three beakers containing 1 l of domestic water. A number of 30 fish were placed in each beaker. On the second day another 10 ml of spore suspension was added to each beaker (5 ml in the morning and 5 ml in the afternoon). Similar additions were made on the third day. A replicate was made for each treatment and another two beakers contained control fish without spores (T.1). The water temperatures at the time of spore administration were 7.1 - 7.5 C. On the eighth day all fish were transferred into eight fibreglass tanks. All beakers were numbered and transfer was made to tanks with identical numbers. The tanks were supplied with water at continuous flow (2.8 - 3.0 l/min) from the same domestic dechlorinated source at seasonal temperatures 5 - 20 C.

The third experiment (June 12, 1981) involved the use of fresh spores

Table 2. Plan of experiment done to transmit a myxosporean parasite, *Myxobolus neurobius* into coho salmon, *Oncorhynchus kisutch*, by force feeding method

Treatments	Numbers of experimental fish	
	Group 1	Group 2
Treatment 1	10	10
0 spore/fish; Control		
Treatment 2	10	10
28×10^3 spores/fish		
Treatment 3	10	10
70×10^3 spores/fish		

Table 3. Plan of experiment done to transmit the fresh spores (T.2), mud-aged spores (T.3), without mud-aged spores (T.4), and control (T.1) of *Myxobolus neurobius* into sockeye salmon, *Oncorhynchus nerka*, by exposing the fish to spore suspension

Treatments	Numbers of experimental fish	
	Group 1	Group 2
Treatment 1	30	30
0 spore/ml; Control		
Treatment 2	30	30
4×10^3 spores/ml		
Treatment 3	30	30
3.62×10^3 spores/ml		
Treatment 4	30	30
4.5×10^3 spores/ml	30	30

Note: T.1 = Treatment 1; T.2 = Treatment 2; T.3 = Treatment 3; T.4 = Treatment 4

obtained from sockeye salmon smolts of Sproat Lake and Henderson Lake caught during seaward migration. Sockeye salmon from the same source as that of experiment 2 were used as experimental fish. Their size range was 53-80 mm. The spores were introduced by force-feeding, as in the first experiment (Table 4). One batch of 30 fish was given 0.3 ml of Henderson Lake spores (55,000 spores) while the other was administered 0.35 ml (75,000 spores) from Sproat Lake. The control fish were given only distilled water. No replicate was made to each treatment in this experiment. The fish were anaesthetized with MS 222 before the administration of the spores. Temperature at the time of spore administration was 12.5 C.

The fourth experiment was conducted on September 9, 1981. Fresh spores and spores kept in a refrigerator at 4 C for five months were used as the infective material. Two batches of fish consisting of 30 individuals each were force-fed with spore suspension. Each fish of the first batch received 0.1 ml of fresh spore suspension containing 75,000 specimens (T.1), whereas that of the second batch received 0.1 ml of refrigerated spore suspension containing 85,000 specimens (T.2). The method of administering the spores was the same as in the previous experiment, at water temperature 18 C. The fish were anaesthetized with MS 222. Table 5 summarizes the treatments.

Regular sampling was conducted at monthly intervals, but some fish were examined eight days postinfection. In addition all fish that died during the course of the experiment were examined immediately for spores or early stages of M. neurobius. The standard sample consisted of one or two fish, but change in the size of the sample was frequent. Both fresh and fixed fish were examined. Hematoxylin-eosin and methylene blue-eosin stains were used on fixed material. All findings were recorded

Table 4. Plan of experiment done to transmit the spores of *Myxobolus neurobius* into sockeye salmon, *Oncorhynchus nerka*, by force feeding method

Treatments	Numbers of experimental fish
Treatment 1	30
0 spore/fish; Control	
Treatment 2	30
Hendersone Lake spores	
55×10^3 spores/fish	
Treatment 3	30
Sproat Lake spores	
75×10^3 spores/fish	

Table 5. Plan of experiment done to transmit the fresh and aged spores of *Myxobolus neurobius* into sockeye salmon, *Oncorhynchus nerka*.

Treatments	Numbers of experimental fish
Treatment 1	30
fresh spores	
75×10^3 spores/fish	
Treatment 2	30
aged spores	
85×10^3 spores/fish	

by photomicrographs. Free-hand drawings were made with the aid of eye-piece graticule under bright field illumination.

RESULTS

A. MORPHOLOGY AND DEVELOPMENT OF MYXOBOLUS NEUROBIUS

1. Morphology

Descriptions of Myxosporea are usually based on the morphological characteristics of their vegetative stages and spores. In many instances, especially among the histozoic forms, species have been described on the basis of spores alone. This is true also of M. neurobius. Schuberg and Schroder (1905) and subsequent investigators (Konovalov, 1971; Arthur, 1975) did not find stages other than spores. Therefore, it was deemed necessary to start the present project with a thorough redescription of the spore stage and initial descriptions of vegetative stages for this species.

(i) Vegetative stages

Vegetative stages in the form of multinucleate trophozoites were found in 22 out of 118 examined specimens of underyearling (0+ aged group) sockeye salmon. Neither the smolt (1+ aged group) nor the adult fish harboured this stage. Among the infected fish, 21 specimens came from the August, 1981 sample, whereas only 1 fish taken in September was infected with this stage. No vegetative stages were found in samples collected in October and subsequent months (Table 6).

All the multinucleate vegetative stages were found in blood capillaries of the brain and spinal cord. In the brain they were located in the telencephalon (forebrain) and in the mid- and hind-brain. In three instances they were found also in capillaries of meninx primitiva. It can be assumed in this latter site that they were in the course of migration to the brain, their ultimate site.

At this stage of development the parasite does not completely occlude

Table 6. The occurrence of multinucleate trophozoites of *Myxobolus neurobius* in Sproat Lake sockeye salmon, *Oncorhynchus nerka*. Data recorded from examination of serial sections

Fish age	Sampling time	Numbers of fish examined	Numbers of infected fish
Underyearling	August	46	21
	September	30	1
	October	12	0
	November	15	0
	December	15	0
Smolts	May (seaward migration)	35	0
Adult	June-September (spawning migration)	16	0

the vessel. No direct contact was observed between the parasite and the capillary membrane, though capillaries were distended at the point occupied by the parasite (Fig. 2). The shape of the parasite appears to be moulded by the blood vessel which contains it. In sections, the shape of the parasite varied from spherical or oval to slender. The average size of the cell was 20.2 $\pm$ 2.3 um (13.8 - 38.3) in length and 13.2 $\pm$ 0.3 um (10.5 - 21.4) in width (n=30).

As mentioned above, the earliest stages found during the course of this study were multinucleate trophozoites. This stage, however, seems to be the final one in the course of the development prior to sporogony, since the nuclei were distributed throughout the cell. Some of the nuclei were present even near the outer cell membrane. At this stage no distinction could be made between ecto- and endoplasm (see Fig. 2,3,4). The peripheral membrane appeared to be devoid of striations. All cytoplasm seemed to have been obscured by nuclei.

The number of cell nuclei (based on counts made from slides cut at 8 um) varied depending on the size of the cell, ranging from 45 to 103. The shapes of nuclei varied from spherical to irregular. Spherical nuclei measured 0.76 - 1.02 um in diameter.

The generative nuclei could not be distinguished from the vegetative. Both types stained dark red with hematoxylin and eosin, and dark blue with methylene blue. The chromatin network could not be seen. It is highly probable that the subspherical nuclei were generative, as seen in Fig. 3. The nucleus was surrounded by a layer of cytoplasm and an outer distinct membrane, all of which indicated the beginning of the formation of sporonts.

In the absence of observations on living organisms it is difficult

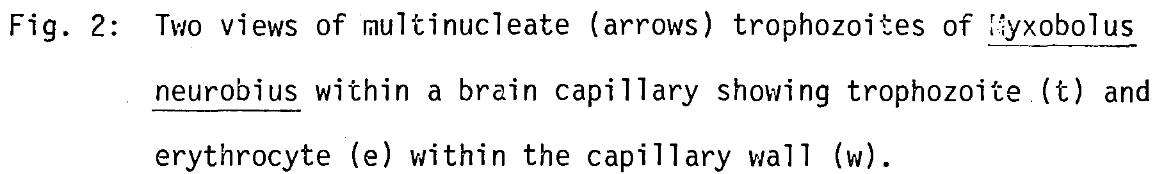
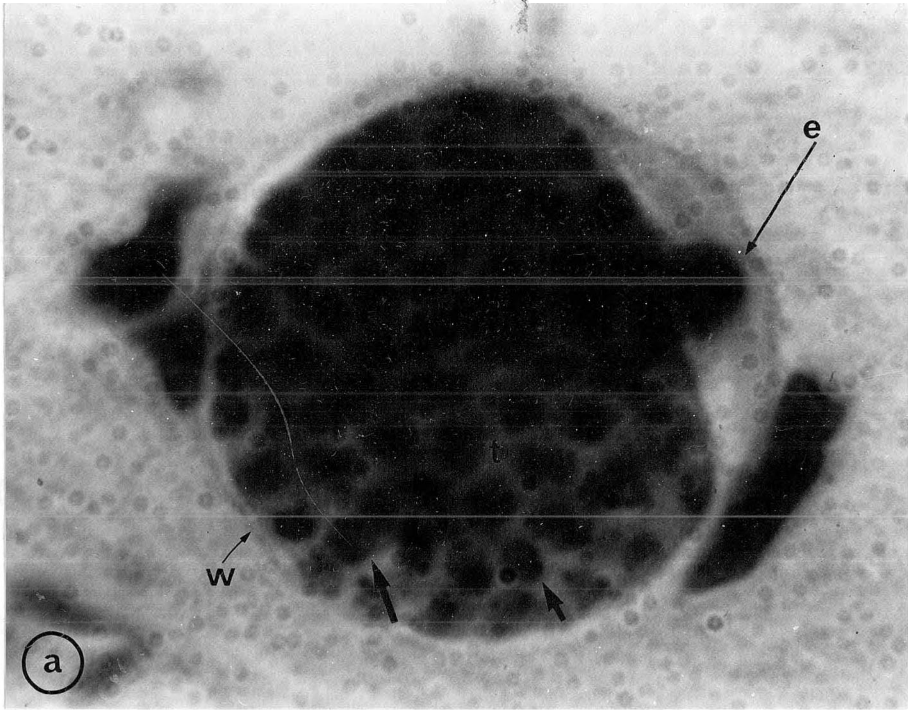


Fig. 2: Two views of multinucleate (arrows) trophozoites of *Myxobolus neurobius* within a brain capillary showing trophozoite (t) and erythrocyte (e) within the capillary wall (w).

Note the variations in shape of the trophozoite and distention of capillary. Hematoxylin and eosin stain (a. x 3500; b. x 2000)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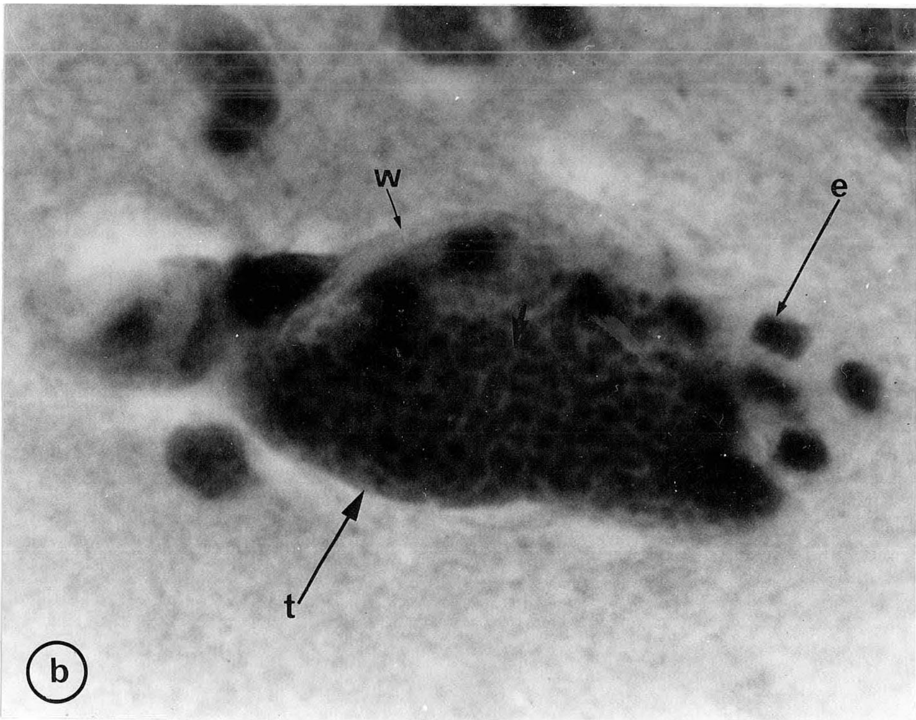
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to comment on the locomotory powers of *M. neurobius*. However, as can be seen in Fig. 4, this species appears to have some locomotory ability. The figure shows two lobes projecting from the cell and curving around an erythrocyte. This strongly suggests amoeboid movement. It seems that such lobe-like extensions form at any point on the surface of the cell.

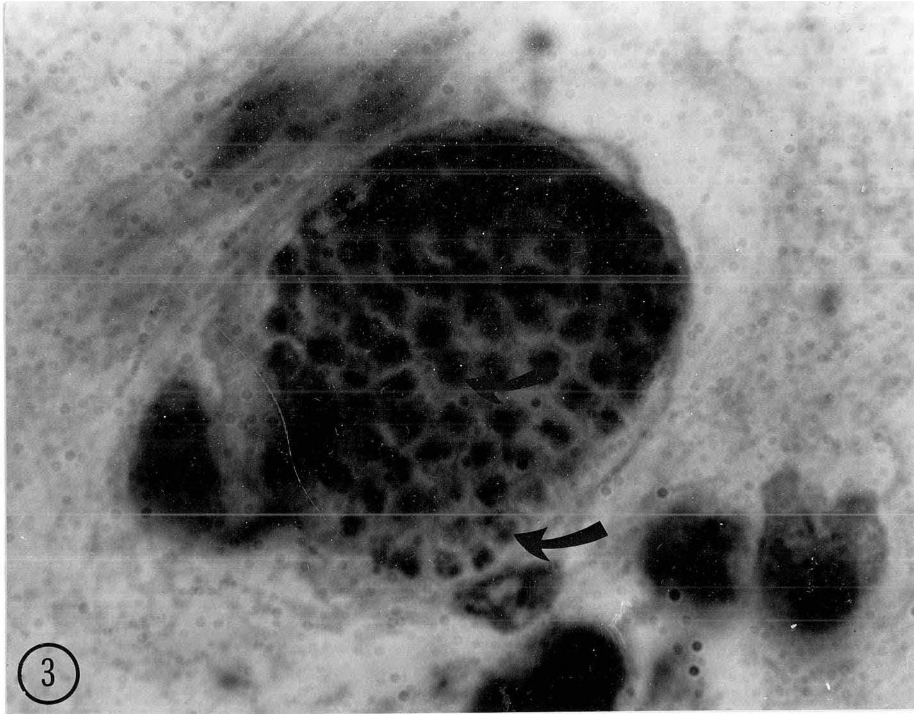
None of the multinucleate cells examined was found to contain a spore. In the August sample, in particular, these multinucleate cells were the only stages found in the capillaries of the brain. No trace of the parasite was found in the brain tissue.

(ii) Spore

In its general characteristics, the spore of *M. neurobius* resembles those of other species of the genus. Like the spores of all *Myxobolus* species it has a transparent spore membrane, the shell, composed of two valves. The valves meet along a sutural ridge running the entire length of the suture between the valves. Inside the spore, two polar capsules are present, occupying the anterior part of the spore. Each polar capsule contains a coiled polar filament. The sporoplasm occupies the posterior part of the spore; and iodinophilic vacuole and sporoplasm nuclei, one in mature and two in immature spores can be seen within it. The surface of the shell is smooth, without ornamentation or processes (Fig. 5). In relation to the total size of the spore, the shell is relatively thick (0.68 um); it is also uniform, except for the prominent sutural ridge (Fig. 5b). The thickness of the shell is clearly visible in polyvinyl-lactophenol-iodine stained spores. Methylene blue imparts a blue colour to the outer membrane of the spore. The shell did not react with Feulgen or hematoxylin and eosin stains. In spores stained with acridine orange, the shell was green under the fluorescent microscope. The application

Fig. 3: Trophozoite nuclei of *Myxobolus neurobius* (arrows) surrounded by a layer of cytoplasm and a distinct outer membrane indicating the beginning of the formation of sporonts. Hematoxylin and eosin stain. x 2500.

Fig. 4: The locomotory ability of the trophozoite of *Myxobolus neurobius* is suggested by the two trophozoite lobes partially surrounding an erythrocyte (e). Hematoxylin and eoxin stain. x 2500.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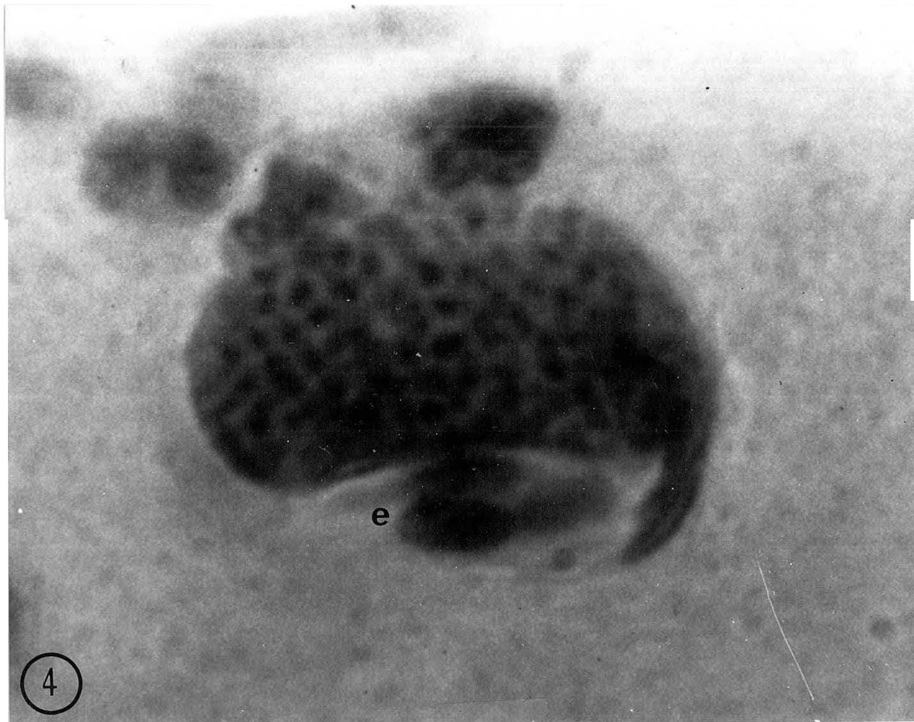
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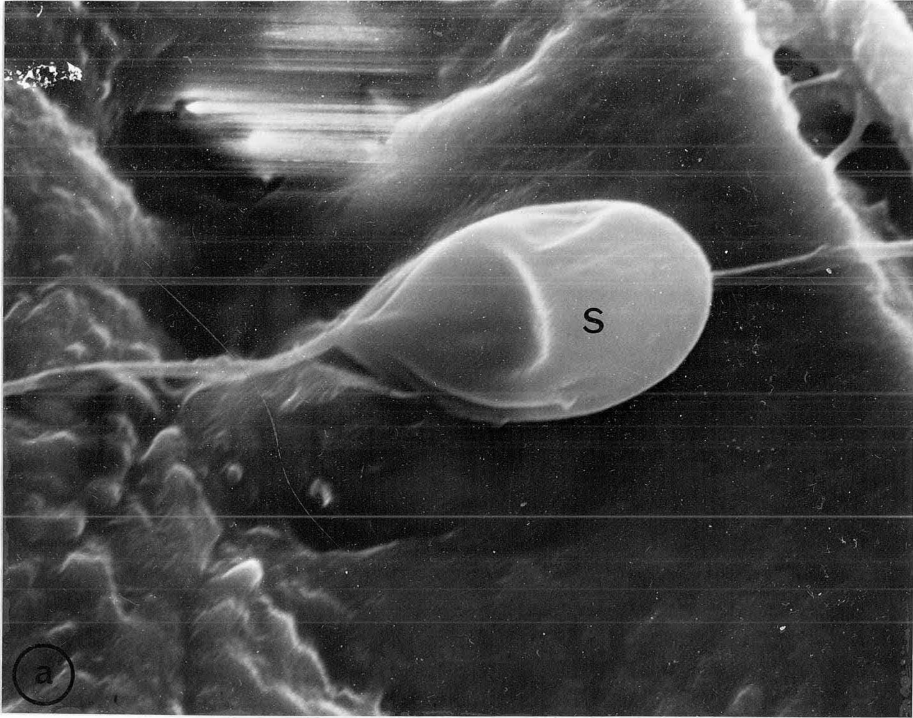
Fig. 5: Scanning electron micrograph of spores of *Myxobolus neurobius* showing:

a) the smooth surface of the shell (s) (x 4000).

b) the prominent sutural ridge (sr). (x 5200).

