Monoclonal antibodies (Mabs) were produced against the myxosporean parasite, *Ceratomyxa shasta*, and were characterized by indirect immunofluorescent techniques, western blot analysis, and immunoelectron microscopy. The resulting Mabs reacted specifically with the trophozoite and sporoblast stages of the parasite, but not with the mature spore, indicating that certain antigens are stage-specific. Two of the Mabs produced also reacted with trout white blood cells as evidenced by indirect fluorescent antibody techniques (IFAT) and Western blot analysis. Further characterization of these cross-reacting antibodies showed that they were directed against carbohydrate epitopes on both the parasite and trout immunoglobulin heavy chain. One hybridoma produced antibodies of high specificity for prespore stages of *C. shasta*. Ultrastructural analysis of the parasite using immunogold labeling
showed that the Mab specifically bound antigen located in the cytoplasm of the trophozoites.

The *C. shasta*-specific antibodies produced by monoclonal technology, standard histological methods, and scanning electron microscopy were used to investigate the host response of *Salmo gairdneri* against infection by the parasite. In infected fish the parasite was first observed in the mucosal epithelium of the posterior intestine using IFAT. As the trophozoites proliferated, the infection spread anteriorally and into the submucosa, muscularis, and serosa of the intestine. Hepatic tissues were also infected early in the disease process. In the terminal stages of the infection, trophozoites had penetrated the stomach, pyloric caeca, pancreas, and adipose tissues and were also observed in the blood and kidney. In fish refractory to infection, the parasite was observed in the lumen of the intestine. Examination of infection in a moribound fish by scanning electron microscopy showed extensive destruction of the mucosal folds of the posterior intestine. Although a vigorous tissue response was evident in infected tissues, trout antibodies specific for the parasite were not detected by either Western blot analysis or IFAT.

The distribution of the infective stage of *C. shasta* in the Columbia River basin was also investigated. The range of the parasite was extended in the Columbia River to its confluence with the Snake River and in the Snake River to Oxbow Dam.
Development of Monoclonal Antibodies against the Myxosporean, *Ceratomyxa shasta*, and Characterization of the Salmonid Host Response

by

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CONTRIBUTION OF AUTHORS

J. S. Rohovec served as advisor to this project and appears on all manuscripts. In Chapter IV, J. E. Sanders and D. G. Stevens helped in conducting field exposures to determine the distribution of *Ceratomyxa shasta*. In Chapter VI, C. E. Smith provided assistance in the histological sectioning of the fish and in the description of the pathology. T. Yamamoto assisted in the embedding, sectioning, and examination of samples for the transmission electron microscopic study in Chapter VII.
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Development of Monoclonal Antibodies against the Myxosporean, *Ceratomyxa shasta*, and Characterization of the Salmonid Host Response

Chapter I

Introduction

The myxosporean parasite, *Ceratomyxa shasta*, is an important pathogen of salmonid fishes in California, Oregon, Washington, Idaho, and British Columbia, Canada. Knowledge of the distribution of the infective stage of the parasite is important because the only means of control is avoidance. Within watersheds where the parasite is endemic, the disease is managed by stocking *C. shasta*-resistant strains of salmonids (Zinn et al., 1977). The impact of this parasite on the salmonid fishery is difficult to assess. Diagnosis of *C. shasta* is made by identifying spore(s) in wet mounts of intestinal tract scrapings, an inefficient method, because spores are not formed until terminal stages of the disease.

The morphology of the infective stage of *C. shasta* is unknown and it is not known if the life cycle is direct or if it requires the participation of an intermediate host. Transmission of the causative agent of ceratomyxosis under controlled laboratory conditions has only occurred when susceptible fish were held with water and substrate from a site endemic for *C. shasta* or when trophozoites from infected fish were injected into susceptible fish. The site of entry of the parasite into the host in a natural infection
has not been identified. Johnson (1975) observed the parasite first in the pyloric caeca, but Yamamoto and Sanders (1979) suggested that the intestine is the initial site of infection. Although the pathology of the infection in susceptible fish has been described (Johnson, 1975), the immune response to the infection and the mechanisms of resistance have not been investigated. In an attempt to resolve some of these problems, I have undertaken the following studies:

(1) Definition of the range of the infective stage of *Ceratomyxa shasta* in the Columbia River basin.

Previous work in this laboratory has established the range of the parasite in the main stream of the Columbia River upriver from the mouth to McNary Dam.

(2) Development of diagnostic procedures.

Previously developed polyclonal antisera were unsatisfactory for use as a diagnostic reagent because of the variability of the immune response between rabbits and between bleedings. Non-specific fluorescence was also a problem when these sera were used in fluorescent antibody techniques. Therefore, a battery of monoclonal and monospecific polyclonal antisera was developed. These were characterized and evaluated for their usefulness as diagnostic reagents.

(3) Determination of the initial site of infection by *Ceratomyxa shasta* and examination of the host response.

The histopathology caused by *C. shasta* has been studied by other researchers but several questions remain. It is not known if
the host produces specific antibody against the parasite. To answer this question, serum was collected from naturally infected fish and probed for the presence of *C. shasta*-specific antibodies. The development of resistance in salmonid strains has been studied by crossing fish from susceptible strains with fish from resistant strains and assessing the resistance of the progeny. These studies have shown that resistance of the progeny of these crosses was intermediate to that of the parental strains (Hemmingson et al., 1986). In this study, histological techniques were used to investigate resistance at the cellular level.

(4) Investigate the life cycle of *Ceratomyxa shasta*.

The inability to establish the conditions for transmission of the causative agent of ceratomyxosis in the laboratory has made the study of this parasite difficult. To investigate these conditions, experimental aquaria containing different substrates and fauna were established. Water samples from areas endemic for the parasite were filtered and examined for life stages using *C. shasta*-specific antisera.
CHAPTER II

Review of Literature

The myxosporean parasite, *Ceratomyxa shasta* (Noble, 1950), invades the intestinal tissues of salmonids and causes mortality in susceptible strains of fish. The disease was first observed in 1948 in fall-spawning rainbow trout (*Salmo gairdneri*) from Crystal Lake Hatchery, Shasta County, California (Wales and Wolf, 1955) and the etiological agent was described as a new species by Noble (1950). The parasite was first observed in Oregon in 1954 in adult spring chinook salmon (*Oncorhynchus tshawytscha*) (Wood and Wallis, 1955). Since these early reports, *C. shasta* has been observed in salmonids from many locations in the Pacific Northwest and is considered endemic in several major river systems. It is recognized as an important pathogen which causes losses in hatchery-reared and wild juvenile salmonids and also contributes to prespawning mortality in adult salmon (Sanders et al., 1970).

Host and tissue specificity of myxosporeans, cytochemical reactions, general morphology, and spore morphometrics have not been included in this review. Reviews on these subjects have been written by various authors (Dunkerly, 1925; Lom, 1970; Rogers and Gaines, 1975; Mitchell, 1977; and Amandi, 1984). Taxonomic monographs have been written by Gurley (1894), Thelohan (1895), Labbe (1899), Auerbach (1910) and Kudo (1920). Histopathology has been discussed by Johnson (1975). Other subjects have been
covered in Chapter I and will not be covered extensively here. They include: external and internal signs of the disease, histopathology, identification, effects of temperature and salinity, host range and susceptibility, geographic distribution, transmission and life cycle, and control.

**Taxonomy**

The taxonomic classification of myxosporeans has been disputed since their initial description. When the taxonomic scheme of the Protozoa was developed, protozoans were considered a phylum and myxosporeans were placed in the class Sporozoa in the subphylum Plasmodroma. In 1964, the Society of Protozoologists revised the original scheme and reclassified myxosporeans and microsporeans as part of the subphylum Cnidospora because of their polar filaments (Honigberg et al., 1964). However, Sprague (1966) disputed this definition as artificial and proposed separation of the two classes and formation of the subphyla Myxospora and Microspora (Sprague, 1969). Advances made by the use of the electron microscope caused another revision of the Protozoa in 1980 (Levine et al.). In this present classification, the protozoa exist as a subkingdom under the kingdom Protista and is divided into seven phyla. The phylum Myxozoa contains organisms having spores of multicellular origin, one or more polar capsules and sporoplasts, and one to three valves. Two classes are contained in this phylum; the Myxosporea Butschli, 1881, and the Actinospora Stole, 1889. Myxosporeans
are characterized by having one or two sporoplasms and one to six polar capsules. Polar capsules contain filaments which may function in anchorage and there are usually two, but as many as six spore valves. Trophozoites are considered the proliferative stage and members of this class are either histozoic or coelozoic in cold-blooded vertebrates. Further classification of the myxosporeans is given below:

Phylum: Myxozoa

Class: Myxosporea

Order: Bivalvulida - spore wall with two valves.
Suborder: Bipolarina - polar capsules at opposite ends of the spore, in the sutural plane.

*Myxidium, Sphaeromyxa*

Suborder: Eurysporina - two to four polar capsules at one pole, perpendicular to the sutural plane.

*Ceratomyxa, Chloromyxum, Sphaerospora*

Suborder: Platysporina - two polar capsules at one pole in the sutural plane; spores bilaterally symmetrical.

*Henneguya, Myxobolus*

Order: Multivalvulida - three or more valves.

*Hexacapsula, Kudoa*

Class: Actinosporea - contains three polar capsules and three valves; several to many sporoplasms; reduced
trophozoite stages; and are parasites of invertebrates, especially annelids.

Subclass: Actinomyxia
Order: Actinomyxidia

*Triactinomyxon*

Although this classification sets myxosporeans apart from other protozoa, they are multicellular organisms and therefore contradict the definition of the subkingdom protozoa. This point has been argued by several researchers (Dunkerly, 1925; Grasse, 1960; Lom, 1973). Electron microscopic studies have shown that the ontogenesis and functional morphology of myxosporeans differs greatly from all protozoans except the Actinomyxidia (Mitchell, 1977). The similarity between the myxosporean polar capsules and the nematocysts of the Cnidaria suggests a relationship between these groups (Lom and de Puytorac, 1965; Lom, 1973); however, myxosporeans have been retained in the Protozoa for convenience.

The family Ceratomyxidae contains two genera, *Ceratomyxa* and *Leptotheca*. *Ceratomyxa* was established by Thelohan in 1892. In 1895 he established the genus *Leptotheca* to include species belonging in the Ceratomyxidae but having oval or ellipsoid spores. Because these classifications are artificial, many species were described which could be placed in either genus. The suggestion to combine the two genera was made (Jameson, 1929), but Kudo (1933) redefined *Leptotheca* as including spores with a width:height ratio of two or less and this system is still used.
Species in each genus are also classified by spore morphometrics and therefore many assignments are arbitrary, especially when preserved specimens are used. Meglitsch (1957) objected to using only spore shape and size in a dichotomous manner and suggested considering host species, target organ, and geographic range in speciation. However, present schemes are still based mainly on morphometrics. Organisms of both genera are usually coelozoic parasites which colonize the gall bladder of marine fishes. *Ceratomyxa shasta* was the first member of its genus identified in freshwater fish and the first recognized as a histozoic parasite (Noble, 1950). Although *Ceratomyxa hongzensis* was described as a parasite of the freshwater catfish, *Pseudobagrus eupogon*, in China, it is coelozoic (Hsieh and Chen, 1984).

*Myxosporean Life Cycles and Replication*

The complete life cycle of *C. shasta*, like that of most myxosporeans, is unknown. Although life stages can be identified in the salmonid host, none have been recognized outside the fish. Early life history studies consisted of examining parasite developmental stages in the host and postulating their relationships. Several descriptions of *Ceratomyxa* life cycles have been made (Awerinzew, 1909, 1911; Georgevitch 1916, 1917 and 1929; and Mavor, 1916). Noble (1941) investigated the life cycle of *Ceratomyxa blennius* from the gall bladder of a marine fish, *Hypsoblennius gilberti*. Although his description varied from earlier researchers, he outlined a general pattern. In the proposed
life cycle, a binucleate cell with haploid nuclei or two uninucleate
haploid cells infects the host, fuse, and produce a diploid
uninucleate zygote. The zygote undergoes nucleogomy, the process
in which a multinuclear cell forms from a uninuclear cell.
Nucleogomy may be accompanied by plasmotomy or budding, the
multiplicative part of the cycle, or by sporogony. Asexual
reproduction in Ceratomyxa, whether budding or plasmotomy, can
be considered a secondary process which accompanies spore
production. Sporogony in Ceratomyxa is usually disporous and
disporoblasts contain twelve generative and one or two vegetative
nuclei. The generative nuclei divide into two groups of six and
form two sporoblasts. Each sporoblast is surrounded by an
envelope developed by the two shell-valve nuclei (sporogenesis);
two form the polar capsules (capsulogenesis) and the remaining two
become the haploid nuclei of the sporoplasm (gametogenesis).

The most widely accepted scenario for infection of the host
was described by Kudo (1966). The spore enters the digestive tract
of the fish and opens. It is thought that the polar filaments are
extruded to serve as an anchor. An infective sporoplasm, termed
the amoebula, is released, penetrates the gut epithelium and
migrates to its target tissue, possibly via the bloodstream. Coelozoic
species found in the gall bladder probably pass from the intestine
to the bile duct (Mitchell, 1977). When a suitable site is reached
the amoebula begins growth as a trophozoite. In most histozoic
species, the trophozoite is surrounded by a host tissue capsule and
forms a cyst and in coelozoic species, trophozoites float free in the
lumen and exhibit pseudopodial movement (Mitchell, 1977). Trophozoites multiply and mature to form spores. If the infection is near the body surface, spores may be liberated when cysts rupture; if internal, spores are released upon the death of the fish. Noble (1941) suggested ripe spores of coelozoic origin are released by way of the bile duct and intestine, and spores from ruptured internal cysts may exit through the intestine and gills (Hoffman et al., 1965).

Attempts to transmit myxosporean infections and to determine the entire life cycle of these parasites have been made. Laboratory transmission of ceratomyxosis has been achieved by intraperitoneal injection and anal intubation of ascites fluid from infected fish (Schafer, 1968; Fryer and Sanders, 1970; Johnson, 1975; Johnson et al., 1979) However, this only represents passive transfer of the infection. Infection by C. shasta has also occurred when susceptible fish were exposed to bottom sediments from an area endemic for the infective stage (Fryer and Sanders, 1970) but that stage was not identified. Other attempts to transmit ceratomyxosis have included cohabitation of infected and uninfected fish, feeding infected viscera to susceptible fish, and exposing fish to spores aged in mud (Schafer, 1968; Johnson et al., 1979; Fendrick, 1980). None of these experiments have resulted in transmission of infections. Attempts to transmit infections of other myxosporeans have been unsuccessful. Bond (1936) could not transmit Myxobolus funduli, Myxobolus subtecalis, Myxidium folium nor Myxobolus bilineatum by feeding infected tissues or
placing infected material in lesions on the fish. [The genus *Myxosoma* has been reduced to synonymy with *Myxobolus* (Lom and Noble, 1984), and that convention will be used in this literature review]. Attempts to transmit *Myxobolus cartilaginous* by feeding infected tissue or cladocerans and algae containing spores have been unsuccessful (Hoffman et al., 1965). Other myxosporeans which could not be transmitted by these or similar methods include: *Myxidium oviforme* (Walliker, 1968); *Myxobolus muelleri* and *Myxobolus dujardini* (Mitchell, 1970); *Myxobolus insidiosus* (Wyatt, 1971); *Henneguya* sp. (Minchew, 1973); *Myxobolus pharyngeus* (Spall, 1973); *Myxobilatus gasterostei* (Lester, 1974) and *Myxobolus pavlovskii* (Molnar, 1979).

