THE BIOLOGY OF INFECTION BY *LOMA SALMONAE* (MICROSPORIDIA)

by

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ABSTRACT

Loma salmonae is an important gill pathogen of Pacific salmon *Oncorhynchus* spp. in the Pacific Northwest. Epizootics of the parasite have caused considerable economic loss in Pacific salmon farming in British Columbia. A study was undertaken on the basic biology of *L. salmonae* since very little information exists to aid fish-farmers and scientists. *Loma salmonae* transmission was examined by challenging fish by *per os* (oral), injection, and cohabitation with infected fish. *Loma salmonae* is transmissible by experimental (e.g. intraperitoneal, intramuscular injection) and natural (e.g. *per os*, cohabitation of naive and infected fish) exposure routes. Autoinfection of hosts is possible as demonstrated by intravascular injections of spores producing infection. The route of infection in salmon was examined using histological techniques. Sporoplasms from extruded spores were detected in epithelial cells of the alimentary canal. The parasite may move through these cells and enter the blood stream eventually reaching the gills. Purified spores were held under various laboratory conditions to examine viability. Spores were still infective after 95 d at 4 °C in fresh or sea water. Spores were killed when frozen or exposed to 100 or 200 ppm iodophor, although some spores survived the latter treatment. Host specificity of *L. salmonae* was examined by *per os* exposure of salmonids and nonsalmonds. All *Oncorhynchus* spp. tested were susceptible, but Atlantic salmon and Arctic char were resistant. All nonsalmonids tested were resistant. A Northern Stream strain of chinook showed higher susceptibility to the parasite when compared to a Southern Coastal or hybrid strains. Macrophage phagocytic ability was investigated as a possible mechanism to explain differences between strains and susceptible or resistant salmonids. No differences were found between strains but Atlantic salmon macrophages have a higher phagocytic response to *L. salmonae* spores than chinook. The possibility of nonsalmonid reservoirs for *L.*

salmonae was examined by collecting different life stages of salmonids and nonsalmonids near Vancouver Island. *Loma salmonae* was present throughout the life cycle of wild salmonids and a new *Loma* sp. is described from *Cymatogaster aggregata* using morphology, transmission studies, and rDNA sequencing.

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PREFACE

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CHAPTER I

Overview Fish-Infecting Microsporidia

INTRODUCTION

About 100 species and 14 genera of phylum Microsporidia Balbiani, 1882 have been described from fishes, and several cause severe disease. Microsporidia of fishes are widely distributed by both host species and geographic location. Whereas most fish microsporidia are host specific, at least at the genus level, a few (e.g. *Glugea stephani* Hagenmuller, 1899) show broad host specificity (Canning & Lom 1986). For comprehensive lists of microsporidian species and their fish hosts see Canning & Lom (1986), Lom & Dyková (1992), and Dyková (1995). Herein I present an overview of economic importance, immunology, and treatment, followed by a review of the most important genera. My review focuses on microsporidia causing significant pathology in cultured fishes. Features of pathology and transmission relevant to individual microsporidia species are included under the genera section. For a general review of microsporidia transmission see Cali & Takvorian (1999).

The ability of some microsporidia to cause disease in fish is well known. Increased mortalities in wild fishes such as rainbow smelt *Osmerus mordax* Mitchill (Haley 1952; Nepszy et al. 1978), young gizzard shad *Dorsoma cepedianum* LeSueur (Putz 1969; Price 1982), and freshwater salmonids (Putz et al. 1965; Urawa 1989) have been attributed to microsporidia infections. In wild fishes, declines of entire commercial fisheries have been attributed to microsporidiosis (Mann 1954; Sindermann 1966; Ralphs & Matthews 1986). *Pleistophora macrozoarcides* Nigrelli, 1946 has been implicated in the collapse of the North American ocean pout *Macrozoarces americanus* Schneider fishery (Fischthal 1944; Sandholzer et al. 1945; Sheehy et al. 1974). Declines in the rainbow smelt fishery of New Hamshire were due in part to infection by

