AN ABSTRACT OF THE THESIS OF

James L. Fendrick for the degree of Master of Science

in Fisheries and Wildlife presented on August 11, 1980

Title: INFLUENCE OF "AGING" ON THE MATURATION, EXAMINED BY SCANNING ELECTRON MICROSCOPY, AND TRANSMISSION OF CERATOMYXA SHASTA (MYXOSPOREA), A PROTOZOAN PARASITE OF SALMONID FISHES

Abstract approved: Redacted for privacy (Lavern J. Weber)

The effects of four natural environmental parameters (time, temperature, substrate, and aeration) on in vitro "aging" and/or maturation of Ceratomyxa shasta spores were studied in an effort to understand the life cycle of this myxosporidan parasite. Mode of transmission was investigated by exposure of rainbow trout (Salmo gairdneri) to "aged" spores by three methods; (1) intraperitoneal injection, (2) stomach tubing, and (3) water borne.

None of the four natural environmental parameters had any detectable effect on "aging" or maturation of C. shasta spores when they were exposed to rainbow trout by the three methods of transmission.

Scanning electron microscopy (S.E.M.) was utilized to study morphological changes in C. shasta spores during the "aging". Unaged spores exhibited a raised sutural border with no evidence of polar filament discharge canals or plugs. After 60 days of "aging" there was evidence that the polar plugs had become raised above the surface of

the spores. Partial protrusion of the polar plugs became prominent by day 120. At this time there was development of ridges surrounding the polar plugs. Two dark bands on either side of the sutural borders were observed on spores "aged" 180 days. The earlier raised sutural borders and ridges encircling the discharge canals became depressed to form grooves at this time. Spores "aged" 240 days were similar in morphology to those observed at 180 days.

The effects of varying concentrations of potassium hydroxide (KOH) or pH on fresh spore structure were studied. A final concentration of 0.25% KOH resulted in rupture greater than 93% of the spores. Spores held for any period of time after removal from fish required a lower concentration of KOH to produce rupture. Variation in the pH from 2.0 to 12.0 yielded no significant number of spores ruptured. The correlation between unstained spores by methylene blue to the percent ruptured spores from KOH treatment were similar. This indicates that methylene blue staining can be used in place of the traditional KOH spore rupture technique for determination of spore "viability".

Influence of "Aging" on the Maturation, Examined by Scanning Electron Microscopy, and Transmission of *Ceratomyxa shasta* (Myxosporea), a Protozoan Parasite of Salmonid Fishes

by

James Leonard Fendrick

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed August 1980

Commencement June 1981

APPROVED:

Redacted for privacy

Professor of Fisheries in charge of major

Redacted for privacy

Chairman of Department of Fisheries and Wildlife

Redacted for privacy

Dean of Graduate School

Date thesis is presented August 11, 1980

Typed by Connie Zook for James Leonard Fendrick

"Die Natur geht ihren Gang, und was uns als Ausnahme erscheint,
ist in der Regel"

in B. G. Chitwood and M. B. Chitwood,
Introduction to Nematology, 1950

ACKNOWLEDGEMENTS

I would like to thank Dr. Raymond E. Millemann for his inspiration and help in planning this research and later in reviewing this manuscript. His enthusiasm for parasitology and science is greatly appreciated.

My special thanks to Dr. Lavern J. Weber for his patience and help in both my research and preparation of this manuscript. His frank and candid opinions about science and life are admired.

I would like to express my extreme gratitude to Dr. JoAnn C. Leong for her interest and assistance with my research and extend my thanks for the use of her laboratory. My special thanks is also extended to Dr. Leong for giving generously of herself and laboratory in the last three years in teaching me virological research. Without her assistance and support I would not be embarking on a career in virology.

To Dr. John L. Fryer I would like to express my thanks for both his time in discussion of my research and the use of his laboratory equipment. I would not have been able to complete certain portions of my research without his assistance.

I would like to extend my thanks to Antonio Amandi for his grammatical and scientific comments in preparation of this manuscript.

To Dr. James E. Sanders I would like to express my appreciation for his reviews and comments of this manuscript.

Oregon Department of Fish and Wildlife (Roaring River Fish Hatchery and Alsea Trout Hatchery) supplied the fish used in the experiments.

My appreciation is extended to Dr. Richard A. Tubb for providing me with funds to conduct the scanning electron microscopy study.

For his untiring assistance and guidance with the scanning electron microscopy I would like to thank Al Soldner.

Lastly, I would like to thank my wife Margaret (Margie) for her assistance with my research and her moral and financial support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

INFLUENCE OF "AGING" ON THE MATURATION, EXAMINED BY SCANNING ELECTRON MICROSCOPY, AND TRANSMISSION OF CERATOMYXA SHASTA (MYXOSPOREA), A PROTOZOAN PARASITE OF SALMONID FISHES

INTRODUCTION

Ceratomyxa shasta is a parasitic protozoan of the class Myxosporea. Noble (1950) recorded the first known epizootic which occurred at Crystal Lake Hatchery, Mount Shasta, California, in the summer of 1948. This epizootic resulted in extensive mortality to rainbow trout (Salmo gairdneri). A second epizootic of C. shasta at the Crystal Lake Hatchery in the summer of 1949, caused 100% mortality of rainbow trout the following September. This is the first report of a species from the genus Ceratomyxa as a histozoic parasite of freshwater fishes. Ceratomyxa occurs widely in marine fishes, parasitizing the lumens of the gall and urinary bladders (Noble, 1950).

Further reports have extended the host list to coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha), steelhead trout (Salmo gairdneri) (Conrad and Decew, 1966), Atlantic salmon (Salmo salar), brown trout (Salmo trutta) (Sanders et al., 1970), cutthroat trout (Salmo clarkii), brook trout (Salvelinus fontinalis), chum salmon (Oncorhynchus keta), and sockeye salmon (Oncorhynchus nerka) (Johnson, 1975). Zinn et al. (1977) showed that there is a wide range in host specificity for C. shasta in nine salmonid species and nine hatchery strains of chinook salmon.

Schafer (1968) found C. shasta to be enzootic in the immediate area of Crystal Lake Hatchery and recorded it from Trinity River

Hatchery, California. Rucker et al. (1953) reported an epizootic of C. shasta from LaCamas Lake, Clark County, Washington. Enzootic areas in Oregon include the Columbia River below the confluence of the Deschutes River and several tributaries of the Columbia, primarily the Cowlitz and Willamette Rivers (Sanders et al., 1970). Gould (1969) showed it to be enzootic in the Deschutes River system and some of the surrounding mountain lakes. Further investigation shows C. shasta to be enzootic in the Nehalem and Rogue Rivers on the Oregon coast. Enzootic areas in California now include the Sacramento River and several of its tributaries plus the Klamath River up to and including Klamath Lake in Oregon (Johnson et al., 1979).

Margolis and Evelyn (1975) reported juvenile chum salmon from coastal waters of southern British Columbia to be infected with C. shasta. The possibility that infected fish might have migrated from enzootic areas of the parasite was considered in their report. Adult salmonids parasitized by C. shasta have been found in several rivers of British Columbia, Washington, Idaho, Oregon, and California. Some of these rivers have been shown to lack the infective stage of the parasite while others have not been tested. Infection may occur by migration through enzootic waters (Johnson et al., 1979).

Myxosporidan spores are thought to be propagative per os with only the fish host involved in the life cycle. Although considerable work has been done to identify the infective stage of many myxosporidans and their mode of transmission, neither has been demonstrated under controlled laboratory conditions.

It is likely the spores of myxosporidans protect the parasite from unfavorable environmental factors. Spores may undergo a kind of "aging" in the environment. When this is achieved, the parasite then becomes infective. Spores ingested by the host, discharge their polar filaments and the spore valves separate at the sutural border releasing the parasite (sporoplasm) into the gut (Kudo, 1930). The two haploid nuclei of the sporoplasm either unite prior to or upon separation of the spore valves, depending on the myxosporidan species, to form a zygote (trophozoite). Trophozoites then migrate to the preferred site of development where they feed, grow, and multiply by nucleogony or plasmotomy and cytoplasmic growth accompanied by sporogony (Noble, 1944).

Spore "aging" of C. shasta may be closely related to water temperature. When the temperature exceeds 10°C, fish become infected (Schafer, 1968; Fryer and Sanders, 1970) with time to death decreasing with increasing temperature (Fryer and Pilcher, 1974; Udey et al., 1975). Enzootic waters for C. shasta become noninfectious below 10°C. If spore maturation occurs during this period, the length of time required is not known because in some years the temperatures reach 10°C sooner in the spring with infective stages of the parasite being present (Johnson, 1975).

This study was done to determine the effects of in vitro "aging" of C. shasta spores on their maturation and infectivity to rainbow trout. Four natural environmental factors (time, temperature, substrate, and aeration) were used in various combinations to affect parasite

maturation. Infectivity was tested by exposure of rainbow trout to *in vitro* "aged" spores. Scanning electron microscopy (S.E.M.) was utilized to examine morphological changes that occurred during the aging process.

LITERATURE REVIEW

Introduction

Myxosporidans are a very successful group of organisms primarily parasitizing fishes but have been described in a platyhelminth, annelid, amphibians, and reptiles (Kudo, 1920; Overstreet, 1976). They have been found in freshwater and marine fishes where virtually all tissues or organs of the hosts are infected (Rogers and Gaines, 1975). Twenty-three genera of myxosporidans were listed from North American freshwater fishes by Hoffman (1970) and over 700 species have been described from freshwater fishes of the U.S.S.R. (Shulman and Shtein, 1962). Myxosporidan infections have been reported from a variety of marine fishes inhabiting tide pools down to depths greater than 3,000 m. Diversity in the freshwater environment is just as great. Myxosporidans have been shown to be an extremely abundant group of parasites (Gurley, 1894; Thélohan, 1895; Labbé, 1899; Auerbach, 1910; Kudo, 1920, 1934; Tripathi, 1953; Shulman and Shtein, 1962; Shulman, 1966) and are adapted to a diversity of their hosts environment.

A comprehensive literature review of myxosporidans would be a monumental task let alone development of a key to all the described species. This literature review will be divided into the following sections:

1. Historical description of myxosporidans and early phylogenetic relationships.
2. Development of the currently accepted myxosporidan mode of reproduction within the host.

3. Sporogenesis
4. Capsulogenesis
5. Evolutionary trends and phylogenetic relatedness of myxosporidans to other taxa, especially coelenterates.
6. Comparison of the effects of chemical agents on stimulating discharge of myxosporidan polar capsules and coelenterate nematocysts.
7. Development of current myxosporidan systematics, more specifically classification, in regards to other taxa of protists in terms of their phylogenetic trends.
8. Ultrastructural studies of myxosporidan spores by transmission and scanning electron microscopy as it relates to the study of C. shasta in this manuscript.
9. Discussion of the literature concerning the life cycles of myxosporidans in relationship to transmission.
10. In vitro cultivation of myxosporidans.

Pathology, epizootiology, diagnosis, and treatment of myxosporidan infections have not been included in this literature review. A number of excellent reviews dealing with these subjects have been written (Dogiel et al., 1958; Hoffman, 1970; Sindermann, 1970; Lucký, 1971; Hoffman and Meyer, 1974; Rogers and Gaines, 1975; Mitchell, 1977).

Historical Description of Myxosporidans

The first observation of a myxosporidan infection is not known. Jurine (1825) recorded the finding of cysts within the muscle of the

white fish Coregonus fera which may represent the first recorded observation of a myxosporidan parasite. He stated that a caseous material was obtained from the cysts but did not describe any morphological stages of the parasite. A myxosporidan was found in the retina of the Crucian carp Carassius carassius (1838) and Henneguya sp. (1840) spores in the gills of perch by Mayer (1864).

Müller (1841, 1843) described Henneguya sp. spores but no trophic stages from the skin and internal organs of fish. Müller called these organisms Psorospermien because of their morphological similarity to spermatozoa, with an oval body and a tail. Parasitic (trophic stages) leading to spore formation within a cyst were first reported by Creplin (1842). He described the similarities and discussed the possible phylogenetic relationship between Psorospermien (myxosporida) and Pseudonavicillen (actinomyxida) that parasitizes the body cavities and gut epithelium of oligochaetes and sipunculids.

Based on the non-cysted branching plasmodial (trophic stages) masses of psorosperms found in the gill tissue of Leuciscus erythrophthalmus by Düjardine in 1845 the relationship of psorosperms to slime-molds was made by Robin (1853). Lieberkühn (1854) was able to break open the myxosporidan spore which resulted in release of an amoeboid sporoplasm. This led Balbiani (1863) to describe psorosperms as being spores. He went on to describe the polar capsules containing the spiral (polar) filaments which could be extruded by specific stimuli.

Myxosporidan Reproduction Within the Host

There was considerable controversy over the mode of reproduction of myxosporidans until Noble (1944) published his study on myxosporidan life cycles. Multicellularity of myxosporidans was first suggested by Bütschli (1881) when he described cell-like organization of myxosporidan polar capsules and their morphological similarities to coelenterate nematocysts. Mutinucleation was first described by Thélohan (1895) in the binucleated sporoblasts and nuclei of capsulogenous cells adhering to the polar capsules. Léger (1906) and Mercier (1906 a,b) described the cellular origin of the myxosporidan spore valves of Chloromyxum truttae and Myxobolus pfeifferi respectively. Multicellularity was established for Myxidium sp., Henneguya sp., and Myxobolus sp. by Léger and Hesse (1906) and for Sphaeromyxa sabrazesi by Schröder (1907).

Plasmodial (trophozoite) and spore development was examined by Schröder (1907) for the polysporous S. sabrazesi. He described fusion of the two sporoplasm nuclei in the developed spores. Plasmogamy was reported to occur with the onset of sporogenesis resulting in pansporoblast formation. Future study showed that plasmogamy did not occur (Debaisieus, 1924). Two large and two small nuclei were found within a single pansporoblast that produced 14 nuclei by mitotic divisions. Division of this sporoplasm resulted in two sporoblasts giving rise to one spore each. Upon completion of spore formation the pansporoblast contained two spores and two residual nuclei.

Georgévitch (1914, 1916, 1917, 1919, 1929, 1935, 1936) extensively studied the nuclear division of the genera Ceratomyxa, Myxidium, and

Zschokkella. He found that the trophic stage in these organisms was diploid and that the sporoplasm nuclei divided by reduction division with completion of sporogenesis. Fusion of sporoplasm nuclei follows.

Davis (1916) was unable to find any evidence of sexual reproduction in Sphaerospora dimorpha. Observations by Mavor (1916) on Ceratomyxa acadiense showed that a uninucleated single cell underwent nuclear division either by mitosis or amitosis to produce one large and one small nuclei. A second nuclear division occurred forming two large and two small nuclei. Each small nucleus has a propagative function and undergoes further nuclear division to form a sporoblast. The large nuclei remain in the trophozoite without further division.

Erdmann (1917) concluded that the residual nuclei of Mercier (1906a, b), Schröder (1907), and Awerinzew (1911) observed in the spore were actually chromatic or glycogenous bodies that function in spore membrane formation and that no true meiosis had been shown to occur in myxosporidans.

Extensive study of the nuclear life cycle of Leptotheca ohlmacheri by Kudo (1922) revealed that both endogenous and exogenous budding or plasmotomy occurred during the trophic stages that resulted in a trinucleated state during sporogony. Naville (1930) studied the life cycles of S. sabrazesi, S. balbianii, and Myxidium incurvatum and concluded that micro- and macrogametes were present and fused during two fertilizations to produce two zygotes during their life cycles. This study has not been supported by the nuclear life cycle studies of Debaisieus (1924) and Noble (1941, 1944).

Noble (1944) concluded that schizogomy was absent from myxosporidan reproduction. The generalized sequence of events occurring in myxosporidan reproduction is outlined in the proceeding discussion. It must be kept in mind that due to the large number of myxosporidans, variation in this reproductive outline may be observed. The two haploid nuclei of the sporoplasm or the two uninucleate sporoplasms unite by autogamy or paedogamy, respectively, within the spore or upon separation of the spore valves depending on the species. This results in a zygotic sporoplasm (trophozoite). Large polysporous species complete their entire life cycle within the original zygote membrane and mainly multiply by nucleogony. Monosporous and disporous species multiply by internal and external budding or plasmotomy. A diploid state exists until formation of sporoblasts which initiates the sporogony cycle. A vegetative cell or nucleus gives rise to a specialized (generative) cell that produces sporoblasts or pansporoblasts. Sporogony may be monosporous, disporous, or polysporous. Sporoblasts form by aggregation of six to eight nuclei or pansporoblasts with fourteen or more nuclei. Each nuclei becomes surrounded by a mass of dense cytoplasm. Six generative nuclei and two somatic residual nuclei are generally found in each sporoblast. Of these six generative nuclei, two form the spore valves (sporogenesis), two produce the polar capsules (capsulogenesis), and two by reduction division form the haploid sporoplasm nuclei (gametes).