Although several reports of experimental transmission have been made, many do not qualify as true transmission and others either did not state the experimental conditions or did not have adequate controls. Transmission of *Myxobolus pavlovskii* (Molnar, 1979), *Myxobolus vanivilasae* (Seenapa and Monohar, 1981), and *Myxobolus insidiosus* (Amandi, 1984) occurred when fish were exposed to contaminated substrates.

The only myxosporean infection for which transmission has repeatedly been achieved under experimental conditions is salmonid whirling disease, caused by *Myxobolus cerebralis*. Uspenskaya (1957) infected trout by pipetting spores aged for four months in spring water into the stomach and Prihoda (1983) described similar results. Hoffman and Putz (1969) did not achieve transmission by this method; however, they did transmit the
disease by aging spores in mud. Taylor and Lott (1978) were able to transmit whirling disease by feeding infected fish to aquatic birds. The excreted spores were aged in mud and infections developed in susceptible fish added to the tanks. This may explain one method by which this organism spreads to non-endemic locations. Keiz (1964) showed that *M. cerebralis* could also be disseminated through contaminated eggs or containers, but O'Grodnick (1979) found it was not transmitted in ovarian fluid. Daniels et al. (1976) found organisms which they believed may represent the infective stage in the epithelium of trout exposed to *M. cerebralis*.

Markiw and Wolf (1983) have proposed that the spore aging process which results in transmission of whirling disease requires participation of tubificid oligochaetes. These tubificids harbor an actinomyxidian of the genus *Triactinomyxon* which reacts by immunofluorescence with polyclonal anti-*M. cerebralis* serum (Wolf and Markiw, 1981). They propose (Markiw and Wolf, 1983; Wolf and Markiw, 1984; Markiw, 1986) that spores of *M. cerebralis* shed from infected fish, either when the fish dies or is eaten by predators, are ingested by *Tubifex tubifex*. Within the tubificid gut, the parasite replicates and undergoes morphologic changes and becomes the actinosporean spore. After three to four months sporocytes release mature *Triactinomyxon* and fish become infected when they feed on infected worms or encounter the waterborne *Triactinomyxon* spore stage. They further supported the relatedness of these two stages in electrophoretic studies (Markiw
and Wolf, 1986). By studying the protein profiles, they determined that at least six antigenic constituents were homologous. Hamilton and Canning (1985) presented evidence contrary to the hypothesis presented by Wolf and Markiw. In fluorescent antibody studies they found the reactivity of \textit{M. cerebralis} antiserum with \textit{Triactinomyxon} extended to genera of other actinomyxidians and was therefore non-specific. They cite other contradictions to the alternate life stage theory: the experimental transmission of \textit{M. cerebralis} in the absence of \textit{Triactinomyxon}, the observation that the addition of \textit{M. cerebralis} spores to mud did not increase the prevalence of \textit{Triactinomyxon}, and the inability to correlate the occurrence of \textit{Triactinomyxon} and \textit{M. cerebralis} in field investigations. Studies by El-Matbouli and Hoffman (1988) with \textit{Myxobolus cotti} and \textit{M. cerebralis}; however, demonstrated that in both species, actinosporan spores were alternate life stages of the parasite. These studies by Wolf and Markiw and El-Matbouli and Hoffman are the first reports of completion of the myxosporean life cycle under controlled laboratory conditions and the first evidence that myxosporeans may have two developmental sequences involving both a vertebrate and invertebrate host.

\textit{Ultrastructure}

Ultrastructural studies of \textit{C. shasta} are limited. Fendrick (1980) studied the influence of aging on the maturation of the spore by scanning electron microscopy. This was the first report showing the polar pores of \textit{C. shasta} filled with a polar cap structure.
located over the filament discharge canal. These polar caps became evident as spores aged and the continuous smooth wall over the area of the polar capsules in fresh spores separated. He also noted a cement-like substance holding the two spore valves together which he suggested may represent the desmosomal junction shown by Gould (1969) in his transmission electron microscopic study of *C. shasta* ultrastructure.

Transmission electron microscopic studies of *C. shasta* ultrastructure have been done by Gould (1969) and Yamamoto and Sanders (1979). The latter studied sporogenesis; however, they were unable to ascertain the initial stages leading to sporogenesis and did not see the union of two cells noted by other researchers (Lom and de Puytorac, 1965; Lom, 1969; Current and Janovy, 1977). They did see the early sporont cells within the larger mother cell. Their description of sporonts deriving from six cells was similar to observations of *Ceratomyxa* life cycles by Noble (1941) and also to descriptions of developmental stages of *Henneguya pinnae* (Schubert, 1968) and *Henneguya exilis* (Current and Janovy, 1977). The formation of the sporoplasm differed slightly from that proposed for *H. exilis* because instead of directly forming a binucleate sporoplasm, the two germinative cells did not fuse until maturation of the spore. The description of the development and structure of the polar capsule was similar to the detailed description by Lom and de Puytorac (1965). Attached to the developing capsule was a long tube which probably produces the material for the polar filament. The filament of *C. shasta*
consisted of five or six coils while those of *Henneguya* species had
twelve (Lom and de Puytorac, 1965; Current and Janovy, 1977). In
cross section, the filament was kidney bean-shaped and had several
electron dense layers, similar to filaments of other myxosporeans.

By transmission electron microscopy, Gould (1969) observed
the formation of a septate desmosome connecting the spore valves.
This characteristic had not been previously reported and he
suggested that the common presence of this feature in
myxosporeans and metazoans may be further proof of their
relatedness. This feature may represent the microtubuli noted by
Lom and de Puytorac (1965).

**In Vitro Cultivation**

One of the difficulties in evaluating the success of *in vitro*
maintenance methods is determining if spores are viable. Extrusion
of polar filaments was the most frequently used criterion until Lom
(1964) reported that dried spores and spores fixed in alcohol for
several years were capable of filament extrusion after treatment
with alkali. Only culture of sporoplasms released by the spores and
artificial transmission of the infection provide evidence of spore
viability. Patashnik and Groninger (1964) and Siau (1977) were
able to release intact sporoplasms by temperature cycling and
mechanical disruption, respectively. Patashnik and Groninger
observed that *Kudoa* spores from samples of milky halibut
disappeared and were replaced by ameboid trophozoites following
temperature cycling.
Only two attempts of *in vitro* cultivation have been reported. Wolf and Markiw (1976) found that trophozoite and other pre-spore stages of *M. cerebralis* proceeded to form spores when cultured in Eagle's minimal essential culture medium. However, spore viability was determined by the ability to discharge polar filaments. Siau (1977) artificially released sporoplasms from *Myxobolus exiguis* and cultured them on rainbow trout gonad 99 cell monolayers and in Stoker's culture medium with 10% calf serum. He was able to see multiplication to the first stage of sporogenesis but not complete development of the spore.

**Biochemical Characteristics**

Cytochemical stains for DNA and RNA have been employed by several researchers to provide evidence for the multicellular nature of myxosporeans, to study the process of sporulation, and to determine when protein synthesis occurs. These studies have been reviewed by Amandi (1984). Chemical composition of spores was first studied by Erdman (1917) who suggested that polar filaments were composed of glycogen. However, Kudo (1921) disagreed with this analysis and also determined that cellulose and chitin were not components of the spore valves. Bond (1937) and Lom (1964) reported that protein was the major spore valve component and they detected no mucopolysaccharides, polysaccharides, or chitin. Siau (1977) determined that the spores contained more calcium than sulfur and that calcium was concentrated in the regions of the suture line, sutural ridge, and in the central spore region.
Amandi (1984) analyzed the amino acid composition of spore valves of *M. insidiosus* and *C. shasta*. They contained 9.5 and 7.8% protein, respectively, and glutamic acid, aspartic acid, and lysine comprised over 40% of the amino acid content.

**Serology**

Serological techniques have been developed mainly for the detection and diagnosis of myxosporean infections. Methods for production of antibodies against myxosporeans have been confined to investigations using the spore stage as antigen because it is the only stage which can be easily purified. Pauley (1974) reported the development of the first antigenic preparation from the spore stage of any histozoic sporozoan parasite. Rabbits formed precipitating antibodies against *M. cerebralis* spores; however, because this antibody cross-reacted with rainbow trout tissue antigens, Pauley concluded that *M. cerebralis* was able to mimic normal host antigens and thus evade the immune response.

Other researchers have also suggested that myxosporean antigens are closely related to those of their hosts. Mitchell (1970) found antigenic similarities between *Myxobolus muelleri* and *Myxobolus dujardini* and their fish hosts when he used hypersensitivity tests in rabbits. McArthur and Sengupta (1982) used immunodiffusion and electrophoresis to demonstrate antigenic similarity between *Myxobolus* sp. and skin and muscle antigens of fish. Halliday (1974) produced polyclonal antiserum against *M. cerebralis* but did not report any cross-reactivity; however, his only
controls were purified spores of Myxobolus sp. from roach. An antibody response was not detected in immunized rainbow trout or in naturally infected fish with clinical whirling disease. He suggested that the lack of response in naturally infected fish was because of the sheltered infection site or the non-pathogenicity of the spores and cited technical inadequacy as a reason for the observation of no response in immunized fish. Siau (1980) used complement fixation techniques to demonstrate that in immunized Mugil there was no production of precipitating or complement-fixing antibodies against Myxobolus exiguus. However, he observed that the fish serum behaved as though it contained antigen when it was reacted with rabbit serum. Griffin and Davis (1978) used indirect fluorescent antibody techniques (IFAT) to detect antibody against M. cerebralis in naturally infected fish.

The application of IFAT for diagnosis of myxosporean infections and methods for detection of spores have been described. Halliday (1974) showed that IFAT could be used to detect spores but did not believe it would be valuable as a diagnostic method. Markiw and Wolf (1978) compared the efficacy of the direct FAT (DFAT) and the IFAT on spores of M. cerebralis and found less cross-reactivity with closely related species when the DFAT was used. Banner et al. (1982) developed a rapid method for labeling antibody with fluorescein and applied this method to rabbit antiserum against C. shasta. Hamilton and Canning (1988) described a method for producing polyclonal mouse anti-M.
cerebralis antiserum from purified spores and the use of immunofluorescent labeling of histological sections.
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CHAPTER III

*Ceratomyxa shasta*, a Myxosporean Parasite of Salmonids

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Introduction

Ceratomyxosis is a disease of salmonid fishes caused by the myxosporean *Ceratomyxa shasta*. The parasite has a tropism for the intestinal tissue of the fish and causes high mortalities in susceptible strains of salmonids. The disease was first observed in 1948 in fall-spawning rainbow trout (*Salmo gairdneri*) from Crystal Lake Hatchery, Shasta County, California (Wales and Wolf, 1955). The etiological agent was established as a new species by Noble (1950), who described *C. shasta* as the first species of the genus *Ceratomyxa* to parasitize freshwater fish and the only member of the genus that is histozoic. Other species of *Ceratomyxa* occur in marine fishes and parasitize the lumen of the gall bladder and urinary bladder.

*Ceratomyxa shasta* is an important parasite in the Pacific Northwest because it not only causes losses in hatchery-reared and wild juvenile salmonids but also contributes significantly to prespawning mortality in adult salmon. Although the parasite has not been detected outside the Pacific Northwest, its distribution in that region has expanded. It is not known whether this increase reflects a true spread of the disease or only improved detection methods.
Diagnosis and Identification

External and internal signs

Clinical signs of ceratomyxosis vary among salmonid species. Infected juvenile rainbow trout (and steelhead) become anorexic, lethargic, and darken in color. Ascites may distend the abdomen, the vent may be swollen and hemorrhaged, and exophthalmia is common (Schafer, 1968). Infected juvenile chinook salmon (Oncorhynchus tshawytscha) first become emaciated and later sometimes develop large fluid-filled blebs and kidney pustules (Conrad and Decew, 1966).

Internally, the intestinal tract of infected juvenile rainbow trout becomes swollen and hemorrhaged and the intestinal contents mucoid, and caseous material lines the intestine and pyloric caeca (Conrad and Decew, 1966). The entire digestive tract, the liver, gall bladder, spleen, gonads, kidney, heart, gills, and skeletal muscle may become diseased, hemorrhaged, and necrotic (Wales and Wolf, 1955). Infected adult chinook salmon may have nodular lesions in the intestine that perforate, causing death. These nodules may be accompanied by gross lesions in the liver, kidney, spleen, and muscle. Infected adult coho salmon (O. kisutch) show grossly thickened intestinal walls and pyloric caeca, and large abscessed lesions in the body musculature (Wood, 1979).
**Histopathology**

In juvenile rainbow trout the first sign of infection appears in the posterior intestine. The progress of the infection is temperature dependent, the first sign of infection appearing between days 12 and 18 post-exposure in fish held at 12°C, and at 7 days in fish held at 18°C (Yamamoto and Sanders, 1979). Trophozoites are first seen in the mucosa; their appearance is followed by a strong inflammatory response in the lamina propria. As the infection progresses, the parasite multiplies in all layers of the intestine and causes severe inflammation and desquamation of the mucosal epithelium. Trophozoites penetrate the intestinal tract, spread into the surrounding adipose and pancreatic tissues, and enter the bloodstream enabling them to reach other tissues and organs. In late stages of infection, the parasite is in most tissues and organs adjacent to the intestine, including the liver, kidney, pyloric caeca, and spleen. The spore stage of *C. shasta* is not evident until the terminal stages of the infection and this sometimes causes delayed diagnosis of the disease.

**Identification**

Diagnosis requires that spores be found and identified by their size, shape, and location. The American Fisheries Society *Fish Health Blue Book* (Amos, 1985) recommends the following procedures: (1) examination of wet mounts from the lower intestinal wall, ascites, gall bladder, and lesions by phase contrast or bright light microscopy at 400X; (2) examination of air-dried
smears stained by the Ziehl-Neelsen method but without heating; or
(3) fixation of smears in Schaudin's fixative and staining with
Heidenhain's iron hematoxylin (for permanent preparations). For
examination of live fish an intestinal lavage technique can be used
(Coley et al., 1983). Spores of *C. shasta* are about 14 to 23 μm long
and 6 to 8 μm wide at the suture line (Fig. III.1). The ends of the
spores are rounded and reflected posteriorly; the suture line is
distinct (Noble, 1950). In smears stained by the Ziehl-Neelsen
method the polar capsules stain red against a bluish sporoplasm
and background. Trophozoites, which are rounded but variable in
shape, mature to form a sporoblast that usually contains two spores
(Fig. III.2). Because of the variability in size and shape of the
trophozoites and their similarity to this stage in other
myxosporeans, observation of trophozoites by light microscopy is
not sufficient for diagnosis. Consequently, serological techniques
have been developed in which monoclonal antibodies are used.
The antibodies produced react specifically with the prespore stages
of the parasite and do not cross-react with trophozoite or spore
stages of other myxosporeans. Use of the monoclonal antibodies
and fluorescein or enzyme conjugated secondary antibodies enables
the reliable detection of early infections (Bartholomew et al.,
manuscript submitted).
Fig. III.1. Phase contrast photomicrograph showing morphology of *Ceratomyxa shasta* spores.