G. hertwigi Weissenberg, 1911 (in Haley 1954). Disfiguring of fish by large cysts or liquefaction of muscle can decrease catch value (Nigrelli 1946; Grabda 1978; Egidius & Soleim 1986; Pulsford & Matthews 1991). Microsporidiosis can also cause indirect mortalities through starvation and reduced growth rates (Matthews & Matthews 1980; Figueras et al. 1992). Sprengel & Lüchtenberg (1991) found that impairment of swimming ability in European smelt, *O. eperlanus* L., infected by *Pleistophora ladogensis* Voronin, 1978 may contribute to higher mortalities due to predation. Wilkund et al. (1996) reported that *P. mirandellae* Vaney & Conte, 1901 which infects gonads, reduced the reproductive capacity in roach *Rutilus rutilus* L. Selective mortalities in juvenile stocks can further reduce reproductive capacity. For example, *Microgemma hepaticus* Ralphs & Matthews, 1986 infects primarily the liver of juvenile grey mullet *Chelon labrosus* Risso, and may contribute to mortality (Ralphs & Matthews 1986).

An increase in aquaculture on a worldwide basis has led to the description of several new microsporidia and outbreaks of microsporidiosis in recent years. Compared to their wild counterparts, cultured fish are particularly susceptible to microsporidian infections because of high stocking densities. Extremely high mortalities have occurred in ornamental fishes as a result of infections by *Glugea* spp. (Lom et al. 1995). *Pleistophora hyphessobryconis* Shäperclaus, 1941 (Fig. 1) well known to fish hobbyists as the source of neon tetra *Paracheirodon innesi* Myers disease, is one of the most common parasites of aquarium fishes (Canning & Lom 1986). Massive mortality of neon tetras held in aquariums is still not uncommon. Morphological abnormalities and mortality due to microsporidiosis in the ornamental zebra danio B*rachydanio rerio* have also been reported (Kinkelin 1980). The zebra fish is now a very important laboratory animal for genetic research, and large colonies of this fish are being maintained at several universities in Europe and North America. ML Kent (pers. comm.) has observed

this microsporidian infection in zebra fish kept in European and North American colonies. The primary site of infection is the central nervous system, where it is often associated with chronic inflammatory changes (Fig. 2). In addition infections and associated severe inflammation in the skeletal muscle surrounding vertebrae and in the kidney have been seen (ML Kent pers. comm.). *Heterosporis* spp. can infect cichlid *Pseudocrenilabrus multicolor* Seegers (Lom et al. 1989), ornamental Siamese fighting fish *Betta splendens* Regan (Lom et al. 1993), and angel fish *Pterophyllum scalare* Curs & Valens (Michel et al. 1989) causing emaciation and distress or severe pathological changes in muscle tissue.

Microsporidia are also important in cultured food fishes. Hatcheries raising salmonids for enhancement of wild stocks or for profit, have experienced epizootics of microsporidia (Putz 1969; Hauck 1984; Urawa & Awakura 1994). Mortality and disease have occurred at both freshwater (Markey et al. 1994) and marine sites where fish are grown to market size (Kent et al. 1989). Chronic low mortality in sea bream *Sparus arurata* L., for example, has been caused by a *Pleistophora* sp. that infects the muscle (Abela et al. 1996). Culture of Japanese eel *Anguilla japonica* Temminck & Schlegel and ayu *Plecoglossus altivelis* Temminck & Schlegel is often plagued by infections of *Heterosporis anguillarum* (Hoshina, 1951) (syn. *Pleistophora anguillarum*), and *Glugea plecoglossi* Takahashi & Egusa, 1977 (Awakura 1974; Kano & Fukui 1982; Kim et al. 1996) respectively. Production of minnows used for bait fishes is often affected by infections of *P. ovariae* Summerfelt, 1964, which damage the ovaries of fish and thereby reduce fecundity (Nagel & Hoffman 1977).

Early investigations of fish microsporidia included observations of host response (Drew 1910; Debaisieux 1920; Nigrelli 1946). Researchers during that time period occasionally confused host reparative events (i.e. phagacytosis) with early parasite