Note a deep furrow along sutural ridge.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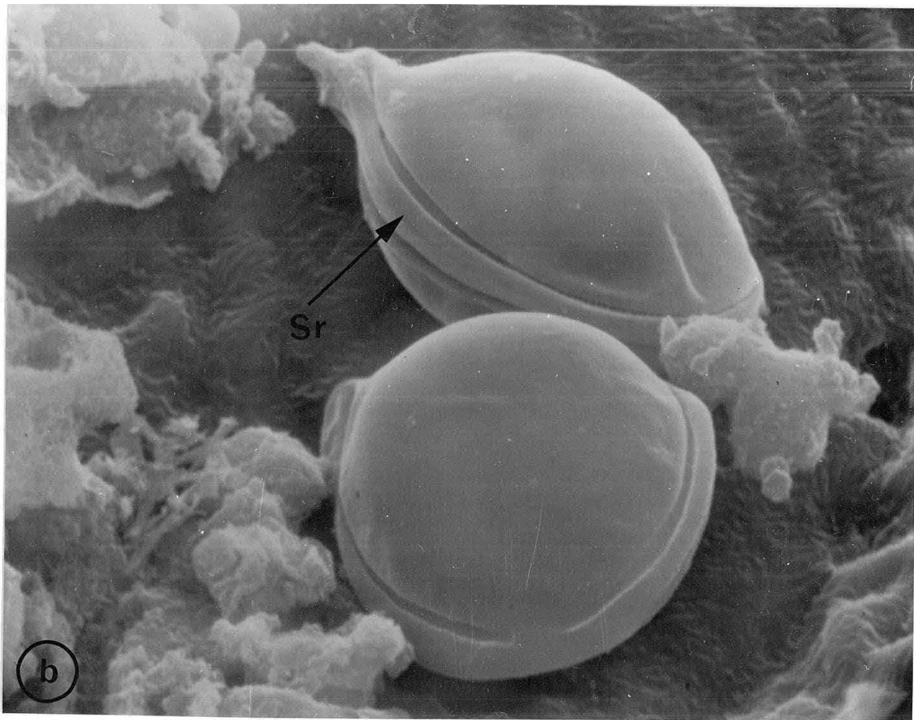
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of India ink stain did not reveal the presence of a mucus envelope.

Fresh spores do not show a prominent sutural line. However, examination under the scanning electron microscope shows it quite clearly, as a straight line dividing the spore symmetrically into two valves (fig. 6a). The prominence of the sutural ridge makes the spore appear thicker in this region. A furrow appears along the sutural ridge. The openings of the polar filament, or so-called capsular foramina, are situated on the suture (Fig. 6b).

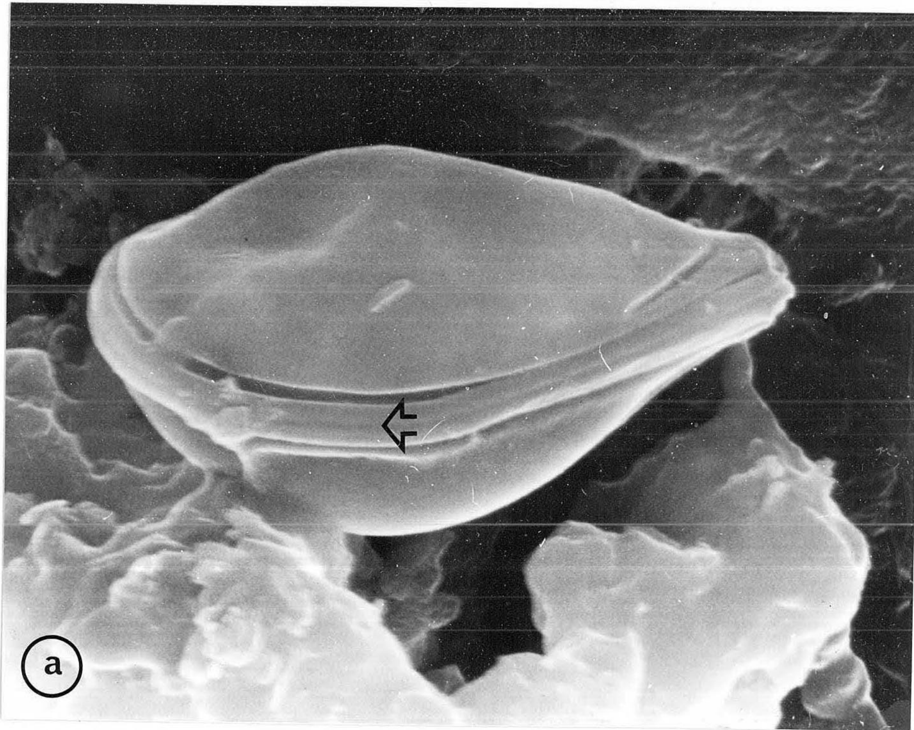
The polar capsules contain spirally wound polar filaments. Each polar capsule develops from a capsulogenic cell during the development of the spore (Fig. 7a). The cell is clearly differentiated from the others by its size and translucent appearance, when seen in fresh material. In the initial stages of development the cell is spherical, one capsule being larger than the other (Fig. 7b). Capsulogenic nuclei can be seen in young spores, but disappear from the mature spores. The polar capsules extend from the anterior end into the second half of the spore, occupying 3/4 of its length. They are arranged in the sutural plane of the spore (Fig. 8) and do not touch each other or the valve walls. The anterior ends of the capsules are neck-like. The typical number of capsules is two, as it is in all species of Myxobolus, but abnormal spores with three polar capsules are occasionally found (Fig. 9). Seen under phase illumination, the capsules are very distinct and birefringent. They stain well with methylene blue and Held test (Fig. 10). Methylene blue stained capsules are clear in outline and definitely blue. The Held test resulted in red polar capsules and distinguished them well from the rest of the spore. The capsules absorbed the dark blue colour of Giemsa stain. Hematoxylin and eosin stain, however, failed to stain them and the capsules

Fig 6: Scanning electron micrograph of spores of *Myxobolus neurobius* showing:

a) the straight sutural line (arrow). (x 5760)

b) the inverted lumen opening of the polar filament, situated on the suture (arrow). (x 10,080)

Note the consistent occurrence of the furrow along the sutural ridge.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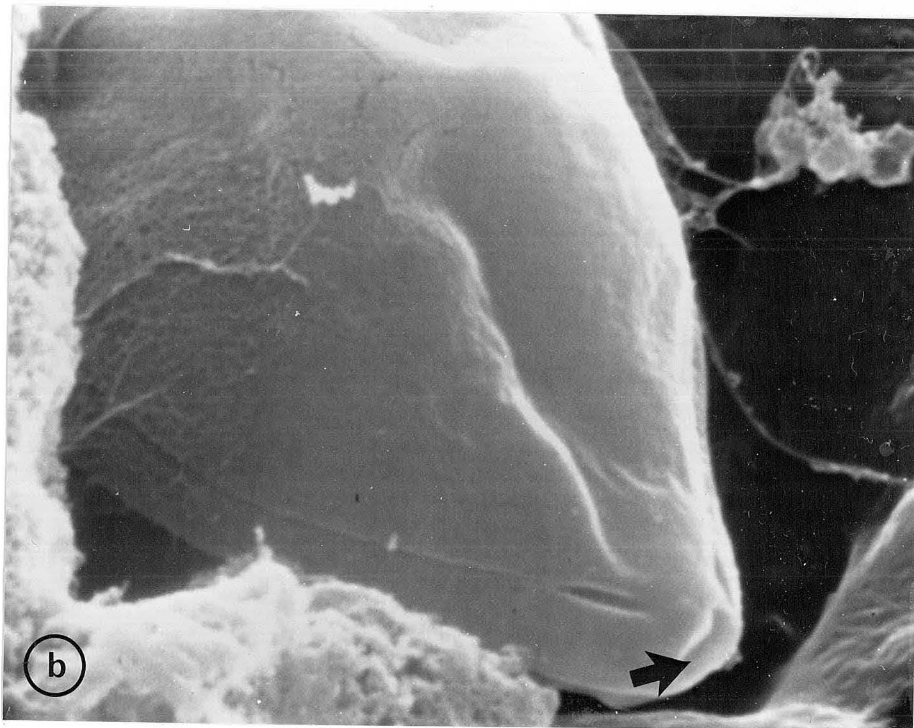
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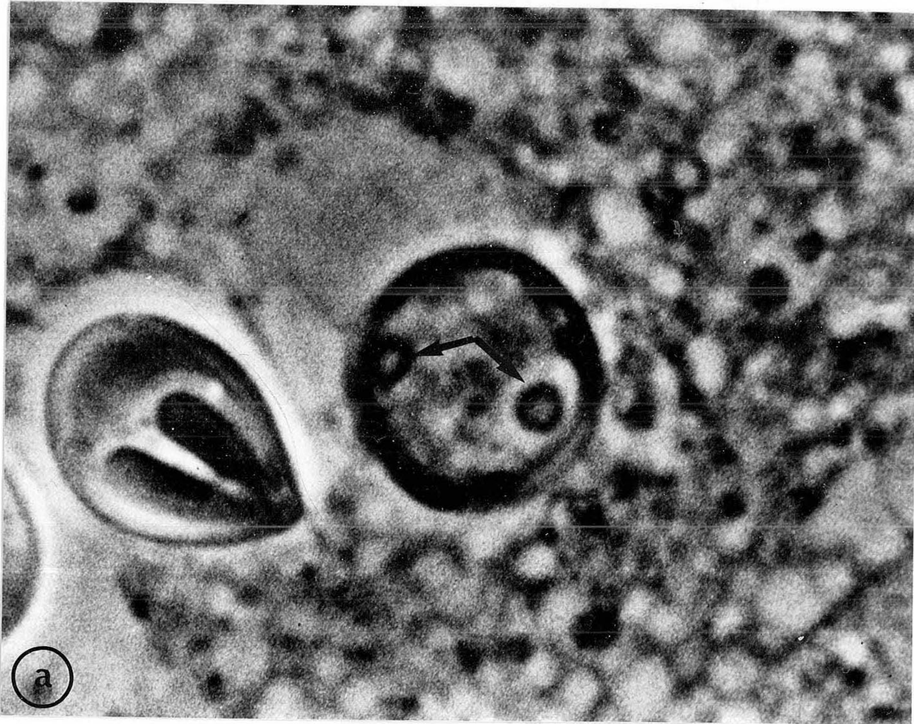
Fig. 7: A squash of brain tissue showing mature spore, sporoblast and also a pair of

a) capsulogenic cells (arrows). (x 2500)

b) polar capsules, one more advanced than the other (arrow). (x 2500)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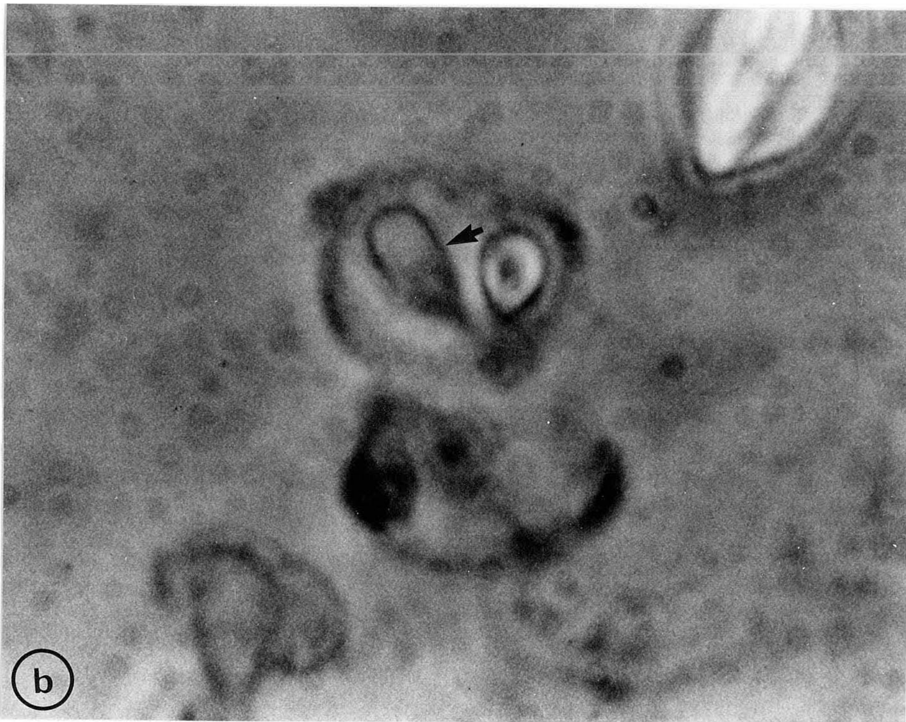
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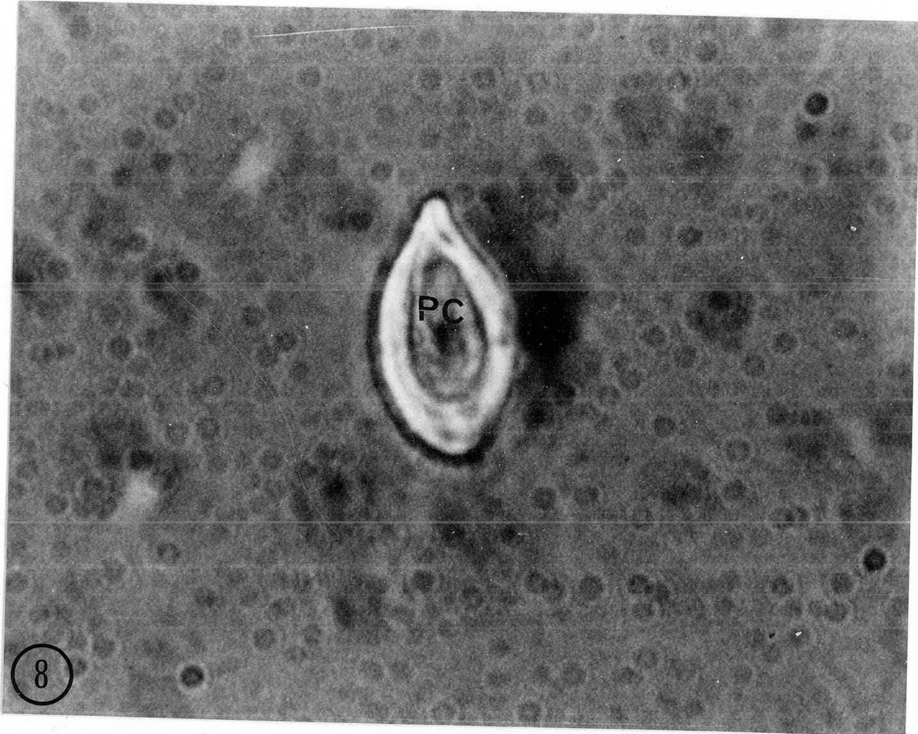
Fig. 8: A spore of *Myxobolus neurobius* showing the relative position of the polar capsules (pc) in the sutural plane. Fresh spore. (x 2500)

Fig. 9: An abnormal spore of *Myxobolus neurobius* containing three polar capsules (pc). Fresh spores. (x 2000)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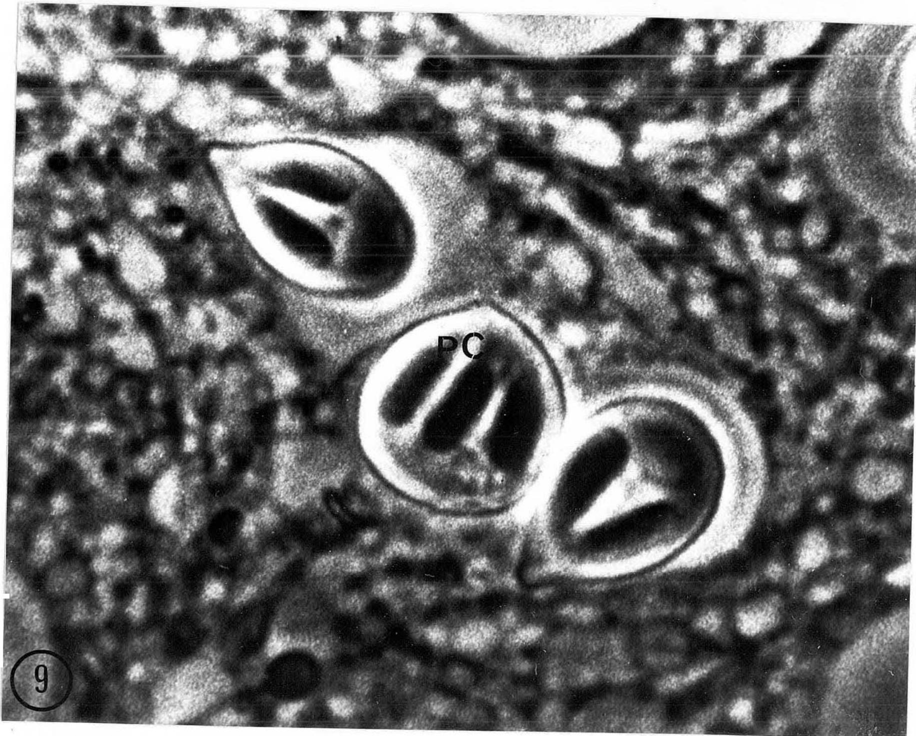
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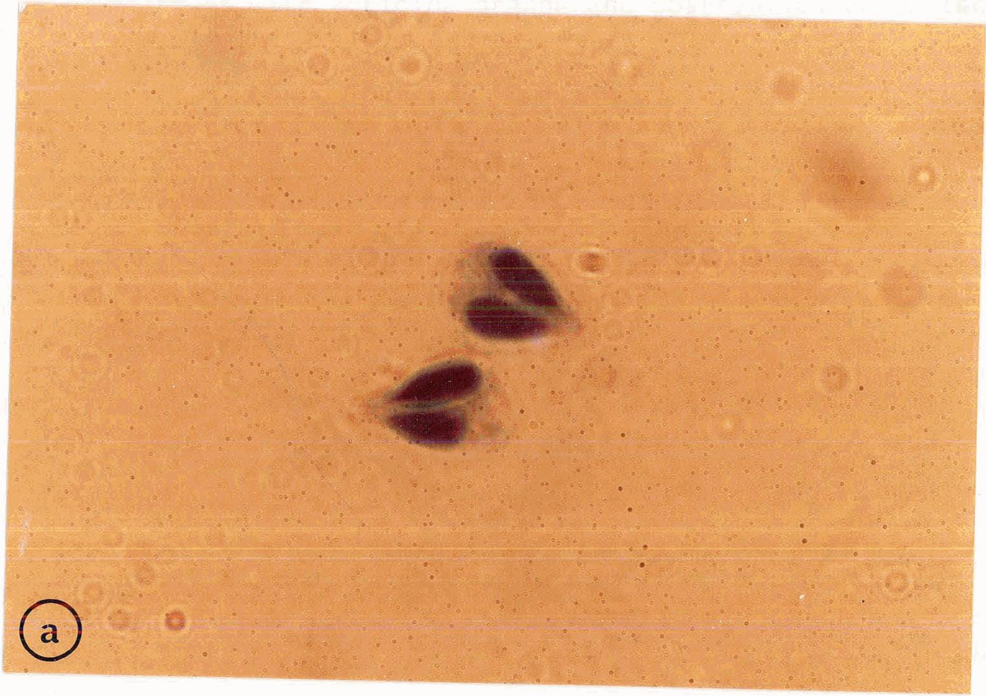
Fig. 10: Spores of *Myxobolus neurobius* showing the affinity of polar capsules for methylene blue and Held stains.

a) methylene blue stain. (x 1840)

b) Held stain.