Sporogenesis (Sporogony)

Ultrastructural studies by electron microscopy have aided in the elucidation of myxosporidan affinities. Differences between the histozoic and coelozoic trophozoites of different myxosporidan species have been summarized by Canning and Vávra (1977). Sporogenesis beginning in the early trophozoites until development of complete spores has been studied in detail (Grassé, 1960; Lom and de Puytorac, 1965 a, b; Lom and Vávra, 1965; Schubert, 1968; Lom, 1969, 1973; Spall, 1973; Morrison, 1974; Schubert et al., 1975; Current, 1977; Desser and Paterson, 1978; Current et al., 1979; Current, 1979; Yamamoto and Sanders, 1979). Summary of the previous references has shown generative cells to form early in development of trophozoites (plasmodia). Bundles of microtubules that participate in polar filament morphogenesis are already present in these generative cells.

Spore formation is shown to occur via two generative cells. Fusion of the two cells does not occur but one cell will envelope (envelope cell or vegetative cell) the other cell (enclosed cell). The enclosed cell divides while the envelope cell exists at the periphery of the forming sporoblast or pansporoblast. No division of the envelope cell has been observed. The enclosed cell now termed the sporoblastogenic cell (sporont) undergoes cellular division to give rise to the sporont progeny found within the sporoblast or pansporoblast. Sporont progeny become compartmentalized within the envelope cell into five separate cell producing units. Two valvogenic cells (from which the spore is formed) surround two capsulogenic cells (in which the polar capsules

are formed) and the binucleated sporoplasm develop from the sporont progeny. All nuclei involved in sporogony are diploid except for the two sporoplasm nuclei which are haploid (Uspenskya, 1976; Siau, 1979). Sporoplasm nuclei undergo autogamy or paedogamy as described earlier, representing the sexual phase. Coupling of the generative cells is indicative of the multicellular origin of the spores (Canning and Vávra, 1977; Siau, 1979). Schubert (1968) demonstrated that the binucleated condition of the sporoplasm occurred without cytoplasmic partitioning.

Capsulogenesis

Polar capsule development has been studied extensively by the same investigators of sporogenesis. Each capsulogenic cell gives rise to a primordium which is connected to an outer capsulogenic tube. Longitudinally placed microtubules surround each tube. Development of the polar filaments occurs in the capsulogenic tubes. As the capsulogenic tube shortens the filament is retracted as a spiral within the capsule primordium. Further differentiation of the capsule wall leads to the formation of an outer opaque layer and an inner electron-lucent layer. Both layers continue into the wall of the tubular filament. The capsule is then sealed at the anterior end by an electron-dense plug.

Phylogenetic Relationship of Myxosporidans to Other Taxa

Due to the individual cell-like structure of the capsulogenic cells, sporogenic cells, and sporoplasm plus morphological similarities to

other organisms many investigators have discussed possible phylogenetic pathways for myxosporidans.

When Bütschli (1881) described the cellular structure of myxosporidan polar capsules he reported the likeness of them to coelenterate nematocysts. The functional complexity of the reproductive and non-reproductive cells of myxosporidans and similarity to various cellular structures of Mesozoa lead Emery (1909) to propose that they were metazoans, more closely related to mesozoans (i.e. Dicyema sp.) although no morphological analogies were made.

Dunkerly (1925) postulated on the previously proposed hypothesis of myxosporidan-mesozoan ancestry when he studied the development of spore forming nuclei of the myxosporidan Agarella gracilis. He felt that the spores of myxosporidans evolved as a protective mechanism of the germ cells and were physiologically similar to the infusion embryo of Dicyema sp. This hypothesis proposed by Emery (1909) and supported by Dunkerly (1925) for the relationship between myxosporidans and mesozoans does not appear plausible since the latter group are thought to be highly modified relatives of the digenetic trematodes (Stunkard, 1954; Manter, 1969).

The relatedness of myxosporidans and actinomyxidans to coelenterates has received considerable attention (Lom, 1973). The lucent layer found in the chitinous polar capsules will withstand 30% KOH at 120°C in tests with Myxobolus sp., Henneguya sp., Kudoa sp., and Myxidium sp. (Lom, 1977). Likewise, nematocysts in the medusa of Aurelia sp. have similar outer opaque and inner layers previously described for polar

capsules. In the former structure the inner layer is resistant to alkaline treatment. Lom and Vávra (1964, 1965) pointed out also that nematocysts differentiated in the presence of golgi complexes while there was no such involvement in the morphogenesis of polar capsules. Myxosporidans exhibit an electron-dense plug in the posterior end of the discharge canal sealing off the polar capsule. A similar structure has been found in coelenterate nematocysts. Chapman and Tilney (1959 a, b), Slautterback (1963), and Westfall (1966) showed that nematocysts were formed in an outer tube surrounded by microtubules and subsequently were withdrawn into a bulbous primordium identical to the primordium in polar capsule formation.

Ormieres (1970) showed the similarities between polar capsule formation in actinomyxidans and myxosporidans. Pansporoblast formation in myxosporidans is by two distinct generative cells, reminiscent of the phorocyte enveloping the generative cell found in neotenic larvae of cuninid (coelenterate) medusae. Lom (1977) points out that some coelenterates have adopted a parasitic habitat which in the case of Polypodium hydriforme is intracellularly parasitic in sturgeon eggs.

Shulman (1966) proposed that myxosporidans originated from parasitic amoeba due to the sarcodine (amoeba) nature of the myxosporidan trophozoites. Evolution of coelenterates are thought to arise from flagellates (Meglitsch, 1972) while the latter have been shown to be evolutionarily related to sarcodines (Corliss, 1968; Meglitsch, 1972; Hanson, 1976; Baker, 1977). The presence of nematocyst-like structures,

multiple fission, and parasitic habitat of some dinoflagellates has given rise to the hypothesis of myxosporidan evolution from flagellates.

The hypothesis of myxosporidan-coelenterate evolution links the former group to organisms having the capacity to differentiate into somatic (non-reproductive) and generative (reproductive) cell proliferation. Grell (1973) points out that differentiation of somatic and generative cells of myxosporidans is cellular with no tissue involvement which favors the evolution of this group from metazoa, primarily the coelenterates. Convergent evolution may account for the sarcodine-myxosporidan evolutionary hypothesis since the multicellularity of some sarcodine cysts show no somatic or generative cell differentiation.

Effect of Chemical Agents on Stimulating Discharge of Myxosporidan Polar Capsules and Coelenterate Nematocysts

Ultrastructural studies of morphogenesis and morphology of myxosporidan polar capsules and coelenterate nematocysts have shown strong similarities between the two groups. Stimulation of filament extrusion in both groups of organisms by a variety of chemicals indicate that they do not respond to the same stimuli. Nematocyst filament extrusion can be accomplished by exposure to sodium thioglycollate, trypsin, pepsin, and extreme pH which had no effect on several myxosporidans tested (Lom, 1964). Studies by Plehn (1924), Lom (1964), Hoffman et al. (1965), and Hoffman and Hoffman (1972) with sodium or potassium hydroxide, saturated urea and host intestinal contents stimulated discharge of polar filaments.

Electron-dense cap-like structures ("stopper mechanism" or cap) have been reported to be associated with the spore wall occurring at the anterior end of the filament discharge canals (Chessin et al., 1961; Lom, 1964; Desser and Paterson, 1978) in most myxosporidans. Bromophenol blue staining of the cap by Lom (1964) has suggested it to be proteinaceous in nature. Polar plugs or stoppers are present in the anterior (posterior end of the discharge canals) end of the polar filament capsules and are somewhat reminiscent of the plugs of coelenterate nematocysts (Yanagita, 1959; Yanagita and Wada, 1953).

Although differences in the chemicals mediating discharge of polar capsules and nematocysts have been shown in vitro, no evidence exists for chemical stimulation of myxosporidan polar filament extrusion in vivo. Nematocysts have been shown to have a modifiable threshold of response. Satiated coelenterates do not discharge nematocysts when they come into contact with food. Starved organisms can have their threshold of activation for nematocysts lowered by exposure to meat juices and glutathione. Concurrent with this lowered threshold a weaker mechanical stimulus will evoke nematocyst discharge (Meglitsch, 1972). Saturated urea was found by Lom (1964) to produce 100% filament extrusion in the myxosporidan Myxobolus muelleri within two minutes after exposure. The percent extrusion was rapidly decreased with weaker solutions of urea. When spores were stored for an undesignated time, filament extrusion with saturated urea only occurred after several minutes and was not effective for all spores.

Development of Current Myxosporidan Systematics

Bütschli (1881) established the subclass Myxosporidia, now the class Myxosporea (Levine et al., 1980) which encompasses only sporozoa with bivalved spores having polar capsules. Classification of myxosporidans within the subclass Myxosporidia was first done by Thélohan (1892). He also gave a description of pansporoblasts as one of the trophic stages. Morrison (1974) discussed in detail the early taxonomy of the class Sporozoa (later elevated to subphylum) and its relationship to myxosporidan classification. Within the class Sporozoa was found the order Cnidosporidia (Doflein, 1901) which included myxosporidia, microsporidia, and sarcosporidia. Although not adopted, on the basis of myxosporidan multicellularity Ulrich (1950) elevated this group of organisms to a phylum that was placed between the phyla Protozoa and Metazoa.

Use of the term sporozoa became very general which lead to it being considered a subphylum or class depending upon the taxonomist's personal preference (Levine, 1970). Honingberg et al. (1964) divided the subphylum Sporozoa into two subphyla, Sporozoa and Cnidospora on the basis of the former being without polar filaments and not always with spores and the latter having one or more polar filaments per spore. The order Myxosporida was put into the class Myxosporea and grouped with the class Microsporea both in the subphylum Cnidospora.

Sprague (1966, 1969) and Levine (1969 a, b) discussed the taxonomic differences within the subphylum Cnidospora and conclude that the cellularity and polar filaments in the two classes were phylogenetically

different. Sprague (1969) proposed to separate the two classes, Microsporea and Myxosporea from the subphylum Cnidospora and place each class under the subphyla Microspora and Myxospora respectively. Levine (1970) accepted the separation which was also adopted by Noble (1977).

Taxonomists have recently divided living organisms into a five kingdom classification; Monera, Protista, Plantae, Fungi, and Animalia (Leedale, 1974; Whittaker, 1977). Canning and Vávra (1977) state that Myxospora should be considered a separate phylum of the subkingdom Protozoa. Further retention within protistan classification is justified by historical and practical reasons only, until further studies on life cycles give a true phylogenetic system of classification for this group of parasites.

Lom (1977) and Noble (1977) both agreed that myxosporidans should not be considered protozoa or as a separate phylum of protistan organisms due to their multicellularity and division of labor. Revised classification of protozoa considers this group a subkingdom of Protista (Levine et al., 1980). The terms sporozoa and cnidospora (or cnidosporidia) have been deleted from protozoan terminology with members of the latter group assigned to four phyla; Apicomplexa, Microspora, Myxosoa, and Ascetospora (Levine et al., 1980). The two classes, Myxosporea and Actinosporea, were assigned to the phylum Myxozoa (Grassé, 1970; Grassé and Lavette, 1978).

Ultrastructural Studies of Myxosporidan Spores

Scanning electron microscopy has allowed a detailed description of myxosporidans by facilitating better visualization of their

ultrastructure and spore surface patterns for taxonomic and developmental studies. Lom and Hoffman (1971) examined Myxosoma cerebralis and Myxosoma cartilaginis by scanning electron microscopy because of the close resemblance of the two species of myxosporidans. Separation of M. cerebralis from M. cartilaginis is based on the larger size of the latter by light microscopy (Hoffman et al., 1965). Scanning electron microscopy showed M. cerebralis to possess a circumsutural groove, prominent polar filament pores, and a mucous envelope which were all absent in M. cartilaginis. Both M. cartilaginis and M. muelleri possess polar canals with the latter having open canals (polar filament pores) to the environment (Lom, 1964; Lom and Hoffman, 1971). Unikapsula sp. has also been shown to possess polar filament pores due to the absence of an electron-dense substance in the spore wall (Canning and Vavra, 1977).

Hine (1975) described three new species of Myxidium infecting freshwater anguillids in which scanning electron microscopy was done on M. zealandicum. Merging lateral striations and the sutural ridge of this myxosporidan species were the only morphological features visible due to the spore orientation. The sutural ridge was distinctly raised with no visible groove near the ridge. Lateral striation appeared to be at the same approximate height above the spore wall when compared to the sutural ridge.

Morrison (1974) in his scanning electron microscopy study of Sphaeromyxa maiyai revealed several parallel longitudinal grooves on the spore surface extending from one truncated spore terminus to the

other. The sutural line was presented as a groove oblique to the longitudinal striations that resulted in bissecting the spore. No polar filament pores were apparent for S. maiyai. Small depressions were found in the center of the truncated terminus where the discharge canal would be situated and was assumed by the author to be plugged or capped.

Extrusion of the polar filaments of S. maiyai and a Myxobolus sp. from the mottled sanddab Citharichthys sordidus indicated that there is two different structural zones of rigidity found in the filaments for both species. The proximal 3-5 micrometers of the filaments found on both species was rigid while distal ends appeared to be pliable, tending to stick to surrounding objects. Sphaeromyxa maiyai filaments when extruded possessed a small protuberance (possibly the cap) at its emergence from the polar capsule similar to that found for M. muelleri (Lom, 1964). A central groove was found extended from the proximal end of the filament although it was not determined if it extended the entire length of the filament.

Ultrastructural observations by scanning and transmission electron microscopy (Desser and Paterson, 1978) of Myxobolus sp. from the common shiner Notropis cornutus indicated that the spore wall became thickened in the region of the lateral suture that resulted in a groove on either side of the suture line. A mucous envelope was found to be associated with the entire spore but in greatest abundance at the posterior end. Air-drying spores instead of fixation in two percent gluteraldahyde caused a wrinkling of the spores and the mucous envelope was absent.

Scanning electron microscopy indicated the absence of filament pores and the electron-dense cap structure was observed by transmission electron microscopy. Polar filaments appeared as hollow tubes.

Current et al. (1979) showed evidence of a polar cap forming in Henneguya adiposa of channel catfish by transmission electron microscopy. The hollow nature of the polar filaments was also observed. Between the valvogenic cells a desmosomal junction was shown to occur. Similar structures were reported by Current (1977) for Henneguya exilis. A desmosomal-like junction was only described in immature spores which developed at a later stage into a series of valvogenic cell microtubules aligned with the suture border.

Spall (1973) reported on the size variation of the developing polar cap between Myxosoma pharyngeus and Myxosoma cyprini. The former was found to be less than 60 nanometers thick while the latter was 200 nanometers wide. No polar capsule pore was observed like that seen in M. cerebralis. Parallel arranged fibrils were described between each valvogenic cell adjacent to the suture border for M. pharyngeus and M. cyprini. Further development indicated that the fibrils coalesced into an amorphous electron-dense material not of desmosomal nature. The hypothesized solid nature of the polar filaments is opposite to the hollow tube eversion theory proposed by Lom (1964) for extrusion of the polar filaments.

Ceratomyxa shasta possesses an electron-dense plug in the mature polar capsule as described by Yamamoto and Sanders (1979). Cross-sections of mature polar filaments indicate that the filaments consist

of a series of concentric rings suggesting the filaments are not of tubule structure. No polar filament discharge canal or electron-dense cap was observed by the authors or Gould (1969). A desmosomal-like junction was reported for C. shasta to occur between the two valves of the spore. The relationship of the desmosomal-like structures found between the spore valves and the cement-like substance that separated the shell valve of Myxobolus uniporus and Myxobolus carassi as reported by Cheissin et al. (1961) is unknown.

Myxosporidan Life Cycles and Transmission

Transmission of myxosporidans from host to host has traditionally been thought to occur by the infectious spore with the route of infection being oral (Plehn, 1904; Kudo, 1930, 1966; Schaperclaus, 1954; Hoffman et al., 1969; Halliday, 1976). Several reports have claimed transmission of myxosporidans by feeding of spores to fish. Auerbach (1909) showed that Myxidium bergense could be transmitted to Gadus virens by feeding of spores. Chloromyxum leydigi spores placed in gelatin capsules were fed to fish in which Erdmann (1911) claimed a 67% infection rate. Shiba (1934) reported similar results for oral ingestion of myxosporidan spores. Hahn (1917) found that Myxobolus musculi was transmittable to experimental fish in all stages of its life history. While transmission was done in the laboratory none of the fish utilized in these experiments were shown to be free of the parasites.