development stages (Canning & Lom 1986). Presently, host-parasite relationships of fish microsporidia can be broadly classified into two groups: those associated with xenoma formation (e.g., *Glugea, Ichthyosporidium, Jirovecia, Loma, Microfilum, Microgemma, Nosemoides, Spraguea, Tetramicra*) (Fig. 3), and those associated with non-xenoma forming microsporidia (e.g., *Nucleospora*, *Heterosporis, Pleistophora, Thelohania*) (Fig. 4). The term "xenoma" was developed from early works of Chatton (1920) and Weissenberg (1922). Cells infected by certain species of microsporidia become transformed and hypertrophied, resulting in a unique host cell-parasite complex. The host cell alters its structure and size, becoming physiologically integrated with the parasite. Cytoplasmic contents are replaced by the parasite, and the surface of the cell, or xenoma, becomes modified for increased absorption. Microvillus-like structures form in cells infected by *Microsporidium cotti* Chatton & Courier, 1923, *Ichthyosporidium*, and *Tetramicra*, while *Glugea* and *Loma* spp. utilize numerous pinocytotic vesicles (Canning & Lom 1986). Hypertrophied cells can reach sizes of 400-500 μm or larger, and are visible macroscopically as white cysts (Matthews & Matthews 1980; Ralphs & Matthews 1986) (Fig. 5). Eventually the cell is totally destroyed by the parasite. Spores released when a xenoma ruptures are easily identified by a large posterior vacuole in wet mounts and histological sections (Fig. 6).

IMMUNOLOGY

The specific and nonspecific defense systems of fish are well reviewed by Kaattari & Piganelli (1996), Secombes (1996), and Dalmo et al. (1997). In comparison to the situation in viral and bacterial pathogens, the response of fish to microsporidian infections is relatively unexplored.

Phagocytosis of spores by macrophages has been described for several

microsporidian species (Dyková & Lom 1980; Canning & Lom, 1986; Pulsford & Matthews 1991; Kim et al. 1996). Macrophages actively ingest and breakdown spores (Fig. 7), playing a crucial role in the host defense mechanism. The spore coat contains an outer protein layer and an inner chitin layer. Spores appear to be digested from the inside out indicating fish macrophages possess a wide repository of chitinases (Dyková & Lom 1980; Canning & Lom 1986). Microsporidia are not without their own defenses, as spores may prevent phagosome-lysosome fusion. Weidner & Sibley (1985) found that anionic components on the spores of *G. hertwigi* may form ionic bonds with the phagosome membrane, increasing rigidity and preventing fusion with a lysosome. It is not surprising that some microsporidia have evolved this protective mechanism as has occurred with other intracellular parasites, such as *Chlamydia psittaci* (Wyrick & Brownridge 1978), and *Toxoplasma gondii* Nicolle & Manceaux, 1908 (Jones & Hirsch 1972).

Suppression of the host inflammatory response by microsporidia has also been documented (Dyková & Lom 1978, 1980). Laudan et al. (1986a,b, 1987, 1989) found that spores of *G. stephani*, when phagocytized, suppressed immunoglobulin (Ig) levels of winter flounder *Pseudopleuronectes americanus* Walbaum by stimulating macrophages to release prostaglandin and/or leukotrienes. This finding was substantiated by injecting serum from infected fish into healthy fish, which resulted in immunosuppression of healthy fish. Administering idomethacin after spore exposure, also failed to produce immunosuppression in flounder. *Glugea stephani* impairs the host's humoral response to other infectious agents, and interferes with initiation, as well as with other levels, of the response. After *G. stephani* becomes established, the host's immune system can recover.

It has been suggested that *Tetramicra brevifilum* Matthews & Matthews, 1980

can immunosuppress turbot *Scophthalmus maximus* L. using a similar mechanism to that found in *G. stephani* (Leiro et al. 1994). Figueras et al. (1992) found that the serum agglutinin titers of naturally infected fish were low, although this did not increase their susceptibility to vibriosis (*Vibrio anguillarum*). These authors also isolated a serum factor from infected fish, concluding that further study was necessary to determine if the factor was an immunomodulator or only a by-product of the parasite. Leiro et al. (1993) injected fish IP with whole spores or a crude extract of *T. brevifilum.* At 30 d after injection they gave the fish a second immunization of whole spores. They found that whole spores lead to higher antibody production initially, but that after 30 d fish responded more to crude extract antigens. Low seropositives were obtained from enzyme-linked immunosorbent assay studies on infected fish, leading them to suggest that this was indirect evidence of immunosuppression.