Note the visibility of coiled polar filament. (x 1840)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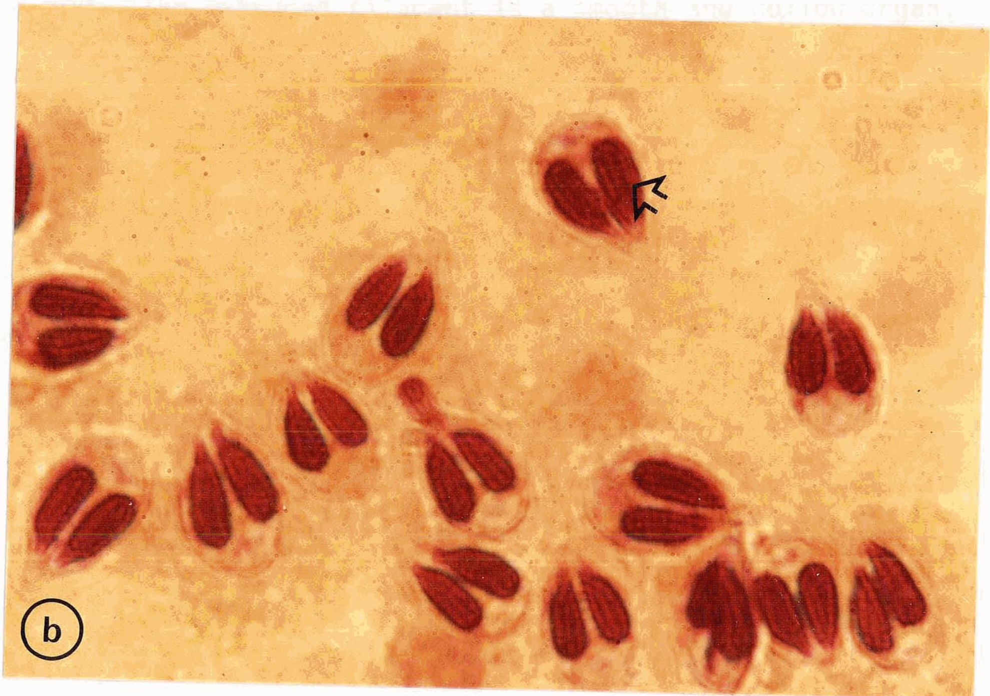

b

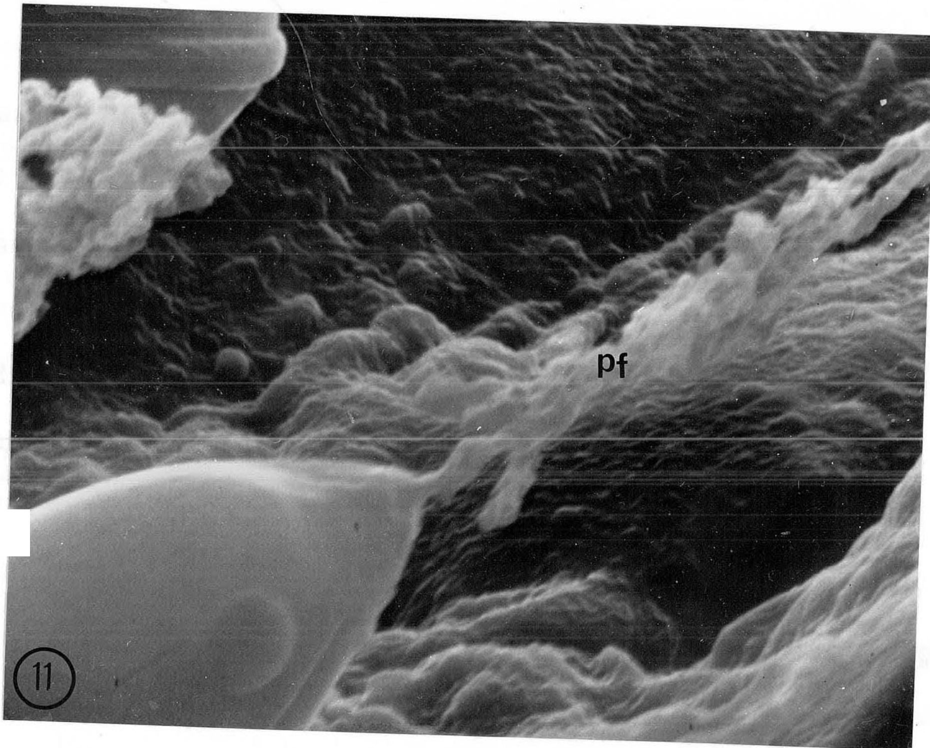
appeared transparent and faint in outline. A dark magenta colour was seen along the points of flexion of the polar filaments stained with Feulgen stain. Treatment with acridine orange and observation under the fluorescence microscope showed the polar capsules as opaque green.

The polar filaments are regularly coiled inside the capsules. They cannot be seen, however, in fresh, unstained material. Filament coils can be distinguished in spores stained with polyvinyl-lactophenol-iodine or with methylene blue. Hematoxylin and eosin, and methylene blue applied to fixed spores failed to show the filaments. Held test, with proper destaining in ferric alum resulted in clearly visible polar filaments. Filaments extruded from the capsules absorb methylene blue. The coils of the polar filament are arranged at right angles to the longitudinal axis of the capsule. The filaments can be extruded by treating the spore with 1-1.5% KOH. The extruded filament is a smooth and hollow organ, when seen under the phase contrast microscope. Examination under the scanning electron microscope, however, shows that it is spirally twisted around its own longitudinal axis (Fig. 11).

The sporoplasm fills the spore cavity posterior to the polar capsules. In fresh material, it is in close contact with both spore membrane and posterior parts of the polar capsules. It does not appear, however, to extend into the anterior part of the spore. The spores of _M. neurobius_ contained only one mass of sporoplasm each. The sporoplasm consists of granular cytoplasm and appears opaque under phase contrast illumination. The nucleus is hard to see in fresh material, although its location can be seen as a darker spot in the sporoplasm. The sporoplasm of young spores contained two nuclei, but only one is found in mature spores. The nuclei are spherical without any distinctive chromatin. They are not limited

Fig. 11: Scanning electron micrograph of *Myxobolus neurobius* spore showing the extruded polar filaments spiralled around their axes. (x 5600)

pf = polar filament


pf
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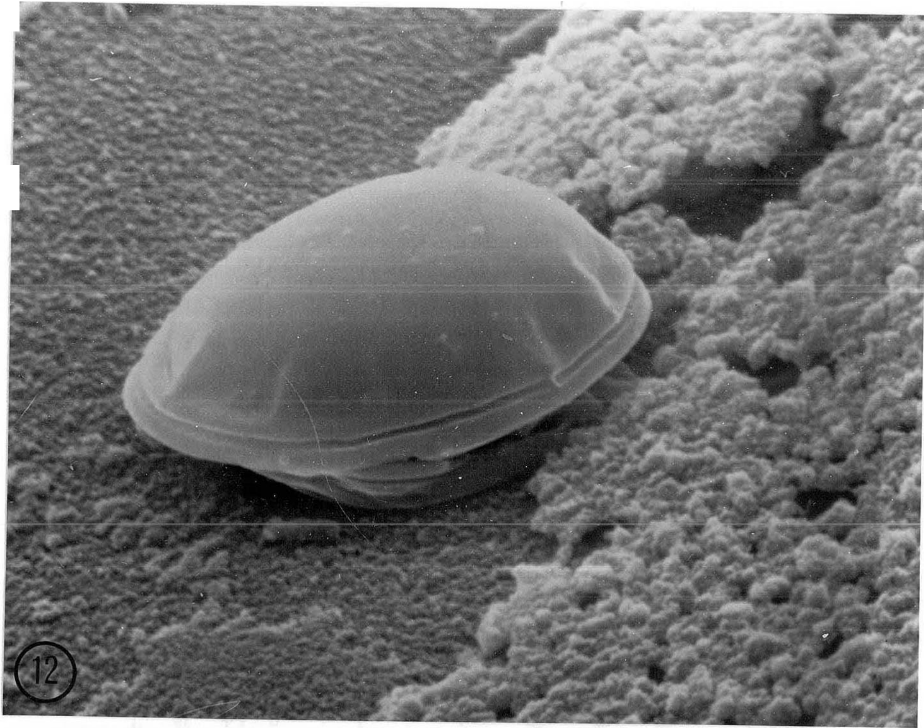
to a strictly defined place in the sporoplasm, being found sometimes in the posterior part and sometimes in lateral positions. Fresh material showed no other inclusions in the sporoplasm. In spores stained with polyvinyl-lactophenol-iodine, however, a spherical vacuole is visible, but it is not universally present. It can also be seen in spores stained with hematoxylin and eosin. It is faintly stained and located in the centre of the sporoplasm. Its appearance is similar in the spores stained with Feulgen's stain.

In general, the shape of the spore of M. neurobius is oviform in the valvular plane, with a tapering anterior end; in sutural view it is rather lenticular. Seen under the scanning electron microscope it has the appearance of a pumpkin seed, the valves being clearly domed (Fig. 12). Figure 13, shows variations in the shape of the spore. Table 7 gives the dimensions of fresh as well as fixed and stained spores.

The young spores (Fig. 14a,b,c,d) are spherical, with an average diameter 13-14 um, which is invariably larger than the mature spore. The stage represented in Fig. 14a is the first in which the cellular organization becomes apparent during the development of the spore, although the component cells are barely distinguishable from one another. However, as the development advances, the cells become apparent. In Fig. 14b an opaque line along the position of the future suture line indicates the formation of the shell valves, whereas Fig. 14c shows the identity of capsulogenic cells. They are rounded in shape and translucent in fresh material. In Fig. 14d the spore is already well defined and distinctly larger than a mature spore. Two sporoplasm nuclei were observed at that stage.

Fig. 12: Scanning electron micrograph of a spore of *Myxobolus neurobius* showing the domed shape of valves and strongly developed sutural ridge. (x 5600)

Fig. 13: Fresh spores of *Myxobolus neurobius* showing variations in their shape. (x 1076)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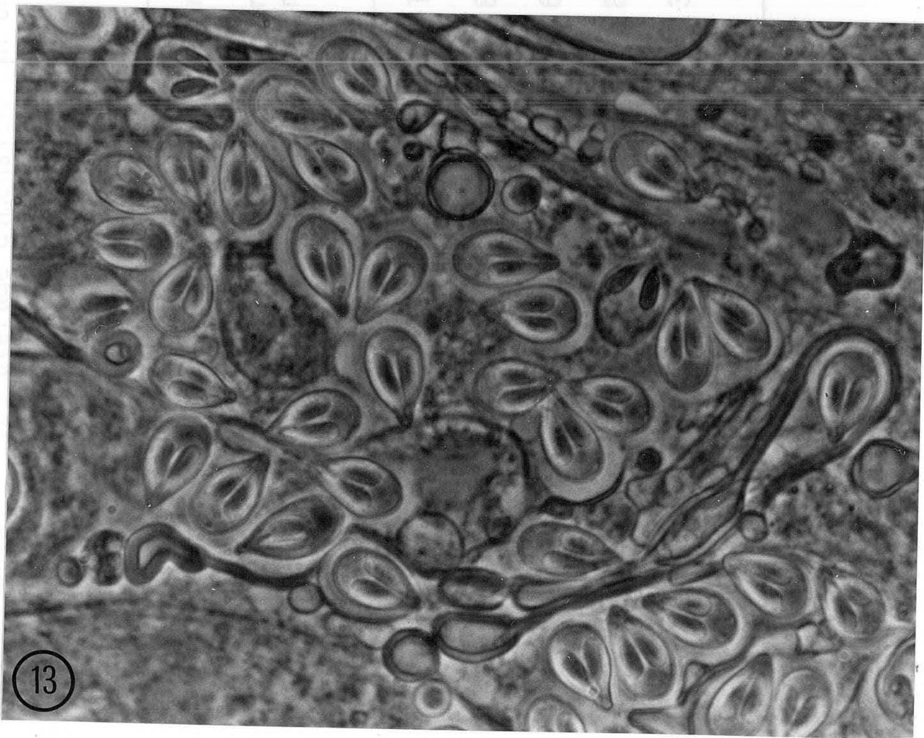
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Table 7. The average dimension (in um) of fresh as well as fixed and stained spores of *Myxobolus neurobius*

Character	Fresh (Mean $\pm$ S.D.) n = 76	Fixed, stained (methanol, Giemsa) (Mean $\pm$ S.D.) n = 100
Spore length	13.01 $\pm$ 0.59	12.20 $\pm$ 0.86
Spore width	8.78 $\pm$ 0.70	7.38 $\pm$ 0.58
Spore thickness	6.09* $\pm$ 0.63	-
Polar capsule length	8.62 $\pm$ 0.48	8.06 $\pm$ 0.87
Polar capsule width	3.05 $\pm$ 0.45	2.96 $\pm$ 0.34

Note: * n - 40

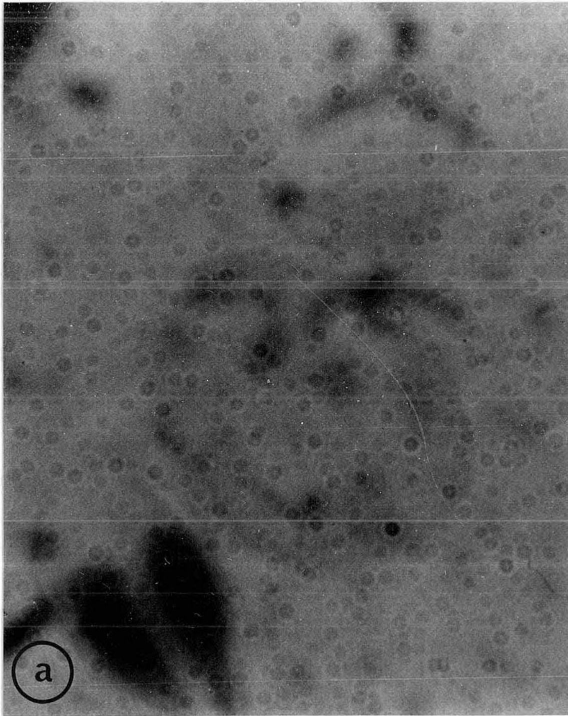
Fig. 14: Various stages of the young spores of _Myxobolus neurobius_.

a) Cellular organization of sporoblast. (x 2500)

b) formation of shell valves (arrow). (x 2500)

c) formation of polar capsule (arrow) (x 1076)

d) well defined spores showing parts of spores (arrows) that have developed from a single sporoblast. (x 1076)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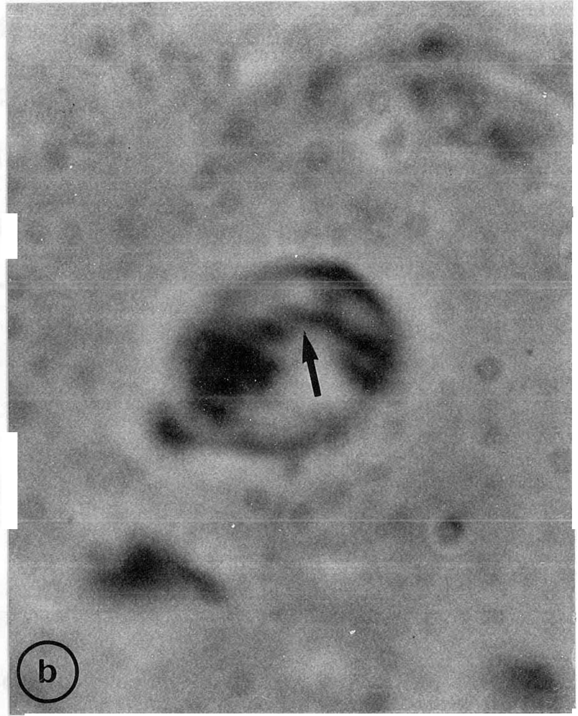
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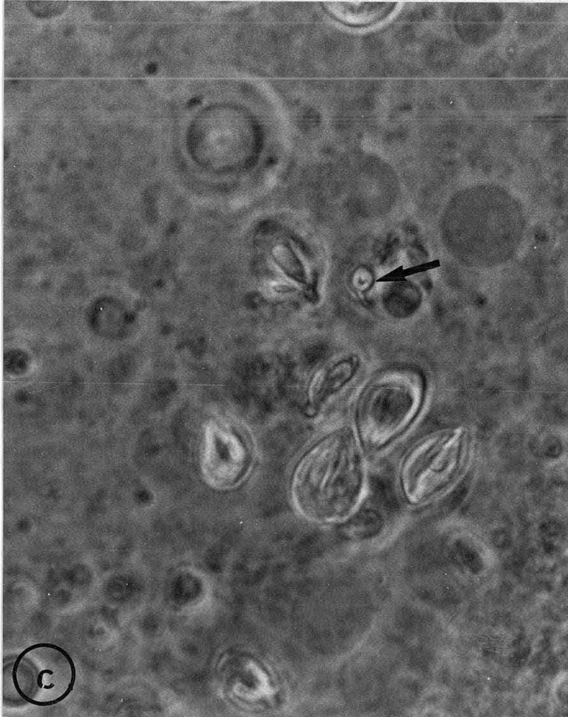
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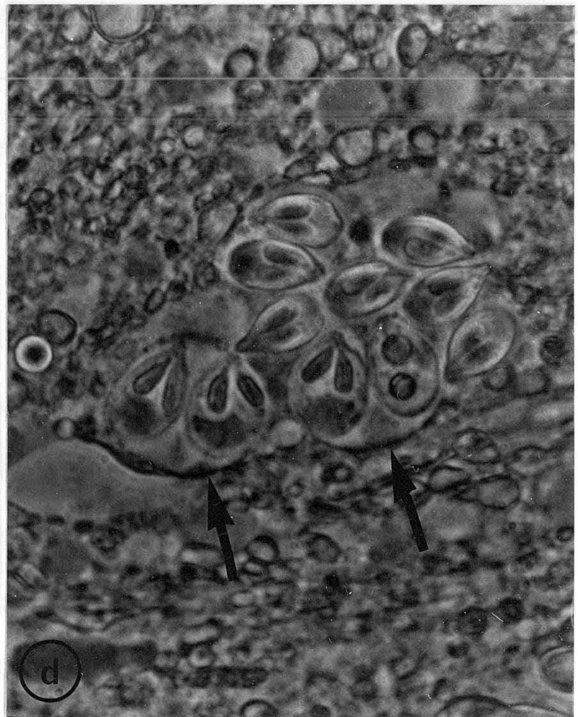
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2. Development cycle of *Myxobolus neurobius*

Examination of naturally infected fish has revealed that the schizogony and sporogony phases of *M. neurobius* development are widely separated from each other, not only in time, but also in space. Parasites undergoing the process of schizogony were found in the blood capillaries of the brain of underyearling sockeye salmon. This, indeed, was the only stage present in the August sample of the fish. During September multinucleate trophozoite was found in only one fish. Most of the September's fish harboured the parasite already undergoing sporogony in the brain tissue. In later months and in other fish the latter phase of development was found comprising sporoblasts, young and mature spores. All these stages were present in the brain tissue, as opposed to the cerebral blood vessels. Table 8 provides a detailed account of the disposition of various developmental stages of *M. neurobius* during the life span of its host.