Fig. III.2. Phase contrast photomicrograph of trophozoite and disporoblast stages of *Ceratomyxa shasta*.
Figure III.1-III.2
Ecology

Effects of temperature

Infection by *C. shasta* was once believed to occur only when water temperatures exceeded 10°C, thus accounting for the seasonal occurrence of the disease; however, later reports indicated that fish can become infected in water at temperatures as low as 4 to 6°C (Ratliff, 1983; Ching and Munday, 1984a). Although fish are infected at these lower temperatures, the progress of the disease is temperature dependent and most infections are detected later, after the water warms. Udey et al. (1975) reported that rainbow trout exposed to the infective stage of *C. shasta* and held at water temperatures of 6.7 to 23.3°C had little or no ability to overcome the infection, and that the mean time from exposure to death was directly correlated to temperature (e.g., about 155 days at 6.7°C and 14 days at 23.3°C). In rainbow trout the disease process was suppressed at 3.9°C; however, when the infected fish held at this temperature were transferred to water at 17.8°C, many died. Coho salmon appeared better able to combat the infection at low water temperatures, but the mean time to death remained temperature dependent.

Effects of salinity

Only limited information is available about the effects of salt water on the progress of a *C. shasta* infection. Johnson (1975) reported that infections were prevented at salt concentrations
greater than 15 ppt. Although this would protect juvenile salmonids from infection in estuarine areas, the fate of fish that were infected in fresh water and then migrated into salt water was not determined. Acute ceratomyxosis has been reported in juvenile chum salmon captured off the coast of British Columbia, Canada (Margolis and Evelyn, 1975), demonstrating that the disease is not attenuated by salt water. Ching and Munday (1984b), who exposed chinook salmon to the infective stage of C. shasta, found that the disease caused 100% mortality when the fish were held in either fresh water or salt water. Similarly designed experiments with steelhead indicated that migration to salt water may reduce the progress of the disease, but the extent of attenuation may be masked in fish overwhelmed by a high number of infectious units (Hoffmaster, 1985).
Host Range and Susceptibility

It is accepted that only salmonids are susceptible to \textit{C. shasta} infection (Table III.1), but this susceptibility may vary within a species. Experiments to test resistance of different strains of the same species to \textit{C. shasta} indicated that juvenile salmonids originating from waters containing the infective stage of the parasite were more resistant than strains from areas free of the infective stage (Johnson, 1975; Zinn et al., 1977; Buchanan et al., 1983; Hoffmaster, 1985). The susceptibility to infection by \textit{C. shasta} in progeny produced from crosses between resistant and susceptible coho salmon is intermediate between that of the parental stocks (Hemmingsen et al., 1986). The management implications of these studies are that relocation of salmonids from areas where \textit{C. shasta} is not present into areas endemic for the parasite is not likely to be successful, and that these introductions may adversely affect the survival of progeny of resident resistant strains, if interbreeding occurs.

Although juvenile salmonids from waters endemic for \textit{C. shasta} are resistant to infection, ceratomyxosis has been determined to be an important cause of prespawning mortality in the adults. Coley et al. (1983) reported that 94% of adult spring chinook salmon at Rapid River Hatchery, Idaho, were infected with \textit{C. shasta}. Similar incidences of infection in adults have been reported by other researchers (Sanders et al., 1970; Yasutake et al., 1986; Chapman, 1986). Although infection by \textit{C. shasta} occurs
Table III.1. Host range of *Ceratomyxa shasta*.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td><em>Salmo gairdneri</em></td>
<td>Noble, 1950</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td><em>Oncorhynchus tshawytscha</em></td>
<td>Conrad and Decew, 1966</td>
</tr>
<tr>
<td>Coho salmon</td>
<td><em>Oncorhynchus kisutch</em></td>
<td>Conrad and Decew, 1966</td>
</tr>
<tr>
<td>Steelhead</td>
<td><em>Salmo gairdneri</em></td>
<td>Conrad and Decew, 1966</td>
</tr>
<tr>
<td>Brook trout</td>
<td><em>Salvelinus fontinalis</em></td>
<td>Schafer, 1968</td>
</tr>
<tr>
<td>Brown trout</td>
<td><em>Salmo trutta</em></td>
<td>Schafer, 1968</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td><em>Salmo salar</em></td>
<td>Sanders et al., 1970</td>
</tr>
<tr>
<td>Cutthroat trout</td>
<td><em>Salmo clarki</em></td>
<td>Sanders et al.,1970</td>
</tr>
<tr>
<td>Sockeye salmon</td>
<td><em>Oncorhynchus nerka</em></td>
<td>Sanders et al., 1970</td>
</tr>
<tr>
<td>Chum salmon</td>
<td><em>Oncorhynchus keta</em></td>
<td>Margolis and Evelyn, 1975</td>
</tr>
<tr>
<td>Pink salmon</td>
<td><em>Oncorhynchus gorbuscha</em></td>
<td>Bell and Traxler, 1985</td>
</tr>
</tbody>
</table>
during the freshwater phase of the fish's life cycle, it is not known whether these fish were infected before they entered salt water or when they reentered fresh water.
Geographic Distribution

*Ceratomyxa shasta* has been identified in salmonids from marine and freshwater environments in northern California, Oregon, Washington, Idaho, and British Columbia. However, waters where infected fish have been found do not necessarily contain the infective stage of the parasite (Johnson et al., 1979). This is exemplified in the Columbia River Basin, where infected adult coho and chinook salmon and steelhead migrate and distribute spores throughout the drainage, but the infective stage of *C. shasta* has not been demonstrated in many tributaries to which these fish have access. This suggests that the presence of spores alone is insufficient to cause transmission and disease.

The presence of the infective stage of *C. shasta* is demonstrated by using sentinel populations of susceptible salmonids and examining them for development of the disease and appearance of spores. The distribution of the infective stage has been documented by Johnson et al. (1979), Ching and Munday (1984a), and Hoffmaster et al. (1988) (Fig. III.3).

The confinement of this parasite to salmonids of the Pacific Northwest is unique. The distribution of many other fish pathogens has been expanded as a result of shipments of eggs or fish. This geographic isolation is compatible with the hypothesis that an as yet unknown factor is required for the completion of the life cycle of this parasite.
Fig. III.3. Distribution of the infectious stage of *Ceratomyxa shasta*.
Transmission and Life Cycle

The life history of *C. shasta*, like that of most other myxosporeans, is unknown. Natural transmission occurs when susceptible salmonids are exposed to water or sediments containing the infective stage (Schafer, 1968; Fryer and Sanders, 1970; Johnson, 1975) and exposure periods as short as 30 min are sufficient for infection to occur. Neither attempts to transmit ceratomyxosis from fish to fish nor the feeding of infected tissues containing spores and trophozoites have resulted in transmission of the disease (Wales and Wolf, 1955; Schafer, 1968; Wood, 1968; Johnson, 1975). But infections developed when susceptible fish were exposed to bottom sediments collected from a site endemic for the parasite (Fryer and Sanders, 1970).

Laboratory transmission of ceratomyxosis has been established by intraperitoneal injection and anal intubation of ascites from infected fish (Schafer, 1968; Fryer and Sanders, 1970; Johnson et al., 1979; Bower, 1985). The natural route of infection has not been established, but Schafer (1968) suggested that the establishment of infection in rainbow trout was not dependent on the ingestion of the spore. Differential filtration of waters endemic for *C. shasta* show that the infective stage is larger than 14 μm.

The inability to transmit ceratomyxosis between susceptible fish has led to speculation that an intermediate host may be involved in the life cycle. As yet there is no conclusive evidence to support this hypothesis.
Control

Because *C. shasta* infections are not transmitted directly between fish, outbreaks of the disease in hatchery fish occur only as a consequence of introducing the infective stage through the water supply. Because no chemotherapeutic agent yet tested has been useful in controlling ceratomyxosis, the most effective means of disease prevention in a hatchery situation is avoidance of water supplies containing the infective stage. In hatcheries where alternative water supplies are unavailable, water treatment methods to eliminate the infective stage have been developed. Bedell (1971) found that ultraviolet irradiation or chlorination of water supplies reduced the number of *C. shasta* infections, but did not eliminate them. Sanders et al. (1972) and Bower and Margolis (1985) determined that sand filtration, in combination with either ultraviolet irradiation or chlorination, was effective in reducing the incidence of disease. Tipping (1986) reported that ozone was effective in controlling ceratomyxosis in hatchery fish. However, the most successful approach for control of ceratomyxosis in both hatchery and wild populations is the introduction of resistant salmonids (Buchanan et al., 1983).
Acknowledgments

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CHAPTER IV

Geographic Distribution of the Myxosporean Parasite, Ceratomyxa shasta in the Columbia River Basin

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Abstract

The geographic distribution of the infective stage of *Ceratomyxa shasta* within the Columbia River basin was studied using sentinel fish populations. Results of this investigation demonstrate that the infective stage of the parasite is present in the Columbia River from its mouth to the confluence with the Snake River, and in the Snake River at least to Oxbow Dam. The extension of the range of this pathogen and the high concentration of infective stages observed from certain sites indicates that ceratomyxosis may be a more important factor causing mortality in Columbia River salmonids than previously recognized.
Introduction

*Ceratomyxa shasta* Noble, 1950, is a myxosporean parasite causing disease and high mortality in susceptible juvenile and adult salmonids in California, Oregon, Washington, and British Columbia. The life cycle of *C. shasta* has not been described but it is known that susceptible fish become infected while residing in, or migrating through, waters that contain the infective stage of the parasite. A distinction is made between waters that harbor the infective stage and those that do not but contain infected anadromous salmonids (Johnson et al., 1979). Although infected fish are found throughout the Columbia River basin, the infective stage of *C. shasta* has only been demonstrated to exist in a restricted number of watersheds, suggesting that the presence of spores alone is insufficient to provide transmission and subsequent disease. Therefore, the infective stage is restricted to a smaller geographic area within the range where infected fish have been observed.

The presence of the infective stage of *C. shasta* can only be demonstrated by exposure of susceptible salmonids to waters endemic for the parasite and subsequently examining these fish for development of disease and appearance of spores. The distribution of the infective stage of *C. shasta* has been documented by Johnson et al. (1979) and Ching and Munday (1984). In the Columbia River basin, the parasite was known to exist in the Columbia River from the mouth to its confluence with the Deschutes River, and in the Deschutes, Willamette and Cowlitz Rivers, and LaCamas Lake and
Creek. This communication reports an extended distribution of the infective stage of *C. shasta* in the Columbia River basin.
Materials and Methods

Susceptible rainbow trout, *Salmo gairdneri* Richardson, were held at selected sites in the Columbia River basin in aluminum liveboxes as described by Udey et al. (1975)(Fig. IV.1). Following a seven (in 1983) to fourteen day (in 1984-86) exposure period, all fish were transported to holding facilities with fish-pathogen-free water for the duration of the incubation period of *C. shasta*. Dead fish were collected daily and were considered infected with *C. shasta* if one or more spores were found in wet mounts of intestinal tract scrapings. After 100-120 days all surviving fish were sacrificed and examined for spores.
Figure IV.1. Map indicating locations where fish were exposed for the detection of the infectious stage of *Ceratomyxa shasta* in the Columbia River basin. ▲ exposure sites; ○ exposure sites where fish were positive for *C. shasta* infections.
Results and Discussion

Exposure of susceptible fish in the Columbia River basin demonstrated that the infective stage of *C. shasta* is present in the Columbia River to its confluence with the Snake River and in the Snake River to Oxbow Dam (Table IV.1, Fig. IV.1). The occurrence of infection was not consistent among fish held at sites in the Columbia and Snake Rivers. Variation in the prevalence of ceratomyxosis was noted at a given exposure site within and between years. These results are consistent with those of other researchers (Ratliff, 1981; Ching and Munday, 1984). Frequency of infection was generally less than 20%, with the exception of a June, 1984 exposure at the Dalles Dam on the Columbia River where the level was 52%, and observations made in July, 1986 at Hell's Canyon and Oxbow Dams on the Snake River where values of 96 and 95% were obtained, respectively. These variations may result from differences in water temperature and flow.

Although infected adult chinook salmon have been reported at Oxbow Dam, they were believed to have contracted the disease in the Columbia River below its confluence with the Deschutes and prior to entering the Snake River (Sanders et al., 1970). The extension of the range of the infective stage into the Snake River drainage suggests that upriver salmonids are exposed to this parasite for a longer period than previously recognized and at higher concentrations than expected. Although strains of upriver salmonids have been considered resistant to infection (Zinn et al., 1977; Buchanan et al., 1983), these conclusions were
Table IV.1. Prevalence of *Ceratomyxa shasta* in susceptible rainbow trout exposed at selected locations in the Columbia River basin.

<table>
<thead>
<tr>
<th>Exposure site</th>
<th>River km</th>
<th>Exposure date</th>
<th>Number of fish recovered</th>
<th>Number of fish infected</th>
<th>Percent of fish infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia River</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonneville Dam</td>
<td>233</td>
<td>9/84</td>
<td>35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/84</td>
<td>25</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Dallas Dam</td>
<td>309</td>
<td>9/84</td>
<td>38</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/84</td>
<td>48</td>
<td>25</td>
<td>52</td>
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<tr>
<td></td>
<td></td>
<td>9/84</td>
<td>50</td>
<td>0</td>
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</tr>
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<td></td>
<td></td>
<td>6/84</td>
<td>67</td>
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<td></td>
<td></td>
<td>5/85</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/85</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>John Day Dam</td>
<td>348</td>
<td>9/84</td>
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The presence of the infective stage was not demonstrated in the following tributaries: Elokomin; Kalama; Lewis River-north fork; Wind; White Salmon; Klickitat; John Day; Umatilla; Yakima; Clearwater-north fork, south fork and mainstream; Grand Rhonde, Wallowa, and Imnaha Rivers and Lookingglass Creek.
drawn from experiments in which fish were exposed to the infective stage of the parasite for only five days. Dawley et al. (1984) calculated that during 1983 the average migration rate for yearling salmon and steelhead trout was 18 and 35 km/day in the Columbia River. This means fish migrating from Oxbow Dam to the mouth of the Columbia River would be exposed to the infective stage of \textit{C. shasta} for approximately 53 and 27 days, respectively. Therefore, our new data indicate that ceratomyxosis may be a more important factor causing mortality in anadromous salmonids in the Columbia River basin than previously recognized.
Acknowledgements

This work was supported by the Bonneville Power Administration under contract No. DE-A179-83BP11987. The authors acknowledge Portland General Electric Company and Oregon Department of Fisheries and Wildlife for supplying the facilities and fish used in this study. Appreciation is extended to individuals who helped with the coordination and placement of liveboxes. Oregon Agricultural Experiment Station Technical Paper No. 8161.