Humoral and/or cellular response of a fish may also be impaired by infection with *Nucleospora salmonis* Hedrick, Groff & Baxa, 1991 (Wongtavatchai et al. 1995b), or *Heterosporis anguillarum* (Hung et al. 1997). Serum factors affecting T and B cells directly may be secreted by these parasites, or parasites may stimulate release of mediators from macrophages. Leiro et al. (1996a) found that *Glugea caulleryi* Van den Berghe, 1940 showed considerable homology of spore surface antigens to those of *T. brevifilum.* However, *G. caulleryi* did not suppress the humoral immune response of turbot, but rather stimulated it during first and subsequent exposures. In general, caution should be exercised in assuming that lyphopenia (low lymphocyte numbers) is caused directly by a concurrent microsporidium infection because this condition may be caused by a wide variety of stressors (Barton & Iwama 1991; Figueras et al. 1992).

The health of fish has often been used as a biological indicator of environmental conditions (Kent & Fournie 1993). Parasitic burdens can have significant impact on

mortality in wild fishes, and in some cases may be related to environmental conditions (Overstreet 1993). There have been very few studies dealing with the effects of anthropogenic contamination on microsporidian infections in fish. For example, Barker et al. (1994) found that abundance of *G. stephani* in winter flounder was significantly higher at a pulp mill effluent site where pollutants accumulated in the sediment. In fish from polluted waters compared to fish from unpolluted waters, Barker et al. (1994) reported larger and more varied cysts forming in visceral organs, in addition to the intestine, which is considered the normal site of infection (Takvorian & Cali 1981). Chronic exposure to pulpmill effluent may have further immunosuppressed the flounder allowing *G. stephani* to proliferate.

Immune responses by fish are often weak or nonexistent when microsporidia evade detection (e.g. by forming xenomas or simply by being intracellular). However, during initial infection, and especially during rupture of xenomas (see section on *Glugea* pathology) or pseudocysts (i.e. *Pleistophora*-type infections), microsporidia are vulnerable to attack by the immune system of fish. Although antibody production in response to infection does occur (Buchmann et al. 1992; Leiro et al. 1993; Hung et al. 1996, 1997), it may not play a protective role (Kim et al. 1996,1997). Resistance to reinfection in recovered fish occurs in *Loma salmonae* Morrison & Sprague, 1981 (syn. *Plistophora salmonae* Putz, Hoffman & Dunbar, 1965) (Speare et al. 1998b).

Different strains of the same fish species can exhibit varying degrees of susceptibility. For example, I have observed marked differences in susceptibility to *L. salmonae* between different strains of chinook salmon *Oncorhynchus tshawytscha* Walbaum (Shaw et al. 1996). Phagocytes may play a primary role in preventing initial and subsequent infection by microsporidia. General phagocytic activity, measured by percent phagocytosis (PP, i.e. the percent of cells showing phagocytic activity or

percent of target particles engulfed), and phagocytic index (PI, i.e. the mean number of objects ingested per phagocyte), usually increases in the presence of serum. It is well known that opsonization by antibody and/or complement of nonself organisms enhances phagocytosis in fish (Griffin 1983; Sakai 1984; Scott et al. 1985; Olivier et al. 1986; Waterstrat et al. 1991; Matsuyama et al. 1992; Rose & Levine 1992; Pedrera et al. 1993; Leiro et al. 1996b). However, Leiro et al. (1996b) found that serum did not increase phagocytosis of *G. caulleryi* or *T. brevifilum* by turbot macrophages. Neither did macrophages from immunized turbot ingest significantly more spores than those from nonimmunized fish. They investigated this further, by treating spores with sodium *m*-periodate to block the binding of macrophages to surface-borne sugars on spores. These spores were ingested less effectively, suggesting that macrophages recognize sugars on the surface of the microsporidia. Lectinophagocytosis, the binding of sugars on the pathogen by macrophage receptors, has also been shown in tilapia *Oreochromis spilurus* Günther by Saggers & Gould (1989). Leiro et al. (1996b) concluded that microsporidia may modify the phagocytic response in fish. I have found that PP and PI of *L. salmonae* spores by macrophages differ significantly between Atlantic salmon *Salmo salar* L. (which is completely resistant to *L.salmonae*) and the very susceptible chinook salmon (see Chapter V).

Temperature can also affect the ability of a fish host to mount an immune response, and phagocytic activity of macrophages is dependent on temperature (Finn & Nielson 1971; Leiro et al. 1995). Development in fish-infecting microsporidia can also depend on ambient temperature (Awakura 1974; Olson 1981; Speare et al. 1998a). For example, Speare et al. (1998a) were unable to detect *L. salmonae* infections in rainbow trout *Oncorhynchus mykiss* Walbaum held at 10°C, although fish held at 14.5°C developed infections.