Bond (1939) and Uspenskaya (1963) reported laboratory transmission of myxosporidans by oral ingestion of fresh or aged spores, respectively,

by non-infected fish. The former work has come into question since numerous investigators have attempted to repeat this experiment without success (Hoffman and Putz, 1968; Schafer, 1968; Fryer, 1971; Spall, 1973; Walliker, 1968).

Experimental transmission has been worked out for M. cerebralis and C. shasta when young salmonids were exposed to water or sediments containing the infectious stage (Schaperclause, 1931; Putz and Hoffman, 1966; Hoffman and Putz, 1968; Schafer, 1968; Hoffman and Putz, 1969; Fryer, 1971; Halliday, 1973, 1974; Johnson, 1975).

Hoffman and Putz (1969, 1971) showed that M. cerebralis spores taken directly from parasitized fish were not infectious to the host, but when aged three to six months in mud the infectious stage was present and transmittable to fish. Putz (1970) and Putz and Herman (1970) were able to confirm the "aging" process. Similar experiments with C. shasta did not result in transmission of the parasite (Schafer, 1968; Fryer, 1971; Johnson, 1975; Johnson, 1980).

Tentative evidence indicates that the portal of entry for myxosporidans may not be oral. Putz and Hoffman (1966) successfully transmitted M. cerebralis to prefeeding sac fry trout by exposing them to water containing the infective stage of the parasite. Transmission of the disease in eggs does not occur (O'Grodnick and Gustafson, 1973; O'Grodnick, 1975). Schafer (1968) demonstrated that establishment of C. shasta infection in trout was not dependent upon ingestion of food organisms.

Hoffman (1976) and Daniels et al. (1976) reported the presence of an intracellular protozoan in the epithelium of rainbow trout exposed to the infective stage of M. cerebralis. The relationship of this unidentified protozoan to M. cerebralis has not been resolved but was not present in unexposed trout. Overstreet (1976) has found the digenetic trematode Crassicutis archasargi from an estuarine fish to be parasitized by the myxosporidan Fabespora vermicola. A Myxobolus sp. from an annelid and Chloromyxum diploxys from an insect (Tortrix viridana) were listed by Kudo (1920). Both the annelid and insect are associated with an aquatic environment.

Schafer (1968) was unable to transmit C. shasta to uninfected trout by force feeding infected viscera containing trophozoites and spores. Intraperitoneal injection of ascitic fluid containing trophozoites and spores obtained from infected fish would transmit the disease to uninfected fish (Schafer, 1968; Johnson, 1975). Myxosoma pharynegus spores and sporogenic stages injected intramuscularly or intraperitoneally into uninfected mosquito fish Gambusia affinis was negative for parasite transmission. Wagh (1961) successfully transplanted Myxosoma ovalis from small buffalo (Ictiobus bubalus) to golden shiner (Notemigonus crysoleucas) by intramuscular injection. It could not be transplanted on the gills or in the alimentary canal of the shiner.

Spall (1977) and Current (1973) examined the possibility of aquatic insects and invertebrates as intermediate hosts. No transmission was achieved when invertebrates and insects, shown to have ingested myxosporidan spores, were fed to susceptible fish. Taylor and Lott

(1978) were able to transmit M. cerebralis to rainbow trout by feeding infected fish to aquatic birds and subsequently exposing uninfected fish to the parasite containing feces. Fish exposed to water from troughs containing mud became infected while in the absence of mud the fish were negative for the parasite upon examination. The authors clearly indicate that fish eating birds can serve as mechanical vectors of the spores and that a maturation period in a mud substrate is required to transmit M. cerebralis. Schaperclaus (1954) and Mitchell (1970) showed that no morphological changes occurred when myxosporidan spores were passed through the intestinal tracts of piscivorous birds.

In vitro Cultivation of Myxosporidans

Lom (1975) reported on a method to store myxosporidan spores in sealed capillary tubes at 4°C with the aid of antibiotics. This method prevents structural changes that occur during fixation or dessication in order to facilitate more accurate taxonomic studies of collected parasites that cannot be examined as fresh material. It was stated that spores would stay unchanged and live for months. Microsamples of Kudoa sp. spores obtained from milky halibut were exposed to cycling temperatures between 21.1°C to 4.4°C for several days, followed by freezing at -17.8°C and very slowly returned to room temperature in approximately an eight hour period by Patashnik and Groninger (1964). This resulted in spore replacement by multiplicative forms undergoing growth, binary fission, budding, and multiple fission. Wolf and Markiw (1976) were able to sporulate in vitro the trophozoites and pre-spore

stages of M. cerebralis in various growth media. Successful removal of sporoplasms from Myxobolus exiguus spores have been shown to develop sporoblasts when cultured in tissue culture medium in the presence or absence of cultured trout cells (Siau, 1977).

MATERIALS AND METHODS

Ceratomyxa shasta Spore Source

Spores were obtained by exposing steelhead trout free of *C. shasta* in a live-box for 48 h in the Willamette River downstream from Corvallis, Oregon. Prior to exposure, the fish were held in 189-liter fiberglass tanks with aerated, flowing (4 liters/min) dechlorinated tap water at 15 ± 2°C. Sulfamethazine and sulfamerazine was administered *per os* with food at a rate of 15 g per 454 kg of fish per day to reduce bacterial infections during their subsequent exposure in the river (Wood, 1974). After exposure, fish were removed from the river and held in the laboratory.

Ceratomyxa shasta Spore Collection

Spores were collected within 24 h after death of the fish. Digestive tracts (stomach, pyloric ceacae, and intestine) were dissected from fish and slit along the longitudinal axis to expose the internal portion. Tissue was then placed in screw-capped culture tubes containing 5 ml of Cortland's salt solution (124.0 mM NaCl, 1.56 mM $CaCl_2 \cdot 2H_2O$, 5.1 mM KCl, 2.97 mM $NaH_2PO_4 \cdot H_2O$, 11.9 mM $NaHCO_3$, 0.93 mM $MgSO_4 \cdot 7H_2O$, 5.55 mM Glucose, pH 7.2) (Wolf, 1963). Tubes were shaken by hand for 1 min to release spores and then the tissue was discarded. Two ml of saline-spore suspension was layered onto 5 ml of 55% (w/v) aqueous dextrose in a 10 ml conical centrifuge tube and centrifuged at 1,200 x g for 30 min, 20°C (Markiw and Wolf, 1974). The aqueous phase was removed from the spore pellet followed by addition of 1 ml

Cortland's salt solution containing 100 µg/ml of gentamicin (Schering Corporation). The pellet was resuspended by trituration with a Pasteur pipette. Concentrated spore suspensions were transferred to a 100 ml graduated cylinder and stored at 4°C for subsequent use.

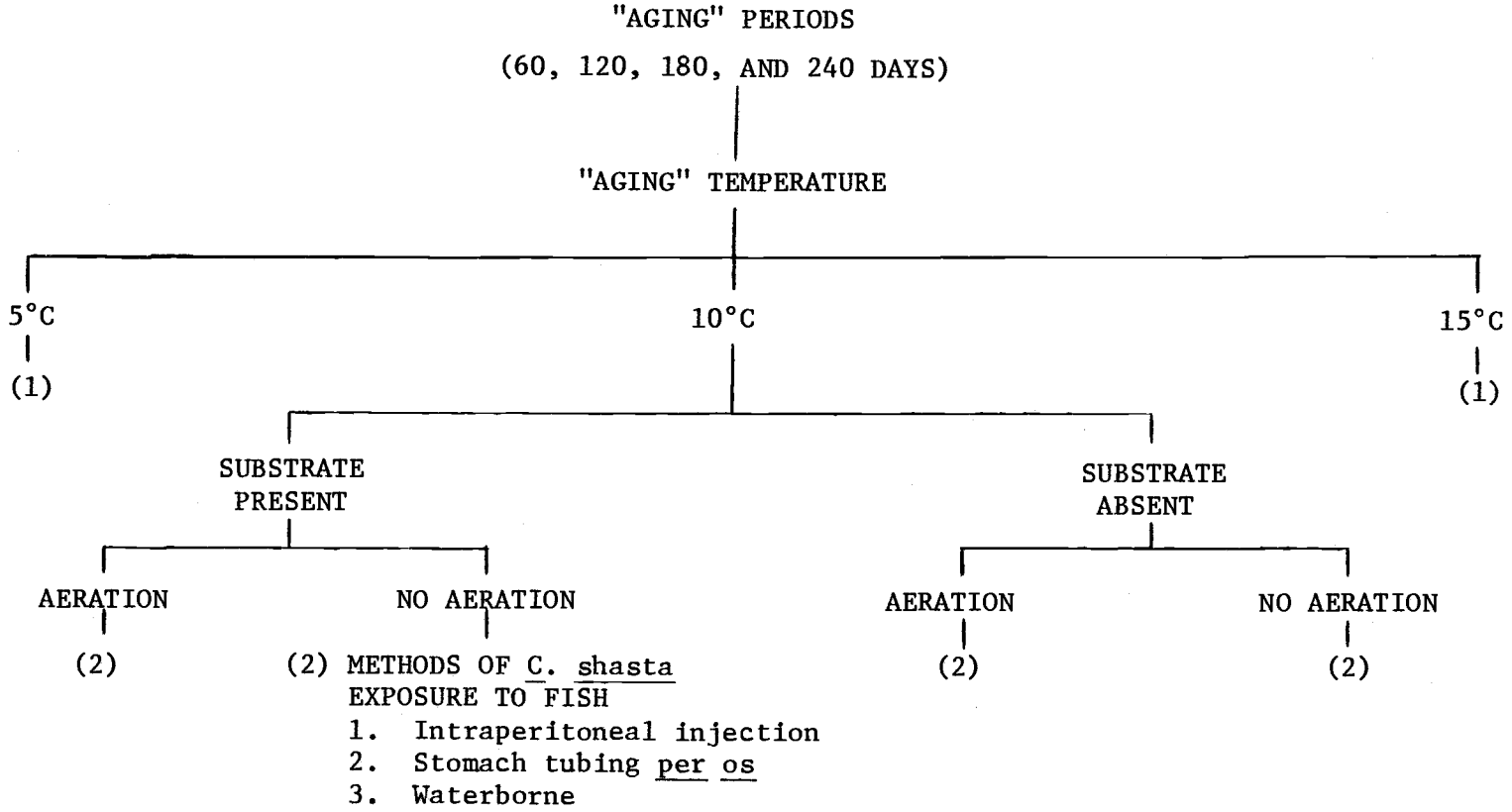
Ceratomyxa shasta Spore Aging

Spores were held for 24 h or less before counting with a hemocytometer and inoculating culture vessels with a final concentration of 0.25×10^6 spores/ml. Culture vessels were 100 ml glass test tubes containing 50 ml Cortland's salt solution with 100 µg/ml gentamicin and 0.002% phenol red. "Aging" time was divided into four periods of 60, 120, 180, and 240 days (Fig. 1). Presence or absence of sand substrate and aeration as "aging" factors were tested at 5, 10, and 15°C. Sand substrate was a commercial white sand washed several times in distilled water and autoclaved 20 min at 121°C, 15 psi.

Source of Fish

Steelhead trout (1 g) were obtained from the Alsea Trout Hatchery (Oregon Department of Fish and Wildlife). Two thousand rainbow trout (0.75 g) were acquired from Roaring River Fish Hatchery (Oregon Department of Fish and Wildlife). Rainbow trout are known to be susceptible to parasitization by C. shasta (Udey et al., 1975) and this was confirmed by exposing 20 fish, in the same manner as described above for steelhead trout. The rainbow trout became heavily infected.

Figure 1. Experimental design for "aging" Ceratomyxa shasta spores. (1) Same experimental design for 5° and 15°C as illustrated for 10°C. (2) Spores incubated by the four different combinations of substrate and aeration for the three "aging" temperatures and four "aging" periods were exposed to fish by these three methods.



Experimental Infections

All rainbow trout were acclimated to 15 ± 2°C before use in the tests. After exposure to "aged" spores, fish were divided into three groups corresponding to the three incubation temperatures and held in 187-liter fiberglass tanks at 15 ± 2°C flowing dechlorinated water (4 liters/min). Fish were fed a commercial (Ore Aqua) moist fish food daily. Before exposure to fish, all C. shasta cultures were removed from their respective aging temperatures and held at 20°C for one week. Fish were exposed to each of the 48 cultures by three methods yielding 144 different test groups. Two fish were exposed per os with a stomach tube to a 0.5 ml spore suspension (0.65 to 1.35 X 10^6 spores/ml) concentrated by centrifugation at 1,200 x g for 10 min, 20°C. Another two fish were injected intraperitoneally with 0.5 ml spore suspension at the same spore concentration. The third method involved exposure of six fish for four days in 16-liters 15°C dechlorinated water containing 80 ml unconcentrated spore-saline solution to give a final exposure concentration of 33 to 675 spores/ml. All fish were examined 50 days postexposure for the presence of C. shasta. Twenty fish were held under the same conditions without spore addition.

Scanning Electron Microscopy (S.E.M.)

Fresh spores which had been centrifuged in 55% (w/v) dextrose or spores from the cultures that were not aerated and without substrate were used. Spores were cleaned by suspending them in distilled water and then centrifuging them three times at low speed (500 x g) for 5 min in a clinical centrifuge. Concentrated spore suspensions were placed

on 15 mm micro-cover glasses with Pasteur pipets. Specimens were then frozen in liquid nitrogen and dried through critical point in a Vir Tis 10-145 MR-BA Freeze-Mobile Freeze-dryer. Micro-cover glasses were then affixed to S.E.M. specimen mounts with conductive silver paint and coated with a thin film (approximately 20 nm thick) of 60/40 gold/ palladium alloy in a Varian VE-10 UPC Evaporator at 1.0×10^{-5} Torr. Specimens were then examined with an International Scientific Instrument's (I.S.I.) Mini-S.E.M. MSM-2 at 15 Kv accelerating potential and 100 μamps beam current. All electron micrographs of fresh and "aged" spores are representative of twenty-five spores viewed by S.E.M. unless otherwise indicated.

Ceratomyxa shasta spore measurements were made directly from scanning electron micrographs. Ten spores were measured from each group including fresh spores and those from each aging period at the three different incubation temperatures. Length was measured between the most distal portions of the spore lateral margins. Measurement along the sutural border from anterior to posterior represented the width. Average widths and length was calculated for each group of spores and the ranges determined. The diameter of ten polar cap plugs were measured from ten different spores "aged" 180 or 240 days and the thickness of the spore wall at the sutural ridge at the lateral and posterior or anterior regions of five unaged ruptured spores were measured. The averages and ranges of these measurements were determined.

Effect of Potassium Hydroxide, Methylene Blue and pH on *C. shasta* Spores

One test involving various concentrations of potassium hydroxide (KOH), was done to determine if the sporoplasm could be artificially released without damage. The percentages of ruptured spores (i.e. separation of the spore valves at the sutural border) was determined microscopically for final concentrations of 0.50, 0.25, 0.20, 0.15, 0.10, and 0.05% KOH. One ml of spore suspension was added to a three ml test tube followed by one ml KOH and agitated with a Pasteur pipet. Two hundred spores were examined 5 min after addition of KOH.

Five separate spore counts were done for each KOH test concentration. Fresh spores were studied after purification in 55% (w/v) dextrose and maintained at 4°C before testing. Spore contrast was enhanced by adding one drop of 0.3% (w/v) methylene blue to the microscope slide before addition of two drops of the exposed spores. Final concentration of methylene blue was approximately 0.1% (w/v).

The effect of holding spores in Cortland's saline solution at 4°C for short periods of time (0, 12, 24, 36 and 48 h) upon the minimum concentration of KOH required to rupture at least 90% of the spores was studied. Spores from each time period were exposed to similar percentages of KOH as in the previous experiment and treated in the same manner. Four separate spore counts were done for each concentration of KOH tested within a given holding period.

The percentages of spores that ruptured at the following concentrations of KOH with time was determined by exposing spores to 0.175% KOH for 1, 3, 5, and 7 min and 0.1% KOH for 1, 2, 3, 4, and 5 min.

Each experiment represented counts of 200 spores each. Five different groups of 200 spores were tested. Methylene blue was used to enhance spore morphology.