The earliest stage of development found during the course of this study was the multinucleate trophozoite. This stage occurs in the blood capillaries of the brain and is characterized by numerous, packed nuclei filling the cell.

The nuclei almost completely fill the cellular space and, consequently, there is little visible cytoplasm and no distinction into endoplasm and ectoplasm. The exact manner in which the nuclei multiplied could not be determined with certainty as they are uniformly distributed throughout the cell. It is highly probable that most nuclear division occurred before the parasite reached the brain, since this stage was also found in the capillaries of meninx primitiva. The exact age of the parasite in this location was impossible to determine. However, in view of the fact that the parasite was found in 4-month-old sockeye salmon, it must be itself 4-month-old or younger. A cytoplasmic layer could be seen around some

Table 8. The disposition of various developmental stages of *Myxobolus neurobius* during the life span of its host, sockeye salmon, *Onchorhynchus nerka*. Numbers in parentheses indicate the number of fish examined

Host age	Sampling time	Number of infected fish	Techniques of preparation	Stage of development	Site of infection
Underyearling	August	21 (46)	serial sections	Sch	bc
	September	22 (30)	"	Sch, Spg	bc,bt
	October	12 (12)	"	Spb,Is,Ms	bt
	November	15 (15)	"	Spb,Is,Ms	bt
	December	15 (15)	"	Is,Ms	bt
	January	10 (10)	"	Is,Ms	bt
Smolt	May	35 (75)	"	Is,Ms	bt
	(seaward migration)	75 (75)	wet mount	Is,Ms	bt
Adult	June-	16 (16)	serial sections	Ms	bt
	September	59 (60)	wet mount	Ms	bt
	(spawning migration)				

Note:
bc = brain capillaries
Is = Immature spore
Sch = Schizogony
Spb = Sporoblast
bt = brain tissue
Ms = Mature spore
Spg = Sporogony

nuclei at this stage (see Fig. 3), suggesting that the parasite has incipient sporonts. It is important to stress that up to this stage no parasite has been found in the brain tissue.

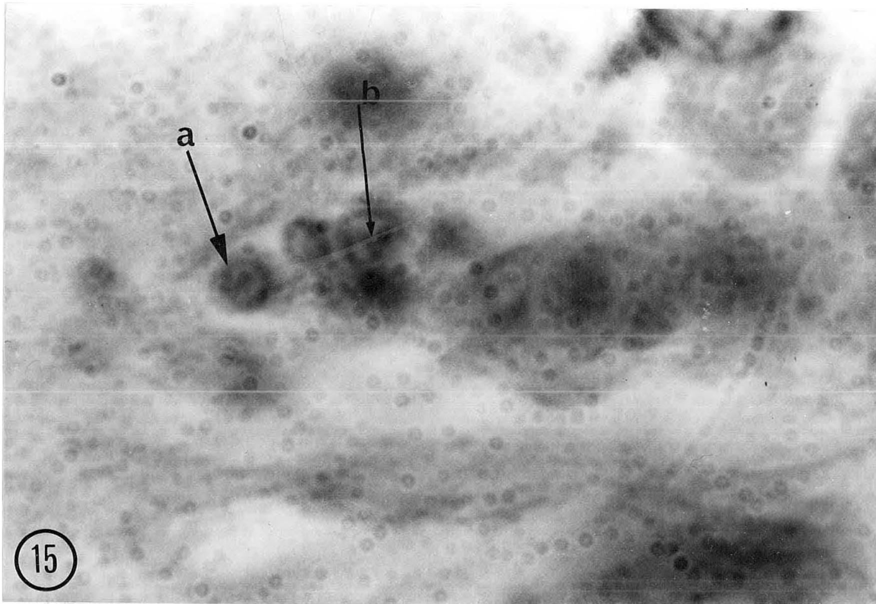
The subsequent stage of development of M. neurobius was obtained by examining fish collected in September, or 55 days after the original August sample had been taken. The parasite in September had developed to a more advanced stage in all infected fish. Furthermore, all parasites at this stage occurred in the brain tissue. Only one fish was found harbouring a multinucleate trophozoite. However, in this fish the later development stage occurred in the brain tissue. The same fish also contained sporoblast and young spores. The earliest stage found in the brain tissue was the uninucleate sporoblast stage. Aggregations of sporoblasts assumed the form of strands or compact groups, being present in all parts of the brain with the exception of the forebrain. Under the microscope, these sporoblasts were subcircular in shape and their size ranged from 2.04 to 2.72 um. The nuclei were excentric in position and stained darkly with hematoxylin, without differentiation of chromatin. The cytoplasm of the uninuclear sporoblasts was rather thin and homogenous.

The next stage of development is the binucleate sporoblast. This stage is the product of nucleate division within the uninucleate sporoblast (Fig. 15b). In this figure the nucleus is almost divided in the equatorial plane.

The binucleate sporoblast stage is followed by a trinucleate stage, which seems to occur rather rarely due to an uneven rate of nuclear division. The most commonly found stage is the sporoblast containing six or more nuclei.

In the fish of the September sample a young spore was also found

Fig. 15: Light micrograph showing the sporoblasts of *Myxobolus neurobius*. Hematoxylin and eosin. (x 2500). Note the uninucleate sporoblast (a) and the nuclear division (b) within sporoblast resulting in a binucleate form.


a
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with two sporoplasm nuclei. Samples from October to December harboured parasites at the stage of multinucleate sporoblast, as well as young and mature spores.

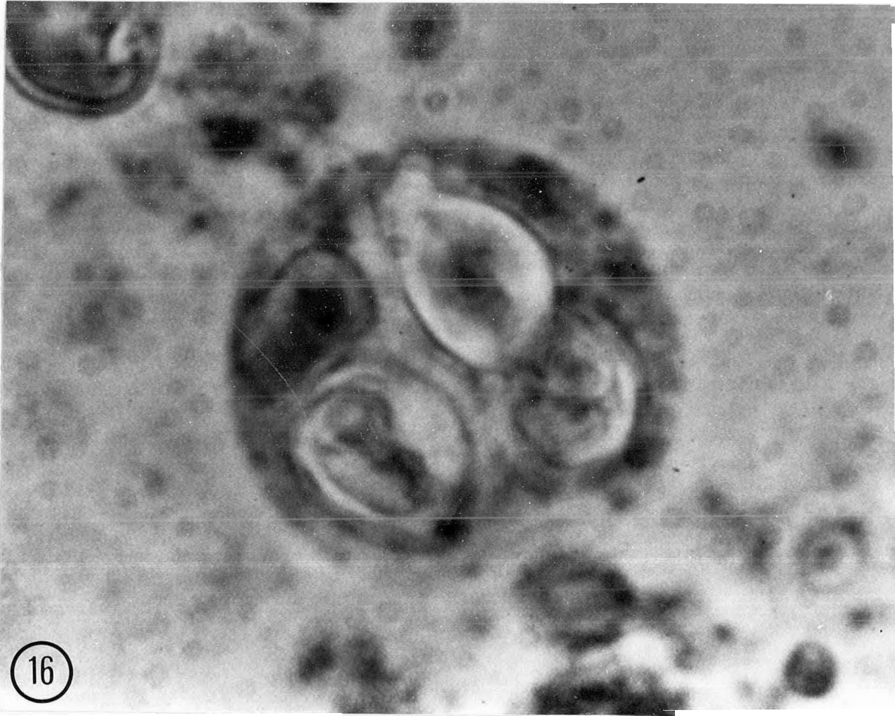
The December sample, in particular, showed many well developed spores, although some young spores, in the form of spherical cells, were still present in the brain tissue.

In smolts (1+ age group), the predominant form of the parasite was mature spore. However, even here young spores were still found together with mature ones within the sporoblast membrane (Fig. 16). In adult fish that have returned to spawn the mature spores were present in the brain tissue; there was no trace of the younger stages of the parasite. The development of the spore from the sporoblast stage was followed by examination of fresh material obtained from smolts. The earliest stage in the development of the spore is a spherical cell. The first characteristic structure of a spore that appeared in the course of spore development were the valves. The two valves met at the line of the suture, the sutural line being clearly discernible at this stage. The original spherical shape of the cell changed into an ovoid. The second recognizeable feature of the spore to appear were the polar capsules. They were at first spherical and birefringent in fresh material examined under phase contrast illumination. With the progress of development the spherical shape of the capsules changed into pyriform, slender necks developing at the anterior ends of the capsules. The development of the two capsules did not seem to proceed at the same rate; frequently one capsule was more developed than the other.

The origin of the development of the polar filament was hard to observe. However, even at the earliest stage of the capsule's development, a roundish concretion was detectable within it (Fig. 17).

Fib. 16: Polysporous sporoblast of _Myxobolus neurobius_ encapsulated in sporoblast membrane. Squash preparation of the brain. (x 3000)

Fig. 17: Light micrograph showing the polar filament (arrow) in the early development of polar capsules. Squash of the brain. (x 1750)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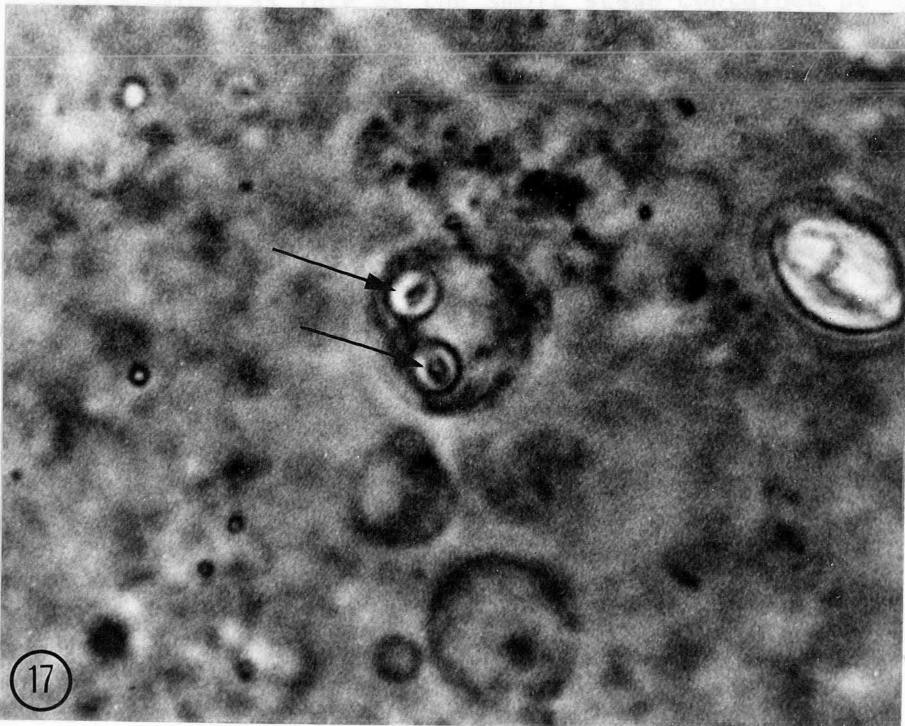
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Spore shape even at the stage when all the architectural features of the spore have appeared, is still rather oval with a rounded posterior and pointed anterior end. The size of young spore was invariably larger than that of the mature one. The main characteristic of the young spore, other than its size and opacity, was the presence of two nuclei in its sporoplasm. As the spore begins to mature its shape becomes more definitely oviform and its size diminishes. At that stage only one nucleus remains in its sporoplasm.

The number of spores produced by sporoblasts varies from one to many. The number of spores that is going to be produced by any sporoblast can not be predicted with certainty. It seems that the disporous and polysporous sporoblasts produce more than one spore due to the continuing division of their nuclei within a single sporoblast membrane (Appendix VIA). The fish sampled as late as the smolt stage contained parasites in which the sporoblast membrane still persisted, at least in the fresh material. This was particularly evident, when the membrane contained spore that was still immature. Becoming gradually distended with fully mature spores, the membrane disintegrated as soon as the material was placed on a slide. This type of spore aggregation contained in an enveloping membrane was never found in fresh preparations of adult salmon. Wet mount preparations from the fish of this age contained spore that were not encapsulated by a sporoblast membrane. None the less, the spores appear to remain permanently at the site of the lesion, since no spores were found in organs other than the brain and spinal cord.

B. THE SPORE AS AN INFECTIVE AGENT

The spore is a central feature of the life cycle insofar as it has

a well defined structure, it exists in the host for several months and probably exists in the water for a variable time. The ability of the spore to resist external factors in the brain tissue and in the water, and to remain viable so as to facilitate transmission is of special interest. Furthermore, there has been much conjecture as to whether the spores must receive an external stimulus before it will initiate a new infection.

It could be determined whether the spore was alive or not by its appearance and by its ability to extrude the polar filaments. The fresh spore of M. neurobius was birefringent under phase contrast illumination, especially at its anterior end where the polar capsules are located, but also at its valves and around the periphery of the sporoplasm. However, the appearance of the spores was similar after they had been fixed in 70% alcohol for 2 months. Consequently, it was impossible to distinguished between fresh and fixed material under phase contrast illumination. The matter was different, when the fluorescence microscope was used. Fresh spores viewed under fluorescence illumination were consistently dark green, after being stained in acridine orange. On the other hand, fixed spores examined in the same way were rather inconsistent in appearance. Examination of 40 fixed spores revealed that all of them had green-orange sporoplasm and polar capsules; however, only 43% of spores had green-orange valves. The remaining spores (57%) had green valves.

Neither fresh nor fixed spores contained any observable inclusions. When fresh spores were treated with 1.5 - 2.0% KOH, 90 out of 100 spores extruded their filaments. When the same treatment was given to fixed spores, no extrusion was observed.

As mentioned earlier, some spores were subjected to ageing and the condition of such spores is summarized in Table 9 and 10.

Table 9. Conditions of *Myxobolus neurobius* spores after being aged (without mud). Numbers in parenthesis indicate the number of spores examined

Ageing period (Days)	Phase contrast (% normal)	Fluorescence mic. % dark green	Polar filament % extruded
30	90.00 (60)	-*	83.08 (65)
60	89.06 (64)	84.00 (50)	84.34 (64)
90	89.55 (67)	85.29 (68)	80.36 (56)
121	86.76 (68)	81.03 (58)	77.92 (77)
131	87.69 (65)	81.03 (58)	87.62 (97)

* no observation

Table 10. Conditions of *Myxobolus neurobius* spores after being aged in mud. Numbers in parentheses indicate the number of spores exampined

Ageing period (Days)	Phase contrast (% normal)	Fluorescence mic. (% dark green)	Polar filament (% extruded)
30	89.23 (65)	-*	83.85 (35)
60	88.31 (77)	85.96 (57)	85.11 (47)
90	78.18 (55)	76.67 (60)	75.61 (41)
109	75.00 (60)	76.67 (60)	72.36 (76)

* no observations

The effect of ageing could be observed under phase contrast illumination as early as 30 days from the beginning of the ageing process because some of the spores (10%) appeared opaque. They contained numerous bacteria and inclusions. Bacterial cells were also found on their surfaces. Some parts of the valves were swollen, presumably due to the action of bacteria. However, even after 131 days of aging, 87% of spores still appeared normal. When aged in the mud, however, 75% of spores were normal after 109 days. When both types of spores were examined under the fluorescence microscope, it was found that 81% of spores aged without mud were dark green, and 76% of those aged in the mud had the same colour.

Some changes were found also in the proportion of the spores that had retained their ability to extrude polar filaments. The extrusion of polar filaments was possible for 78-88% of spores aged without mud, and for 73-83% spores aged with mud. The movement of the sporoplasm inside the spore was never seen in spore subjected to ageing, the position of the sporoplasm remained the same as that in fresh spores. They still retained their contact with the polar capsules and with the valve walls of the spore. The appearance of the aged spores under the fluorescence microscope was the same as that of fresh spores. Only dead spores of the two groups could be distinguished from one another, those of the aged group being heavily infiltrated by bacteria that were absent from the fresh material.

The Feulgen-positive substances in fresh spores of M. neurobius were generally found inside the polar capsules and in the sporoplasm. In the polar capsules these substances were visible along the inflections of the polar filaments. The filament and polar caps themselves were only faintly stained. In the sporoplasm, these substances were scattered

in the form of granules. The iodinophilous vacuole was often present as a hollow rounded structure with faint colour along its borders. The shell valves were also only faintly stained.

Fig. 18 (A,B,C) is a composite illustration of the distribution of Feulgen-positive substances, based on the examination of 30 spores. Fig. 18A shows the distribution of these substances in about 60% of the examined spores. The prominent accumulation of these substances occurs in the polar capsules, along with the inflections of the coiled polar filaments. The sporoplasm of these spores contained varied amounts of these substances, from weak to strong accumulations. The iodinophilous vacuole in these spores appeared consistently as a hollow, faintly stained sphere. Fig. 18B shows these substances in 20% of spores examined. The pattern of distribution was similar to that shown in Fig. 18A, but the iodinophilous vacuole was absent. The pattern in the remaining 20% of the spores is shown in Fig. 18C. These spores were only faintly stained, both in the polar capsules and in the sporoplasm.

The appearance of the aged spores treated by the Feulgen technique was similar to that of the fresh spores. It is summarized in Table 11.

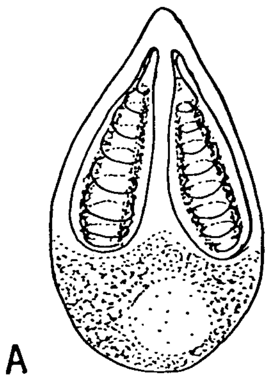
As Table 11 makes clear, the three types of distribution of Feulgen positive substances that occurred in the fresh spores, are present also in the aged spores. The proportions of the three types are, however, different. The differences are particularly extensive in spores aged for 121 days. In these spores no distribution type A was present and type C was more common. In the sample of these spores one remained completely unstained and was obviously dead. It did not appear to have the polar filament left.

Fig. 18: A diagrammatic representation of three different spores types of Myxobolus neurobius classified according to distribution of Feulgen positive substances. (Composite drawing, not to scale.)

A. Spores with a consistently prominent accumulation of Feulgen positive substances in polar capsules, but a weak or strong accumulation of Feulgen positive substances in the sporoplasm leaving a consistent clear iodinophilic vacuole.

B. Spores with well stained polar capsules but without a distinct iodinophilic vacuole.

C. Spores of faintly stained.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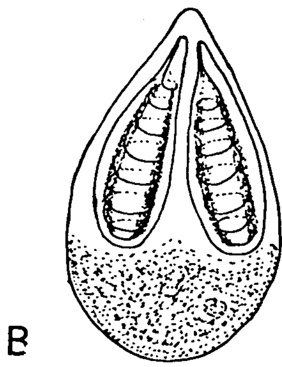
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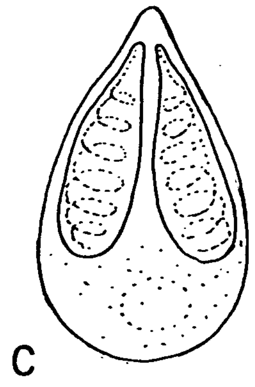
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Table 11. The distribution of three differently staining (Feulgen positive) types of spores of *Myxobolus neurobius* expressed as percentage occurrence. Numbers in parenthesis indicate the number of spore examined

Ageing period (Days)	Type A (%)	Type B (%)	Type C (%)
30	46.7 (30)	33.3 (30)	20.0 (30)
60	53.3 (30)	43.3 (30)	3.4 (30)
90	33.3 (30)	16.7 (30)	50.0 (30)
121*	0	36.7 (30)	60.0 (30)

Note: Type A = Spores with prominent accumulation of Feulgen positive substances in polar capsules, and that of weak or strong in sporoplasm, but consistent appearance of iodinophilous vacuole.

Type B = Spores as Type A, but without distinct iodinophilous vacuole

Type C = Spores as Type A, but faintly stained

* = 1 specimen unstained

C. TRANSMISSION OF MYXOBOLUS NEUROBIUS

1. Temporal coordination between the life cycle of *M. neurobius* and that of its host in nature.

Most of the information needed to elucidate the transmission problem was obtained form the examination of the life cycle of *M. neurobius* in nature and from a series of experiments. Most of the remaining gaps in our knowledge were filled with the help of T. Gjernes (1982, personal communication) on the life cycle and habits of sockeye salmon in Sproat Lake. A diagram of the probable cycle of *M. neurobius*, constructed from earlier known data and that derived from the present study, is shown in Fig. 19.