TREATMENTS

Several drugs have been used to treat microsporidian infections in fish, mostly on an experimental basis. Fumagillin, an antimicrobial agent developed for treating the microsporidian *Nosema apis* Zander, 1902 infections in honey bees, is the drug most widely used to treat microsporidiosis in fishes. The drug apparently acts by inhibiting RNA synthesis (Jaronski 1972). Kano et al. (1982) reported that fumagillin was effective against the microsporidian *H. anguillarum* in eels *Anguilla japonica*. The drug has been used also against *N. salmonis* infections in chinook salmon (Hedrick et al. 1991b) and *Loma salmonae* infections in chinook salmon (Kent & Dawe 1994). Fumagillin has also been used successfully to control several myxosporean diseases in fish (e.g., whirling disease, proliferative kidney disease, and sphaerosporosis) (Molnár et al. 1987; Hedrick et al. 1988; Székely et al. 1988; Laurén et al. 1989; Wishkovsky et al. 1990; Yokoyama et al. 1990; El-Matbouli & Hoffmann 1991; Sitjà-Bobadilla & Alvarez-Pellitero 1992; Higgins & Kent 1996).

Various concentrations of the drug were employed in these studies, and on the basis of these reports, 3 to 10 mg fumagillin kg^{-1} of fish day^{-1} is the recommended dose for salmonids. Higher concentrations or prolonged treatment (e.g., 30 to 60 d) may cause anorexia, poor growth, anemia, renal tubule degeneration and atrophy of hematopoietic tissues in salmonids (Laurén et al. 1989; Wishkovsky et al. 1990). Lower doses may be efficacious for some microsporidian infections in fish (e.g., Hedrick et al. (1991b)) controlled *N. salmonis* infections with oral fumagillin treatment at 1 mg of drug kg^{-1} of fish day^{-1} for 4 wk). Kent & Dawe (1994) reported positive results for controlling *L. salmonae* infections at 10 mg of drug kg^{-1} fish day^{-1} with a 4 wk treatment. Recent experiments with the drug demonstrated that the infections can be controlled with lower doses of 2 or 4 mg of drug kg^{-1} of fish day^{-1} (Higgins et al. 1998).

Fumagillin is not heat-stable. Therefore, it is recommended that feed be coated with the drug, instead of incorporating it into feed during milling. The drug is not very soluble in water, but is very soluble in alcohol. In most studies, fumagillin was mixed with alcohol and sprayed on the feed; then the feed was coated with oil.

An analog of fumagillin, TNP-470 (Takeda Chemical Industries, Ltd.), is a potent antiangiogenesis agent (Kusaka et al. 1994). Fumagillin and its derivatives appear to work by binding and inhibition of methionine aminopeptidase type II (Liu et al. 1998) which is the target for angiogenesis inhibitors AGM-1470 and ovalicin (Sin et al. 1997). Furthermore, in laboratory studiesTNP-470 has been shown to be effective against the mammalian microsporidia pathogens *Encephalitozoon intestinalis* Hartskeerl, Gool, Schuitema, Didier & Terpstra, 1995, *Vittaforma corneae* Silveira & Canning, 1995 (syn. *Nosema corneum* Shadduck, Meccoli, Davis & Font, 1990), *E. cuniculi* Levaditi, Nicolau, & Schoen, 1923 and *E. hellem* Didier, Didier, Friedberg, Stenson, Orenstein, Yee, Tio, Davis, Vossbrinck, Millichamp & Shadduck, 1991. Higgins et al. (1998) tested the efficacy of this drug in controlling *L. salmonae* and *N. salmonis* in experimentally infected salmon. Fish treated orally at 1.0 mg or 0.1 mg of drug kg^{-1} of fish day^{-1} for 6 wk showed markedly reduced infections by both microsporidia, even at the low dose. No significant toxic side effects were associated with the treatment. Higgins & Kent (1996, 1998) also found that treating fish at this low dose prevented infections in fish naturally exposed to the PKX myxosporean.

The effects of a systemic triazinone, Toltrazuril (Bayer AG) on *Glugea anomala* Moniez, 1887 infections in sticklebacks *Gasterosteus aculeatus* L. have been investigated at the ultrastructure level (Schmahl & Mehlhorn 1989; Schmahl et al. 1990). Bath exposure of the drug was found to cause destruction of all life stages of the parasite, but the overall effects of this drug in reducing the prevalence or intensity of microsporidian infection in fish

have not been reported.