CHAPTER V

Development, Characterization, and Use of Monoclonal and Polyclonal Antibodies against the Myxosporean, *Ceratomyxa shasta*

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Running Head: Antibodies against *Ceratomyxa shasta*.

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Abstract

Both monoclonal and polyclonal antisera were produced against Ceratomyxa shasta. Ascites containing trophozoites of the parasite was collected from infected fish and used as antigen for immunization of mice. The resulting monoclonal antibodies reacted specifically with trophozoite and sporoblast stages but did not react with C. shasta spores by either indirect fluorescent antibody techniques or in Western blots. This indicates that C. shasta contains unique antigens and that some of these antigens are specific to certain life stages of the parasite. Polyclonal antiserum was produced in a rabbit by injecting a spore protein electroeluted from an SDS-polyacrylamide gel. This antiserum reacted with both trophozoites and spores by indirect fluorescent antibody techniques and in Western blots. All antisera were tested for cross-reactivity to trout white blood cells, a contaminant of the ascites, and to other myxosporeans. Two monoclonals antibodies reacted with white blood cells and myxosporeans of the genera Sphaerospora and Myxobilatus. One hybridoma produced antibodies of high specificity for C. shasta prespore stages. This is the first report of a monoclonal antibody produced against a myxosporean parasite.
Introduction

The myxosporean parasite, *Ceratomyxa shasta* (Noble, 1950) causes an intestinal disease in susceptible salmonid fish in the Pacific Northwest region of the United States and Canada. This parasite has been responsible for epizootics in both wild and hatchery populations of salmonids. At present there is no effective means of control other than avoidance of the infectious stage of this organism and stocking of resistant strains of salmonids in endemic areas. Spores of *C. shasta* are easily detected in moribund fish; however, the infectious process is temperature dependent, with mortality occurring about 56 days post-exposure among fish held at 12°C, (Udey et al., 1975). Development of mature spores occurs just prior to death of the host, but before spore formation, trophozoite stages of the parasite are abundant in the intestinal tract. While these stages may be identified using histological techniques, they often go undetected by microscopic examination of intestinal tract scrapings, the standard diagnostic procedure. The similarity of the multinuclear trophozoites to the same life stages of other myxosporeans also complicates diagnosis.

The life cycle of *C. shasta*, like that of most myxosporeans, remains unknown. The morphology of the infective stage and the initial site of infection in the host have not been determined. Attempts to transmit the disease by feeding infected tissues, cohabitation of infected and susceptible fish, and exposing susceptible fish to a mixture of mud and infected tissues have
failed (Johnson et al., 1979). The inability to transmit the disease between susceptible fish has led to speculation that an intermediate host may be necessary. The life cycle of another myxosporean parasite, *Myxobolus cerebralis*, was shown by Wolf and Markiw (1984) to require *Tubifex tubifex* as an intermediate host. They were also able to demonstrate, that within the oligochaete, the myxosporean spore transforms into a form previously identified as a triactinomyxon. This alternation of life stages and intermediate host involvement has not yet been demonstrated for *C. shasta*.

The usefulness of serological techniques for identifying different life stages has been shown for several human parasites. Results of those studies indicate that the presence of stage-specific antigens is common to parasites such as *Plasmodium vivax* and *P. ovale* (Andrysiak et al., 1986), and *Trypanosoma cruzi* (Wrightsman et al., 1986). This paper presents immunological evidence of stage-specific *C. shasta* antigens which accompany the morphological change from trophozoite to spore and is the first report of monoclonal antibodies produced against a myxosporean parasite. The use of specific antibodies for diagnostic purposes is also described.
Materials and Methods

**Antigen**

Ascites containing prespore (trophozoite and disporoblast) stages of *C. shasta* was collected from naturally infected rainbow trout. Cells were pelleted by centrifugation (1500 g) and washed twice in 0.1 M phosphate-buffered saline, pH 7.6 (PBS). This antigen was used both for injection of mice for hybridoma production and for all screening procedures.

Spore stages of *C. shasta* were collected from the intestinal tract of infected rainbow trout. After homogenization of the tissue and low-speed centrifugation (1500 g) to remove fish tissue, the spores were layered onto a 12-75% (v/v) gradient of modified colloidal silica (Percoll, stock density 1.13 g/ml) (Sigma Chemical Co., St Louis, MO). Gradients were centrifuged at 1500 g for 35 min in a swinging bucket rotor. Spores layered on top of the 75% Percoll. This band was removed and the spores washed twice in PBS. Spores were used for screening hybridoma supernatants and for polyclonal antiserum production.

To obtain white blood cells, rainbow trout were bled from the caudal vein. Blood was stored in heparin (15 IU/ml) for one hour, cells were pelleted by centrifugation at 2500 g, washed twice and resuspended in PBS. The buffy coat was collected after overnight refrigeration.
Hybridoma Production

Monoclonal antibodies (Mabs) were produced by the method of Campbell (1984). Briefly, for the production of antibodies specific to *C. shasta*, freshly collected prespore stages (10^6 total/mouse) suspended in PBS were injected intraperitoneally into Balb/c mice. Two booster injections were given at one month intervals. Antibody production was evaluated by indirect fluorescent antibody techniques (IFAT) on cells from the ascites of infected rainbow trout. Four days after the second booster, the mice were primed with an injection of 10^6 parasites. Four days later the spleen cells were removed and fused with a non-secreting tumor line, SP2/0, at a ratio of 5:1, using polyethylene glycol (80% PEG 1500, 20% PEG 4000). The cells were distributed in 96 well microtiter plates (Costar, Cambridge, Mass) in selective medium (RPMI with 10% (v/v) fetal calf serum plus hypoxanthine, aminopterin, and thymidine), and incubated at 37°C in 5% CO₂. After 14 days, supernatants were screened for antibodies specific for *C. shasta*. A dot-immunobinding assay (Hawkes et al., 1982) using prespore stages from ascites as antigen was the primary test for reactivity. Supernatants eliciting a positive reaction were screened again by IFAT using cells from the ascites of infected fish and fish blood cells from uninfected fish as antigens. Those hybridomas producing antibodies positive only for cells from infected rainbow trout were cloned by limiting dilution. The supernatants from wells with single clones were tested for antibody as described; positive hybridomas were again cloned by limiting
dilution and re assayed after 10-14 days. Selected hybridomas were expanded into 75 cm$^2$ flasks and maintained in RPMI (Sigma Chemical Co.) plus 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, Utah). The immunoglobulin class and subclass produced by each hybridoma was determined by an enzyme-linked immunoassay (Mouse monoclonal subisotyping kit; HyClone Laboratories).

Western blot analysis was performed using antibodies from the expanded clones. Following electrophoretic separation of *C. shasta* spores, prespore stages, and fish WBCs, on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), the antigens were transferred to nitrocellulose by electroblotting in a Trans-blot cell (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose was then probed with hybridoma supernatants and peroxidase-labeled goat anti-mouse serum (Hyclone Laboratories). Antibodies which cross-reacted with fish WBC antigens were further characterized by Western blot analysis, using immunized fish sera as a source of antigen, and by enzyme-linked immunoassay (ELISA). In the ELISA, the cross-reacting Mabs were used as a second antibody. Plates were coated with trinitrophenyl-bovine serum albumin (TNP-BSA) and incubated with trout anti-TNP antiserum as primary antibody. In both Western blot and ELISA, a noncross-reacting Mab was used as a negative control and a Mab produced against fish immunoglobulin (cell line 1-14, a gift of Dr. G. Warr, Dept. of Biochemistry, Univ. of South Carolina) served as a positive control. Antibodies specific for carbohydrate epitopes were detected by
periodate oxidation of the antigens on nitrocellulose (Woodward et al., 1985). Briefly, *C. shasta* prespore stages and fish sera were separated by electrophoresis on a polyacrylamide gel and then transferred onto nitrocellulose. After exposing to periodate concentrations from 5-20 mM, blots were incubated with the monoclonal antibodies and probed as described.

Antibodies were also screened for cross-reactivity with other myxosporeans by IFAT on histological sections from fish infected with the following parasites: *Henneguya exilis*, *Sphaerospora* sp., PKX, *Chloromyxum majori*, *Myxobilatus* sp., and *Myxobolus cerebralis*, and also on purified spores of *Henneguya salmincola* and *Myxobolus insidiosus*.

*Polyclonal Antiserum*

To obtain the antigen used for the production of polyclonal antisera, 1 x 10^8 purified spores were diluted 1:1 with sample buffer (Schleif and Wensink, 1981) and the proteins were separated by SDS-PAGE. The gel was stained with Coomassie blue (0.1% in 40% methanol and 10% acetic acid) and the polypeptide profile examined for bands specific to the spore stage. A preparative SDS-PAGE was used to purify a single protein band. After electrophoresis of the spores, a narrow vertical strip of the gel was excised and stained in order to identify the protein bands. The portion of the gel corresponding to the band of interest was excised and the protein electroeluted (Elutrap, Schleicher and Schuell, Keene, NH). A sample of the eluent was diluted 1:1 in
sample buffer and run on a gel to assess the purity of the antigen. Antigen was mixed 1:1 with Freund's complete adjuvant and injected into New Zealand white rabbits; 0.5 ml in each footpad and 0.5 ml subcutaneously between the scapula. After four weeks, a booster was administered by injection of the antigen mixed 1:1 in Freund's incomplete adjuvant. Two weeks later the rabbit was bled and the antiserum tested for specificity by IFAT and Western blot analysis.

IFAT

Monoclonal or polyclonal antibodies were incubated for 15 minutes at room temperature with cells fixed in acetone-xylene (1:1). Specific antibodies were detected using biotinylated horse anti-mouse IgG or anti-rabbit Ig and fluorescein isothiocyanate conjugated avidin D (Vector Laboratories, Burlingame, CA). Methyl green dye (1% in distilled water) was used as a counterstain. Cells were examined using a Zeiss standard microscope with an IV Fl epi-fluorescence condensor.

Histology

For serological diagnosis of infections in tissue sections, viscera of fish exposed to the infectious stage of *C. shasta* were fixed in either Bouin's or 10% (v/v) neutral buffered formalin, processed routinely for histology to 6 µm and mounted on gelatin coated slides. Sections to be examined by IFAT were stained as described. Sections for examination by bright light microscopy
were incubated with specific antibody, then biotinylated anti-mouse IgG, and finally with an avidin DH - biotinylated alkaline phosphatase H complex (Vectastain ABC-AP kit,; Vector Laboratories). The enzymatic activity was localized with an insoluble substrate (Alkaline Phosphate Substrate Kit II; Vector Laboratories).
Results

Monoclonal Antibodies

One fusion resulted in four hybridomas which produced antibodies that reacted positively by dot immunobinding and IFAT to fixed, whole trophozoites from ascites. Isotypes of the immunoglobulins produced by each hybridoma were determined; one Mab was an IgM and three were IgGs, one each IgG1, IgG2a, and IgG3. Hybridoma supernatants were also tested for their reactivity to C. shasta spores and to fish WBCs by IFAT (Table V.1). The Mabs did not react with spores. The pattern of fluorescence of the parasite was similar after reacting with antibodies from each hybridoma.

In Western blot analysis all Mabs recognized antigens from prespore stages of C. shasta and there were none that reacted with spore antigens. Two Mabs reacted with fish WBC antigens, recognizing a protein with a molecular weight (MW) of approximately 80 kilodaltons (kdal) in both buffy coat preparations and in fish sera (Fig. V.1). These Mabs also reacted specifically with fish immunoglobulins in an ELISA. To determine if these cross-reacting antibodies reacted with a protein or carbohydrate epitope, Western transfers of antigens in prespore stages and in fish sera were exposed to varying concentrations of periodate. Complete loss of binding of Mabs 1 and 3 occurred at a concentration of 10 mM periodate, indicating that these antibodies
Table V.1. Characteristics of monoclonal antibodies (Mab) produced against prespore stages of *Ceratomyxa shasta*.

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<th>Western Blot Reactivity</th>
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<td>prespores spores WBCs</td>
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<td>B1</td>
<td>IgM</td>
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\(^1\)Indirect fluorescent antibody test
Figure V.1. Western blot analysis showing the patterns of antigen recognition by monoclonal antibodies (Mab) D8, B1, and 3 (antigen recognition by Mab 1 was not shown because it was identical to that of Mab 3). Lane representations are: A, molecular weight markers, with weights in kilodaltons to the left of the gel; B, lysates of *C. shasta* spores, C, lysates of cells from the ascites of infected fish, and D, lysates of trout white blood cells.
Figure V.1
are directed against carbohydrate antigens. Binding of Mab D8 to prespore antigens was not inhibited even at the 20 mM periodate concentration.

The cross-reactivity of the monoclonal antibodies with other myxosporeans was examined using IFAT on either histological sections from infected fish or purified spores. Two Mabs, 1 and 3, reacted with *Sphaerospora* sp. and *Myxobilatus* sp. from sticklebacks (*Gasterosteus* sp), and their use resulted in high background fluorescence of fish tissues. Although no specific cross-reactivity was detected between Mabs B1 and any myxosporeans, high background fluorescence of fish tissues was again noted. Mab D8 did not cross-react with other myxosporeans nor cause non-specific fluorescence of fish tissues. In addition to reacting specifically to *C. shasta* in IFATs, Mab D8 was also useful in detecting the parasite when an alkaline phosphatase conjugated second antibody was used (Fig. V.2).

**Polyclonal Antiserum**

The protein chosen as antigen for production of polyclonal antisera was shown by SDS-PAGE to be a major component of *C. shasta* spores and present in lower concentration in prespore stages (Fig. V.3). The MW of the purified protein was approximately 84 kdal (Fig. V.4). Antiserum against this antigen reacted by IFAT to both prespore and spore stages of the parasite, but fluorescence of later life stages was more pronounced (Fig. V.5). In Western blot analysis the antisera reacted with the 84 kdal protein of both
spores and prespores and also with a 180 kdal protein from spores. No specific cross-reactivity between the polyclonal antiserum and other myxosporeans was detected by IFAT.
Figure V.2. Histological sections of infected rainbow trout intestines showing reaction of Mab D8 with trophozoites (T) by indirect fluorescent antibody techniques (a), and antibody-conjugated alkaline phosphatase (b).
Figure V.2
Figure V.3. SDS-PAGE of lysates of *C. shasta* spore (B) and prespore (C) stages, and trout white blood cells (D). Weights of molecular weight standards (A) are indicated at left.