Stage 3 of the parasite's cycle was observed in August. This was the earliest developmental stage found in the course of this study. It is represented by multinucleate trophozoites in the brain and meninx primitiva capillaries of 4 months old sockeye salmon in early August.

Due to lack of material from the months preceding August, the earlier stages of the cycle were not directly observed. From the information provided by T. Gjernes, it is known that the eggs of sockeye salmon hatch in March and April and the alevins, after several weeks in the gravel, during which they are nourished by their store of yolk, emerge in the first week of May. Their initial diet consists of microscopic organisms, but they change eventually to feeding on larger organisms or particles, such as insect larvae and adults, and worms. As suggested by stage 2 Fig. 19, it is likely that at this stage the young fish swallow the spores released by (i) recently dead spawners, or (ii) spawners of the previous season or (iii) smolts that were infected previously and died while residing in the lake. It should be stressed that this suggestion is conjectural,

Fig. 19: A diagrammatic representation of a probable life cycle of *Myxobolus neurobius*. (The stages of the life cycle that were observed in the course of this study are indicated by solid lines. Those outlined in interrupted lines are conjectural).

1. A. September-November. Spawning season of host, sockeye salmon (*Oncorhynchus nerka*).

   B. November-April. Decomposition of dead spawners and release of the spores into the environment.

   C. March-April. Hatching of the sockeye salmon.

2. May, June, July, a period during which spores are probably picked up by the sockeye salmon.

3. August. Schizogony developmental phase of *M. neurobius*. Multinucleate trophozoites in the capillaries of the brain.

4. September. Sporogony developmental phase of *M. neurobius* in the brain tissue of sockeye salmon (forebrain excepted).

5. October-November. Maturation of spores in the brain tissue.

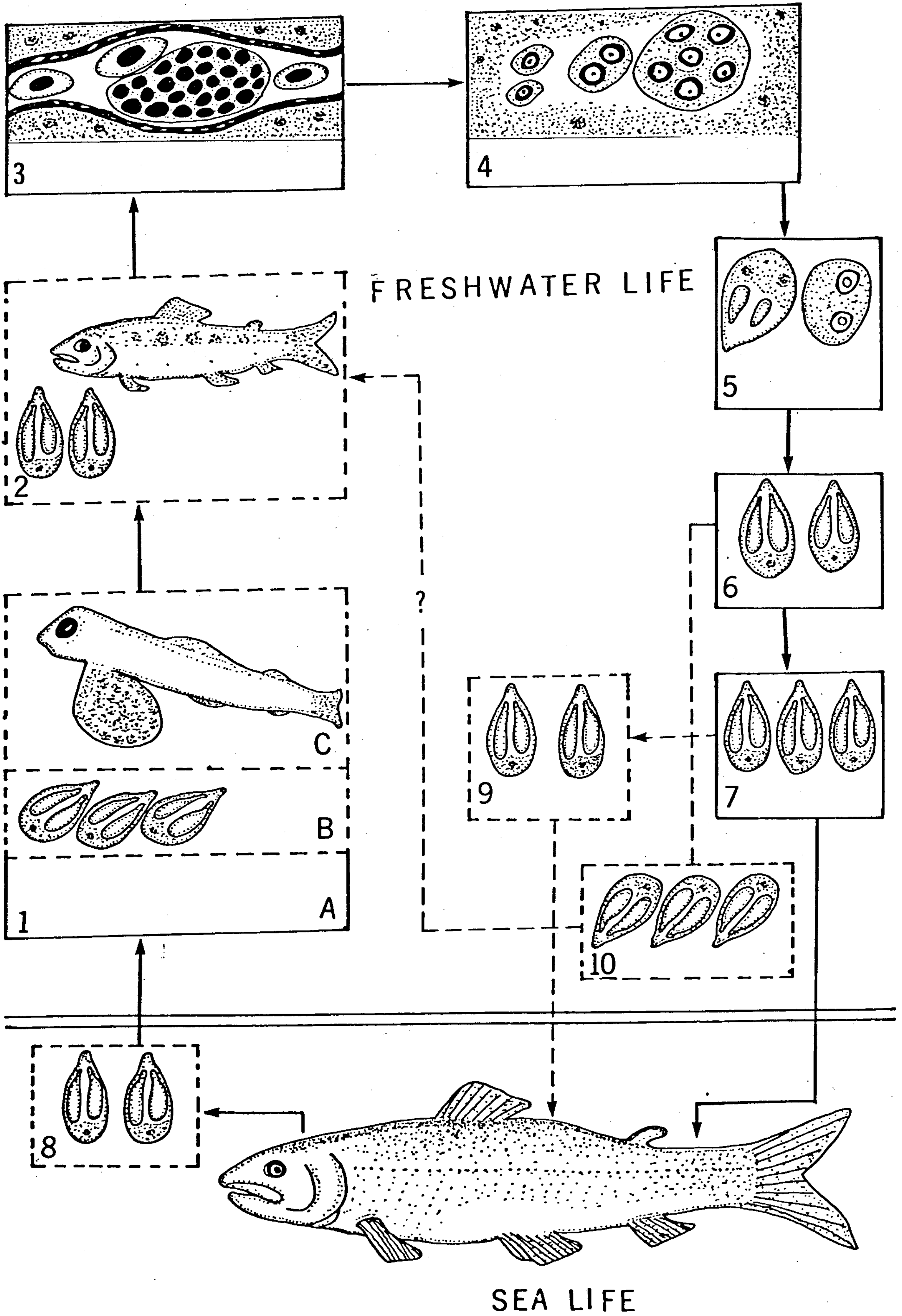
6. December. Immature and mature spores in the brain tissue.

7. May. Immature and mature spores in the brain tissue of the fish caught during its seaward migration.

8. Fish with mature spores during its sea life.

9. Infected fish containing mature and immature spores resident in the lake in the second year of their life.

10. Spores released by smolts dying during their residence in the lake.


3
4
FRESHWATER LIFE
5
2
6
?
C
7
9
B
1
A
10
8
SEA LIFE

since fish at that stage of the life cycle were not available for examination. However, experimental evidence shows that the fish swallow the spores when placed in a spore suspension. It is quite possible that similar events can take place in the natural environment, soon after the fish emerge from the gravel, i.e. in May or between May and July. The ingestion is followed by the extrusion of the polar filaments in the intestine, the opening of the shell valves and the emergence of the sporoplasm. The sporoplasm, having abandoned the spore, makes its way to the site of infection, the central nervous system, via the bloodstream. It is highly probable that the sporoplasm grows, the nuclei divide continually, during their transit in the lumen of the blood vessels. It is in that form that they were located in the blood vessels of the meninx primitiva. The sporoplasm, to which the name trophozoite might be applied at this stage, reaches the brain in the form of a multinucleate trophozoite (stage 3, Fig. 19). It is at this stage that was found in the capillaries of brain. Hence, from what has been said above it can be inferred that this multinucleate stage lasts for 2-3 months.

At this time, two other generations of fish reside in the lake. They are the spawners that have returned to the lake early and smolts born during the previous season that did not migrate during their first year of life. All these fish might be sources of spores for the new infection, continuously adding to the reservoir of infective material in the lake.

Stage 4, late September. The fish were then 5-6 months old. Most of the infected fish by this time carry the parasite in the brain tissue (except forebrain) rather than in the blood vessels. Only one fish of the 30 examined carried multinucleate trophozoites in its brain capillaries.

The parasite had by now advanced morphologically to the so-called sporogony stage. Both uninucleate and multinucleate sporoblasts occur in the brain tissue. It should be noted that at this stage the parasite does not occur in the forebrain. This is in marked contrast with the preceding stage. At this time of the year the environment is very likely to contain many free spores that have been released by the earliest spawners.

Stage 5, October-November. By this time the fish is about 7-8 months old and the parasite is mainly at the spore stage, although some sporoblasts are still present. Immature and maturing spores were found together in the brain tissue. At this time, the late spawners were still present in the lake and could be expected to release more spores into the water after they die.

Stage 6, December. The fish are about 9 months old and the young and mature spores become more abundant during this month. The multinucleate sporoblasts are rare.

Stage 7, Fig. 19, shows the condition of the parasite caught during its seaward migration in May. This fish is about 1+ year old. The fish sampled at this stage did not belong to the generation in which all the earlier stages were found. However, it can be taken as representative of the usual life cycle stage occurring at that time of the year. It belongs to the same population (Sproat Lake) and, as far as known, the life cycles of the sockeye salmon of this lake are almost perfectly cyclical in time and events. Consequently, this fish can be taken as representative of the normal situation, as well as normal condition of *M. neurobius* at this stage. By now, no more sporoblasts are present. The immature spores, however, are still encountered. The main characteristic feature of the parasite at this stage is the presence of polysporous and even

monosporous sporoblasts when examined in fresh mounts.

Stage 8, Fig. 19, shows the condition of the parasite during the sea life of its host. The host was not examined at any stage during its life in the sea. However, sockeye salmon were examined soon after their return to the fresh water habitat to spawn. At this stage, the spores found in the brain tissue were no longer surrounded by the sporoblast membrane. Smear preparations made from the material collected at this time contain spores spread individually throughout the sample, in contrast to smears made from the tissues of brain sockeye smolts in which most of the spores were still surrounded by the membrane and did not spread freely. There were no immature spores, normally recognizeable by their opaque appearance and bigger size. It appears that all spores were completely mature.

Stages 9 and 10, Fig. 19, illustrate the events presumably occurring at the time when the young salmon live in the lake or in its tributary streams before they reach the age of 1+ and 2+. Stage 9 refers to spores that have matured and remained in smolts that have not migrated at the end of the their first year of life but stayed in the lake. Stage 10 represents spores released into the lake by smolts that have died at some stage during their residence in the lake. They constitute a reservoir of infection.

2. <u>Experimental evidence corroborating observations in the natural habitat</u>

(i) Experiment 1

The duration of this experiment was 8 months and it was concluded in March, 1981. However, four fish (two from Treatment 3 batch, and two from Control) were kept for 10 months, and another 1 fish from Treatment

3 batch (replicate tank) was retained for 18 months.

During the course of the experiment, three fish from Treatment 3 batches died as the result of fungal infection. Deaths occurred one and two months post infection. To prevent further losses, the water supply was changed to sea water. No more fungal infections were observed. The fish were healthy and responded actively to food.

A detailed account of the results of fish examination during the course of the experiment is tabulated in Appendix II. Both fresh tissues as well as fixed and stained tissues were examined. During the first 4 months, no parasite was discovered as a result of these examinations. The blood, intestine and brain tissue were all free from M. neurobius.

In December 1980, 5 months post infection cells believed to be sporoblasts of M. neurobius (Appendix VIB) were observed in brain tissue, on slides from two fish each injected with 28,000 spores (Treatment 2). The slides had been stained with methylene blue and eosin. Only one sporoblast was found on a single slide (out of five examined) from each of two fish. Other samples were parasite free. The two sporoblasts contained six and seven nuclei respectively. The sporoblasts cytoplasm stained darkly with eosin, whereas the nuclei stained faintly. The nucleoli were distinctly visible, and were centrally located. The sporoblasts were subspherical and measured 10.3 um in diameter.

As the result of these findings, and on the suggestion of Dr. N. Boyce (1981, personal communication), further examination of the fish was delayed to give more time for the sporoblasts to develop into spores. Examination of the fish was resumed in February, 1981. However, no further evidence of the parasite was found in either February or March 1981, by which time the sample was finished, and the experiment completed. Some fish, however

were retained (two from Treatment 3, one from its replicate and two from the Control group). These fish were examined in May 1981 and January 1982.

In May 1981, four fish (two treated and two controls) were examined fresh in squash and smear preparations. One of the treated fish was found to carry a pansporoblast containing developing spores (Fig. 20). The pansporoblast was 14.24 um in diameter and almost spherical. At least three spores could be distinguished inside the pansporoblast, arranged in parallel, in a "head-to-toe" configuration. Neither the other treated fish nor the control fish contained any pansporoblasts.

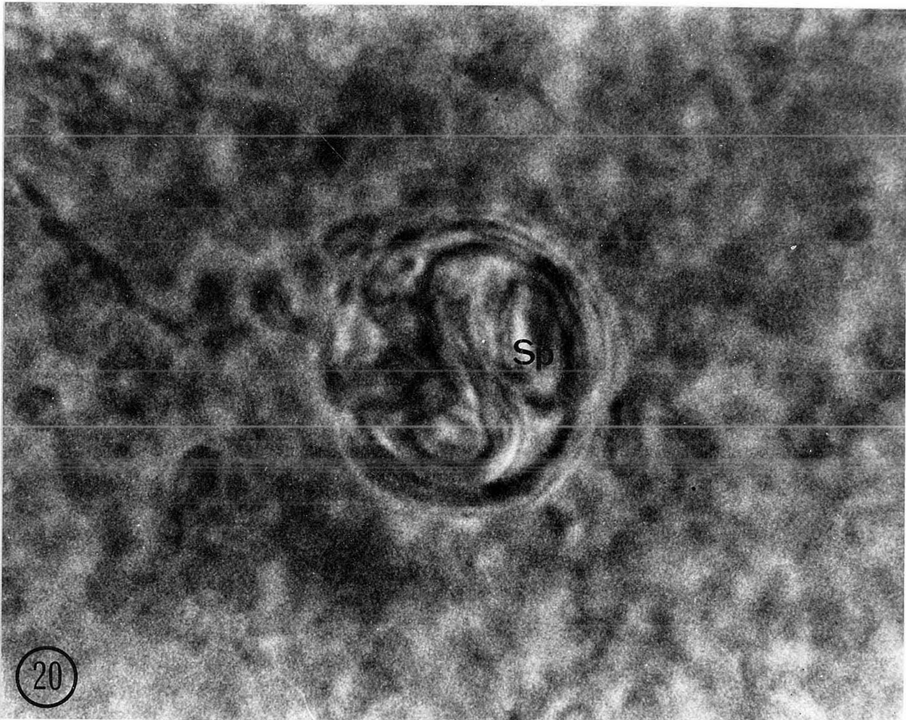
The last fish from the Treatment 3 batch was examined in January 1982, 18 months post infection. No developmental stage of Myxobolus was found.

(ii) Experiment II

As mentioned in the section on materials and methods this experiment was conducted in beakers. Fish were kept for 8 days before being transferred to holding tanks and during this period, ten fish from the Treatment 4 batch died, 2 days after initiating the administration of the spores. The dead fish were replaced by a new batch. On the sixth day, another fourteen fish from various beakers died. The fish were weak and some of them died during transfer to the holding tank. A detailed account of examination dates and the fatality dates during the 10 months of the experiment's duration is tabulated in Appendix III.

Examination of the ten fish that died 2 days after having been placed in the spore suspension revealed that 60% (6 out of 10) of them contained spores in the intestine. Of the six fish that had ingested spores, five did not contain spores with extruded polar filaments; the sixth fish did

Fig. 20: Pansporoblast of *Myxobolus neurobius* containing developing spores (sp) in the brain tissue of an experimentally infected fish at 10 months post infection. (x 1750)


Sp
20

contain such spores. The total number of spores ingested by each fish was not determined. However, the fish that contained spores with extruded filaments, had four out of ten spores in this condition, in a single field of vision.

The appearance of the ingested spores was closely similar to that of free, live spores. The birefringent appearance of the polar capsules and valves of the ingested spores was retained. Migration of the sporoplasm from the spore to, and through, the intestinal wall was not detected. Furthermore, no movement of the sporoplasm within the spores was observed.

Of the fourteen fish that died 6 days after being placed in beakers, only two contained spores in their intestines and the polar filaments of the spores were extruded. These fish had been exposed to the suspension of spores aged without mud, Treatment 4.

Examination of the stomach of both samples failed to disclose any spores.

This experiment took 10 months. The fish were examined at regular monthly intervals, both fresh and stained. Up to 73 days post infection no _Myxobolus_ at any stage of development was found. This applied also to the brain tissue and the brain capillaries.

On May 26, 1981, 84 days post infection seven fish exposed to suspension of fresh spores (Treatment 2) died in one of the tanks. Examination on squash and smear preparations of their tissues showed that four of them contained various structures resembling myxosporean sporoblasts. The identity of these structures could not be determined with absolute certainty, since no well defined spores accompanied them. One fish, however, carried an elongate body with a very distinct outer membrane and containing other cellular organelles (Fig. 21). This structure was the only of its type

found in the course of this experiment. It, too, was not accompanied by and did not contain spores.

In the course of further examination of monthly samples no more early spore stages of M. neurobius were found up to August 31, 1981. On that date an unforeseen disturbance of the water supply caused death of most fish in four experimental tanks. Only two fish of Treatment 3 experiment survived. Thereafter, observations were concentrated on examining fish of the Treatment 2 experiment (exposed to fresh spores).

The fish that died due to the interruption of their water supply contained no spores or early developmental stages of the parasite.

In September 1981, 6 months post infection, one of the two fish examined in a routine monthly sample contained young spores with distinct polar capsules (Fig. 22). The young spores were rounded and contained two rounded polar capsules each. Four such spores, each 14.24 um in diameter, were found on one slide and all at a similar stage of development. No mature spores were found on that slide. This was the only observation of developing spores. Further examination of samples up to the end of the experiment in January 1982 were negative.

(iii) Experiment III.

This experiment lasted 7 months and no parasites were found (see Appendix IV).

(iv) Experiment IV.

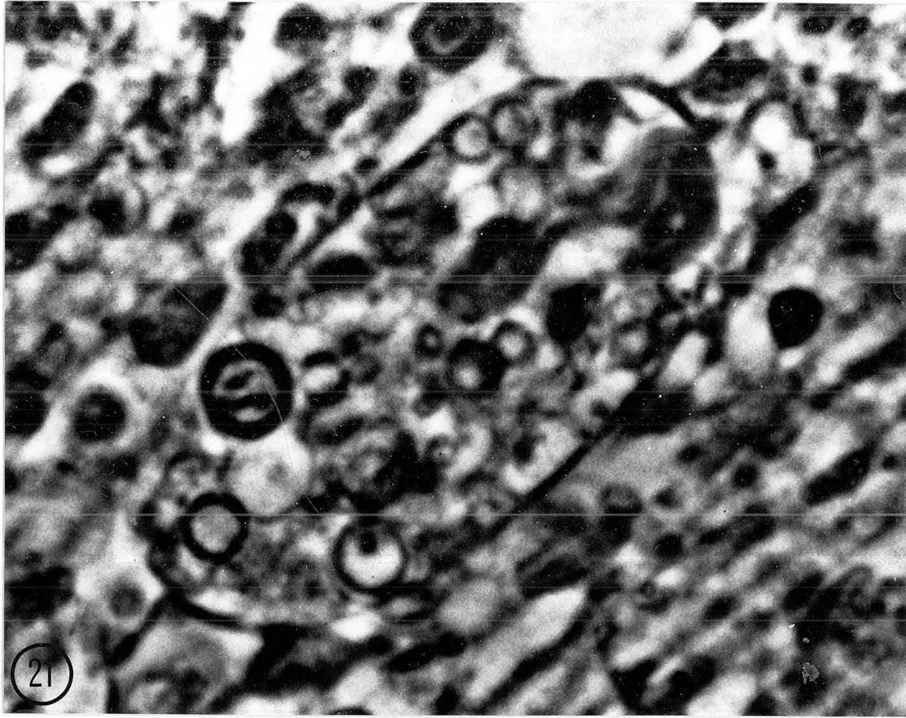
This experiment lasted 4 months, ending in January 1982 (see Appendix V).