Spores were exposed to a pH range from 2 to 12 in one unit intervals to observe the effect of pH on spore rupture. Britton and Robinson Type Universal Buffer Solution (KH_2PO_4, $C_6H_8O_7$, H_3BO_3, $C_8H_{12}N_2O_3$, NaOH) (Dawson et al., 1975) was used for the variable pH source. Two hundred spores were treated and examined for each pH unit. The experiment was repeated four separate times with different groups of spores.

Use of methylene blue staining to determine spore "viability" was tested against the traditional "viability" method of exposing spores to KOH and observing rupture. One group of 100 spores were exposed to a final concentration of 0.1% (w/v) methylene blue and a second group of 100 spores were treated with KOH at a final concentration of 0.5%. Each experiment was repeated five times and the number of unstained spores was compared to the number of KOH ruptured spores.

RESULTS

Exposure of Rainbow Trout to "Aged" *Ceratomyxa shasta* Spores

Ceratomyxa shasta was not found in fish exposed to spores "aged" for 60 days. Spore viability tests with 1% KOH at the time of the experimental exposure indicated that all culture contained "live" parasites. Similarly, spores "aged" 120 days were found viable but not infective when exposed to fish.

All cultures "aged" 180 days contained viable spores. Fish exposed to these spores did not contain parasites but some showed signs of the disease, darkening of the body, exopthalmia, listlessness, and loss of appetite. Two of these were exposed to the parasite in 16-liters of water, that had been "aged" 180 days at 5°C with aeration and no substrate. They showed initial signs of listlessness and loss of appetite 40 days postexposure. Darkening of the body and exopthalmia appeared after the initial signs. Casts of epithelium and mucosa were not found in the holding tank. Peritoneal dropsy was not observed and upon examination the fish contained no ascitic fluid or any stages of *C. shasta*. The same signs were observed in one fish exposed *per os* to spores incubated at 5°C in substrate without aeration and two other fish exposed by the same method to spores incubated at 5°C with no substrate or aeration. All fish showing signs of the disease and controls were negative for *C. shasta*.

Control fish treated identically as the *C. shasta* treated fish exposed to 180 day "aged" spores did not exhibit any of the described

signs. Both control and challenged fish of the 180 day spore aging experiment were negative upon examination for other parasites and bacteria. Spores "aged" for 240 days were found viable but not infective upon exposure to fish.

Scanning Electron Microscopy (S.E.M.) of *Ceratomyxa shasta* Spores

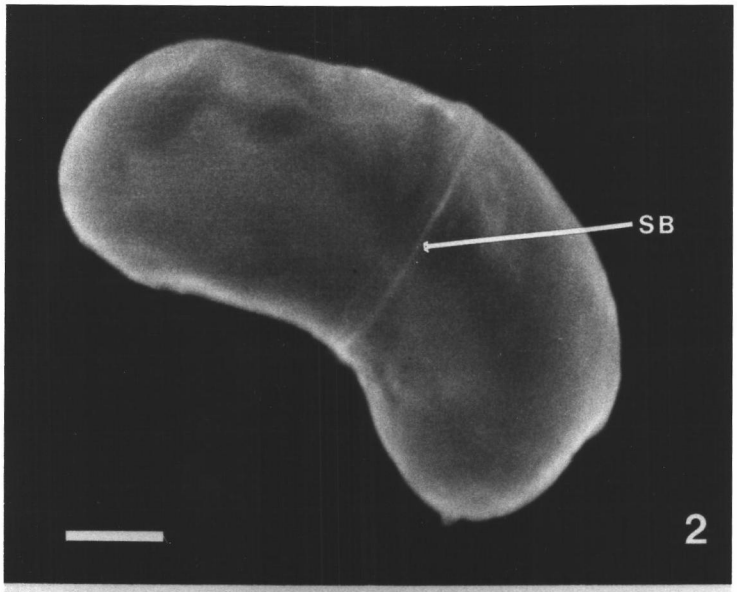
Spores taken from exposed steelhead trout and viewed by scanning electron microscopy appear amorphous with the only discernable structure being the raised sutural border composed of two ridges, one for each spore valve (Fig. 2). No discharge canals (pore) for the polar filaments or polar caps over the pore were evident. Examination by scanning electron microscopy indicated there was no mucous envelope. Shrinkage of spores was present at the anterior and posterior margins (Fig. 3). A lateral ridge along the spore wall was also visible. The marginal shrinkage and ridge development is due to improper fixation of the spores which occurred with different spore preparations and is thought to occur from dessication. When extreme spore disfigurement was observed, the spore preparation was repeated.

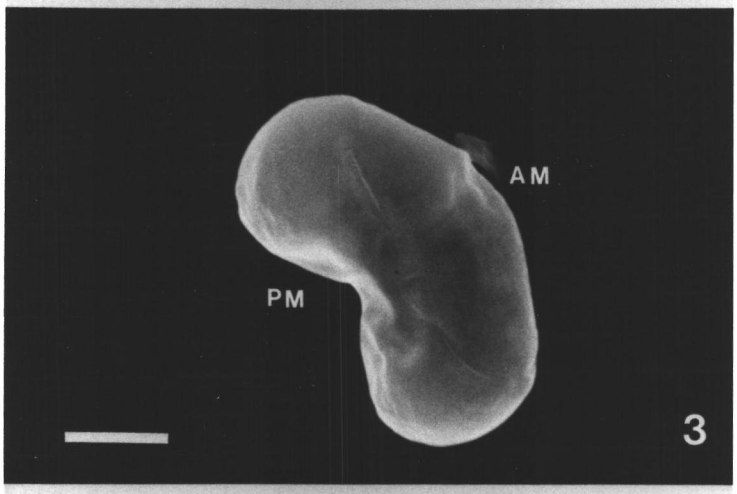
Sixty day old spores "aged" at 5° and 15°C showed two new changes in their morphology. The sutural border extending around the circumference of the spore from anterior to posterior in the median position had become more distinct (Figs. 4 and 5). A prominent groove was observed between the two sutural ridges where the valves separate. Both micrographs show circular raised areas that represent the polar caps found directly over the discharge canal. Spores "aged" for 60

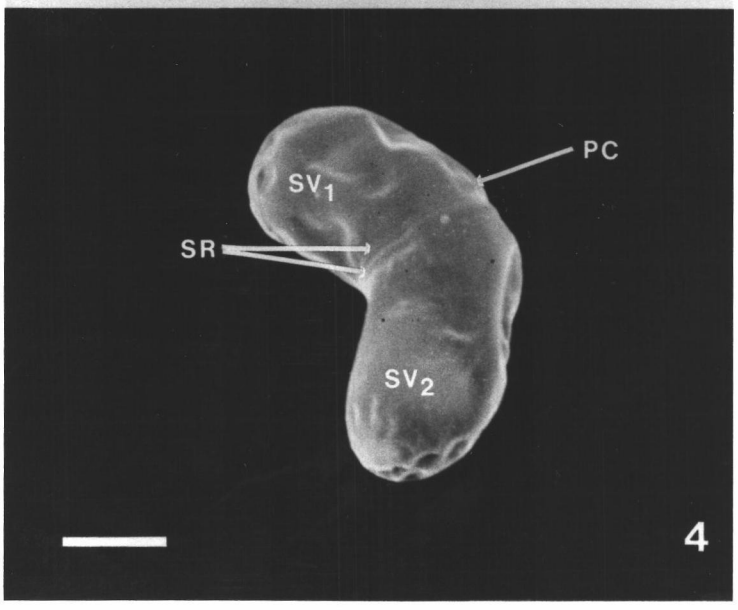
Figure 2. Raised sutural border encircling a fresh Ceratomyxa shasta spore, (x7,000). Sutural Border, SB. Bar = 2 μm.

Figure 3. Fresh Ceratomyxa shasta spore exhibiting spore wall shrinkage due to fixation, (x5,000). Anterior Margin, AM; Posterior Margin, PM. Bar = 3 μm.

Figure 4. Ceratomyxa shasta spore "aged" 60 days, 5°C, showing developed sutural ridge on each spore valve and polar cap, (x5,000). Sutural Ridges, SR; Polar Cap, PC; Spore Valves, SV_1 and SV_2. Bar = 3 μm.


SB
2


AM
PM
3


PC
SV1
SR
SV2
4

days at 10°C showed extreme disfigurement from fixation which was not observed for spores incubated at 5° and 15°C.

Delineation of the polar caps and its circular form can be seen in spores "aged" 120 days at 5°C (Fig. 6). A ridge appears to have formed around the protruding cap. In only one spore was it possible to see a polar filament discharge canal or pore (Fig. 7). This was a spore "aged" 120 days at 5°C. Surrounding the spore can be observed a ridge formed from the spore valve. Depressed polar caps were observed in spores "aged" for 120 days at 10°C (Fig. 8). Aging of spores for 120 days at 15°C (Fig. 9) show no change in morphology when compared to spores "aged" for the same length of time at 10°C.

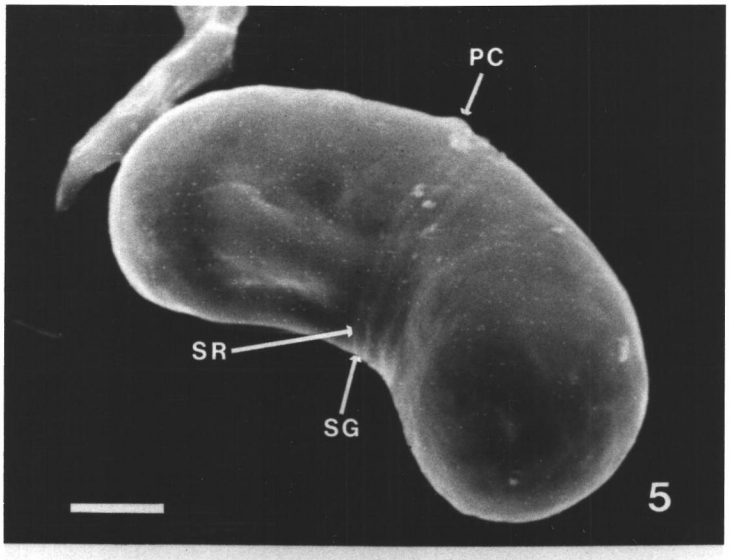
Spores "aged" for 180 days at 5 (Fig. 10) and 10°C (Fig. 11) again show the same morphological structures of the sutural borders and raised polar caps which now lack the polar cap ridges. The presence of two dark bands, or outer sutural grooves, one on each side of the sutural border (Figs. 10, 11, and 12) was not observed in spores "aged" less than 180 days. Anterior to posterior orientation of the outer sutural groove next to the sutural ridge on each spore valve with the inner sutural groove dividing the valves was clearly delineated for spores "aged" 180 days, 10°C (Fig. 13).

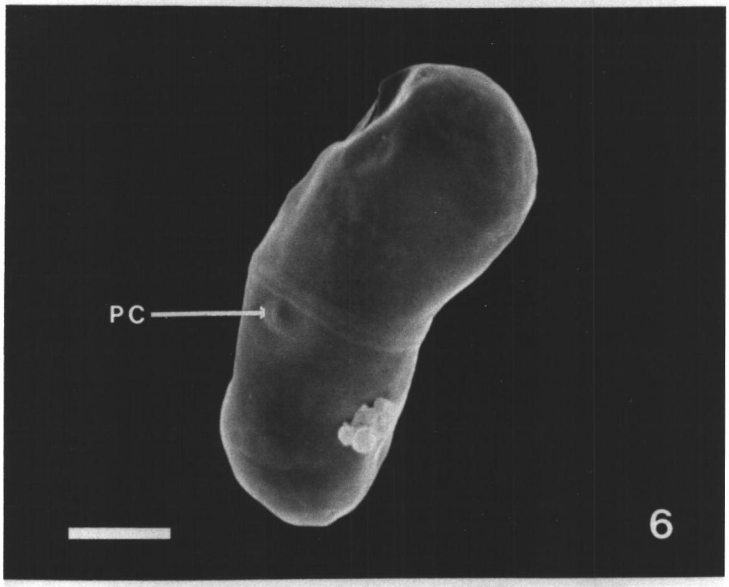
"Aged" spores for 180 days at 15°C (Fig. 14) indicate that both the sutural border region and the ridge around the polar caps have sunken to form grooves. This was not seen on other "aged" spores until this group including spores "aged" for the same time period at

Figure 5. Development of distinct ridges on each valve resulting in a defined groove at the sutural border. Delineation of one polar cap can be observed for the Ceratomyxa shasta spore "aged" 60 days, 15°C, (x7,000). Sutural Ridge, SR; Sutural Groove, SG; Polar Cap, PC. Bar = 2 μm.

Figure 6. Demarcation of the circular polar cap in a Ceratomyxa shasta spore "aged" 120 days, 5°C, (x5,000). Polar Cap, PC. Bar = 3 μm.

Figure 7. Ceratomyxa shasta spore "aged" 120 days, 5°C, (x5,000) with an open polar filament discharge pore surrounded by a ridge. Polar filament discharge pore, P. Bar = 3 μm.


PC
SR
SG
5


PC
6

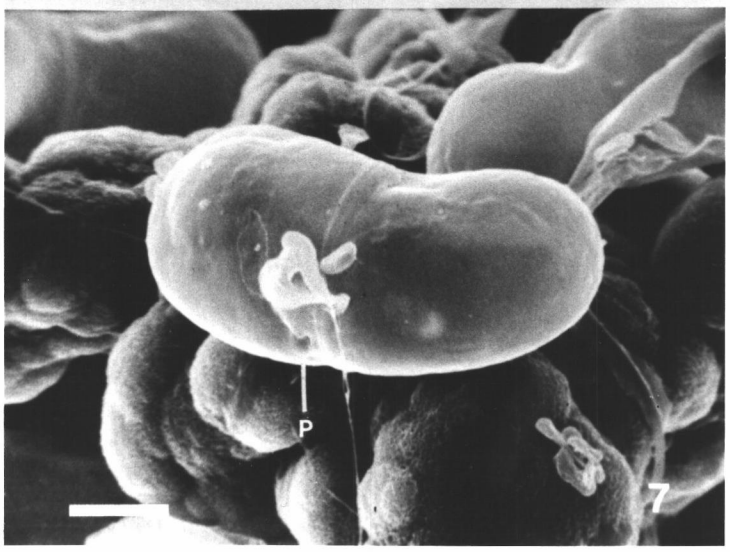
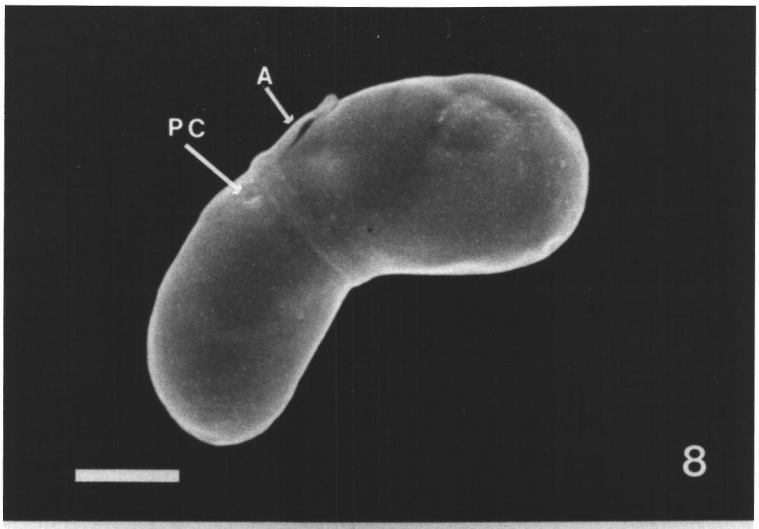
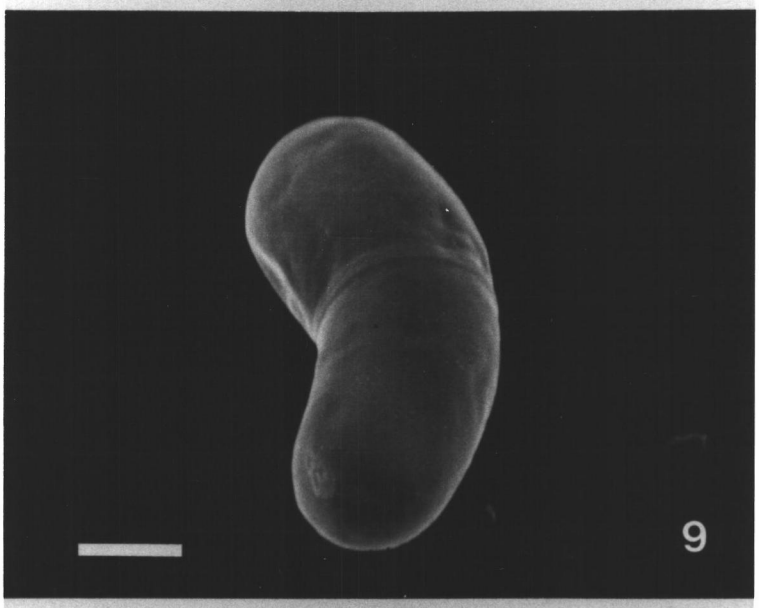

P
7

Figure 8. Ceratomyxa shasta spore "aged" 120 days, 10°C with spore anomaly branching from sutural line along the anterior edge, (x5,000). Anomaly, A; Polar Cap, PC. Bar = 3 µm.