Figure V.4. SDS-PAGE of the *C. shasta* antigen used for production of polyclonal antiserum (C) and the lysate of spores from which the antigen was purified (B). Weights of molecular weight standards (A) are indicated.
Figure V.5. Indirect fluorescent antibody reaction of whole spores probed with polyclonal antiserum produced against an antigen from *Ceratomyxa shasta* spores.
Discussion

Four hybridomas synthesizing monoclonal antibodies which reacted against trophozoite and disporoblast stages of *C. shasta* were produced. Reactivity to *C. shasta* prespore stages was determined by indirect immunofluorescence and by Western blot analysis of electrophoresed trophozoite antigens. None of the hybridomas produced antibodies that reacted with *C. shasta* spores by dot immunobinding, IFAT, or in Western blots. These data suggest the presence of stage-specific antigens associated with different life stages of *C. shasta*. SDS-PAGE analysis demonstrated that some proteins present in high concentration in *C. shasta* spores are either absent or in low concentration in earlier life stages. It is likely that these noncross-reactive antigens are associated with the formation of the spore coat. Because of difficulties in physically separating *C. shasta* life stages, all prespore stages were grouped together. This caused some problems in the analysis of electrophoretic profiles because disporoblast stages might have formed some of the antigens found in mature spores.

Two of the hybridomas (Mabs 1 and 3) produced antibodies which cross-reacted with the white blood cell population of uninfected fish and with a high MW protein in fish sera. The cross-reacting protein had a MW of approximately 80 kdal and was determined to be fish immunoglobulin heavy chain by Western blot analysis and ELISA. Because no attempt was made to purify *C. shasta* prespores, it was not unexpected to find antibodies that
reacted with trout antigens; however, these antibodies were also found to react specifically by IFAT with cells that could be positively identified as *C. shasta*. One possible explanation for this cross-reactivity is that *C. shasta* had evoked an immune response and was coated with fish antibody. However, when cells were incubated with fluorescein-conjugated anti-trout Ig there was no reactivity. It has been suggested (Pauley, 1974) that cross-reactivity of a specific anti-parasite antibody with uninfected tissue antigens may indicate that an antigenic component of the parasite has been able to mimic normal host antigens and thus evade an immune reaction. Profiles of antibody-antigen recognition seen in Western blots (Fig. V.1) suggested that the antibodies recognized a common epitope which may indicate the presence of carbohydrate antigens. Mild periodate oxidation, which destroys carbohydrate determinants without affecting either proteins or lipids, caused a complete loss of binding of both Mabs 1 and 3. There has been evidence indicating that antisera produced against phylogenetically distant immunogens may cross-react by virtue of their carbohydrate moieties (Bayne et al., 1987; Yamaga et al., 1978). Whether or not this cross-reactivity represents a mechanism by which the parasite avoids the host immune system, the presence of common carbohydrates may be effective in facilitating parasitism by *C. shasta*.

In addition to Mabs which reacted with both *C. shasta* and host antigens, we also produced one monoclonal antibody specific for the prespore stages of the parasite. Hybridoma D8 secreted
antibodies which did not recognize any other myxosporeans and which were effective in detecting low numbers of *C. shasta* both in histological sections and in smears of intestinal material from infected fish. Its specificity makes it suitable for use as a diagnostic reagent, either in the IFAT or in enzyme immunohistochemistry. In addition to its applications in diagnostics, this Mab will be useful in studying the life history and pathogenesis of this parasite.

The polyclonal antiserum produced against an antigen predominant in the spore stage of *C. shasta* was shown by IFAT to react most specifically with these later life stages. This provides additional evidence for the presence of stage-specific antigens associated with the parasite. The abundance of this antigen in the spore makes it a good candidate for production of either monoclonal or polyclonal antibodies against a single defined antigen. The production of these antibodies may be useful not only in diagnostics, but in understanding the biology of *C. shasta*. 
Acknowledgements

We thank Cindy Arakawa for her assistance in the production and screening of the hybridomas and Mary Arkoosh for testing the Mabs in the ELISA. This research was supported by Bonneville Power Administration under contract No. DE-A179-83 BP 11987; G. R. Bouck, Contracting Officer's Technical Representative. Oregon Agricultural Station Technical Paper No. 8568.
Literature Cited


Characterization of the Host Response to the Myxosporean Parasite, *Ceratomyxa shasta*, by Histology, Scanning Electron Microscopy, and Immunological Techniques

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Abstract

The tissue response of *Salmo gairdneri* against the myxosporean parasite, *Ceratomyxa shasta*, was investigated using histological techniques, scanning electron microscopy, and immunological methods. The progress of infection in *C. shasta*-susceptible and resistant steelhead and rainbow trout was examined by standard histological techniques and by indirect fluorescent antibody methods using monoclonal antibodies directed against *C. shasta* antigens. Trophozoite stages were first observed in the posterior intestine and there was indication that resistance was due to the inability of the parasite to penetrate this tissue rather than to an inflammatory response. Examination of a severely infected intestine by scanning electron microscopy showed extensive destruction of the mucosal folds of the posterior intestine. Western blotting and indirect fluorescent antibody techniques were used to investigate the immunological component of the host response. No antibodies specific for *C. shasta* were detected by either method.
Ceratomyxa shasta is a histozoic myxosporean which parasitizes the intestinal tissues of salmonids. Its geographic range is limited to northern California and the Pacific Northwest region of the United States and Canada (Hoffmaster et al., 1988). The parasite is an important cause of mortality among susceptible salmonids. Fish are infected while in fresh water; however, anadromous salmonids may continue to die of ceratomyxosis during the salt water phase of their life cycle (Ching and Munday 1984). The life cycle of C. shasta has not been defined, but its apparent discontinuous distribution and the difficulty in achieving laboratory transmission of the infectious agent suggests that one or more unknown factor(s) is required. The involvement of an intermediate host has been suggested; however, no life cycle has been proposed.

Initial signs of infection by C. shasta may include darkening, lethargy, and loss of appetite. As the disease progresses, the descending intestine and anus become swollen and hemorrhaghic and ascites may collect in the coelom (Wales and Wolf, 1955; Schafer, 1968; Johnson, 1975). Definitive diagnosis is made by observation of the mature spore in wet mounts or histological sections. However, before sporogenesis is complete, trophozoites can be identified by their multicellular ameboid morphology and characteristic nuclei
containing a large karyosome and peripheral chromatin (Noble 1944). Multiplication of trophozoites is by nucleogony followed by either budding or plasmotomy (cytoplasmic division)(Noble, 1941).

Many reports describe infections of fish by myxosporeans, but the view that most species cause little host response is commonly held. Coelozoic myxosporeans cause little host reaction and have been considered the most harmless (Lom 1970); however, Fantham (1912) described an inflammatory reaction consisting of leucocyte infiltration, desquamation and necrosis of epithelial cells, and increased mucous secretion caused by myxosporeans parasitizing the gall bladder. Lom (1969) also reported hyperemia and hypertrophy associated with coelozoic myxosporeans.

A lack of tissue response has also been attributed to cyst forming myxosporeans (Lom 1969). Dykova and Lom (1978) reported histopathological changes in gills of fish infected with two species of *Henneguya* and proposed that the tissue response to myxosporeans invading soft tissue occurred in two phases. First, as the plasmodium grew, there occurred alterative changes in the tissue: displacement, atrophy and hyperplasia. Although organ function may have been impaired, there was no host defense reaction. In the second stage, cysts were full of mature spores which evoked an inflammatory reaction leading to replacement of the cyst by granulomatous tissue. Amandi and Fryer (1985)
recognized a similar host reaction against *Myxobolus insidiosus* which infects the muscle of salmonids. Contrary to the lack of inflammatory response to immature plasmodia, Duhamel (1986) reported that trophozoites of *Henneguya exilis* were responsible for severe granulomatous branchitis in channel catfish.

The tissue response against non-cyst forming histozoic myxosporeans is also varied. *Myxobolus cerebralis* infects young salmonids by invading and causing destruction of cartilage cells. A granulomatous response with infiltration by macrophages and mononuclear leucocytes has been reported (Roberts and Elson 1970; Taylor and Haber 1974; Halliday 1976). Other myxosporeans cause complete degeneration of invaded areas accompanied by hypertrophy of connective tissue cells and invasion by fibroblasts. Kent and Hedrick (1985) described the reaction of salmonids against the etiological agent of proliferative kidney disease as a granulomatous nephritis with an infiltration of macrophages and mononuclear cells and suggested that the severity of the inflammatory reaction is because salmonids are abnormal hosts for this myxosporean.

Investigations of the immune component of the host reaction against myxosporeans has produced varied results. Some researchers (Lom 1969; Dykova and Lom 1978) suggest that regression of *Henneguya* infections at high temperatures is probably caused by an increase in antibody production and
enhanced cellular defenses. However, Siau (1980) found no antibody response in *Mugil* immunized with *Myxobolus exiguus* by gel precipitation or complement fixation assays. Halliday (1974) and Pauley (1974) were unable to detect antibodies to *Myxobolus cerebralis* spores in either naturally infected or immunized fish. Halliday suggested that a lack of antibody response indicates either a privileged site of infection or non-pathogenicity of the spore stage. Pauley presented evidence suggesting that parasite antigens mimic host antigens. However, Griffin (1978) was able to detect trout antibodies against *M. cerebralis* spores by an indirect fluorescent antibody test.

The host range of *C. shasta* includes a number of salmonid species; however, different strains of the same species vary in susceptibility to the disease. Development of resistance appears to be a selective factor in waters where *C. shasta* is present (Johnson 1975; Zinn et al., 1977; Buchanan et al., 1983). Fish from watersheds endemic for the parasite are resistant but those from areas free of *C. shasta* are susceptible and may experience serious mortality when exposed to the infective stage of the organism. In this study the histopathology of infection was observed in resistant and susceptible strains of salmonids, the immune reaction of the host was investigated, and a scanning electron microscopic examination was made of a heavily infected intestine.
Materials and Methods

*Infection of Fish.*

Two strains of steelhead trout (*Salmo gairdneri*), *C. shasta*-susceptible Siletz River and *C. shasta*-resistant North Santiam, were exposed simultaneously to the infective stage of *C. shasta* for three days in the Willamette River at Corvallis, Oregon. One hundred-fifty fish (1-4 g in weight) of each strain were exposed to the parasite. During the three days in the river, 10 fish were removed from each group at each of eight sampling periods which were 30 min, and 1, 2, 4, 8, 24, 48, and 72 h. After three days, the remaining fish were returned to holding tanks and maintained at 12°C in a pathogen-free water supply. Fish were taken at 5, 8, 18, and 30 days post-exposure. From each exposure period, five whole fish were fixed in Bouin's fixative and five in 10% neutral buffered formalin. Unexposed control fish from each strain were obtained in the same manner.

Susceptible Shasta rainbow trout (*Salmo gairdneri*) were exposed to the parasite as described; however, following the three day exposure they were held at 21°C. Ten fish were removed at each of the following time intervals: 0, 2, and 8 hours, and 1, 2, 4, 7, 10, 14, 18, and 20 days. Visceral organs and gills of the fish were removed and fixed in either Bouin's or 10% neutral buffered formalin. Additionally, two sets of
blood smears and kidney imprints were made from each fish; one set was fixed in Schaudinn's fixative and stained with May-Gruenwald Giemsa and one set was air dried and methanol fixed for examination by fluorescent antibody techniques.

**Preparation and Examination of Specimens for Histology.**

Whole fish and organs were embedded in paraffin, sectioned, and mounted on gelatin coated slides. Sections for light microscopy were stained with either May-Gruenwald Giemsa or haematoxylin-eosin (H&E). Sections were also examined serologically using either indirect fluorescent antibody techniques (IFAT) or alkaline phosphatase (AP) immunoenzymatic staining techniques. For both immunostaining procedures, sections were deparaffinized in two, 15 sec changes of xylene and hydrated in three, 15 sec changes of 95% ethanol followed by two, 5 min washes in phosphate buffered saline (PBS). Sections and methanol fixed smears and imprints were incubated with monoclonal antibodies directed against antigens of *C. shasta* trophozoites (Bartholomew et al., submitted), then with biotinylated horse anti-mouse IgG (diluted 1:100 in PBS)(Vector Laboratories, Burlingame, CA). Slides to be examined by IFAT were incubated with fluorescein isothiocyanate-conjugated avidin D
(diluted 1:200 in PBS)(Vector Laboratories), counterstained with methyl green dye (1% in distilled water), and examined using a Zeiss standard microscope with an IV Fl epi-fluorescence condensor. Sections to be labeled immunoenzymatically were incubated with avidin DH-biotinylated AP H complex (Vector Laboratories). The enzymatic activity was localized with an insoluble substrate (Alkaline Phosphate Substrate Kit II; Vector Laboratories) which was visualized by bright light microscopy.

Preparation and Examination of Specimens for Scanning Electron Microscopy

Intestines from three heavily infected and one uninfected Shasta rainbow trout were dissected and fixed in 3% glutaraldehyde in cacodylate buffer pH 7.0, 0.2 M for 3 h then transferred through solutions of increasing concentrations of acetone in water and trichlorofluoroethane (TF) in acetone, 30 min per change. From absolute TF, tissues were critical point dried following the method of Cohen et al. (1968) in a Balzers CPD 020 CP dryer. Specimens were then mounted on aluminum plancets using DUCO adhesive and coated with 200 A of 60:40 wt % Au:Pd alloy in a Varian VE-10 vacuum evaporator of 1 x 10⁻⁵ Torr. Examination was made using an AMRAY 1000A SEM, operated at 20 kv.
Detection of Antibody Response.

Shasta rainbow trout were exposed to the infective stage of *C. shasta* for three days, returned to holding facilities, and held at 15°C. When signs of infection were evident, fish were killed, bled, and examined for presence of the parasite. Blood from positive individuals was pooled, allowed to clot at room temperature, and the serum was collected. Antibodies were detected by Western blotting, using electrophoresed spore and prespore proteins as antigen. Following electrophoretic separation of these antigens on a 12% sodium dodecyl sulfate-polyacrylamide gel, they were transferred onto nitrocellulose by electroblotting in a Trans-blot cell (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose was then probed with the fish antiserum, mouse monoclonal antiserum produced against trout immunoglobulin (cell line 1-14, a gift of Dr. G. Warr, Dept. of Biochemistry, Univ. of South Carolina), and finally with goat anti-mouse antibodies conjugated with horseradish peroxidase (Hyclone Laboratories, Logan, Utah).

Attempts were also made to detect trout immunoglobulin on the surface of trophozoites. Methanol fixed smears of trophozoites in ascites were examined by IFAT as described previously, using monoclonal antibodies directed against trout immunoglobulin as the primary antibody.
Results

*Histopathology in Salmonids Susceptible to Infection by* *Ceratomyxa shasta.*

Infection by *C. shasta* in Siletz River steelhead trout was first detected 18 d post-exposure when fish were held at 12°C. Trophozoites were first seen in the descending intestine and were located between or at the base of mucosal epithelial cells. An inflammatory response, consisting of an infiltration of lymphocytic cells, was usually seen in the submucosa adjacent to the trophozoites (Fig. VI.1). These foci were easily detected in histological sections stained by Giemsa or H&E and the identity of the trophozoites was confirmed by IFAT. The number of foci was limited and mucosal folds adjacent to the infected site appeared normal. The epithelium was usually intact, but cell nuclei showed signs of karyolysis and cells were often necrotic (Fig. VI.2).