Only one of all fish injected with the spores developed an infection. The fish was given a dose of 85,000 spores that had been refrigerated for 5 months. The infection was found in December 1982, three months post infection. The parasite, located in the brain tissue of the fish,

Fig. 21: Pansporoblast of *Myxobolus neurobius* in the brain tissue of an experimentally infected fish at 84 days post infection. (x 2000)

Fig. 22: Young spore of *Myxobolus neurobius* in the brain tissue of an experimentally infected fish at 6 months post infection. Hematoxylin and eosin. (x 2500)

pc = polar capsule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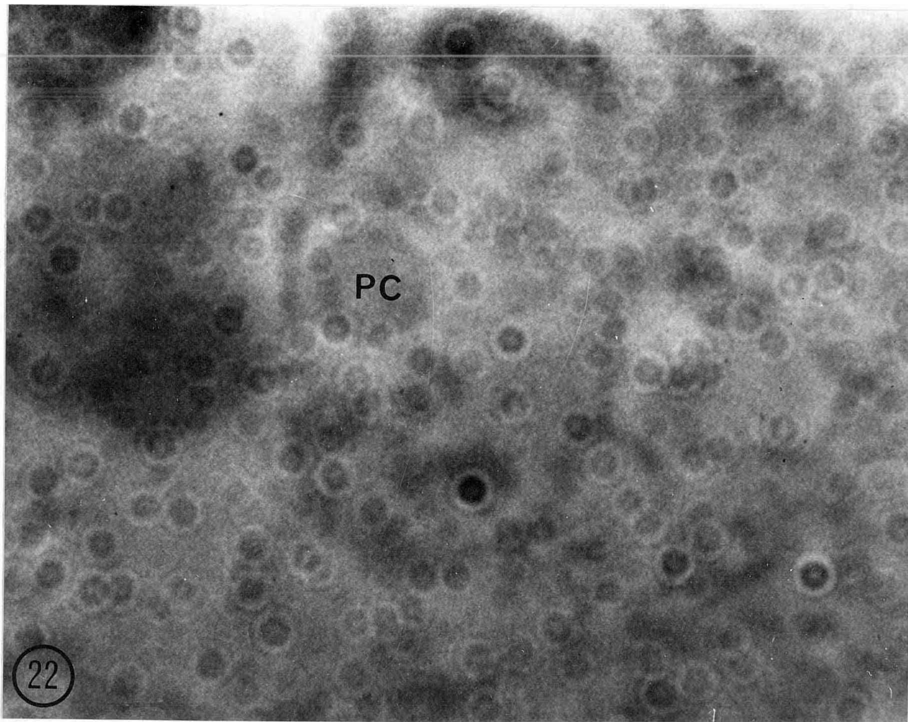

PC
22

was at the sporoblast stage. The sporoblast contained five cells (Appendix VIC) was subcircular and was 24.64 x 26.72 um in size. The identity of the cells within the sporoblast could not be determined, due to the early stage of their development and a relatively low degree of definition. Only one sporoblast was found in an examination of five slides with serial sections of the head cut at 8 um.

D. DISTRIBUTION AND PATHOGENICITY

1. Parasite distribution in the brain

As stated earlier, the schizogony and sporogony phases of *M. neurobius* development are spatially and chronologically separated from each other. The former occurs in the capillaries of the brain and spinal cord, but the latter occurs in the tissues of these parts of the central nervous system. As well as being separated in this manner, these two phases of development are not distributed in the brain in the same way. Schizogony occurs in the capillaries throughout the brain and spinal cord. Sporogony, however, was restricted to tissues of the midbrain, hindbrain and spinal cord. The forebrain was completely free of spores, although spores were found in the optic nerves and even in the nerve fibres within the eyeball. The details of the distribution of *M. neurobius* in the brain of underyearling and adult sockeye salmon are shown in Table 12.

Of the fish carrying *M. neurobius* at the schizogony phase of development, 17.39% contained it in the forebrain. Its prevalence in mid- and hindbrain, in contrast, was 45.65%. Thus, there is a difference in the prevalence of infection in different parts of the brain. The difference becomes more pronounced at the sporogony phase which is completely absent from the forebrain, whereas 73.33-100% of fish examined contained it in the

mid- and hind-brain.

As with many protozoans it is very difficult to estimate the intensity of infection of M. neurobius. It is more difficult with this brain parasite because it does not produce cysts within the tissue. Figures 23A and 23B illustrate semidiagrammatically the longitudinal and transverse sections of a highly infected brain and show the pattern of spore distribution in the brain. The spores are not evenly distributed (Fig. 23A). In diencephalon the spores are concentrated in areas between the lobus inferior and valvulla cerebelli, but spores are scarce within the valvulla cerebelli and in lobus inferior themselves. Only few spores were found within mesencephalon. No spores were found in the saccus vasculosus.

The pattern of spore distribution in the metencephalon follows that of the midbrain where the spores are not evenly distributed. They are rare in that part of the brain and do not occur in all its regions. The only part of metencephalon in which the spores are found is within the granular layer of the metencephalon. Their distribution there closely follows the line of corpus cerebelli granular layer.

The density of the groups of spores in the myelencephalon (Fig. 23A) is considerably higher than that in the corpus cerebelli. The distribution is linear as it is in the corpus cerebelli. The groups of spores are arranged in the shape of a horizontal "V". In lower part of the myelencephalon the groups of spores are arranged in a thick line. In the upper part of the myelencephalon the spores are less abundant and, consequently the upper arm of the "V" is thinner than the lower.

In the transverse section of the brain, groups of spores are clearly distributed throughout the brain. It can also be seen that they rarely occur in the lobus inferior.

Table 12. Prevalence, expressed as percentage of sample examined, of *Myxobolus neurobius* schizogony and sporogony phases in various parts of the brain of sockeye salmon, *Oncornynchus nerka*

Host age	Stage of Development	Forebrain	Midbrain	Hindbrain
4+ months (n = 46)	Schizogony	17.39	45.65	45.65
	Sporogony	0.0	0.0	0.0
5+ months (n = 30)	Schizogony	3.33	3.33	3.33
	Sporogony	0.0	73.33	73.33
6+ months (n = 12)	Schizogony	0.0	0.0	0.0
	Sporogony	0.0	100.0	100.0
7+ months (n = 15)	Schizogony	0.0	0.0	0.0
	Sporogony	0.0	100.0	100.0
8+ months (n - 15)	Schizogony	0.0	0.0	0.0
	Sporogony	0.0	100.0	100.0
1+ year (n = 35)	Schizogony	0.0	0.0	0.0
	Sporogony	0.0	100.0	100.0
Adult (n = 16)	Schizogony	0.0	0.0	0.0
	Sporogony	0.0	100.0	100.0

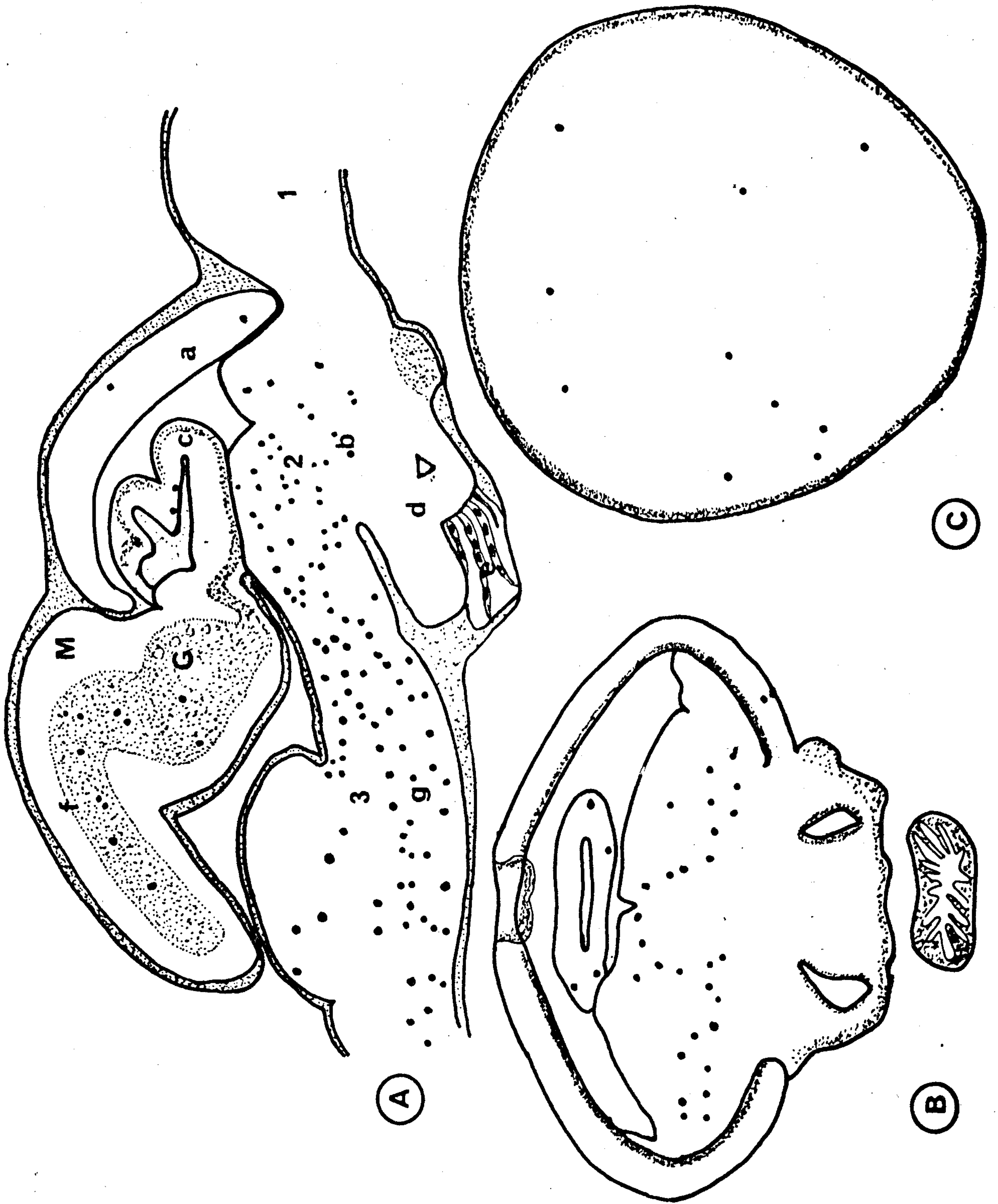
Fig. 23: Diagrammatic representations of distribution pattern of *Myxobolus neurobius* spores (shown as solid dots) in the brain (A and B) and Spinal cord (C); The labelling of the brain follows the terminology used by Healy, 1966; Grizzle and Rogers, (1976)

A. Median longitudinal section.

1. Forebrain
2. Midbrain
   - a. mesencephalon
   - b. diencephalon
   - c. valvula cerebelli
   - d. lobus inferior
   - e. saccus vascularis
3. Hindbrain
   - f. metencephalon
   - g. myelencephalon
   - G. granular layer
   - M. molecular layer

B. Transverse section throughout part of midbrain.

C. Transverse section cut at the level of pectoral fin.


A
B
C
M
G
a
b
c
d
f
g
1
2
3

Spores are distributed throughout the entire length of the spinal cord, but their distribution does not follow a well defined pattern. Figure 23C shows a transverse section of spinal cord and the pattern of spore distribution within it.

2. Pathogenicity

(i) Effects of *Myxobolus neurobius* infection on host histology

In view of the fact that two different developmental phases of *M. neurobius* are located in two distinct locations within the host, the description of the effects this species has on the histology of the host tissues is treated in two parts.

The vegetative stages of the parasite occur in the brain capillaries which are distended at the point of the parasite's location (Fig. 2). At no stage is the parasite attached to the inner wall of the blood vessel. In some instances the capillaries are not completely occluded by the parasite and so the blood cells, including erythrocytes, are capable of squeezing their way past the parasite. This is facilitated by the elasticity of the erythrocytes which become distorted during their passage along the infected capillaries. It is also possible that the parasite itself is capable of some degree of contraction or distortion at this stage as it moves along the blood vessel, so that at some period there is a sufficiently broad lumen open to the passage of the blood cells. Some blockages are apparent, however, and accumulation of blood cells on the afferent side of the occlusion can be observed. The occlusions do not appear to be so severe as to cause rupture of the capillaries and extravasation of blood. No such damage has been observed in the vicinity of the parasite. The parasite has never been found to contain blood cells and, consequently, an amoebic type of feeding on blood cells can probably be discounted.

During the sporogony phase, which occurs within the brain tissue, groups of parasite cells are arranged in strand like formation or in compact aggregations. The brain tissue is frequently eroded in the area immediately surrounding the parasite, but this damage is not uniformly present (Fig. 24a,b). Brain cell damage is due to displacement, and especially to the pressure exerted on the surrounding cells by the parasite. The integrity of the cells is not affected in any other way. There is no cyst formation and no inflammatory processes are provoked by the parasite.

(ii) Effect of M. neurobius on its host in nature.

It is difficult to judge the pathogenic effect of M. neurobius on its host in nature. No observations were made on the earliest developmental stages. Hence, nothing can be said about possible harmful effects of these early stages. However, it can be presumed that capillaries of the brain of these young fish were partially or completely occluded by the parasite and that some disruption of the blood supply to the brain may occur. This suggestion needs to be verified.

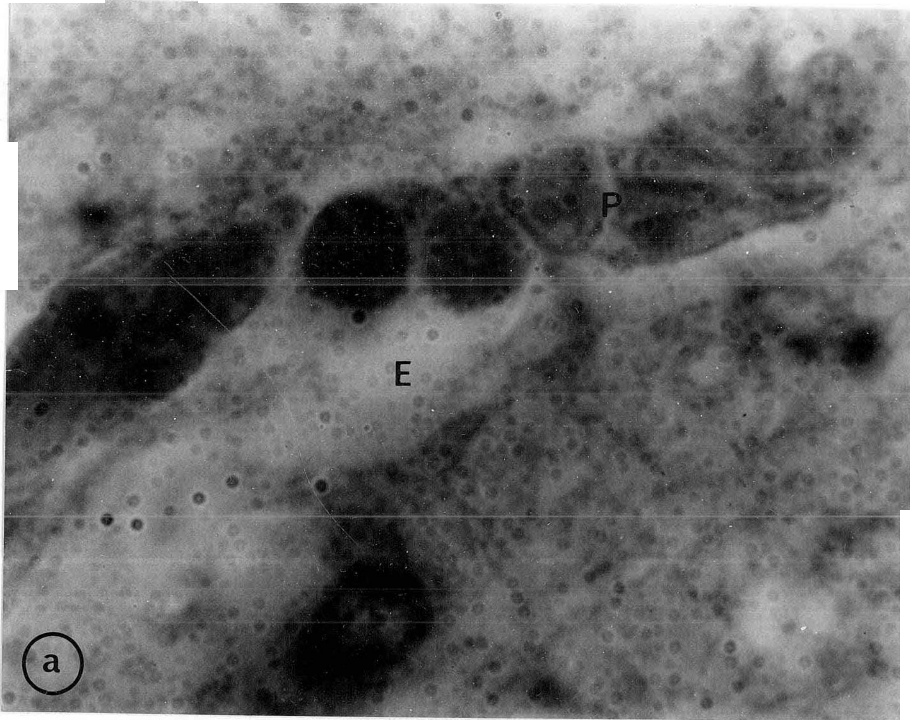
It is easier to deduce that during the sporogony stage, when the parasite is situated in the brain tissue, that there is no great damage to the central nervous system. Certainly infected fish appear to behave normally and undertake normal seaward and spawning migrations, together with uninfected fish. There are no external morphological or behavioural signs of the parasite's presence.

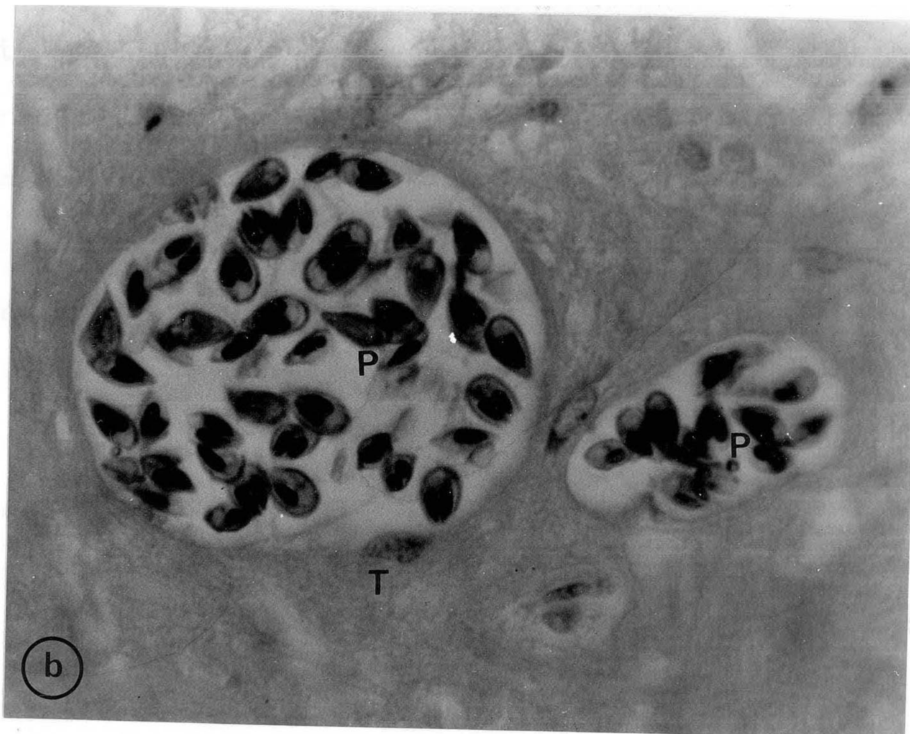
Fig. 24: Histological appearance of the brain tissue due to *Myxobolus neurobius* infection

a) Note eroded area (E) of the tissue adjacent to the parasite (P).

b) The brain tissue (T) surrounding the parasite (P) still intact.

Methylene blue and eosin. (x 900)


P
E
a


P
P
T
b

DISCUSSION

A. MORPHOLOGY AND DEVELOPMENT OF MYXOBOLUS NEUROBIUS

1. MORPHOLOGY

Kudo and Meglitsch (unpublished) emphasized the importance of information of the morphology of the vegetative stages of members of the Myxosporea because it can be used to supplement information about the spores, which themselves are often difficult to differentiate. The importance of vegetative morphology is particularly pertinent for the genus Myxobolus where interspecific differentiation of spores within the genus is difficult.

(i) Vegetative stages

Despite the fact that M. neurobius was discovered in 1905 (Schuberg and Schroder, 1905) and despite many records of this species, such as in the U.S.S.R, United Kingdom, Canada and the U.S.A., its vegetative stages have never before been described. The failure of previous investigators to find the vegetative stages may be attributed to one or more of several reasons: such as time of sampling, the size and/or age of the fish examined, and the unexpected location of the vegetative stages (in the capillaries). In the course of this study the vegetative stage, a multinucleate trophozoite, was found in the brain capillaries of a 4 month-old-fish in an August sample. The brain tissue itself was entirely free of the parasite and, notably, no spores were present. In fish in which the parasite has developed to the sporogony stage, almost no other vegetative stages were to be found. In fish that contained completely mature spores no vegetative stages were found. There have been many previous records of M. neurobius spores in tissue and so it can be assumed, with a high degree of probability, that the fish examined did not contain any vegetative stages of this species as otherwise they would have been

recorded. Arthur (1975), whose study covered the period from June to September, (i.e. the period during which vegetative stages were found in the course of the present study) failed to discover any vegetative stages. However, he did find mature spores.

The multinucleate vegetative stages of M. neurobius bear close resemblance to multinucleate trophozoites of Myxosoma cerebralis (cf. Hoffman et al., 1962, Fig. 8). However, the location of these stages in the host differs markedly for the two species. There are no records, or descriptions, of the early stages of M. cerebralis in the bloodstream of its host. Daniels et al. (1976) found similar structures in the skin from a region anterior to the eye, in the epithelium lining the buccal cavity, and in the base of the gill filaments, of young rainbow trout (Salmo gairdneri) exposed to water known to contain infective stages of M. cerebralis. Although the identity of that structure is not known with certainty, the authors suggested that it might be an early developmental stage of M. cerebralis. They failed to find any trace of the parasite in the capillaries. Stensaas et al. (1967) reported structures in the axons of a toad, Bufo arenarum , that resembled the cells discovered in the present study. They suggested that the observed organism was probably a sporozoan of the order Eucoccidia. Further identification of the parasite was not attempted. Wu et al. (1979) reported, but did not describe, early vegetative stages of M. dryagini in the nervous tissue of a silver carp, H. molitrix but no developmental stages of any kind were reported in the capillaries.