Figure 9. Ceratomyxa shasta spore "aged" 120 days, 15°C, (x5,000). Bar = 3 µm.

Figure 10. Development of the outer sutural groove in Ceratomyxa shasta spore "aged" 180 days, 5°C, (x5,000). Polar Cap, PC; Outer Sutural Groove, OSG; Inner Sutural Groove, ISG. Bar = 3 µm.


A
PC
8


9

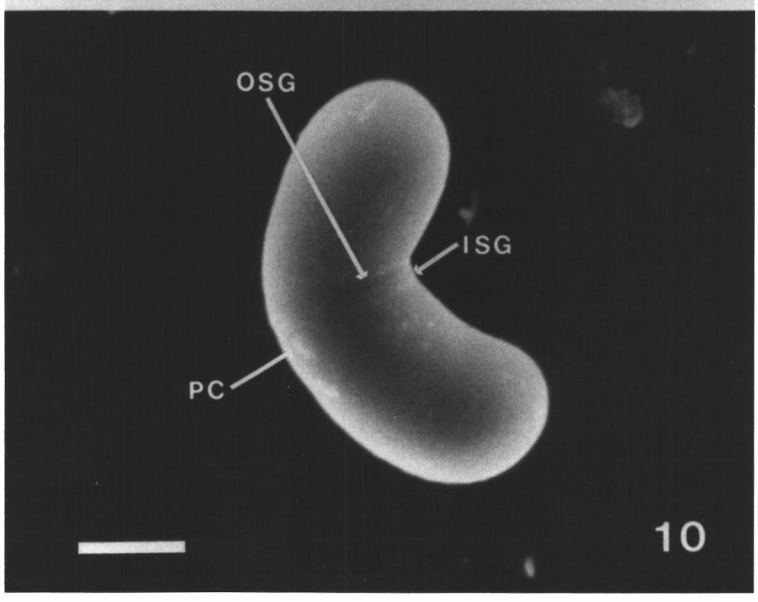
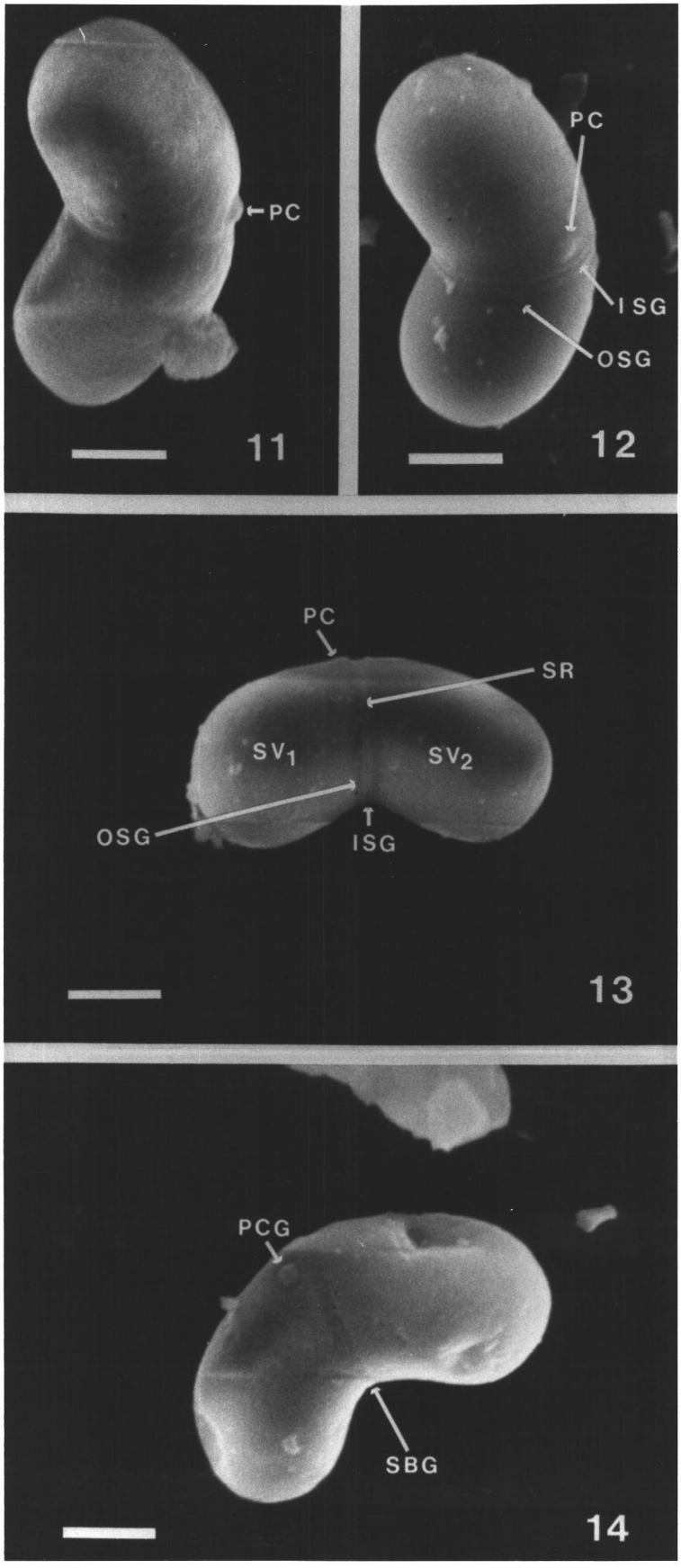

OSG
ISG
PC
10

Figure 11. Ceratomyxa shasta spore "aged" 180 days, 10°C, (x5,000) with prominent polar cap. Polar Cap, PC. Bar = 3 µm.

Figure 12. Outer sutural groove of Ceratomyxa shasta spore "aged" 180 days, 10°C, (x5,000). Outer Sutural Groove, OSG; Inner Sutural Groove, ISG; Polar Cap, PC. Bar = 3 µm.

Figure 13. Development of outer sutural grooves in a Ceratomyxa shasta spore "aged" 180 days, 10°C, (x5,000). Outer Sutural Groove, OSG; Inner Sutural Groove, ISG; Sutural Ridge, SR; Spore Valves, SV_1 and SV_2; Polar Cap, PC. Bar = 3 µm.

Figure 14. Depression of the polar cap ridge and sutural ridge on a Ceratomyxa shasta spore "aged" 180 days, 15°C, (x5,000). Sutural Border Groove, SBG; Polar Cap Groove, PCG. Bar = 3 µm.


PC
11
PC
ISG
OSG
12
PC
SR
SV1
SV2
OSG
ISG
13
PCG
SBG
14

different temperatures. The sutural ridges are still evident in this groove. The majority of spores observed exhibited this type of morphology.

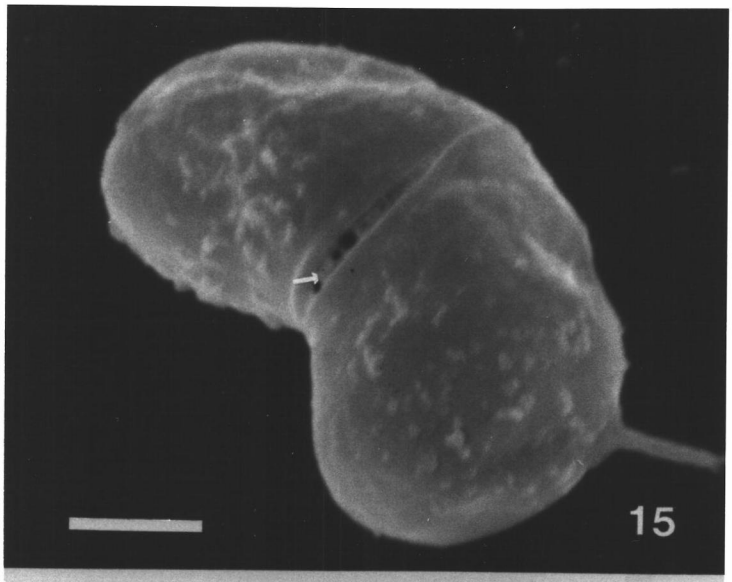
Only one spore "aged" 240 days at 15°C (Fig. 15) indicates that an internal cement-like substance holds the two spore valves together. Morphology of the spore with the distinct sutural ridges, and absence of the large sutural groove that was observed in spores "aged" 180 days at 15°C suggests that this spore was not viable at 180 days. Other spores "aged" 240 days at 15°C (not shown) and 10°C (Fig. 16) show that the groove encompassing the sutural region has become narrower and deeper displacing the sutural ridges and outer sutural grooves. Spores "aged" 240 days at 5°C (Fig. 17) have not undergone the morphological changes seen for spores held the same period at 15° and 10°C. These spores still possess the outer sutural grooves and cap grooves.

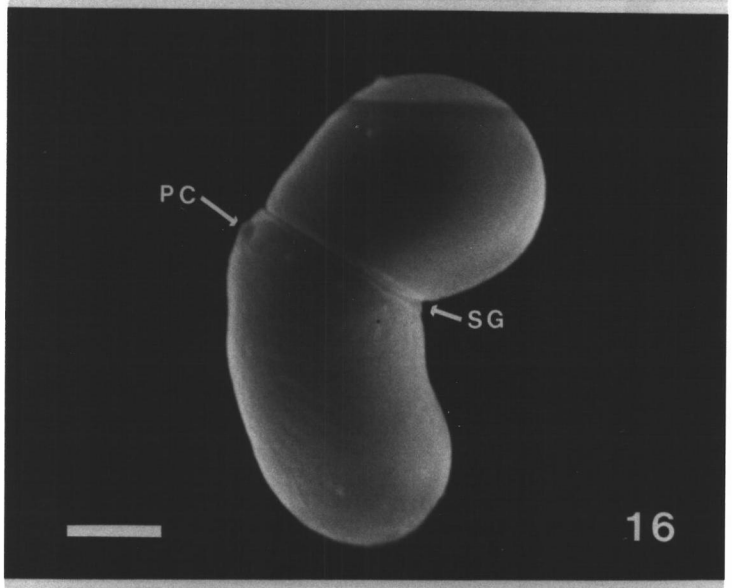
Morphological spore anomallies were not common. One spore "aged" for 120 days at 10°C showed an anomally (Fig. 8). Part of the spore wall apparently branches from the sutural border and extends laterally along the anterior side. The significance of this spore variation cannot be ascertained since only one spore exhibited any wall abnormality for all the fresh and "aged" spores observed. Possible depression of the polar cap may be present in the left valve. One spore "aged" 240 days at 10°C (Fig. 16) was observed to be asymmetrical with one valve shorter in lateral length than the other valve. This was a rare observation, since the spores have symmetrical valves.

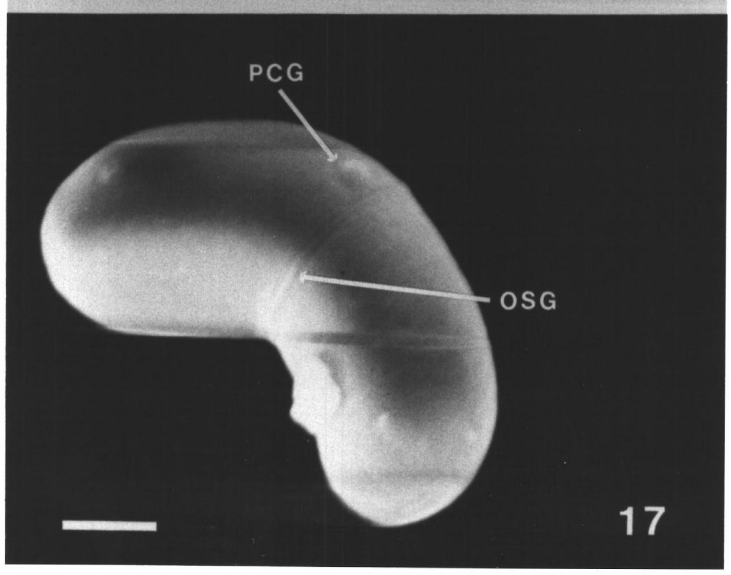
Figure 15. Internal cement-like substance (arrow) holding the two spore valves together in a Ceratomyxa shasta spore "aged" 240 days, 15°C, (x10,000). Bar = 2 µm.

Figure 16. Asymmetrical Ceratomyxa shasta spore with a narrow, deep sutural groove displacing the two outer sutural grooves and ridges "aged" 240 days, 10°C, (x7,000). Sutural Groove, SG; Polar Cap, PC. Bar = 2 µm.

Figure 17. Ceratomyxa shasta spore "aged" 240 days, 5°C, (x7,000). Outer Sutural Groove, OSG; Polar Cap Groove, PCG. Bar = 2 µm.


15


PC
SG
16


PCG
OSG
17

Dimensions of fresh and all groups of "aged" C. shasta spores are shown in Table 1. Fresh spores had dimensions of: length, 12.6(11.6-13.7)µm; width 5.2(3.9-6.0)µm. Spores "aged" 60 days at 5° and 15°C showed similar length and width measurements as that of fresh spores. "Aged" spores for the same period of time at 10°C were not measured due to improper fixation. Aging of spores for 120 days at 5° and 10°C indicates that the respective lengths of 13.9(13.0-14.6)µm and 14.4 (11.8-14.8)µm are greater than lengths recorded for fresh and 60 day "aged" spores. Length measurements for spores "aged" 120 days at 15°C were similar to fresh and all 60 day "aged" spores. Measurements of widths on spores "aged" for 120 days at 5°, 10°, and 15°C were similar to those of fresh and 60 day "aged" spores.

Aging of spores for 180 days at 5°, 10°, and 15°C resulted in the respective decreases of both length and width dimensions as follows: length, 12.0(11.4-12.6)µm and width, 4.8(4.4-5.6)µm; length, 11.9(10.7-12.6)µm and width, 4.9(4.0-5.6)µm; length, 12.0(11.3-12.6)µm and width, 5.0(4.2-5.9)µm. A further decrease in length and width measurements for all groups of spores "aged" 240 days was observed, although it was greater for spores incubated at 10° and 15°C than at 5°C. Spores incubated at 5°C had a length of 11.0(10.2-11.4)µm and width of 4.6(4.0-5.5)µm. Spores incubated at 10° and 15°C had dimensions of: length 10.0(9.6-10.4)µm; width, 4.3(3.9-5.4)µm and length of 9.9(9.6-10.3)µm; width of 4.1(3.7-5.1)µm, respectively.

Table 1. Morphometric measurements of fresh and "aged" Ceratomyxa shasta spores. Average dimensions represents measurements of ten different spores from scanning electron micrographs for each group examined.

Treatment of spores (fresh or "aged")	Measurements in microns			
	Average Spore Length	Range	Average Spore Width	Range
Fresh Spores	12.6	11.6-13.7	5.2	3.9-6.0
"Aged" Spores				
60 Days, 5°C	12.0	11.0-12.2	5.6	5.1-5.8
60 Days, 15°C	12.1	11.8-12.6	5.4	5.0-5.7
120 Days, 5°C	13.9	13.0-14.6	5.3	4.0-5.7
120 Days, 10°C	14.4	11.8-14.8	5.3	4.0-5.7
120 Days, 15°C	12.6	11.9-13.0	5.4	4.4-5.9
180 Days, 5°C	12.0	11.4-12.6	4.8	4.4-5.6
180 Days, 10°C	11.9	10.7-12.6	4.9	4.0-5.6
180 Days, 15°C	12.0	11.3-12.6	5.0	4.2-5.9
240 Days, 5°C	11.0	10.2-11.4	4.6	4.0-5.5
240 Days, 10°C	10.0	9.6-10.4	4.3	3.9-5.4
240 Days, 15°C	9.9	9.6-10.3	4.1	3.7-5.1

Spores "aged" 180 and 240 days had polar cap plugs with diameters of 0.72(0.50-0.80)μm. No difference in plug diameter range was observed between the two different aging periods. Polar cap diameter was not measured on spores "aged" 60 and 120 days due to the inability to clearly delineate the entire plugs.