By 30 d, the entire intestinal tract was infected and trophozoites were proliferating in all layers of the mucosa, the submucosa, and in the muscularis (Fig. VI.3). Trophozoites were diffusely scattered throughout the tissues and were surrounded by necrotic host cells (Fig. VI.4). The lamina propria was thickened due to the inflammatory response and numerous parasites. The epithelium was no longer intact; epithelial cells were rounded and many had pycnotic nuclei.
Many cells had sloughed so that trophozoites and necrotic epithelial cells were present in the lumen of the intestine. Trophozoites penetrated the muscularis and serosa and invaded the adjacent adipose tissue in the coelomic cavity. Trophozoites were not found in the stomach, pyloric caeca, spleen, pancreas, or kidney but they were found in the blood sinusoids of the liver. Trophozoites were present in the gill capillaries and epithelial tissue of one fish (Fig. VI.5).

Fish began to die at 52 d post-exposure and it was at this time that spores were first noted. Portions of the descending intestine were severely necrotic and completely occluded. Fibroblasts formed a network which enmeshed trophozoites, lymphocytes, and necrotic host cells. Exudative material was found throughout the intestine and the ascending intestine, pyloric caeca, and stomach were heavily infected. Trophozoites were present in all layers of the pyloric caeca; in some sections the epithelial cells were intact and in others they were sloughed and caeca were occluded (Fig. VI.6 and VI.7). Adipose tissue surrounding the caeca was destroyed and the pancreatic tissue heavily infected and sometimes necrotic. Trophozoites were found in hematopoietic tissue of the kidney; also, in the kidney tubules, between epithelial cells and in their lumens (Fig. VI.8). Foci of infection were seen in the liver, some were necrotic with exudative material and others were characterized by a lymphocytic infiltration. Proliferating trophozoites were observed throughout the organ and sporogenesis was occurring (Fig. VI.9).
Figure VI.1. Section of intestinal tissue from a susceptible Siletz River steelhead trout showing an inflammatory response (arrow) 18 d after exposure to the infective stage of *Ceratomyxa shasta*. H&E stain, 100X.

Figure VI.2. *Ceratomyxa shasta* trophozoites (arrows) in the mucosal epithelium at 18 d post exposure. Giemsa stain, 1000X.

Figure VI.3. Intestinal tract at 30 d post-exposure. Trophozoites are proliferating in all intestinal layers and portions of the mucosal epithelium have sloughed. Giemsa stain, 100X.

Figure VI.4. Trophozoites (arrows) are diffusely scattered throughout the tissues and are surrounded by necrotic host cells. Epithelial cells are rounded (white arrow) and are being sloughed. Giemsa stain, 250X.

Figure VI.5. Trophozoites in the gill capillary and adjacent epithelial tissue (arrows). Giemsa stain, 40X.

Figure VI.6. Infected pyloric caeca and surrounding adipose and pancreatic tissue 52 d post-exposure. Muscularis of caeca is infected. Note necrosis and sloughing of mucosal epithelium from underlying submucosa. Giemsa stain, 250X.

Figure VI.7. Severe necrosis and occlusion of the lumen of a pyloric caeca. Numerous trophozoites are multiplying in all tissue layers. Giemsa stain, 400X.
Figure VI.1-VI.7
Figure VI.8. Kidney of Siletz River steelhead trout 52 d post-exposure. Trophozoites (white arrows) are present in the hematopoietic tissue and in necrotic kidney tubules (black arrows). Giemsa stain, 250X.

Figure VI.9. Liver tissue at 52 d showing area of focal necrosis. Trophozoites proliferated throughout the tissue and sporogenesis was occurring (arrows). Giemsa stain, 250X.
In Shasta rainbow trout held at 21°C after exposure to the infective stage, the parasite was first detected at 7 d post-exposure. Trophozoites were most easily detected by IFAT because of limited inflammation (Fig. VI.10). Multicellular trophozoites were detected mainly at the base of epithelial cells in the posterior intestine and some were also observed in hepatic blood sinusoids (Fig. VI.11). By 10 d, focal inflammatory reactions had developed in some fish and trophozoites were detected between epithelial cells or in the submucosa of the inflamed area. Parasites were observed throughout the posterior intestine (Fig. VI.12) and some appeared in the anterior intestine and connective tissue surrounding blood vessels in the pancreatic tissue. The livers of several fish were also infected and hepatic cells were undergoing degeneration. Fish sampled at 14 and 18 d post-exposure showed varying degrees of infection and host response. Intestines were mildly to severely infected and lumens were occluded in certain areas (Fig. VI.13). In severely infected fish, trophozoites proliferated in the mucosa, submucosa, muscularis, and serosa. Infected tissues were hemorrhaged and infiltrated by eosinophilic granular leucocytes and lymphocytes. The epithelium was necrotic and had sloughed in certain areas so that necrotic host cells and trophozoites were observed in the intestinal lumens. Parasites penetrated the muscularis and serosa and were free in the peritoneal cavity. Adipose tissue adjacent to the
intestine was parasitized and infiltrated by lymphocytes and there was a hyperplasia of connective tissue (Fig. VI.14). At 18 d, sporogenesis was evident in the intestine. The muscularis of the pyloric stomach was infected and trophozoites were separating muscle fibers, leaving spaces filled with necrotic material (Fig. VI.15). Nuclei of smooth muscle fibers were pycnotic and the fibers were degenerating. Pyloric caeca in close proximity to the infected stomach and intestine were also infected (Fig. VI.16). Although trophozoites penetrated the submucosa, muscularis, and serosa of these tissues, the epithelium was still intact in certain areas. The liver in most fish had sites of focal infection (Fig. VI.17). Hepatocytes were necrotic and lymphocytes infiltrated the infected tissues. Blood smears and kidney imprints were also positive for trophozoites (Fig. VI.18 and VI.19). The spleen remained uninfected. At 20 d post-exposure, as fish were dying of the disease, intestinal lumens were completely occluded and most adjacent tissues and organs were infected to varying degrees. The cardiac stomach and spleen remained uninfected.

One Shasta rainbow, sampled at 18 d post-exposure, did not show typical histopathology. In this fish, trophozoites were observed in the lumen of the intestine and within the epithelial layer (Fig. VI.20 and VI.21). Those in the lumen were both free and attached to the epithelial surface. There was no inflammatory
Figure VI.10. Fluorescent antibody stain of intestinal tissue from Shasta strain rainbow trout 7 d post-exposure showing a multicellular trophozoite in the mucosal epithelium. 400X.

Figure VI.11. Trophozoite (arrow) in the blood sinusoids of the liver at 7 d. Giemsa stain, 1000X.

Figure VI.12. Intestine at 10 d with parasites (arrows) in or at the base of the mucosal epithelium. Alkaline phosphatase immunostain, 100X.

Figure VI.13. Occluded lumen of posterior intestine from a Shasta strain rainbow trout sampled 18 d post-exposure. Epithelial cells are necrotic and sloughed and trophozoites exist in all intestinal layers. Giemsa stain, 100X.

Figure VI.14. Adipose tissue (A) adjacent to the intestinal serosa (S). The intestine has been penetrated, there is hemorrhaging, and trophozoites (arrows) are multiplying in the adipose tissue. Giemsa stain, 250X.

Figure VI.15. Trophozoites (arrows) in the muscularis of the stomach were found between muscle fibers. Giemsa stain, 400X.

Figure VI.16. Infected stomach and adjacent pyloric caeca. Trophozoites appear in all tissue layers but the epithelium is intact. Giemsa stain, 100X.

Figure VI.17. Liver at 14 d post-exposure showing numerous trophozoites (arrows). Giemsa stain, 250X.

Figure VI.18. Blood smear with multicellular trophozoite (arrow) surrounded by blood cells. Giemsa stain, 400X.

Figure VI.19. Trophozoite (arrow) in kidney imprint. Giemsa stain, 400X.
Figure VI.20. Trophozoites (arrows) in the lumen of the intestine of a Shasta strain of rainbow trout 18 d post-exposure. Giemsa stain, 100X.

Figure VI.21. Trophozoite (arrow) on the surface of the intestinal mucosal epithlium. Giemsa stain, 400X.

Figure VI.22. Trophozoite in the lumen of the intestine of a North Santiam strain steelhead trout 30 d post-exposure. Giemsa stain, 250X.
inflammatory response. The identity of these trophozoites as early stages of *C. shasta* was confirmed by IFAT.

**Histopathology of Infection in Salmonids Resistant to *Ceratomyxa shasta*.**

*Ceratomyxa shasta* was detected in only two resistant fish, both at 30 d post-exposure. In one fish, a single trophozoite was observed in the lumen of the posterior intestine (Fig. VI.22). It was not attached and there was no sign of a host response. The second fish was severely infected. Trophozoites had penetrated the intestinal epithelium and the infection appeared the same as in severely infected susceptible fish.

**Scanning Electron Microscopy.**

Comparison of infected and uninfected posterior intestines by scanning electron microscopy showed complete destruction of the mucosal epithelium and different degrees of damage to the structure of the mucosal folds in infected fish (Fig. VI.23 - VI.28). Lymphocytes, macrophages, and red blood cells were numerous in infected areas. Sporogenesis
Figure VI.23. Scanning electron micrograph of the posterior intestine from an uninfected control fish showing parallel mucosal folds with secondary folds. Bar indicates scale in micrometers.

Figure VI.24. Posterior intestine of an infected fish at the same magnification. The secondary structure of the mucosal folds has been destroyed in the lower portion, and there is little left of any primary structure in some areas (arrow). Bar indicates scale in micrometers.

Figure VI.25. Control intestine, showing smooth mucosal folds. Outlines of columnar epithelial cell surfaces are visible. Bar indicates scale in micrometers.

Figure VI.26. Infected intestine at the same magnification. Destruction of the intestinal mucosa and penetration into the underlying layers. Little identifiable structure remains except at the top of the folds, where trophozoites and inflammatory cells are not as numerous. Bar indicates scale in micrometers.

Figure VI.27. High magnification of the mucosal surface in an uninfected fish. Outlines of cell surfaces are visible. Depressions mark the pores of discharging mucous cells. Bar indicates scale in micrometers.

Figure VI.28. High magnification of an infected intestine showing extreme tissue damage. A variety of cell types are present, including lymphocytes, red blood cells, and *C. shasta* trophozoites and spores (arrow). Bar indicates scale in micrometers.
Figure VI.23 - VI.28
was also observed. Desquamation of the columnar epithelial cells caused loss of secondary structures of the folds and in certain areas the underlying connective tissues were also destroyed.

Antibody Response.

The antibody response of naturally infected rainbow trout was investigated by Western blot analysis and IFAT. Specific antibody against *C. shasta* was not detected by either method.
Discussion

Myxosporeans are generally known to parasitize fish without damaging tissues; however, certain species cause extreme tissue reactions and death of the host. In concurrence with this, descriptions of host responses to these parasites range from a complete lack of tissue reaction to a severe inflammatory response. *Ceratomyxa shasta* belongs to this latter group, causing extensive intestinal damage and death in susceptible salmonid fishes.

The first location that trophozoites were detected was between epithelial cells of the descending intestine. This observation was in agreement with that of Yamamoto and Sanders (1979). Although Johnson (1975) suggested that the pyloric caeca were the primary site of infection, we did not detect trophozoites in this tissue by examination of Giemsa stained sections or by IFAT until late in the infectious process. Furthermore, Johnson’s descriptions of trophozoites in the pyloric caeca did not coincide with those of trophozoites in the intestine, suggesting that they may not have been the same organism. The inability to detect *C. shasta* until 7 d post-exposure in fish held at 21°C suggests that very few organisms are required to initiate infection. An alternate explanation is that the infective stage may enter the fish by a route other than the intestine and was not observed using our detection methods.
A vigorous host response, consisting mainly of a lymphocytic infiltration, accompanied the proliferation of parasites in the intestine. The observation of an inflammatory response against these early stages differs from the observation of Dykova and Lom (1978) that inflammatory reactions occur only with the development of mature spores. However, their conclusions were based on examining the pathology caused by cyst-forming histozoic species. The trophozoite is the pathogenic stage of this parasite and once established in the host is proteolytic and invasive.

Although *C. shasta* has a trophism for the intestine, it differs from the tissue specificity shown by most myxosporeans by effectively invading and multiplying in most other tissues and organs. Noble (1950) noted the parasite in the entire alimentary tract, gill capillaries, kidney, spleen, liver, gall bladder, and gonads; Schafer (1968) reported infections of the eyes; Conrad and DeCew (1966) found the parasite in muscle; and Margolis and Evelyn (1975) reported lesions in the kidney, pyloric caeca, intestine and on the flanks below the dorsal fin. Trophozoites multiply extensively in the intestinal tract and several days after initial detection, they are present in the mucosa and submucosa throughout the intestine.

In this study, appearance of trophozoites in the liver closely followed their presence in the intestine. The route of invasion into the liver was probably venous blood which
enters the hepatic portal system directly from the alimentary tract. Blood transport via the hepatic portal system was also observed by Jakowska (1979) for *Henneguya* species. While necrotic degradation and inflammatory responses were seen in the hepatic tissues, this organ was not as extensively infected as the alimentary tract.

The infection proceeded anteriorly in the intestine, but at the same time it penetrated the muscularis and serosa and became free in the peritoneal cavity. This made it difficult to determine whether the caeca became infected internally or by penetration of the serosa from the outside. It is possible that both occur, as some caeca were infected only in the muscularis and serosa and had an intact epithelium, while other caeca were completely destroyed. Adipose and pancreatic tissue adjacent to the pyloric caeca were also infected. Penetration of the stomach did appear to be external or via the bloodstream, as trophozoites were always found between muscle layers while the mucosa was intact, with no parasites or inflammation.

At the same time that trophozoites penetrated the intestinal wall, they were also detected in blood smears and kidney imprints. The presence of *C. shasta* in the bloodstream allowed its spread to other tissues and organs which had not been penetrated by the parasite from the body cavity.

Scanning electron microscopic studies of heavily infected posterior intestines allowed assessment of the
amount of structural damage caused by this parasite. Secondary mucosal folds were destroyed throughout the entire intestine and primary folds were severely eroded in areas. Spores and trophozoites were numerous, as were inflammatory and red blood cells.

Examination of the contents of the gall bladder has been suggested in diagnosis of an infection (Amos, 1985). Johnson (1975) noted that infection of the gall bladder occurred late in the infectious process and in this study little or no infection of that organ was observed. When the gall bladder and spleen were infected, trophozoites appeared to have entered from the body cavity. Because the posterior intestine is the primary site of infection and because infection of this tissue always precedes invasion of other areas, and is more severe than infections in other tissues, we believe examination of the intestine is sufficient for diagnosis of the disease.