Records of vegetative stages of members of the Myxosporea in the blood capillaries are very rare. Davis (1923) reported Lentospora (= Myxosoma) ovalis as occurring in the capillaries of the gill filaments

of its hosts, the buffalo fishes (Ictiobus bubalus and I. cyprinella). Lom and de Puytorac (1965) found vegetative stages of H. psorospermica, parasite of Perca fluviatilis, in the same habitat. Both these species, however, complete their life cycles in the lumen of the blood vessels and produce cysts in these vessels. The spores are produced within the cysts, quite unlike the situation in M. neurobius.

The above facts lead to two conclusions. (1) The similarity between the vegetative stages of Myxosporea described previously and the cells discovered in the course of this study strongly indicate that these latter cells are vegetative stages of Myxosporea.(2) The fact that these cells occur in the lumen of blood vessels, unlike those of any previously described myxosporean suggests that they do not belong to species other than M. neurobius.

(ii) Spore

All morphological characteristics of M.neurobius spores observed under the light microscope are in agreement with the species description by Schuberg and Schroder (1905). There are, however, some differences in dimensions of the spores but these differences are within the ranges determined by Schuberg and Schroder (1905), and by Pavlovskii (1962). Kudo (1921) pointed out that the size of myxosporean spores might be affected by the treatment used to prepare the material for observation. In addition, Lom and Hoffman (1971) stressed that spore size is subject to differences resulting from host species. Similar observations were made by Davies (1968). Furthermore, she stated that the size of the myxosporean spore might be affected also by the location within the individual host. In the present study, M. neurobius spores are described from sockeye salmon, whereas that of the original description (see Schuberg and Schroder,

1905) is from trout.

According to Schuberg and Schroder (1905), there is only one nucleus in the sporoplasm. In the present study, two nuclei were frequently seen in the sporoplasm of immature spores. The literature contains many references to either one or two sporoplasm nuclei in various species of Myxosporea (cf. Shulman, 1966). It appears that in M. neurobius the conjugation of the sporoplasm nuclei occurs before the spore is ingested by its new host.

The openings of the polar filaments (capsular foramina) could not be seen under the light microscope in the course of this study, although they were described by Schuberg and Schroder (1905). However, examination under the scanning electron microscope showed distinct capsular foramina in the suture (Fig. 6B).

There are only few publications describing the structure of the myxosporean spores as seen under the scanning electron microscope. Those that have been published refer most commonly to the following structural features: capsular foramina, sutural ridge (lines), a groove running parallel to the sutural ridge, mucus membrane covering the posterior part of the spore, and ornamentation of the valve surface (Lom and Höffman, 1971; Komourdjian et al., 1977; Desser and Paterson, 1978; Wold and Iversen, 1978; Kabata and Whitaker, 1981). Not all species have spores with all these characteristics. M. neurobius has all these characteristics except for the mucus membrane and shell ornamentation. The sutural ridge is quite thick, with straight suture line. The capsular foramina, as mentioned earlier, are located in the sutural ridge, in contrast with a species like Kudoa paniformis (cf. Kabata and Whitaker, 1981, Fig. 2B) in which they open in the polar region of the valves. This position closely resembles

that occurring in Myxosoma cerebralis. The spores of these two species resemble each other in the presence of the groove running along the sutural ridge. The functional significance of this groove is unknown, particularly in view of the fact that it is absent from some closely related species, e.g. Myxosoma cartilaginis.

In view of the limited data presently available on spore structure, as observed under the electron microscope, it is not yet possible to assess the significance of the individual features for the purposes of taxonomy.

The nature of the iodinophilous vacuole, as well as its value for separating the genera Myxobolus and Myxosoma, is still debatable. It appears that the occurrence of the vacuole is not consistent and it might depend on the treatment of the spore. Some investigators (Walliker, 1968; Lom, 1969; Desser and Paterson, 1978) considered that the presence or absence of the vacuole is not a reliable criterion for separating Myxosoma from Myxobolus. Others (Shulman et al., 1978; Donets et al., 1978) remain firm in the belief that the vacuole is a morphologically significant structure and must be taken into account in the systematics of Myxosporea. Their arguments are based on the ecological significance of the vacuole, particularly for the freshwater Myxosporea. Spores containing the iodinophilous vacuole are heavier, a characteristic of potentially great importance in the freshwater habitat (cf. Shulman et al., 1978).

In the fresh and aged spores of M. neurobius treated with Feulgen's stain, the vacuole did not occur consistently. This agrees with the findings of Walliker (1968), Lom (1969) and Desser and Paterson (1978). The spores of M. neurobius are released into the environment after death

of the host fish which takes place on the bottom of a river or near the banks of a lake. Consequently, they do not require any mechanism that would make them heavier and cause them to settle more rapidly on the bottom. The same is true, however, of the spores of Myxosoma cerebralis, which are released under similar circumstances from decomposing host and have no iodinophilous vacuoles. It would appear, therefore, that the use of the vacuole for taxonomic purposes is at least difficult and requires a great deal of additional study before its value can be determined.

2. Comparison of developmental cycles

Four features of the developmental cycle of M. neurobius warrant discussion, namely:

(i) development of vegetative stages in the lumen of blood vessels;

(ii) liberation of sporonts from the trophozoite cell;

(iii) method of sporoblast formation;

(iv) time scale of spore formation.

(i) Development of vegetative stages in the lumen of blood vessels

Although there is very little knowledge about the earliest stages in the development of Myxosporea, there are various opinions about the location of the route along which the parasite proceeds to its definitive target site. It is commonly held that the bloodstream acts as one of the main, if not the only, pathway. Some species, such as Myxosoma (= Lentospora) ovalis and Henneguya psorospermica, continue their development up to the spore stage in the capillaries of the gill filaments (Davis, 1923; Lom and de Puytorac, 1965; Dykova and Lom, 1978).

The discovery of the multinucleate vegetative stages of M. neurobius in the brain capillaries can be taken as confirmation of earlier suggestions

that the bloodstream acts as one of the pathways in the course of myxosporean development. What is more interesting, is that it shows that M. neurobius passes through the trophozoite stage and develops to the sporont stage in the lumen of the capillary. This has not previously been reported in any species of Myxosporea.

The possible occurrence of an early intracellular stage in Myxosporea has been suggested by some investigators (e.g. Kudo, 1922). Such a stage, however, has been found on only rare occasions. Joseph (see Kudo, 1922) found young intracellular stages of Chloromyxum protei in the uriniferous tubules of its host, Proteus anguinus. Auerbach (after Kudo, 1922) observed uninucleate "amoebula" of Myxidium bergense and Zschokella hildae enter into epithelial cells of the gall gladder of the host fish, although he did not express any views on the importance of that process to the development of the parasite.

It is impossible to assess accurately the significance to the parasite of the fact that it develops its vegetative stages in the lumen of the brain capillaries. However, the fact that the host does not seem to be impaired by the infection of its brain with M. neurobius suggests that this type of development might be an adaptation enabling the host and the parasite to reach an equilibrium that would greatly limit or completely eliminate the pathogenic effect of the parasite.

Discussing the evolution of Myxosporea, Shulman (1966) suggested that they are phylogenetically young, although they are well adapted to their hosts. Most of them appear to have reached a physiological equilibrium with their hosts. Coelozoic forms are considered primitive stages in the evolution of Myxosporea, whereas the histozoic forms are more advanced. As a rule, the coelozoic forms are relatively nonpathogenic. M. neurobius, with its vegetative stages in the lumen of the capillaries and spores in the tissue of the brain appears to occupy an intermediate position in the evolutionary sequence of

Myxosporea, connecting the coelozoic with the histozoic forms.

(ii) Liberation of sporonts from trophozoite cell

Kudo and Meglitsch (unpublished) stated that sporonts are released from the trophozoite in only a few species of Myxosporea. Furthermore, Noble (1944) mentioned that plasmotomy and budding (endo- and exogenous) occur frequently in Myxosporea during the growth phase. Kudo (1922) observed these phenomena in Leptotheca ohlmacheri and Noble (1941) recognized similar phenomena in Ceratomyxa blennius. Budding is considered to be one of the methods of multiplication, leading to the formation of either new trophozoite or to spores. The fact that no new trophozoïtes of M. neurobius were found in brain tissues strongly suggests that this species has a different course of development. It releases sporonts individually from the trophozoite in the lumen of a capillary into the tissue of the brain.

(iii) Method of sporoblast formation

Various methods of sporoblast formation have been described in the literature and have been reviewed by Noble (1944) and Shulman (1966). However, there is a distinct possibility that these differences are more apparent than real, and are due to difficulties in the study of the morphological changes of the very small nuclei, as well as to the asynchronous character of sporogenesis progressing inside a trophozoite (Uspenskaya, 1976). The most widely accepted hypothesis of sporoblast formation is that it is a process of differentiation and growth of a single cell, without previous union of nuclei or cytoplasm. If this hypothesis is accepted, it must be assumed that only a single sexual process takes place in the sporoplasm. Meiosis occurs in only the development of the sporoplasm, the nucleus of which is haploid. Other nuclei of the spore are diploid. Uspenskaya (1976), who used a cytophotometric method

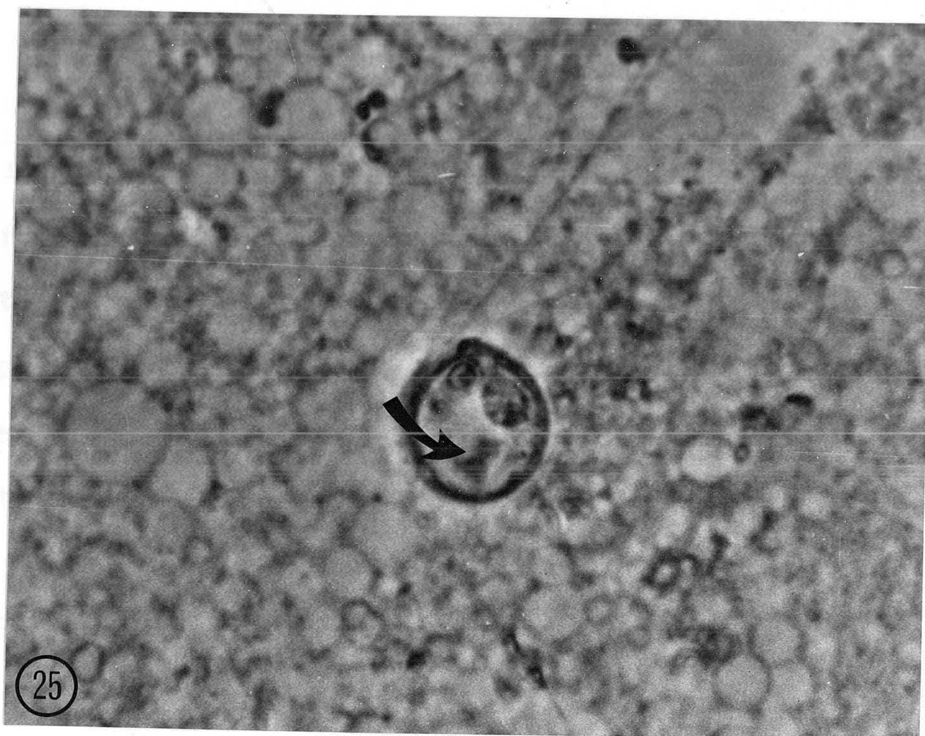
for studying the nuclear cycle of Sphaeromyxa eligini, discovered that the nucleus of the spore as well as that of the sporoplasm are haploid. This discovery can be used to formulate another hypothesis of the nuclear cycle.

Little can be said of the nuclear cycle of M. neurobius, in view of the fact that these methods have not been employed in the course of the present study. However, the fact that the sporoplasm of an immature spore contains two nuclei, while that of a mature one contains only one, strongly suggests that the sexual process occurs in the sporoplasm. This fusion of nuclei is clearly visible in Fig. 25. The exact way in which it progresses during the growth phase could not be ascertained. However, by the time a sporont is formed (Fig. 3) only one nucleus could be seen within the cytoplasmic layer of sporont cells. Thus uninucleate sporonts could be traced as they emerged from the blood vessels and penetrated the host brain tissue (Fig. 15a). The next stage of development, the sporoblast, is binucleate, presumably as the result of nuclear division of the uninucleate sporoblast (Fig. 15b). The next developmental stage is difficult to follow and it is uncertain whether it is a process of meiotic or mitotic division. However, at least up to the stage of the binucleate sporoblast, the nuclear cycle of M. neurobius conforms to those of other members of Myxosporea, as described by Noble (1944), i.e. it represents differentiation and growth of a single cell.

In the course of the spore formation of M. neurobius which occurs after the differentiation of component cells of the spore, the shell valves are the first visible structures to develop in the spore. They are followed by the polar capsules. The valves also develop first in Ceratomyxa auerbachi (cf. Kabata, 1962) and Henneguya vitiensis, a parasite of the heart of Leiognathus fasciatus (cf. Laird, 1950).

Fig. 25: Fusion of sporoplasm nuclei inside the spore of *Myxobolus neurobius* (arrow).

Fresh spore. (x 1500)


25

(iv) Time scale of spore formation

The time scale of the development of Myxosporea appears to vary from species to species. The development might be concluded in 21 days, as in *Ceratomyxa shasta* (cf. Yamamoto and Sanders, 1979), or it might take as long as 4 months, as in *Myxobolus dryagini* (cf. Wu *et al.*, 1979). Various factors have been considered as possibly affecting the duration of the development of Myxosporea. For example, Halliday (1973) observed that spores of *Myxosoma cerebralis* can be detected in 52 days post infection at 17 C. At 12 C and 7 C, spores took 101 and 120 days respectively to develop. According to Schafer (1968) *C. shasta* cannot become infectious at temperatures below10 C.

The factors that affect the duration of the development of *M. neurobius* are hard to determine. It appears, however, that the rate of development of this species is faster than that of *Henneguya salminicola*, which infects the same population of sockeye salmon in Sproat Lake (Boyce, personal communication).

B. TRANSMISSION OF *MYXOBOLUS NEUROBIUS*

1. In nature (Sproat Lake)

At least three facts derived from the study of the developmental cycles of *M. neurobius* in the population of sockeye salmon resident in Sproat Lake warrant discussion. They are:

(i) Vegetative stages were found in four-month-old sockeye salmon;

(ii) Vegetative stages were absent from 6, 7, 8, and 9 month old fish;

(iii) There were no vegetative stages in yearling fish.

It was unfortunate that the sampling of salmon in Sproat Lake could not distinguish between fish collected from different parts of the lake.

Such a distinction may have been helpful in determining some aspects of the mode of transmission of M. neurobius. However, available evidence suggests at least two possibilities of its transmission in Sproat Lake.

The first hypothesis is that the transmission of M. neurobius takes place in only certain parts of the lake, most probably near the banks. This hypothesis is supported by the fact that no vegetative stages (indicating recent infection) were found in larger fish. The fish are distributed in the lake in such a manner that the progressively larger fish occupy progressively deeper water. Hence, the larger fish have lost contact with the source of infection. Kennedy (1975) stated that close contact between the potential host and the source of infection is among the most important factors in the transmission of the parasite. Further support of this hypothesis is given by the work of Sanders et al. (1970), who demonstrated that the occurrence of Ceratomyxa shasta is restricted to well defined areas of the river. A similar opinion was also expressed by Knight et al. (1980) as regards the transmission of Myxosoma funduli.

Differential age susceptibility is not uncommon in host-parasite relationships. Dogiel et al. (1958) stated that the change in the qualitative composition of parasitic fauna is directly proportional to the extent of the changes in the ecology of the host with age.

The second hypothesis presupposed an even earlier infection of the fish. In accordance with this one, the transmission of M. neurobius occurs at a very early age of the host, possibly directly after the fish emerge from the gravel. These young fish ingest the spores present in the surrounding areas whilst feeding. To maintain this hypothesis, one must also postulate that the older fish are immune to infection by this parasite because young stages of the parasite are not found in older

fish.

As mentioned earlier, there are two sources of spores in the lake. Firstly, the spores that have been released by dead spawners. The spores coming from this source are more likely to be distributed along the banks of the lake, close to the spawning grounds. Secondly, the spores in the infected young fish that have died during their period of residence in the lake. These spores might be scattered along the route taken by migrating fish from the place of hatching to the final destination in the lake.

The author is inclined to believe that the infection occurs during the very early stage of the host's life, as suggested in the second hypothesis.

2. Laboratory experiments

The four transmission experiments conducted during this study and involving the use of both fresh and aged spores, force fed or ingested by the fish in the spore suspension, produced the following pertinent facts:

(i) the fish ingested the spores, when placed in spore suspension;

(ii) a considerable proportion (though not all) of spores ingested in this manner extruded their polar filaments;

(iii) both fresh and aged spores (latter aged in refrigerator, a 4 C for 5 months were infective;

(iv) the fish did not become uniformly infected.

The fact that the fish ingested the spores provides some clue as to the method of transmission of M. neurobius. By itself, however, this fact is not sufficient to lead to the conclusion that the spore ingestion is the mode of transmission of M. neurobius. Young fish are likely to

ingest any particular matter of a suitable size, occurring in suspension in their environment. When combined with the fact that at least some spores did extrude their polar filaments upon ingestion, it becomes more convincing. It must also be taken into account that ingestion as the mode of transmission of Myxosporea has been generally accepted by the investigators. In accordance with present understanding, ingestion is followed by the extrusion of polar filaments in the intestine of the host, exactly as has occurred in the course of these experiments.

A question that occurs at this point of the discussion is: Why do not all the spores extrude their polar filaments in the intestine? A correct answer to this question might provide the key to the success of laboratory transmission of Myxosporea. In view of the fact that no published report exists on the complete success in transmitting the myxosporean, this question is still hard to answer. It is reasonably certain that such an answer must take into account all factors concerned with the infection, such as the spores, the host, and the environment.

The numerous failures of previous transmission experiments has led some investigators (Yamamoto and Sanders, 1979) to speculate that an infective agent other than the spore must be present. However, as demonstrated by the present study and by some other experiments (see Uspenskaya, 1978), infection was transmitted by spores. Hence, I am convinced that there is no reason to look for an infective agent other than the spore.

One must now raise the question of the conditions necessary to maintain the successful growth of the parasite. Since spores are ingested by the fish, it is obviously necessary that the conditions within the intestine must permit spores to germinate. These conditions are the quality and

quantity of the intestinal fluid. The next step that must be successfully accomplished is the penetration of the intestinal wall by the sporoplasm, which then breaks into the circulatory system. The sporoplasm must, therefore, be able to resist the defence mechanisms of the host. These two barriers having been overcome, the parasite must find tissues capable of supporting its development and growth. Not all host tissues are capable to fulfill this role. For example, it has been establisehd in this study that the forebrain is not favourable for M. neurobius development. The fact that multinucleate trophozoites of M. neurobius were discovered mainly in four month old sockeye salmon, indicates that this early stage of the fish offers to the parasite favourable developmental conditions. In this association there appears to be the confirmation that the parasite is able to overcome the barriers presented by the fish, while the fish, in its turn, is able to support the parasite's development. The exact mechanisms involved in establishing this host-parasite system are unknown and urgently need further study.

The environmental conditions at the time of infection must be such as to allow the infection to proceed. For example, Schafer (1968) showed that the infection with C. shasta cannot occur, when ambient temperature is below 10 C. If the fish become infected, temperatures below that level probably act as a retardant, but do not totally prevent further development of the parasite.