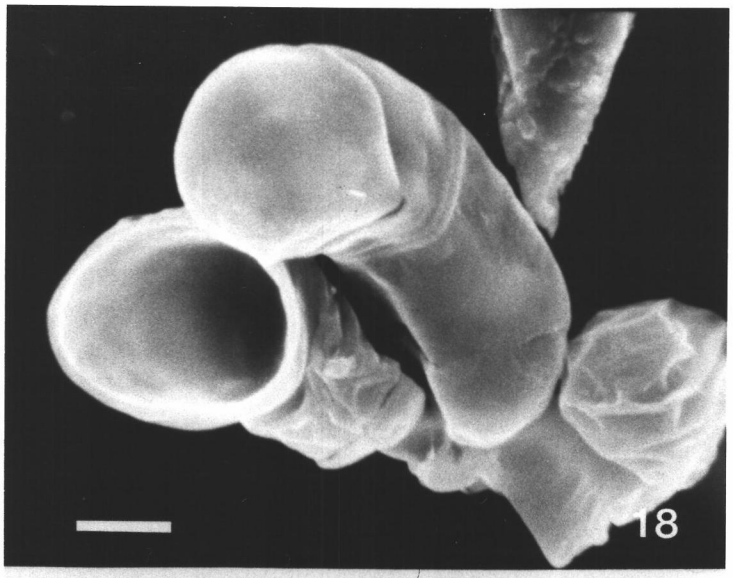
Measurement of the wall thickness at the sutural ridge of unaged spores showed that the lateral margins had a dimension of 0.43(0.36-0.50)μm and the posterior margins were 0.29(0.22-0.36)μm. Posterior and anterior margins were found to have the same thickness.

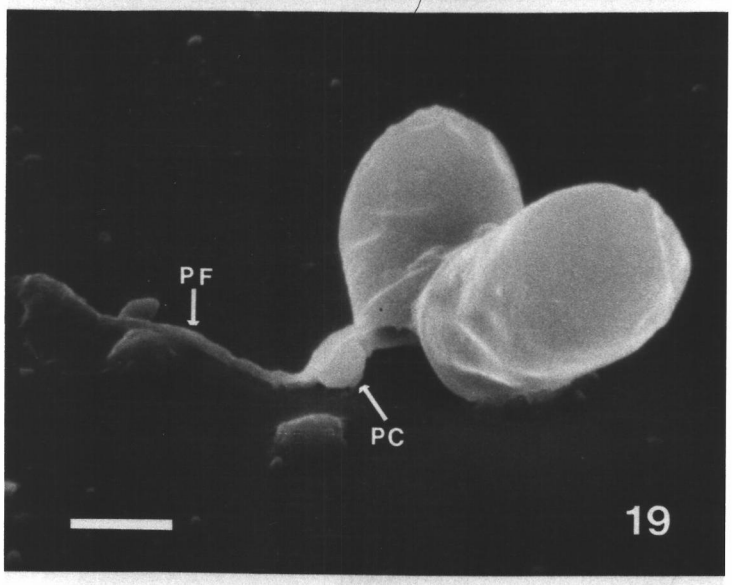
The internal wall structure of a fresh spore valve appears smooth and amorphous (Fig. 18) like the external surface. No evidence of the polar cap was observed on the inner side of the spore valve correlating with its absence on intact fresh spores. Partial detachment of the two spore valves has shown that some internal cement-like substance appears to have a function in maintaining the integrity of the spore prior to maturation (Figs. 19 and 20). Ruptured fresh spores show the polar capsule embedded in the cement-like substance with partial extrusion of the polar filaments (Figs. 19 and 20). The polar filament did not extend through the polar canal. Few ruptured spores were observed in this condition. Exposure of fresh spores to KOH resulted in destruction of the internal sporoplasm and polar filament. Such spores also showed no definite evidence of polar canals or caps.

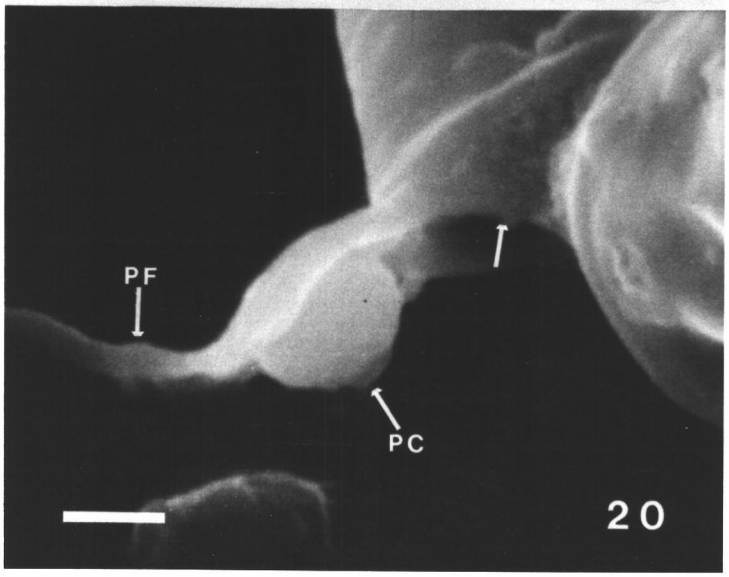
Figure 18. Internal spore wall structure of a fresh Ceratomyxa shasta spore, (x7,000). Bar = 2 µm.

Figure 19. Fresh Ceratomyxa shasta spore with partially extruded polar filament and polar capsule embedded in cement-like substance (arrow), (x5,000). Polar Capsule, PC; Polar Filament, PF. Bar = 3 µm.

Figure 20. Polar capsule and extruded polar filament of a fresh Ceratomyxa shasta ruptured spore, (x15,000). Polar Filament, PF; Polar Capsule, PC; Cement-like substance, arrow. Bar = 1 µm.


18


PF
PC
19


PF
PC
20

Effect of Potassium Hydroxide, Methylene Blue and pH on Spores

A 54% increase in ruptured spores was found when comparing the percent average spore rupture of 0.05% to 0.1% KOH (Table 2). All other increases in KOH concentration resulted in only a gradual increase of ruptured spores. At 0.5% KOH the percent rupture was 96. In all concentrations of KOH, once the spore ruptured exposing the sporoplasm, the sporoplasm became amorphous and assumed dead. Final percentages of KOH were achieved after mixing equal volumes of KOH and spore suspensions.

Table 2. Rupture of Ceratomyxa shasta spores at various concentrations of potassium hydroxide. Two hundred spores were examined for each KOH test concentration. The average spore rupture represents five different counts of 200 KOH exposed spores. Methylene blue, M.B.

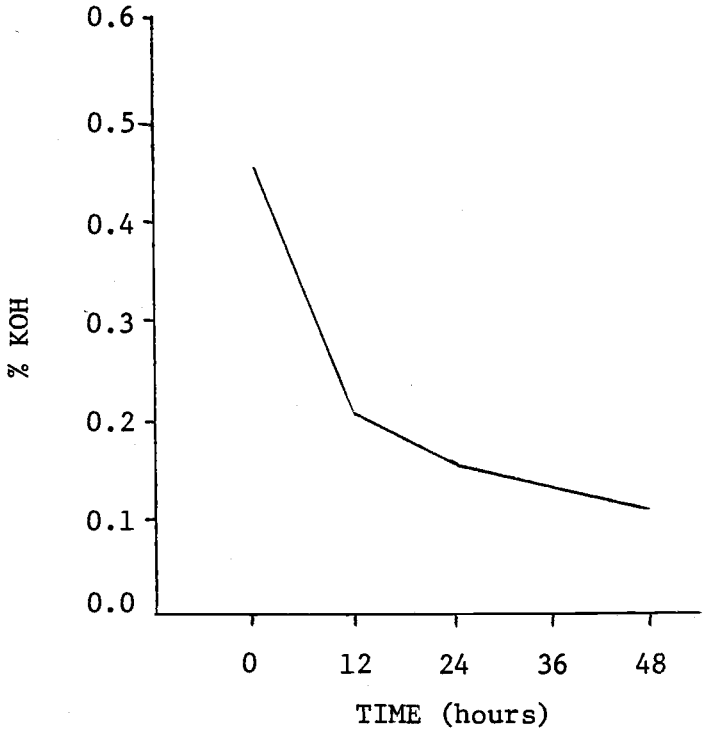
	Percent Concentration of Potassium Hydroxide						
	0.50	0.25	0.20	0.15	0.10	0.05	M.B.
Average Spore Rupture	192	187	172	145	121	11	0
Percent Spore Rupture	96	93	86	76	60	6	0
pH	12.5	12.3	12.2	12.1	11.9	11.5	7.0

Exposure of spore suspensions to a final concentration of 0.1% (w/v) methylene blue did not rupture any spores and showed no internal staining of the sporoplasm. Spores not ruptured when exposed to 0.5% KOH stained dark blue internally with no internal morphology visible. Spores not rupturing when exposed to 0.25% KOH again stained blue but showed some integrity of the internal structures. Internal morphology

of non-ruptured spores exposed to 0.2% KOH was more distinct and closely resembled untreated spores. Only at concentrations of 0.2% KOH or lower was extrusion of polar filaments without rupture of the spores observed.

Spores held for twelve hr or longer after removal from the fish required a lower concentration of KOH to rupture the majority of spores than spores removed directly from fish (Fig. 21). A final minimum concentration of 0.45% KOH was required for 90% or greater spore rupture. By the twelvth hr after removal of spores from fish 90% or greater spore rupture was achieved by a minimum final concentration of 0.2% KOH. Subsequent time periods produced 90% or greater spore rupture with decreasing minimum concentrations of KOH but did not produce the large decrease in KOH observed between time zero and twelve hours.

Figure 21. Effect of holding Ceratomyxa shasta spores, in Cortland's saline solution at 4°C for 0, 12, 24, 36, and 48 hr of time, upon the minimum concentration of potassium hydroxide (KOH) required to rupture at least 90% of the spores. Each time period represents the average of 200 spores examined in four separate tests.



An exposure period of two to four min at a given concentration of KOH is required before the actual number of ruptured spores can be calculated. Exposure time after this period resulted in little increase in ruptured spores (Table 2). Little difference was observed in this time period when the KOH concentration was varied so that it resulted in a high or low percentage of spore rupture.

Table 3. Timed exposure of Ceratomyxa shasta spores to rupture by 0.175% and 0.1% potassium hydroxide. Two hundred spores were examined for each time period. The percent spore rupture represents five different counts of 200 KOH exposed spores.

	0.175% KOH				0.1% KOH				
	Time (minutes)				Time (minutes)				
	1	3	5	7	1	2	3	4	5
Percent Spore Rupture	36	77	78	87	5	8	7	16	15

Varying the pH from 2.0 to 12.0 at an exposure time of five minutes (data not shown) only resulted in a six percent spore rupture at pH 12.0. All other pH values produced no spore rupture or polar filament extrusion.

Spores either exposed to a final concentration of 0.1% (w/v) methylene blue or 0.5% KOH showed a correlation between the percent of unstained spores and percent ruptured spores (Table 3). Exposure of spores to methylene blue gave an unstained spore percentage of 92 while KOH exposed spores resulted in 95% rupture. This indicates that all unstained spores are "viable".

Table 4. Comparison of methylene blue staining and potassium hydroxide in determining "viability" of Ceratomyxa shasta spores. One group of 100 spores were exposed to 0.1% (w/v) methylene blue and a second group of 100 spores were treated with 0.5% KOH. Each experiment was repeated five times.

	0.1% Methylene Blue	0.5% KOH
Percent Unstained Spores	92	--
Percent Ruptured Spores	--	95

DISCUSSION

Using the hypothesized life cycle of myxosporidans (Kudo, 1930), an attempt was made to partially simulate the environment and study the effects of temperature, aeration, substrate, and time on the development of C. shasta spores under controlled conditions.

Even though experimental aging of C. shasta spores did not result in maturation or development of an infective stage for fish, it did eliminate this form of spore "aging" as a possibility for the life cycle of C. shasta. It also showed that at the end of a 240 day aging period all inoculated cultures contained "viable" spores. This demonstrates that the spores are very resistant to environmental factors.

Possible reasons for failure to transmit C. shasta may be; (1) a longer aging period than 240 days is required, (2) escapement of histozoic myxosporidans by degradation of host tissue may provide some maturation stimuli for the parasite, such as an anaerobic environment and/or liberated host enzymes from decomposing tissue, (3) maturation of the parasite may require cycling water temperatures, (4) a more complex life cycle outside the host than that already hypothesized, or (5) a combination of these factors or others.

Aging periods longer than 240 days for C. shasta spores does not seem probable since enzootic waters for the infective stage of the parasite are not infectious for periods of five to eight months (Johnson et al., 1979). During this time the supposed maturation takes place. This non-infectious period, while encompassing the 240 day

aging time, is also correlated with the time natural water temperatures are approximately 10°C or lower (Schafer, 1968; Johnson et al., 1979). The other alternative is that an aging period of a year or longer is required, which is only speculative.

Hoffman and Putz (1969, 1971) were successful in transmitting M. cerebralis to susceptible fish by first "aging" the spores four to six months in a mud substrate seeded with infected tissue containing spores. Similar experiments by Johnson (1975) using infected intestinal tracts containing C. shasta spores "aged" in mud did not result in transmission of the parasite. Johnson (1975) did not account for the effect of temperature on "aging" of the spores since the water temperature in which he incubated the material was 12°C or higher. Wyatt (1978) was also unable to transmit Myxobolus insidiosus by this method. The water temperature used to incubate the substrate containing M. insidiosus infected host tissue was 12.2°C where the transmission of this parasite has become more difficult in the natural environment. Natural infections of fish by M. insidiosus occurs at lower water temperatures of 8.9°C. With a rise in water temperature to 11.1°C the infective stage becomes less numerous and at 13.3°C is not present. It should also be noted that C. shasta spores experimentally incubated at 5°, 10°, and 15°C did not consider the effect of cyclic water temperatures that occur during the non-infectious period in enzootic waters. Patashnik and Groninger (1964) were able to obtain multiplicative forms from Kudoa sp. spores by exposing them to cyclic temperatures.

Seasonal cycles of infection and epizootics is common to many myxosporidans in temperate climates. Infection of C. shasta occurs only at temperatures above 10°C (Schafer, 1968; Johnson et al., 1979). Markevish (1951) observed that boil disease of barbels caused by Myxobolus pfeifferi occurred only in the spring and summer months. Henneguya sp. infections in hatchery-reared channel catfish were reported by Meyer (1970) to happen in the early spring. Infections of Henneguya psorospermica in perch (Lom, 1970) and Myxobolus dujardini infections in juvenile northern squawfish (Mitchell, 1970) are in the winter months. Both Wyatt (1978) and Meyer (1970) correlated the appearance of infective stages of these myxosporidans with the presence of young susceptible juvenile fish. The effect of seasonal water temperatures on spore maturation may be an adaptation of the parasites to be present in their infective stage when susceptible hosts are present.

The possibility of intermediate hosts were examined experimentally by Current (1973) and Spall (1977) with no transmission of the myxosporidans tested. Sindermann (1970) suggested that zooplankton might be intermediate hosts for myxosporidans infecting marine pelagic fishes. The role of myxosporidan transmission and dissemination may occur by piscivorous birds (Schaperclaus, 1954; Mitchell, 1970; Taylor and Lott, 1978).

Evidence is accumulating that C. shasta (Schafer, 1968) and M. cerebralis (Putz and Hoffman, 1966) may not be transmitted by oral ingestion of the spores or infective stages. Johnson (1975) showed

that when water naturally containing the infective stage of C. shasta was held in the laboratory it was infectious to fish. However, by the third day after removal from the environment fish exposed to this water did not become infected with the parasite. Fish were exposed to "aged" C. shasta spores by three different methods (stomach tubing, intraperitoneal injection, and water-borne challenge) which encompassed several of the possibilities for parasite portal of entry. However, if the infective stage and/or spores were viable for only very short periods of time as shown by Johnson (1975) it would have been fortuitous to have experimentally infected fish.