The progress of infection in resistant strains of fish was difficult to determine because only two fish of the resistant, North Santiam strain became infected. No evidence of the parasite or any inflammatory response was observed in uninfected, North Santiam steelhead. The appearance of the infection in one of the two fish was similar to that in susceptible fish, indicating that the primary site of infection is the same and once the parasite has penetrated the intestine, the infection proceeds as in susceptible fish. Detection of a trophozoite in the lumen of the intestine of the second fish
supports the hypothesis that resistance is at the site of entry. If resistance was due to a cellular or humoral response it would be expected that parasites would be in the tissue. One individual from a susceptible strain also appeared refractory to infection. Many trophozoites were detected in the lumen of its intestine, several were attached to the epithelial surface, and some were present between epithelial cells. Although this fish may have eventually succumbed to the infection, it provided additional evidence that the parasite first enters the fish from the lumen of the descending intestine and that exclusion of the parasite from entering at this site may be the mechanism of resistance.

The progress of the infection is temperature dependent, with mean time to death inversely correlated to temperature (Udey et al., 1975). The histopathology of the infection paralleled this; trophozoites were detected at an earlier time and the inflammatory response developed earlier as temperature increased. Although trophozoites multiplied faster, the progression of events was similar to that in fish held at lower temperatures. These observations are contrary to those of Lom (1969) and Dykova and Lom (1978) who noted a regression of infection by Henneguya species as temperatures increased. It was suggested that antibodies become active at higher temperatures and help destroy the parasite.
No immune response was detected in rainbow trout naturally infected with *C. shasta* by either Western blotting or IFAT, even though the fish had harbored the parasite for over thirty days and were dying from the infection. This supports the work of other researchers (Siau, 1980; Halliday, 1974; Pauley, 1974) who were unable to detect antibodies against myxosporeans by complement fixation, gel precipitation, or immunoelectrophoresis. One criticism of these studies is that they were trying to detect antibodies against the spore stage of the parasite. Spores form later in the infectious process, and spore antigens may differ from those of trophozoites (Bartholomew et al., submitted). Therefore, fish antibodies against these early stages may go undetected. Griffin and Davis (1978) were able to demonstrate antibodies against *M. cerebralis* in naturally infected trout by IFAT; however, their results did not show absolute correlation between the presence of spores and the presence of antibody, and their results required some interpretation because of background fluorescence. Also, results of monoclonal antibody studies (Bartholomew et al., submitted) indicate that *C. shasta* trophozoites and trout immunoglobulin have cross-reacting carbohydrate epitopes. If this is true for other myxosporeans, polyclonal antibodies against trout immunoglobulin may be reacting against the parasite itself rather than against antibody on its surface. It appears that if an immune response is directed against *C. shasta*, it is ineffective in
halting the infection. Infection by *C. shasta* fits the description by Plehn (1932) of a superinfection in which invasion leads to a generalized myxosporidiasis and subsequently to death.
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We thank Elizabeth MacConnell and Oliva Nunez for their assistance in sectioning fish for the histological study and Al Soeldner for preparation of samples for the scanning electron microscope. This research was supported by Bonneville Power Administration under contract No. DE-A179-83 BP 11987; G. R. Bouck, Contracting Officer's Technical Representative. Oregon Agricultural Experiment Station Technical Paper No.
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CHAPTER VII

Characterization of a Monoclonal Antibody against *Ceratomyxa shasta* Using Immunocytochemistry

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Abstract

The antigenic specificity of a monoclonal antibody (Mab) against *Ceratomyxa shasta* was investigated using immunogold labeling techniques and transmission electron microscopy and by immunoenzymatic labeling. Specific binding by the Mab was observed in the cytoplasm of the primary cell. Label was not observed in the cytoplasm of the developing secondary cells or in the nucleus of either the primary or secondary cells. A hypothesis explaining the specificity of this Mab for prespore stages of the parasite is presented.
Introduction

A monoclonal antibody (Mab) specific for the myxosporean salmonid parasite, *Ceratomyxa shasta*, has been developed and characterized (Bartholomew et al., submitted). This Mab reacts specifically with the trophozoite and sporoblast stages of the parasite in indirect fluorescent antibody tests (IFAT). In Western blot analysis it recognizes many polypeptide bands from these prespore stages, suggesting that it is formed against a common epitope. The Mab did not react with *C. shasta* spores by immunoblotting procedures, Western blot analysis, or IFAT. These data indicate that the parasite has stage-specific antigens and that Mabs may not react against all life stages.

Antibodies that recognize early life stages of *C. shasta* would be helpful in studying the biology and life cycle of the parasite. Presently, the only life stages identified are those that exist within the salmonid host. A Mab recognizing trophozoites may also recognize stages of the parasite prior to infection of the fish and could be used to look for these stages in water and invertebrates collected from endemic sites. In this study the specificity of the Mab was investigated by using immunogold electron microscopic techniques and immunoenzymatic labeling of histological sections.
Materials and Methods

Immunogold Electron Microscopy

To determine the location of the antigens against which the monoclonal antibodies are directed immunogold labeling procedures were used. Prespore stages of the parasite from the ascites of infected rainbow trout were fixed for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.6 (PBS). The suspension was centrifuged to pellet the cells which were resuspended in PBS. Cells were embedded in Spurr's resin (Spurr, 1969) without OsO4 staining. Sections were cut on an ultramicrotome and mounted on nickel grids. The embedding medium was etched with sodium metaperiodate (saturated solution in distilled water) for 30 min, washed with distilled water, then blocked with 100 mM glycine + 5% bovine serum albumin in PBS for 15 min. Sections were incubated with undiluted monoclonal antibody for 1 h at room temperature. After washing three times with PBS, grids were incubated with biotinylated anti-mouse IgG (1:100 in PBS)(Hyclone Laboratories, Logan, Utah) for 1 h, washed, and incubated for 30 min with Streptavidin-gold (1:200 in PBS)(particle size, 20 nm)(Polysciences Inc., Warrington, PA). In control experiments, the incubation conditions were identical except that non-immune serum or PBS was substituted for specific antibody in the first incubation step. All grids were washed thoroughly, first with PBS then with distilled water, and examined with a Philips EM-301 transmission electron microscope. Some
grids were post-stained with 2% OsO₄ (5 min), uranyl acetate (2 min) and lead citrate (5 min).

**Immunoenzymatic Staining**

For examination of the parasite in tissue sections, viscera of fish exposed to the infectious stage of *C. shasta* were fixed in 10% neutral buffered formalin, processed routinely for histology to 6 μm, and mounted on gelatin coated slides. Sections were dewaxed and hydrated in 2 changes each of xylol, absolute ethanol, 95% ethanol, and were transferred to water. Sections were incubated with undiluted anti-*C. shasta* monoclonal antibody for 1 1/2 to 3 h at room temperature then rinsed with PBS. Sections were then incubated with biotinylated goat anti-mouse Ig streptAvidin-peroxidase conjugate (Biogenex, Dublin, CA). The enzymatic activity was localized with the substrate hydrogen peroxide-AEC-acetate buffer (Biogenex, Dublin, CA). Sections were counterstained with a non-alcoholic solution of hematoxylin (Meyer's) to provide a contrasting background stain.
Results and Discussion

Specific binding by the Mab was observed by electron microscopy to occur in the cytoplasm of the prespore stages of *C. shasta*. Gold particles were randomly distributed in the cytoplasm and on the cell membrane of the primary (mother) cell but did not appear to bind to any specific organelle. Label was not observed in the cytoplasm of the developing secondary (daughter) cells or in the nucleus of either the primary or secondary cells. No gold binding occurred in prespore stages incubated with either normal mouse serum or PBS which served as negative controls (Fig. 1-3). Immunoenzymatic staining of trophozoites in histological sections also showed that the antibody binding sites were on the surface and in the cytoplasm of the primary cell (Fig. 4). Secondary cells were clearly outlined and were not stained. Chromogen was also observed outside the trophozoite, suggesting that the antigen may be secreted.

Association of the gold particles with the cytoplasm of the primary cell may explain why this Mab recognizes only prespore stages of *C. shasta* and not mature spores. The first recognizable stages of the parasite in the fish are unicellular. This cell undergoes nucleogomy, the process of forming a multinuclear cell from a uninuclear cell, and eventually consists of a primary cell enveloping two sporoblasts. Each sporoblast contains two sporogenic, two capsulogenic, and two gametogenic cells (Noble, 1941; Yamamoto and Sanders, 1979). The primary cell is present
until mature spores are released; therefore, antigens present only in the cytoplasm of this enveloping cell may not be present in the mature spore.

Observation of the antigen recognized by this Mab gives additional support for its usefulness as a diagnostic reagent for prespore stages of *C. shasta*. The location of the antigen in the cytoplasm of the early stages offers some hope that this antigen would be present in life stages that exist outside of the salmonid.
Figure VII.1. Transmission electron micrograph of a *Ceratomyxa shasta* trophozoite incubated with normal mouse serum and immunogold reagents. 5700X.

Figure VII.2. *Ceratomyxa shasta* trophozoite incubated with monoclonal antibody and immunogold reagents. Arrows indicate gold label on cell surface and in the cytoplasm. 7100X.

Figure VII.3. *Ceratomyxa shasta* trophozoite incubated with monoclonal antibody and immunogold reagents. Gold label diffusely scattered in primary cell cytoplasm. Unstained, 11000X.
Figure VII.1–VII.3
Figure VII.4. Immunoenzymatic stain of *Ceratomyxa shasta* trophozoites in a histological section. Chromogen stains only the cytoplasm of the primary cell, secondary cells are unlabeled. x1000
Acknowledgements.

The authors wish to thank Jim Winton for arranging use of the facilities used in this study and Cindy Arakawa for her assistance. This research was supported by Bonneville Power Administration under contract No. DE-A179-83 BP 11987; G. R. Bouck, Contracting Officer's Technical Representative. Oregon Agricultural Experiment Station Technical Paper No.
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SUMMARY AND CONCLUSIONS

1. The known range of the infectious stage of *Ceratomyxa shasta* in the Columbia River basin was extended to include the mainstem of the Columbia River to its confluence with the Snake River, and the Snake River to Oxbow Dam. Demonstration of the parasite in the upper reaches of the watershed indicates that ceratomyxosis may be a more important factor causing mortality than previously recognized.

2. Monoclonal antibodies (Mabs) were produced against trophozoite and sporoblast stages of *C. shasta*. The resulting antibodies reacted with prespore stages of the parasite, but not with mature spores in IFAT or Western blot analysis. This suggests that *C. shasta* contains stage-specific antigens. One hybridoma produced antibodies of high specificity for *C. shasta* and is useful as a diagnostic reagent.

3. The antigenic specificity of the *C. shasta*-specific Mab was investigated using immunogold labeling techniques and transmission electron microscopy and by immunoenzymatic labeling. Specific binding by the Mab was observed in the cytoplasm of the primary cell.

4. Polyclonal antiserum was produced against a protein purified from *C. shasta* spores. This antiserum recognized both spore and prespore stages, but reacted most strongly with the later
stages. This evidence supports the hypothesis of the presence of stage-specific antigens in this parasite.

5. The progress of infection was investigated using histological techniques in combination with IFAT. In susceptible fish, trophozoites were first detected in the posterior intestine. The parasite evoked a severe inflammatory response which consisted mainly of an infiltration by lymphocytic cells. There was some indication that resistance was due to the inability of the parasite to penetrate the intestinal epithelium.

6. Examination of a severely infected intestine by scanning electron microscopy showed extensive destruction of the mucosal folds of the posterior intestine.

7. The antibody response of naturally infected fish was investigated. No antibodies specific for C. shasta were detected by either Western blot analysis or IFAT.
Purification Method for Spores of *Ceratomyxa shasta* and *Myxobolus squamalis*

Introduction

*Ceratomyxa shasta* and *Myxobolus squamalis* are myxosporean parasites which infect the intestinal tissues and scale pockets of salmonids, respectively. Because these organisms have not been successfully cultured in an *in vitro* system, it is necessary to purify spores directly from the fish. Uncontaminated preparations of spores are necessary for the production of specific antiserum, characterization of the spore, isolation of genetic material, and other *in vitro* studies.

Myxosporean spores have been purified by various methods, including sucrose gradients (Amandi, 1984), aqueous polymer two-phase systems (Kozel et al., 1980), and ether extraction (Landolt, 1973; Contos and Rothenbacher, 1974). Purification methods for *Myxobolus cerebralis* have been extensively studied and Hamilton and Canning (1988) recently reported the use of Percoll (Pharmacia Laboratories, Uppsala, Sweden) in purifying spores for use as antigen. This paper reports methods for the purification of *C. shasta* and *M. squamalis* spores using Percoll.
Materials and Methods

Rainbow trout (*Salmo gairdneri*) and chinook salmon (*Oncorhynchus tshawytscha*) that were infected with *C. shasta* and *M. squamalis*, respectively, were obtained. For collection of *C. shasta* infected tissues, the intestinal tracts were removed from dead or moribund fish. Intestinal tissue was mechanically disrupted in a stomacher (Tekmar Company, Cincinnati, OH) for 1 min. The homogenate was seived through Tyler screens (0.833 and 0.088 mm) and rinsed with 0.1 M phosphate buffered saline, pH 7.6 (PBS). The homogenate was centrifuged at 4000 g for 15 min, the supernatant discarded, and the white portion of the pellet carefully collected. Cysts of *M. squamalis* were collected from under the scales of adult chinook salmon. Spores obtained in this manner were relatively free of tissue debris. Unpurified spores of both species were resuspended in a small amount of PBS and 1.0 ml was layered on top of a gradient consisting of 2.0 ml layers of 12.5, 25, 50 and 75% Percoll made in PBS. For separation of *C. shasta*, the gradient was centrifuged at 430 g for 35 min in a swinging bucket rotor and for *M. squamalis*, at 770 g for 40 min. Spores of both species layered on top of the 75% layer of Percoll and were removed with a Pasteur pipette. To remove the Percoll, spores were diluted in PBS and centrifuged at 4000 g. The supernatant was discarded and the pellet resuspended in PBS. Two additional washes were done in this manner. Spores were resuspended in PBS and counted using a haemocytometer.
Results and Discussion

Separation of *C. shasta* spores occurred between the 50 and 75% layers of Percoll when the gradient was centrifuged at 430 g for 35 min. *M. squamalis* spores layered at the same position in the gradient but required a centrifugation force of 770 g for 40 min.

Use of Percoll as a separation medium for purification of myxosporean spores has several advantages over other methods. Percoll is iso-osmotic and unable to penetrate biological membranes and therefore it does not cause the osmotic changes in spores caused by sucrose gradients. Sucrose gradients are also easily contaminated and act as a good culture medium for yeasts. Because Percoll is sterile and not metabolized, contamination problems are minimized. Percoll has physiological ionic strength and pH and is non-toxic; therefore, spore antigens are unaltered by harsh isolation techniques as with ether extraction.