C. DISTRIBUTION IN THE BRAIN AND PATHOGENICITY

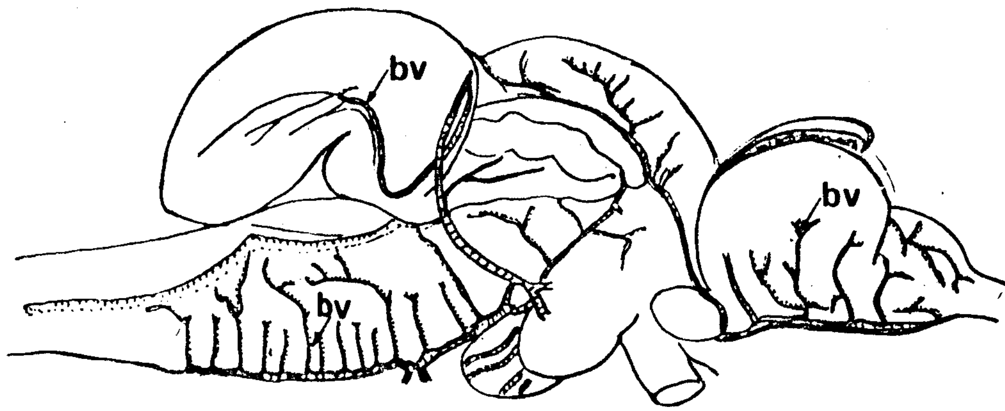
1. Distribution

The evidence derived from the distribution of the multinucleate trophozoites within the lumen of the blood vessels leads one to expect

that M. neurobius is distributed throughout the entire brain of the fish. In fact, these stages of the parasite do occur in all parts of the brain, as well as in the spinal cord. The examination of the pattern of spore distribution and its comparison with the pattern of the brain capillaries (Fig. 26, after Grodzinski, 1947, Fig. 3B) provides a good argument in favour of the bloodstream being the pathway for distribution of M. neurobius within the host. Comparison of Fig. 23A with Fig. 26 clearly shows that the spores concentrate mostly in the highly vascularized areas of the brain. The low vascularization on the valvula cerebelli and the lobus inferior is accompanied by the scarcity of spore aggregates. On the other hand, in the myelencephalon, which has a relatively rich vascularization, the density of spore aggregates is high. This correspondence between the spore distribution and the pattern of the capillaries exists only in the mid- and hind- brain. It does not hold true for the forebrain, a part of the nervous system completely free of spores, in spite of its abundant blood supply and in spite of the fact that multinucleate trophozoites are common in it.

The absence of spores from the forebrain suggests that it does not present a favourable environment for the development of the parasite. Some form of defence mechanism might be present and be suppressing the parasite. The nature of this mechanism is completely unknown. The forebrain does not contain sporoblast, and there are no traces of phagocytosis in progress. Alternatively, the sporonts might not be released from the blood vessels in the forebrain due to some unknown and unfavourable factor. It is far from clear, what prevents the parasite from infecting the forebrain. Comparison of the histology of the forebrain with that of the other parts of the nervous system showed (Fig. 27) that they are

Fig. 26: Distribution pattern of main blood vessels (bv) of trout (*Salmo irideus* Gibb).
(after Grodzinski, 1947).


bv
bv
bv

not identical. Fig. 27A shows the compact nature of the brain tissue in the forebrain, while that of the other parts (Fig. 27B,C) are rather loose. Perhaps this difference accounts for the absence of spores in the forebrain.

2. Pathogenicity

The course of infection of sockeye salmon by M. neurobius was followed from the stage of multinucleate trophozoite occurring in the capillaries of the brain of 4 month-old-fish, up to the stage of mature spores occurring in the brain tissue of adult fish, caught during their spawning migration. Neither gross clinical signs nor histological changes in the infected brain were observed. The infected and uninfected fish migrate together to the sea and return together. The former fish shows no impairment that could be attributed to Myxobolus infection.

Histological examination revealed neither a defensive response of the tissues nor any of necrosis. Some erosion and displacement of the brain tissue occurred, as well as distension of the brain capillaries. It seems, however, that these changes produce no harmful effects on the fish. These observations are in agreement with those of Konovalov (1971). The opinion of Schuberg and Schroder (1905), therefore, is not confirmed. The mechanism involved in the relationship between Myxosporea and the brain of their hosts are not clear. Some species, such as Myxobolus kisutchi (cf. Yasutake and Wood, 1957) and M. neurobius, are harmless to their hosts. However, M. dryagini produced in its host, Hypophthalmichthys molitrix, the so-called whirling disease (Wu et al., 1979). (Not to be confused with the classical whirling disease caused by Myxosoma cerebralis).

Shulman (1966) stated that the majority of Myxosporea are nonpathogenic.

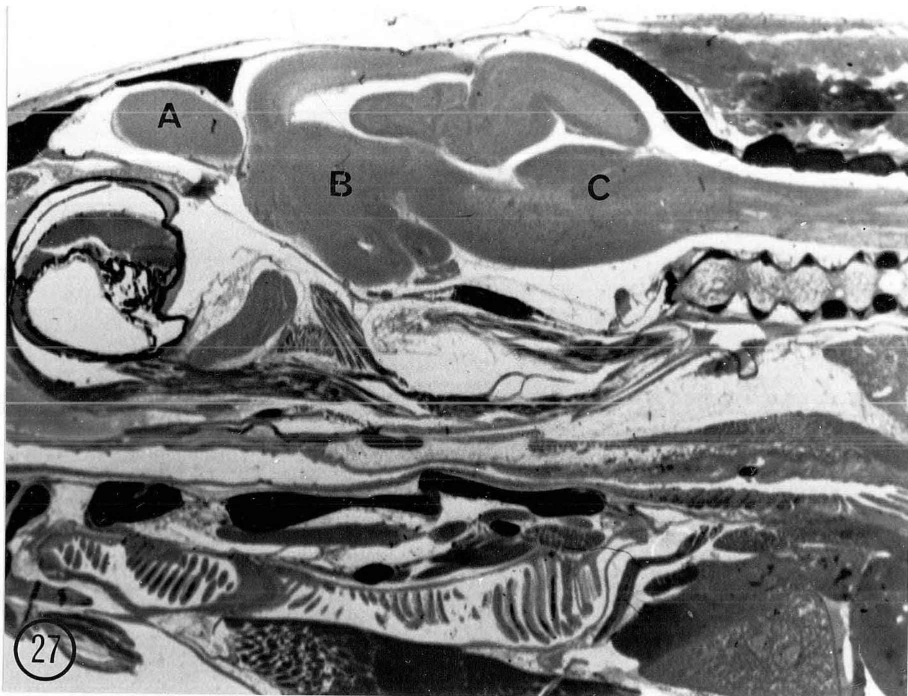
Fig. 27: Histological appearance of various parts of the brain of sockeye salmon.

Methylene blue and eosin. (x 8)

A. Forebrain

B. Midbrain

C. Hindbrain


A
B
C
27

He believed that the Myxosporea are well adapted parasites in view of the state of equilibrium with their hosts and producing well-balanced host-parasite systems. According to Shulman (1966), the evidence of this adaptiveness of Myxosporea is provided by their morphology, life cycle, and physiology. Current et al. (1979) suggested that any studies aimed at discovering the features of the parasite that are responsible for its pathogenic effect should involve a close examination of the plasmodial wall structure and function. Ultrastructural studies (Lom and de Puytorac, 1965; Current and Janovy, 1976, 1978; Current, 1979) have demonstrated that the plasmodial wall of Myxosporea varies from species to species and, even within a single species, according to the pathogenic effect exerted on the host. Current and Janovy (1978) demonstrated that the plasmodium wall of Henneguya exilis that inhabits inter- and intralamellar spaces on the gills of channel catfish (Ictalurus punctatus) is quite different. The interlamellar plasmodia are bounded by two outer unit membranes and covered by a fine granular coat of highly variable thickness and in some regions there is direct contact between the parasite and cells of the host. The intralamellar plasmodia, however, are bounded by a single outer unit membrane and covered by a fine granular coat of almost uniform thickness. These authors uncovered evidence that the host cell cytoplasm and interstitial material are taken by interlamellar plasmodia. In contrast, no direct contact was observed between the parasite and the cells of the host inside the lamella, indicating the absence of pathogenic influence at that site.

Since no ultrastructural study of the plasmodiaum wall of M. neurobius was undertaken in the course of this project, it is not possible to say anything about the structure of its wall. In addition, no true cysts

or plasmodial walls were found in M. neurobius. The high degree of adaptation of M. neurobius to its hosts is evident from the type of its life cycle. As mentioned earlier, the vegetative stages of this parasite develop within the capillaries up to the sporont stage. The spores are formed by individual sporonts that have been released from trophozoites. It can be postulated that this type of development tends to minimize the damage to brain tissues. An indication of this effect can be obtained from a simple arithmetical calculation. Let us assume that M. neurobius develops within its original zygote membrane until the formation of mature spores. Since a multinucleate trophzoite 20 um in diameter contains some 100 generative nuclei, and since each of these nuclei develops into a polysporous form about 20 um in diameter, that trophozoite would produce a spore aggregate as big as 2 mm in diameter. This is sufficiently big to produce severe damage to the brain of a small fish, or to destroy its spinal cord completely. The extent of possible injury would have to be multiplied by the number of the trophozoites present. M. neurobius, however, does not develop in this manner. Its spore aggregates, produced by single sporonts, never develop to the size that would be injurious to the brain of the fish. Thus, it can be concluded that this part of the life cycle of M. neurobius does not induce any pathogenic effects on the fish.

SUMMARY

1. Morphology, developmental cycle, pathogenicity, and mode of transmission of a myxosporean parasite, *Myxobolus neurobius* from sockeye salmon, *Oncorhynchus nerka* is described.

2. Vegetative stages in the form of multinucleate trophozoites were found in the blood capillaries of the brain and spinal cord of 4 month-old sockeye salmon. Its shape varied from spherical or oval to slender, and its average size was 20.2 um in length and 13.2 um in width.

3. The number of cell nuclei varied ranging from 45 to 103 and measured 0.76-1.02 um in diameter. The generative and vegetative nuclei could not be distinguished from each other.

4. The morphology and structure of *M. neurobius* obtained from present study are in agreement with that originally described by Schuberg and Schroder (1905). However, it is established that the sporoplasm has two nuclei in the immature and one in the mature spores whereas Schuberg and Schroder, 1905, recorded only a single sporoplasm nucleus.

5. Scanning electron microscope observations of spores revealed that the spore surface is smooth and without ornamentation or a mucus envelope. A parallel furrow occurs along a prominent sutural ridge. The capsular formamina are situated at the anterior on the sutural ridge.

6. The schizogony and sporogony phases of *M. neurobius* development are separated from each other in time and space. The schizogony phase up to sporont formation is developed in the brain capillaries, whereas the sporogony phase occurs in the brain tissue.

7. The sporonts are individually liberated from the trophozoite cell into brain tissue as uninucleate sporoblasts. The next development is differentiation and growth of this uninucleate sporoblast leading to formation of either monosporous or polysporous forms. Six nuclei are needed to form a spore and it takes about 6 months for spore maturation. This type of development has not been reported for other Myxosporea species.

8. The vegetative stages of *M. neurobius* are distributed throughout the brain and spinal cord, whereas the spore stage occurs everywhere except the forebrain.

9. No gross clinical and behavioural signs are observed in *M. neurobius* infected fish. No histological changes are found in the infected brain, though erosion and displacement of brain tissue occurred. Distension of brain capillaries, however, is observed at the site of the parasite.

10. The type of development of *M. neurobius* (separation of schizogony from sporogony phase, and liberation of individual sporonts) is considered to result in the absence of pathogenic effects on the host.

11. It is established from experimental evidence that the fresh as well as the stored spores (5 months in refrigerator at 4 C) are infective. Furthermore, it reveals that ingestion of spores is a mode of M. neurobius transmission.

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LIST OF PERSONAL COMMUNICATIONS

Boyce, N. P. 1981. Nanaimo, B.C., Canada

Gjernes, T. 1982. Nanaimo, B.C., Canada

Mcdonald, T. 1980. Nanaimo, B.C., Canada

O'Grodnick, J. 1980. (letter)

APPENDIX I

THE GLOSSARY OF TERMS USED

Term	Definition
Anterior end of spore	The end of the spore where the polar capsules open.
Capsular foramen	The opening of the polar capsule through which the filament is extruded.
Capsular view	The aspect of the spore with capsular foramina towards the observer.
Capsulogenous cell	A small, nucleated island of cytoplams in which the polar capsule becomes differentiated.
Capsulogenous nucleus	The nucleus of capsulogenous cell, becoming pycnotic as capsular differentation progresses
Cyst	A resistant cover about a parasite produced by the parasite or the host
Disporous	Term applied to a trophozoite producing only two spores
Iodinophile vacuole	A vacuole in the sporoplasm, the contents of which are stained brown with iodine, characteristic of the family Myxobolidae.
Spore length	The diameter of the spore in sutural plane.
Monosporous	Term applied to a trophozoite producing a single spore.
Polar capsule	The hollow body in the spore enclosing the polar filament.
Polar filament	The coiled filament inside the polar capsule.
Polysporous	Term applied to trophozoite producing more than two spores.

Shell	The envelope of the spore
Shell valve	One of the pieces of which the spore membrane or shell is composed. The valves meet at the suture.
Sporoblast	A developmental stage containing primordial cells of the shell valves, capsules and the sporoplasm, leading up to the spore.
Sporont	A nucleated, specialized island of protoplasm within a trophozoite, which gives rise to one or two sporoblasts.
Sporoplasm	The cytoplasmic mass found within the spore.
Striation	A fine ridge ornamenting the surface of the shell.
Suture line	The line on the shell marking the union of the valves.
Sutural ridge	A ridge marking the sutural line.
Thickness of spore	The diameter of spore at right angles to length.
Trophozoite	The vegetative or multiplicative stage of a myxosporean.
Valvular view	Aspect of spore in the plane parallel to the sutural line.
Width of spore	Diameter of the spore measured between the lateral faces of the shell valves and at right angles to the sutural lines.

APPENDIX II

Result of an experiment to infect the yearling coho salmon with *Myxobolus neurobius* in experimental tanks (Experiment I). Numbers in parentheses indicate the number of fish examined

Days post infection		Treatment I		Treatment II		Treatment III	
		1	2	1	2	1	2
11	(August, 1980)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
22*	(August, 1980)	-	-	-	-	0 (2)	-
31	(September, 1980)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
49*	(September, 1980)	-	-	-	-	-	0 (1)
62	(October, 1980)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
94	(November, 1980)	0 (2)	0 (2)	0 (2)	0 (2)	0 (1)	0 (1)
124	(December, 1980)	0 (2)	0 (2)	0 (2)	2 (2)	0 (2)	0 (2)
187	(February, 1981)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
194	(March, 1981)	0 (2)	-	0 (2)	0 (2)	-	-
271	(May, 1981)	-	0 (2)	-	-	-	0 (1)
275	(May, 1981)	-	-	-	-	-	1 (1)
516	(January, 1982)	-	-	-	-	0 (1)	-

Note: * fish died due to fungal infection

APPENDIX III

Result of an experiment to infect the underyearling sockeye salmon with *Myxobolus neurobius* in beakers (Experiment II). Numbers in parentheses indicate the number of fish examined

Days post exposure to the infection	Treatment I		Treatment II		Treatment III		Treatment IV	
	1	2	1	2	1	2	1	2
2* (March, 1981)	-	-	-	-	-	-	0[a] (10)	-
6 (March, 1981)	-	-	0 (3)	0 (3)	0 (3)	-	0 (2)	0[b] (3)
8 (March, 1981)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)
9* (March, 1981)	-	-	0 (3)	0 (2)	-	-	-	-
10*(March, 1981)	0 (8)	0 (27)	0 (1)	-	-	-	-	-
14*(March, 1981)	-	-	0 (5)	0 (8)	-	-	-	-
34 (April, 1981)	0 (4)	-	0 (2)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
73 (May, 1981)	0 (1)	-	0 (1)	0 (1)	0 (2)	0 (2)	0 (2)	0 (2)
84 (May, 1981)	-	-	-	1 (7)	-	-	-	-
98 (June, 1981)	0 (5)	-	0 (2)	-	0 (3)	0 (2)	0 (2)	0 (2)
129 (July, 1981)	0 (2)	-	0 (2)	-	0 (2)	0 (2)	0 (2)	0 (2)
170 (August, 1981)	0 (1)	-	0 (1)	-	0 (1)	0 (1)	0 (1)	0 (1)

(continued)

Appendix III continued

Days post exposure to the infection	Treatment I		Treatment II		Treatment III		Trestment IV	
	1	2	1	2	1	2	1	2
183** (August, 1981)	-	-	-	-	0 (10)	0 (16)	0 (14)	0 (13)
208 (September, 1981)	0 (2)	-	1 (2)	-	-	-	-	-
239 (October, 1981)	0 (2)	-	0 (2)	-	-	-	-	-
270 (November, 1981)	0 (1)	-	0 (3)	-	-	-	-	-
297 (January, 1982)	0	-	0	-	-	-	-	-

Note: * fish died (fish were weak during spore administration held in beakers)
** fish died (water supply disturbance)
a six fish contained spores in their intestine; one fish with spores that extruded polar filaments
b two fish contained spores in their intestine

APPENDIX IV

Result of an experiment to infect the underyearling sockeye salmon with Myxobolus neurobius in experimental tanks (Experiment III). Numbers in parentheses indicate the number of fish examined

Days post exposure to the infection	Treatments		
	Treatment I	Treatment II[1]	Treatment III[2]
33 (July, 1981)	0 (2)	0 (2)	0 (2)
34* (July, 1981)	0 (1)	-	-
61 (August, 1981)	0 (2)	0 (2)	0 (2)
105 (September, 1981)	0 (2)	0 (2)	0 (2)
131 (October, 1981)	0 (3)	0 (3)	0 (3)
148 (November, 1981)	0 (5)	0 (5)	0 (5)
179 (December, 1981)	0 (10)	0 (10)	0 (10)
221 (January, 1982	0 (5)	0 (5)	0 (3)

Note: * one fish died
1 one fish missing
2 three fish missing

APPENDIX V

Result of an experiment to infect the underyearling sockeye salmon with *Myxobolus neurobius* in experimental tanks (Experiment IV). Numbers in parentheses indicate the number of fish examined

Days post exposure to the infection	Treatment I	Treatment II
40* (October, 1981)	-	0 (3)
47 (October, 1981)	0 (2)	0 (2)
60 (November, 1981)	0 (10)	0 (10)
69 (November, 1981)	0 (1)	-
99 (December, 1981)	0 (10)	1 (7)
135 (January, 1982)	0 (7)	0 (8)

Note: * fish died

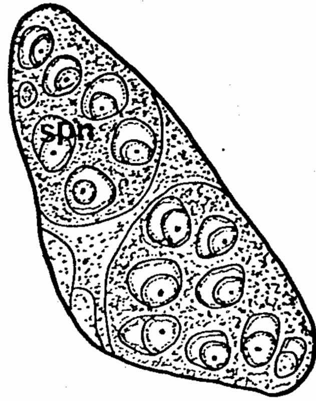
APPENDIX VI

Diagrammatic representations of sporoblasts of *Myxobolus neurobius*

A. Continuing division of the sporoblast nuclei (spn) of *Myxobolus neurobius* results in the formation of polysporous forms.

B. Sporoblasts of *Myxobolus neurobius* from the brain tissue of an experimentally infected fish at 5 months post infection.

C. Sporoblasts of *Myxobolus neurobius* from the brain tissue at 3 months post infection.



A

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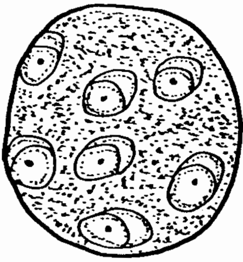

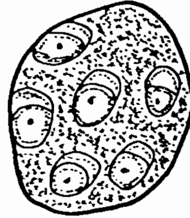

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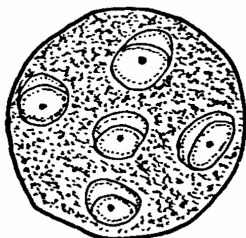

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