Many experiments have been done in an attempt to elucidate the life cycle of myxosporidans. Experiments as controlled as feeding spores to susceptible hosts to the more complex undefined "aging" of infected tissues in mud have produced limited success. Myxosporidans are found worldwide, inhabiting the many different environments of the hosts. It appears that the investigator will need to place more emphasis on the variables such as, seasonal water temperatures, appearance of susceptible juvenile fish, and possible role of intermediate hosts or freeliving life stages of the specific myxosporidan being studied in order to elucidate their complete life cycle.

Scanning electron microscopy of fresh C. shasta spores showed an absence of polar filament pores which have only been reported from M. cerebralis (Lom and Hoffman, 1971), Unikapsula sp. (Canning and Vavra, 1977), and M. muelleri (Mitchell, 1977). The only distinct structures present were the two sutural ridges comprising the sutural

border, but no polar caps were observed. No reports utilizing scanning electron microscopy have studied the morphological changes that occurred during "aging" of C. shasta spores.

A sequence of ultrastructural changes occur with "aging" of C. shasta spores. The first step in "aging" is the development of more distinct sutural ridges of the two spore valves. These ridges are divided by a prominent inner groove. Polar caps on the spore valves are present at this time. Further "aging" produces a ridge surrounding the polar cap. Eventually the two sutural ridges and its inner sutural groove become enclosed by two dark bands or outer sutural grooves that run parallel to the sutural border on each spore valve. The polar cap ridges are no longer evident.

Extended "aging" results in the sutural region developing into a sunken groove, containing the less distinct sutural ridges and inner sutural groove. The polar caps are now surrounded by grooves. Final "aging" brings about the disappearance of the sunken sutural region and development of one narrow, deep groove between the two spore valves. The grooves encircling the polar caps are now more distinct and deeper. The morphological changes just described occurred at a faster rate and were more morphologically pronounced in spores "aged" at 15°C. Spores incubated at 10°C also exhibited the same features as described for spores incubated at 15°C, but at a later aging period and were not as pronounced. Spores held at 10° and 5°C formed the outer sutural grooves at the same time but 5°C spores never developed the single narrow sutural groove by the end of the "aging" study.

A cement-like substance was observed to hold the spore valves together. Gould (1969) showed the presence of a desmosomal junction between the two spore valves of C. shasta by transmission electron microscopy. This may represent the cement-like substance seen in partially ruptured spores viewed by scanning electron microscopy. Cheissin et al. (1961) also found a cement-like substance separating the spore valves of M. uniporous and M. carassi. Electron-dense cap-like structures have been reported for several species of myxosporidans by transmission electron microscopy ultrastructural studies (Cheissin et al., 1961; Lom, 1964; Spall, 1973; Current, 1977; Desser and Paterson, 1978; Current, 1978). Gould (1969) and Yamamoto and Sanders (1979) did not report this cap-like structure for C. shasta spores when viewed by transmission electron microscopy. Polar caps were observed in "aged" but not fresh C. shasta spores by scanning electron microscopy as reported in this thesis.

Anomalies of C. shasta spores were not very common. One spore had a branch of the spore wall extending laterally from the sutural border along its anterior side. Another spore exhibited an asymmetrical spore valve. Lom and Hoffman (1971) reported on the divided polar filament pore they observed in M. cerebralis. The function of these anomalies in C. shasta or M. cerebralis is not known.

A correlation between the sequence of ultrastructural and dimensional changes was observed with "aging" of C. shasta spores. Initial "aging" resulted in similar measurements of the lengths and widths as that found for fresh spores. Further "aging" resulted in an

increase in spore length while the width remained approximately the same. Subsequent "aging" showed spore length returning to similar spore dimensions of fresh and initially "aged" spores, while spore width decreased. Final "aging" resulted in further decreases of both spore lengths and widths.

The appearance of a ridge surrounding the polar cap and development of the two outer sutural grooves corresponded to the same "aging" period in which C. shasta spore lengths increased. Further "aging" resulted in a sunken sutural region with its less distinct sutural ridges and inner groove which was concomitant with a decrease in both spore length and width. Final "aging" resulted in development of a narrow, deep groove with continued decrease in spore length and width. Only in the final "aging" period at 15°C was the greatest decrease in spore dimensions observed. Spores incubated at 10°C for the same period of time showed a lesser decrease in spore dimensions, while spores held at 5°C had the smallest reduction in measurements.

Sanders (1967) found that the length and width of live C. shasta spores measured by light microscopy was 17.9(14.0-21.0)μm and 7.9 (6.2-9.8)μm, respectively. When he measured the dimensions of spores fixed in Schaudinn's and stained with Giemsa, a decrease in both measurements was observed: length, 16.5(11.4-20.1)μm; width, 7.7(4.2-9.7)μm. Measurement of fixed and stained C. shasta spores by Noble (1950) gave a length of 14.0μm and width of 6.0μm. The nature of the fixative and stain were not stated. Kudo (1921) demonstrated that all

the fixatives and stains he tested on L. ohlmacheri spores resulted in a decrease in all spore dimensions measured.

Dimensions calculated from scanning electron micrographs of C. shasta spores in this work were less than those determined by Noble (1950) and Sanders (1967), regardless of the method of specimen preparation they used. Whether the smaller spore dimensions represent the actual size of the spores or due to the fixation process required for scanning electron microscopy cannot be ascertained since no measurements were made on unfixed, live spores.

Polar cap plugs diameters showed no change in size between spores "aged" 180 and 240 days. Thickness of the wall at the sutural ridge of ruptured unaged spores were found to be approximately 0.14μm thicker at the lateral margins than at the posterior or anterior margins.

Spores of C. shasta exposed to varying concentration of KOH did not allow escapement of viable sporoplasms. Potassium hydroxide at 0.25% to 0.5% was required to give maximum spore rupture.

Upon holding spores any period of time it was found that lower concentrations of KOH were required for 90% or greater rupture. Lom (1964) observed somewhat different results for extrusion of M. muelleri polar filaments by exposure to saturated urea. Fresh spores would have 100% filament extrusion in two minutes after exposure. Spores held any length of time would require several minutes before maximum filament extrusion occurred with the percent extrusion being reduced significantly below 100%. Both results indicate that there is

some type of process (i.e. biochemical) taking place in the spores upon their removal from the host.

A two to four minute exposure period of C. shasta spores to KOH was required before all "viable" spores rupture. Treatment of spores with varying pH only brought about a 6% spore rupture at pH 12.0. This gives evidence that spore rupture and polar filament extrusion is by osmotic pressure and not high pH stimulation.

Hoffman and Markiw (1977) used methylene blue (0.08%) to give presumptive evidence that M. cerebralis spores killed by heating became stained. Exposure of C. shasta spores to 0.1% methylene blue or 0.5% KOH indicated that the percent unstained spores was similar to the percent ruptured spores. Use of methylene blue for "viability" tests would be more suitable since it does not destroy the parasite which could then be used for subsequent experiments. Until an appropriate method is developed to determine viability by infectivity tests one must view quantitative results of spore death or "viability" by the KOH test with caution. This was pointed out by Gurley (1894) who reported that small numbers of myxosporidan spores preserved in alcohol were able to extrude polar filaments by stimulation with sulfuric acid or iodine-water.

Janus Green B, a supravital stain of mitochondria, failed to stain any fresh spores of C. shasta. Spores preserved in 10% formalin exhibited 100% staining with 0.1% methylene blue, but no spore rupture with 1.0% KOH. Kudo (1921) showed that fixation brought about changes in the size and structure of myxosporidan spores which could result in the above results.

Exposure of "aged" spores to digestive fluids from freshly killed uninfected fish failed to bring about polar filament extrusion or rupture. This was only a casual observation and not conducted under defined experimental conditions.

SUMMARY AND CONCLUSIONS

1. "Aging" of C. shasta spores by various combinations of four environmental factors (time, temperature, substrate, and aeration) did not result in development of the infective stage for susceptible fish.
2. Exposure of susceptible fish to the "aged" spores by three different methods (stomach tubing, intraperitoneal injection, and water-borne challenge) was unsuccessful in transmission of the disease.
3. This is the first report showing the polar pores to be filled with a polar cap structure. Fresh spores have a continuous smooth wall over the area of the polar capsules giving no indication of polar caps. Upon "aging" the polar caps separate from the spore and become raised above the wall.
4. Extended "aging" brings about a series of morphological changes in the sutural border region. Sutural ridges first become very prominent. This is followed by development of two dark bands in the spore wall located on the outer side of the ridges. The dark bands then develop into grooves and the entire sutural region becomes sunken below the spore wall. Depressed sutural ridges are still present until a narrow, deep groove replaces the entire sunken sutural region.
5. Concomitant with the series of morphological changes observed during the "aging" process there was a change in spore dimensions. Spore length first increased upon "aging" followed by a decrease

to a length less than that for fresh spores. Width measurements showed a decrease with an increase in the spore "aging" period.

6. Similar results were observed around the polar caps. A ridge develops and encircles the raised polar cap which then changes into a distinct polar cap groove during "aging".
7. A cement-like substance (desmosomal junction) holds the two spore valves.
8. A higher concentration of KOH was required to rupture the majority of spores removed directly from fish than was required to rupture the same lot of spores when held for several hours. The lower concentration of KOH required to rupture the majority of spores that had been held for a period of time after removal from the host may indicate some biochemical process occurring within this period.
9. Methylene blue can be used as a substitute for KOH "viability" tests. All spores unstained by methylene blue were equivalent to the number of spores ruptured by KOH.

LITERATURE CITED

Auerbach, M. 1909. Bericht uber eine Studienreise nach Bergen (Norway). Verh. Naturw. Ver. Karlsruhe. 21:37-73.

Auerbach, M. 1910. Die Cnidosporidien (Myxosporidien, Actinomyxidien, Microsporidien). Eine Monographische Studie. Leipzig, W. Klinkhardt. 261 p.

Awerinzew, S. 1911. Studien uber parasitische. Protozoen. VII. Uber Sporenbildung bei Myxidium sp. aus der Gallenblase von Cottus scorpius. Arch. Protist. 23:199-204.

Baker, J. R. 1977. Systematics of parasitic protozoa. Pages 37-56 in J. P. Kreier (ed.). Parasitic protozoa. Vol. 1. Academic Press, New York, New York.

Balbiani, G. 1863. Sur l'organization et la nature des psorospermies. C.R. Acad. Sci., Paris 57:157-161.

Bond, F. F. 1939. Experimental studies on myxosporidiosis of Fundulus heteroclitus (Linn.) and F. diaphanus (Le Sueur). Trans. Amer. Microsc. Soc. 58:164-169.

Butschli, O. 1881. Beitrage zur Kenntnis der Fischsporospermien. Zeitschr. wiss. Zool. 35:629-651.

Canning, E. U. and J. Vávra. 1977. Microspora and Myxospora. Pages 42-50 in S. H. Hunter. (ed.). Proc. 5th Int. Cong. Protozool.

Chapman, G. B. and L. G. Tilney. 1959a. Cytological studies of the nematocysts of Hydra. I. Desmoemes, Isorhizas, Cnidocils, and supporting structures. J. Biophys. Biochem. Cytol. 5:69-79.

_____. 1959b. Cytological studies of the nematocysts of Hydra. II. The Stenoteles. J. Biophys. Biochem. Cyto. 5:79-85.

Cheissin, E. M., S. S. Shulman and L. P. Vinnitchenko. 1961. Stroenie spor Myxobolus. Tsitologia 3:662-667.

Conrad, J. F. and M. Decew. 1966. First report of Ceratomyxa in juvenile salmonids in Oregon. Prog. Fish-Cult. 28:238.

Corliss, J. O. 1968. The value of ontogenetic data in reconstructing protozoan phylogenies. Trans. Am. Microsc. Soc. 87:1-20.

Creplin, J. C. H. 1842. Beschreibung der Psorospermien des Kaulbarsches nebst einigen Bemerkungen uber die der Plotze und andere. Arch. Naturgesch. 8:61-66.

Current, W. L. 1977. The biology of Henneguya exilis. Doctoral thesis, University of Nebraska, Lincoln, Nebraska. 74 p.

Current, W. L. 1979. Henneguya adiposa Minchew (Myxosporida) in channel catfish: Ultrastructure of the plasmodium wall and sporogenesis. J. Protozool. 26:209-217.

Current, W. L., J. Janovy and S. A. Knight. 1979. Myxosoma funduli Kudo (Myxosporida) in Fundulus kansae: Ultrastructure of the plasmodium wall and of sporogenesis. J. Protozool. 26:574-583.

Daniels, S. B., R. L. Herman and C. N. Burke. 1976. Fine structure of an unidentified protozoon in the epithelium of rainbow trout exposed to water with Myxosoma cerebralis. J. Protozool. 23:402-410.

Davis, H. S. 1916. The structure and development of a Myxosporidian parasite of the squeteague, Cynoscion regalis. J. Morphol. 27:333-377.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott and K. M. Jones, eds. 1969. Data for biochemical research. Oxford Univ. Press, London, England. 654 p.

Debaisieux, P. 1924. Sphaeromyxa sabrazesi Laveran et Mesnil. La Cellule 35:269-301.

Desser, S. S. and W. B. Paterson. 1978. Ultrastructural and cytochemical observations on sporogenesis of Myxobolus sp. (Myxosporida: Myxobolidae) from the common shiner Notropis cornutus. J. Protozool. 25:314-326.

Dogiel, W. A., G. K. Petrushevski and Yu. I. Polyanski, eds. 1958. Parasitology of fishes. Leningrad Univ. Press, Leningrad, U.S.S.R. (Transl. by Z. Kabata. Oliver & Boyd, Edinburgh, England, 1961.)

Doflein, F. 1901. Die Protoxoen als Parasiten und Krankheitserreger nach biologischen Gesichtspunkten dargestellt. Jena, Gustav Fischer. 274 p.

Dŭjardine, M. F. 1845. Histoire naturelle des Helminthes ou vers intestinaux. Paris, Roret. 654 p.

Dunkerly, J. S. 1925. The development and relationships of the Myxosporidia. Quart. J. Microsc. Sci. 69:185-216.

Emery, C. 1909. Myxosporidia sono protozoi. Monit. Zool. Ital. 20:247-249.

Erdmann, R. 1911. Zur Lebensgeschichte von Chloromyxum leydigi, eine miktosporen Myxosporidie. Teil I. Arch. Protist. 24:149-162.

Erdmann, R. 1917. New facts and views concerning the occurrence of a sexual process in the Myxosporidian life cycle. Amer. Natur. 51:719-739.

Fryer, J. L. 1971. Ceratomyxa in salmonids. FAO Aquaculture Bull. 3:12-13.

Fryer, J. L. and J. E. Sanders. 1979. Investigation of Ceratomyxa shasta, a protozoan parasite of salmonid fish. J. Parasitol. 56:759.

Fryer, J. L. and K. S. Pilcher. 1974. Effects of temperature on diseases of salmonid fishes. Ecolog. Res. Ser. EPA-660/3-73-020. Office of Research and Development, U.S. Environ. Prot. Ag., Washington, D.C. 114 p.

Georgévitch, J. 1914. Étude du cycle évolutif chez chez les Myxosporidies. Arch. Zool. Exp. Gen. 54:387-409.

_____. 1916. Sur le cycle evolutif de Ceratomyxa herouardi Georgévitch. C.R. Acad. Sci., Paris 163:983-985.

_____. 1917. Recherches sur le development de Ceratomyxa herouardi. Georgévitch. Arch. Zool. Exp. Gen. 56:375-399.

_____. 1919. Études sur le développement de Myxidium gadi Georgévitch. Arch. Zool. Exp. Gen. 58:251-289.

_____. 1929. Recherches sur Ceratomyxa maena nov. sp. Arch. Protist. 65:106-123.

_____. 1935. Uber diplo und haplophase im Entwicklungskreise des Myxosporidien. Arch. Protist. 84:419-428.

_____. 1936. Uber diplo und haplophase im Entwicklungskreise des Myxosporids Zschokkella rovig nensis Nemeczek. Arch. Protist. 84:419-428.

Gould, R. W. 1968. Occurrence of the protozoan fish parasite Ceratomyxa shasta in the Deschutes river basin and the ultra-structure of the spore. Master thesis, Oregon State Univ., Corvalis, Oregon. 51 p.

Grassé, P-P. 1960. Les Myxosporidies sont des organismes pluricellulaires. C.R. Acad. Sci., Paris 251:2638-2640.

_____. 1970. Embranchement de Myxozoaires. Pages 107-112 in Grassé, P-P., R. A. Poisson, O. Tuzet (eds.). Precis de Zoologie 1. Invertébrés (2nd. ed.) Masson et Cie., Paris.