This method is simple and easily adaptable to purifying different spore types by varying Percoll concentration, centrifugation speed, or length of centrifugation. Although this method works well for spore purification, it is not as effective in purifying trophozoites. Trophozoite size and density varies greatly and their properties are similar to those of some host cell types. However, with modification of the above technique, it is possible that different sizes of trophozoites may be separated.
Literature Cited


Evaluation of a Tangential Flow Filtration Technique for Detection of *Ceratomyxa shasta* in Water

Introduction

The geographic range of the salmonid parasite, *Ceratomyxa shasta*, is confined to certain regions of California, Oregon, Washington, Idaho, and British Columbia. Fish infected with this parasite may be found in many water systems, but the distribution of the life stage of the parasite capable of infecting fish is limited. Presence of the infective stage is demonstrated by holding sentinel populations of fish in waters and observing them for development of the disease and the appearance of spores in the intestinal tissues. The life cycle of this parasite is unknown and therefore waterborne stages other than the spore have not been identified.

A technique that makes possible the detection of *C. shasta* in water supplies would have several applications. First, it would be valuable in the study of the life cycle of this parasite. It is not known whether the life cycle of *C. shasta* is direct or involves an intermediate host and the morphology of the infective stage and initial site of entry into the fish are undetermined. It is known that in waters containing the infective stage, exposure periods as short as 30 min are sufficient for infection to occur in susceptible fish, and the infective stage is retained on membranes of 14 μm
mean pore size (Johnson et al., 1979). This information suggests that the infective stage is relatively abundant and that it remains viable after filtration. Development of filtration methods may also allow screening of watersheds for the parasite without holding sentinel fish populations. Because the time from exposure to the infective stage to development of spores in fish may exceed 60 days, filtration combined with serological techniques could provide a rapid screening method. Direct fluorescent antibody techniques have been developed (Banner et al., 1982) and could be used to examine filtrates.

The filtration method evaluated in this study was the tangential flow filtration system. In this system, fluids flow parallel to the filter surface and compounds smaller than the exclusion size of the filters pass through and are collected in the filtrate. Larger particles are retained and recycled to the original reservoir (retentate). The retentate volume decreases with continued filtration until the desired volume is reached. Johnson (1975) determined that the size of the infective stage was larger than 14 μm; therefore, a filter was chosen that would retain particles this size. Tangential flow techniques have been used successfully by Issac-Renton et al. (1986) for detection of *Giardia lamblia* cysts in water. In that study, water samples were seeded with cysts, filtered through the tangential flow system, and cyst recovery was evaluated by microscopic examination. Because the infective stage of *C. shasta* has not been identified, its recovery was
evaluated by attempting to transmit ceratomyxosis to susceptible fish by exposure to, or injection with, the filtered samples.
Methods

Water samples were collected from a location in the Willamette River near Corvallis, Oregon, where the presence of the infective stage of *C. shasta* could be demonstrated by using sentinel fish. Water was either collected from the surface or was pumped from the bottom. Samples were returned to the laboratory and held on ice during the entire filtration process.

Initial samples were prefiltered to remove large particles. Water was filtered through Tyler screens (sizes 0.833, 0.088 and 0.043 mm) directly into a 20 liter dispensing pressure vessel (Millipore Corp., Bedford, Mass). The water was forced out of the vessel with a vacuum/pressure pump through a 30 μm polypropylene pleated filter (Pall Trinity Micro Corporation, Cortland, NY). Using a low-shear peristaltic pump, prefiltered and unfiltered samples were passed through a Millipore cassette system fitted with a 0.5 μm durapore filter packet (Millipore Corp) (Fig. 2.1). The sample was separated by the unit into a filtrate and a concentrated sample (retentate). The latter would contain the infective stage of *C. shasta* which is too large to pass through the filter. The retentate volume was reduced to 300-500 ml by recirculation. Procedures for filter preparation, use, and storage were described by Millipore Corp. (1981).

The retentate of each sample was divided into two portions. Half was centrifuged at 16300 g for 15 min. The pellet was examined by bright light microscopy, smears were made for
examination by fluorescent antibody techniques, and the remainder was fixed in buffered formalin. The other half of the retentate was used in attempts to transmit the disease to susceptible trout. This was done either by exposing the fish to a dilution of the retentate or by injecting the retentate into the fish. Fish were exposed to the sample in an 18 l tank in which the retentate had been diluted to approximately 4 l, a level that would support 15 fish with aeration. The fish were held in this manner for 5 h before the water was turned on and the sample further diluted. In other samples, the material was pelleted by centrifugation as described and 15 trout were injected intraperitoneally with 0.5 ml each. After exposure or injection, fish were held in pathogen-free water, observed for signs of the disease, and dead fish were examined for the presence of spores. Ninety days following exposure or injection with the sample, all surviving fish were killed and examined for spores.

In one sample set an additional 160 l of water, equal to the sample volume filtered, was collected for each of the seven samples. Fifteen susceptible fish were held in each control sample, with aeration, for 6 h, and fifteen fish were injected with portions of each filtrate. After exposure or injection, they were held in pathogen-free water and observed for signs of the disease as described.

Wet mounts of material from the retentates were examined by bright light microscopy and smears were air dried and fixed in acetone/xylene (1:1) for examination by fluorescent antibody
techniques (FAT). Fixed smears were incubated with rabbit antiserum directed against *C. shasta* for 15-30 min, then with fluorescein-conjugated goat anti-rabbit serum. After thorough rinsing they were counterstained with Evan's blue, mounted in buffered glycerol, and examined using a Zeiss standard microscope with an IV Fl epi-fluorescence condensor.
Figure 2.1. Millipore cassette system.
Results and Discussion

Twenty-one samples were processed by tangential flow filtration. Six of these samples were prefiltered to exclude particles larger than the infective stage of C. shasta, the remaining 15 samples were not prefiltered. The method of exposing fish to the retentate was also varied. Subsamples of retentates from five samples were diluted and susceptible fish were held in them and subsamples of the remaining 16 retentates were injected intraperitoneally into susceptible fish. In the last six samples processed, an identical volume of water was collected and fish were held in this water to assess whether viability of the infective stage was affected by the filtration procedure.

There was no difference in the recovery of the infective stage of C. shasta between samples that were prefiltered and those that were not. Recovery of the infective stage was extremely low in both cases (Table 1). Changing the method of assaying recovery from exposure of susceptible fish to the retentate to injection of fish with the retentate also made no difference. The apparent low recovery of the infective stage indicated that either viability was lost in the filtration process or the number of infective units in the samples was extremely low. To determine which, samples of identical size were collected and used as controls. Ten fish (10% of the total number of fish exposed) held in the control samples died of ceratomyxosis, while only one fish (1% of the total sample)
injected with the filtrate became infected. These results indicate that viability was lost in the filtration process.

Portions of the retentate were also examined by bright light microscopy and fluorescent antibody techniques. Although transmission results discouraged exhaustive examination of each sample, a single spore was observed in each of two samples. Although this is the first report of any stage of the parasite being visualized in a field sample, it is not surprising that spores are present in the water because they are probably shed from resident infected fish.

Although this method has proved applicable to detection of certain other pathogens in water supplies (Issac-Renton et al., 1986; Watanabe, 1986), it is not as effective in recovering viable infective *C. shasta* from field samples. Reasons for this may include the fragility of the infective stage, the relatively small sample size used, and the inefficiency of the methods used to assess recovery. Sample sized could be increased; however, this means longer filtration times and increased recirculation of *C. shasta* through the system. Both of these manipulations may decrease the viability of a fragile organism. There are other areas in this study that could be improved upon. Addition of protein and amino acid supplements may stabilize a fragile infective stage and pretreatment of the filters may prevent adsorption of the organism to the membrane. Also, recent development of a monoclonal antibody against *C. shasta* trophozoites (Bartholomew et al., submitted) should increase the effectiveness of examination of samples by FAT. However,
without knowledge of the nature of the infective stage, it is difficult to determine what modifications of this system are necessary.
Table 2.1. Comparison of recovery of *C. shasta* infective stages between prefiltered and nonprefiltered samples and between different methods for assaying recovery.

<table>
<thead>
<tr>
<th>Sample</th>
<th># Liters Filtered</th>
<th>Prefilter Used</th>
<th># of Fish Infected by Injection Method&lt;sup&gt;1&lt;/sup&gt;</th>
<th># of Fish Infected by Exposure Method&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>+</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>+</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>+</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>+</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>+</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>340</td>
<td>+</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>273</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>227</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>227</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>227</td>
<td>-</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>160</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>182</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>160</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>160</td>
<td>-</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

1. 15 fish were used in each experimental group.
Literature Cited


APPENDIX 3

Experimental Transmission of *Ceratomyxa shasta* Infections

Introduction

Experimental transmission of ceratomyxosis in a laboratory environment has occurred when susceptible fish were exposed to water and substrate from areas endemic for the parasite (Sanders and Fryer, 1970) or by direct injection of *C. shasta* trophozoites (Johnson et al., 1979). The identity of the infective stage and involvement of any intermediate hosts are undetermined, but the ability to experimentally reproduce the conditions necessary for transmission would make investigation of these problems possible.

The only myxosporean infection which has repeatedly been transmitted in the laboratory is salmonid whirling disease, caused by the myxosporean *Myxobolus cerebralis*. Uspenskaya (in Walliker, 1968) and Hoffman and Putz (1969) reported that *M. cerebralis* spores required an aging process to be infective. However, Markiw and Wolf (1983) have proposed that the spore aging process which results in transmission of the disease requires the participation of tubificid oligochaetes. These tubificids harbor the actinomyxidian, *Triactinomyxon*, which they propose as an alternate life stage of *M. cerebralis*. They have suggested that this requirement for an intermediate host may be necessary for transmission of other myxosporean infections. This paper
describes several attempts to achieve transmission of ceratomyxosis in laboratory conditions.
Materials and Methods

Two experiments were designed to transmit ceratomyxosis. In the first experiment, tanks contained combinations of washed sand from a source free of *C. shasta*, mud from a site endemic for *C. shasta*, viscera from *C. shasta* infected fish, and the oligochaete worm *Kinkaidiana hexathea*. Control tanks contained either sand and infected viscera (tank 1), or sand and oligochaete worms (tank 2). Tank 3 contained sand, infected viscera, and oligochaetes, and tank 4 held mud from an area endemic for *C. shasta* and infected viscera, but no oligochaetes. Infected viscera (10 infected intestines per tank) was added to tanks 1, 3, and 4 at two intervals 1 month apart. All tanks received 20 uninfected, susceptible rainbow trout 1 month after the second addition of infected tissues (Table 3.1). Fish were observed for signs of infection and fish that died during the experiment were examined for spores. After 9 months, all remaining fish were killed and examined for spores.

In the second experiment, three different substrates were used. Tanks 1 and 2 were duplicate tanks containing autoclaved mud. Tanks 3 and 4 contained sterile mud and *K. hexathea* from a non-endemic source. Tanks 5 through 8 contained mud and natural fauna from sites endemic for *C. shasta*, either the Willamette River or LaCamas Lake. Five uninfected, susceptible fish were added to each tank 1 d after the substrates were settled. Two weeks later, five live infected fish were added to each tank except 6 and 8 (Table 3.2). Infected fish were held in pathogen-
free water at least 3 d before addition to the tanks and were fed a diet with 3% terramycin incorporated in the form of TM 50 (Pfizer). They were also treated for parasites and fin clipped for identification. Infected and uninfected fish were added at intervals so that there were always five of each in every tank except 6 and 8 which did not receive infected fish. Freshly collected oligochaetes were added to tanks 3 and 4 after 6 months, and after 1 year, tanks 5 - 8 were replenished with fresh mud and native fauna from the two endemic sites. As fish died, they were removed from the tanks. Fin clipped, infected fish were returned to the tanks to decay and unclipped fish were examined for spores. After 22 months, remaining unclipped fish were killed and examined for spores.
Results and Discussion

In the initial experiment, in which susceptible fish were held in tanks containing oligochaete worms and *C. shasta*-infected tissues (Table 3.1), no infections developed over a nine month exposure period. In the second experiment using artificial environments with mud and aquatic organisms from areas endemic for *C. shasta* plus live infected fish (Table 3.2), transmission occurred only in tanks containing substrate and water freshly collected from the Willamette River. Only three fish became infected, and one of these was held in the tank which did not contain infected fish as a source of the parasite. No transmission occurred in tanks seeded with oligochaete worms from an uninfective source, mud from LaCamas Lake where the parasite is endemic, nor control tanks containing sterilized mud.

These experiments did not result in transmission of *C. shasta* infections in a manner that would allow us to draw conclusions about the life cycle of the parasite, but they did support the work of earlier researchers. Transmission did not result when infected fish were held with susceptible fish or when susceptible fish were allowed to feed on infected tissues. Studies by Uspenkaja (in Walliker, 1968) and Hoffman and Putz (1971) on *Myxobolus cerebralis* showed that the infective stage was present only after spores were aged three to six months in mud. However, in these experiments infected viscera was allowed to decay and spores age for as long as 22 months and no transmission occurred. The only
cases of transmission of the parasite in these studies were traced to the addition of fresh sediments from an endemic area. These results are similar to those of Sanders and Fryer (1970) who reported infections in fish exposed to bottom sediments collected from LaCamas Lake.

The inability to transmit ceratomyxosis between infected and susceptible fish has led to the speculation that an intermediate host may be involved in the life cycle. Markiw and Wolf (1983) have shown that the spore aging process of *M. cerebralis* requires the participation of tubificid oligochaetes. They were able to show that specifically, *Tubifex tubifex* was the intermediate host for the alternate life stage of the parasite, an actinomyxidian of the genus *Triactinomyxon*. In our experiments the only oligochaete identified was *K. hexatheca*. No transmission occurred in the presence of this organism; however, results of *M. cerebralis* studies indicate that the actinomyxidian is species specific for its host. Therefore, although these experiments indicate that *K. hexatheca* is not involved in the life cycle of *C. shasta*, they do not preclude the possibility that another oligochaete may be the intermediate host.
Table 3.1. Contents of experimental transmission tanks. Twenty uninfected, susceptible fish were added to each tank.

<table>
<thead>
<tr>
<th>Tank</th>
<th>Substrate</th>
<th>Oligochaetes</th>
<th>Infected Viscera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sand</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>sand</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>sand</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>mud</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3.2. Contents of experimental transmission tanks. Five uninfected, susceptible fish were maintained in each tank.

<table>
<thead>
<tr>
<th>Tank</th>
<th>Substrate</th>
<th>Organisms</th>
<th>Infected Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&amp;2</td>
<td>sterile mud</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3&amp;4</td>
<td>sterile mud</td>
<td>oligochaetes</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>LaCamas L. mud</td>
<td>natural fauna</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>LaCamas L. mud</td>
<td>natural fauna</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Willamette R. mud</td>
<td>natural fauna</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Willamette R. mud</td>
<td>natural fauna</td>
<td>-</td>
</tr>
</tbody>
</table>
Literature Cited