Grasse, P-P. and A. Lavette. 1978. La Myxosporidie Sphaeromyxa sabrazesi et le nouvel embranchement de Myxosoaires (Myxozoa). Recherches sur l'état pluricellulaire primitif et considérations phylogénetiques. Ann. Sci. Nat. Zool., Paris 20:193-285.

Grell, K. G. 1973. Protozoology. Springer-Verlag, Berlin. 554 p.

Gurley, R. R. 1894. The Myxosporidia, or psorosperms of fishes, and the epidemic produced by them. Rep. U.S. Fish Comm. 26:65-304.

Hahn, C. W. 1917. On the sporozoan parasites of the fishes of Woods Hole and vicinity. I. Further observations on Myxobolus musculi from Fundulus. J. Parasitol. 3:91-104.

Halliday, M. M. 1973. Studies on Myxosoma cerebralis, a parasite of salmonids. II. The development and pathology of Myxosoma cerebralis in experimentally infected rainbow trout (Salmo gairdneri) fry reared at different water temperatures. Nord Veterin. 25:349-358.

Halliday, M. M. 1976. The biology of Myxosoma cerebralis: The causative organism of whirling disease of salmonids. J. Fish Biol. 9:339-358.

Hanson, E. E. 1976. Major evolutionary trends in animal protists. J. Protozool. 23:4-12.

Hine, P. M. 1975. Three new species of Myxidium (Protozoa: Myxosporidia) parasitic in Anguilla australis Richardson, 1848 and A. dieffenbachii. Gray, 1842 in New Zealand. J. Roy. Soc. New Zeal. 5:153-161.

Hoffman, G. L. 1970. Parasites of North American freshwater fishes. Univ. of California Press, Los Angeles. 486 p.

Hoffman, G. L. 1976. Whirling disease of trout. Fish Dis. Leaf. 47. U.S. Dept. Interior, U.S. Fish Wildl. Serv., Washington, D.C. 10 p.

Hoffman, G. L. and G. L. Hoffman. 1972. Studies on the control of whirling disease (Myxosoma cerebralis): The effects of chemicals on spores in vitro, and of calcium oxide as a disinfectant in simulated ponds. J. Wildl. Dis. 8:49-53.

Hoffman, G. L. and M. E. Markiw. 1976. Control of whirling disease (Myxosoma cerebralis): use of methylene blue staining as possible indicator of effect of heat on spores. J. Fish Biol. 10:181-183.

Hoffman, G. L. and F. P. Meyer. 1974. Parasites of freshwater fishes, a review of their control and treatment. T.F.H. Publications, Neptune, New Jersey. 224 p.

Hoffman, G. L. and R. E. Putz. 1968. Progress in sport fishery research, 1967: Parasites. Div. Fishery Res., Bur. Sport Fish Wildl. (Washington, D.C.). Resource Publ. 64:27-28.

Hoffman, G. L. and R. E. Putz. 1969. Host susceptibility and the effect of aging, freezing, heat and chemicals on spores of Myxosoma cerebralis. Prog. Fish-Cult. 31:35-37.

Hoffman, G. L. and R. E. Putz. 1971. Effect of freezing and aging on the spores of Myxosoma cerebralis, the causative agent of salmonid whirling disease. Prog. Fish-Cult. 33:95-98.

Hoffman, G. L., C. E. Dunbar, and A. Bradford. 1969. Whirling disease of trouts caused by Myxosoma cerebralis (Protozoa: Myxosporida) in the United States. U.S. Fish Wildl. Serv. Spec. Sci. Rep. Fish. No. 427. 15 p.

Hoffman, G. L., R. E. Putz, and C. E. Dunbar. 1965. Studies on Myxosoma cartilaginis n. sp. (Protozoa: Myxosporidea) of centrarchid fish and a synopsis of the Myxosoma of North American freshwater fishes. J. Protozool. 12:319-332.

Honigberg, B. M., W. Balamuth, E. C. Bovee, J. O. Corliss, M. Gojdics, R. P. Hall, R. R. Kudo, N. D. Levine, A. R. Loeblich, J. Weiser, and D. H. Wenrich. 1964. A revised classification of the phylum Protozoa. J. Protozool. 11:7-20.

Johnson, K. A. 1975. Host susceptibility, histopathologic, and transmission studies on Ceratomyxa shasta, a Myxosporidan parasite of salmonid fish. Doctor thesis, Oregon State Univ., Corvallis, Oregon. 134 p.

_____. 1980. Host susceptibility, histopathologic and transmission studies on Ceratomyxa shasta, a Myxosporidan parasite of salmonid fish. Fish Pathol. 14:183-184.

Johnson, K. A., J. E. Sanders, and J. L. Fryer. 1979. Ceratomyxa shasta in salmonids. Fish Dis. Leaf. 58. U.S. Dept. Interior, U.S. Fish Wildl. Serv., Washington, D.C. 11 p.

Jurine, L. L. 1825. Histoire abregee des poissons du lac Leman. Mem. Soc. Phys. Hist. Naturel. Geneve 3:133-232.

Kudo, R. 1920. Studies on Myxosporidia. A synopsis of genera and species of Myxosporidia. Illinois Biol. Monogr. 5:1-265.

Kudo, R. R. 1921. On the effect of some fixatives upon Myxosporidian spores. Trans. Am. Microsc. Soc. 40:161-167.

_____. 1922. On the morphology and life history of a myxosporidian, Lepthotheca ohlmacheri, parasitic in Rana clamitans and R. pipens. Parasitology 14:221-244.

_____. 1930. Myxosporidia. Pages 303-324 in R. W. Hegner and V. M. Andrews (eds.). Problems and methods of research in protozoology. Macmillan Co., New York, New York.

_____. 1934. Studies on some protozoan parasites of fishes of Illinois. Illinois Biol. Monogr. 13:1-44.

_____. 1966. Protozoology. 5th ed. C. C. Thomas, Springfield, Illinois. 1174 p.

Labbe, A. 1899. Sporozoa. Das Tierreich. 180 p.

Leedale, G. F. 1974. How many are the kingdoms of organisms? Taxon 23:261-270.

Léger, L. 1906. Sur une nouvelle maladie Myxosporidienne de la truite indigene (Chloromyxum truttae n. sp.) C.R. Acad. Sci., Paris 142:665-666.

Léger, L. and E. Hesse. 1906. Sur la structure de la paroi sporale des Myxosporidies. C.R. Acad. Sci. 142:720-722.

Levine, N. D. 1969a. Problems in systematics of parasitic protozoa. Pages 109-121 in G. D. Schmidt (ed.). Problems in systematics of parasites. University Park Press, Baltimore, Maryland.

_____. 1969b. Taxonomy of the Sporozoa. Pages 365-367 in A. A. Strelkov, K. M. Sukhanova, and I. B. Raikov (eds.). Prog. Protozool., Proc. 3rd Int. Cong. Protozool.

_____. 1970. Taxonomy of the Sporozoa. J. Parasitol. 56:II: 208-209.

Levine, N. D., J. O. Corliss, F. E. G. Cox, G. Deroux, J. Grain, B. M. Honigberg, G. F. Leedale, A. R. Loeblich, J. Lom, D. Lynn, E. G. Merinfeld, F. C. Page, G. Poljansky, V. Sprague, J. Vavra, and F. G. Wallace. 1980. A newly revised classification of the Protozoa. J. Protozool. 27:37-58.

Lieberkuhn, N. 1854. Notice sur les psorospermies. Bull. Acad. Roy. Sci., Lett., Beaux-Arts Belg. 21:21-23.

Lom, J. 1964. Notes on the extrusion and some other features of Myxosporidian spores. Acta Protozool. 2:321-327.

_____. 1969. Notes on the ultrastructure and sporoblast development in fish parasitizing Myxosoporidia of the genus Sphaeromyxa. Zeit. Zellforsch. Mikrosk. Anat. 97:416-437.

_____. 1970. Some aspects of the host-parasite relation to Myxosporidians of the genus Henneguya. J. Parasitol. 56:II:442.

_____. 1973. Current status of Myxo- and Microsporidia. Page 254 in dePuytorac, P. and J. Grain (eds.). Prog. Protozool., Proc. 4th Int. Congr. Protozool.

_____. 1975. A capillary tube method for storage of Myxosporidian and Microsporidian spores. Folia Parasitol. 22:275-277.

_____. 1977. Microspora and Myxospora. Pages 42-50 in Hunter, S. H. (ed.). Proc. 5th Int. Cong. Protozool.

Lom, J. and P. dePuytorac. 1965a. Studies on the Myxosporidian ultrastructure and polar capsule development. Protistol. 1:53-65.

_____. 1965b. Observations sul l'ultrastructure des trophozoites de Myxosporidies. C.R. Acad. Sci. 260:2588-2590.

Lom, J. and G. L. Hoffman. 1971. Morphology of the spores of Myxosoma cerebralis (Hofer, 1903) and M. cartilaginis (Hoffman, Putz and Dunbar, 1965). J. Parasitol. 57:1302-1308.

Lom, J. and J. Vávra. 1964. Parallel features in the development of Myxosporidian polar capsules and coelenterate nematocysts. Proc. 3rd European Reg. Conf. Elect. Microsc. 191-192.

_____. 1965. Notes on the morphogenesis of the polar filament in Henneguya (Protozoa, Cnidospora). Acta Protozool. 3:57-60.

Lucký, Z. 1971. Methods for the diagnosis of fish diseases. (Transl. by Franklin Book Programs, Inc., Cair, Egypt, 1977.)

Manter, H. W. 1969. Problems in systematics of trematode parasites. Pages 93-105 in G. D. Schmidt (ed.). Problems in systematics of parasites. University Park Press, Baltimore, Maryland.

Margolis, L. and T. P. T. Evelyn. 1975. Ceratomyxa shasta (Myxosporida) disease in chum salmon (Oncorhynchus keta) in British Columbia. J. Fish. Res. Bd. Can. 32:1640-1643.

Markevich, A. P. 1951. Parasitic fauna of freshwater fish of the Ukrainian S.S.R. Akad. Nauk Ukr. SSR. Inst. Zool. (Israel Program for Scientific Translations, 1963.)

Markiw, M. E. and K. Wolf. 1974. Myxosoma cerebralis: Isolation and concentration from fish skeletal elements--sequential enzymatic digestions and purification by differential centrifugation. J. Fish. Res. Bd. Can. 31:15-20.

Mavor, J. W. 1916. On the life history of Ceratomyxa acadiensis, a new species of Myxosporidia from the eastern coast of Canada. Proc. Amer. Acad. Sci. 51:549-578.

Mayer, A. F. 1864. Uber psorospermien. Arch. Anat. Physiol. Wissens. Medic. 3:1-264.

Meglitsch, P. A. 1972. Invertebrate Zoology. Oxford University Press, New York, New York. 834 p.

Mercier, L. 1906a. Phénomènes de sexualité chez Myxobolus pfeifferi. C.R. Soc. Biol. 110:427-428.

_____. 1906b. Contribution à l'étude du développement des spore chez Myxobolus pfeifferi. C.R. Soc. Biol. 110:763-764.

Meyer, F. P. 1970. Seasonal fluctuations in the incidence of disease on fish farms. Pages 21-29 in S. F. Snieszko (ed.). A symposium on diseases of fishes and shellfishes. Spec. Publ. No. 5. Amer. Fish. Soc., Washington, D.C.

Mitchell, L. G. 1970. Studies of the Myxosporida (Protozoa: Amoebogena) of fishes of western Montana. Doctoral thesis. University of Montana, Missoula, Montana.

_____. 1977. Myxosporida. Pages 115-154 in J. P. Kreier (ed.). Parasitic protozoa. Academic Press, New York, New York.

Morrison, N. D. 1974. Sphaeromyxa maiyai sp. n. (Protozoa: Myxosporidea): A light microscopic and ultrastructural analysis employing the scanning and transmission electron microscopes. Doctoral thesis. Oregon State University, Corvallis, Oregon. 170 p.

Müller, J. 1841. Krankhafter hautsusschlag mit speefisch organisierten körperchen (Psorospermien). Ber. Preuss. Akad. Wiss. Berlin 4:212-221.

_____. 1843. Recherches sur une variete remarquable de production parasitique morbide avec des corpuscules seminaux specifiquement organises. Arch. Med. Comp. 6:219-234.

Naville, A. 1930. Recherches sur la sexualité chez les Myxosporidies. Arch. Protist. 69:327-400.

Noble, E. R. 1941. Nuclear cycles in the life history of the protozoan genus Ceratomyxa. J. Morphol. 69:455-479.

_____. 1944. Life cycles in the Myxosporidia. Quart. Rev. Biol. 19:213-235.

_____. 1950. On a Myxosporidia (protozoan) parasite of California trout. J. Parasitol. 36:457-460.

_____. 1977. Microspora and Myxospora. Pages 42-50 in S. H. Hunter (ed.). Proc. 5th Int. Cong. Protozool.

O'Grodnick, J. J. 1975. Egg transmission of whirling disease. Prog. Fish-Cult. 37:153-154.

O'Grodnick, J. J. and C. C. Gustafson. 1975. A study of the transmission, life history and control of whirling disease of trout. Pa. Fish Comm., Fed. Aid Fish Restoration, Prog. Rep. F-35-R-5. 22 p.

Ormieres, R. 1970. Formation des capsules polaires dans la spore de l'Actinomyxidie Aurantiactinomyxon eiseniellae Orm. Frea. (Etude ultrastructurale). C.R. Acad. Sci., Paris 271:2326-2328.

Overstreet, R. M. 1976. Fabespora vermicola sp. n., the first myxosporidan from a platyhelminth. J. Parasitol. 62:680-684.

Patashnik, M. and H. S. Groninger. 1964. Observations on the milky condition in some Pacific coast fishes. J. Fish. Res. Bd. Can. 21:335-346.

Plehn, M. 1904. Über die Drehkrankheit der Salmoniden (Lentospora cerebralis) (Hofer, Plehn). Arch. Protist. 5:145-166.

_____. 1924. Praktikum der Fischkrankheiten. Schweizerbartsche, Stutgart.

Putz, R. E. 1970. Experimental transmission of Myxosoma cerebralis (whirling disease) and effect of freezing on the spores. Progress in Sport Fishery Research 1969, Bureau Sport Fish Wildl., Washington, D.C. Resource Pub. 88:55-57.

Putz, R. E. and R. L. Herman. 1970. Studies on earliest susceptibility of rainbow trout to Myxosoma cerebralis and exposure time. J. Parasitol. 56:II:460.

Putz, R. E. and G. L. Hoffman. 1966. Earliest susceptible age of rainbow trout to whirling disease. Prog. Fish-Cult. 28:82.

Robin, C. 1853. Histoire naturelle des vegetaux parasites. C.R. Soc. Biol. 5:82-257.

Rogers, W. A. and J. L. Gaines. 1975. Lesions of protozoan diseases in fish. Pages 117-141 in W. E. Ribelin and G. Migaki (eds.). The Pathology of fishes. University of Wisconsin Press, Madison.

Rucker, R. R., B. J. Earp, and E. J. Ordal. 1953. Infectious diseases of Pacific salmon. Trans. Amer. Fish. Soc. 83:297-312.

Sanders, J. E. 1967. Occurrence of the protozoan parasite Ceratomyxa shasta among salmonid fishes in Oregon waters. Masters thesis. Oregon State Univ., Corvallis, Oregon. 60 p.

Sanders, J. E., J. L. Fryer, and R. W. Gould. 1970. Occurrence of the Myxosporidan, parasite Ceratomyxa shasta, in salmonid fish from the Columbia River basin and Oregon coastal streams. Pages 133-144 in S. F. Snieszko (ed.). A symposium on diseases of fishes and shellfishes. Spec. Publ. No. 5. Amer. Fish. Soc., Washington, D.C.

Schafer, W. E. 1968. Studies on the epizootiology of the myxosporidan Ceratomyxa shasta Noble. Calif. Fish Game 54:90-99.

Schäperclaus, W. 1931. Die drehkrankheit in der forellenzucht und ihre bekampfung. Z. Fisch. Hilfswiss 29:521-567.

_____. 1954. Fischkrankheiten. Akademie-Verlag, Berlin. 708 p.

Schröder, O. 1907. Beiträge zur Entwicklungsgeschichte der Myxosporidien Sphaeromyxa sabrazesi (Laveran et Mesnil). Arch. Protist. 9:359-381.

Schubert, G. 1968. Elektronenmikroskopische untersuchungen zur sporentwicklung von Henneguya pinnae Schubert (Sporozoa, Myxosporidea, Myxobolidae). Z. Parasitenkd. 30:57-77.