Awakura & Kurahashi (1967) reported that amprolium inhibited merogony in *Microsporidium takedai* administered at 0.06 % body weight day^{-1} for up to 48 d, but the treatment was associated with toxic side effects. There are a few other reports of treatment of fish microsporidia. For example, Andodi & Frank (1969) (cited in Canning & Lom 1986) claimed that they cured *P. hyphessobryconis* infections in aquarium fish by adjusting the pH to 7.5-8.0 and introducing ozone at a rate of 1 mg h^{-1} 100 L^{-1}. Nagel & Summerfelt (1977) reported that nitrofurazone reduced infections of *Pleistophora ovariae* in golden shiners *Notemigonus chrysoleucas* Mitchill when used in oral treatments at 2.2 - 3.3 g of drug kg^{-1} feed. Speare et al. (1998c) reported delayed *L. salmonae* xenoma formation in rainbow trout fed to satiation with quinine hydrochloride treated feed (5 g kg^{-1}).

Elevated water temperatures have also been used to treat some fish-infecting microsporidia. Takahashi & Ogawa (1997) reported an effective treatment for *G. plecoglossi* in ayu was to keep fish at 29°C for 5 d 10 to 20 d after initial infection and repeat this treatment a week later. Kim et al. (1997) noted *H. anguillarum* in Japanese eel could also be treated with elevated water temperatures resulting in morphologically abnormal spores in the musculature. Normal spores were also recovered from treated fish suggesting relapses of infection might occur.

SIGNIFICANT MICROSPORIDIAN DISEASES

Xenoma-Forming Genera

GLUGEA

Species of *Glugea* are some of the most intensively studied fish microsporidia. They infect the submucosal intestinal cells of a variety of wild and cultured fishes.

Disease in the food fish sand smelt *Atherina boyeri* Risso, pike perch *Stizostedion lucioperca* Cuvier, and bait fish *D. cepedianum*, can be caused by *Glugea atherinae* Berrebi, 1978, *G. luciopercae* Dogiel & Bykhowsky, 1939, and *G. cepedianae* Putz, Hoffman & Dunbar, 1965 respectively (Price 1982; Canning & Lom, 1986). However, most research has focused on *G. anomala* in stickleback (*Gasterosteus* and *Pungitius* spp.), *G. hetwigi* in smelt, *G. plecoglossi* in ayu, and *G. stephani* in flatfish (*Pleuronectes* spp. and *Platessa* spp.). *Glugea anomala* represents classical developmental and pathological features (Weissenberg 1968; Dyková & Lom 1980), while mortality of fishes have been documented in the other species (Olson 1976; Nepszy et al. 1978; Cali et al. 1986; Kim et al. 1996).

Glugea anomala and *G. plecoglossi* develop in almost all body organs of the host, forming large (2 to 3 mm) white xenomas, which can cause serious injury to organs (Fig. 8). *Glugea hertwigi* (Fig. 9) and *G. stephani* (Figs. 10 & 11) primarily infect the subepithelial connective tissue of the intestine, although in heavy infections, *G. hertwigi* can spread to all other organs, even the skeletal muscle and gills (Canning & Lom 1986).

Pathology. *Glugea* spp., like *Loma*, and *Tetramicra*, form xenomas that can be classified by stages of development (Dyková & Lom 1980). Early or young xenomas have a cytoplasm filled uniformly with developmental stages of the parasite (Fig. 12). As they grow, the developmental stages occupy the center of the xenoma surrounded by peripheral host cytoplasm. For *Glugea*, maturing xenomas exhibit a light refractile wall, and peripheral host cell cytoplasm with spores in the center. A fully developed xenoma is filled with spores and few host cell components (Fig. 13). The host's reaction to xenoma formation varies depending on the stage of xenoma development, the tissue infected, and the age and species of host. However, some generalizations can be

made. The stages of host reaction, described below, were originally developed by Dyková & Lom (1978) for *G. anomala, G. aculeatus*, *G. hertgiwi*, *G. plecoglossi*, and *G. stephani*. Further study and clarification was made by Dyková & Lom (1980), and Canning & Lom (1986).

Weakly Reactive Stage.

Early or very young xenomas begin to exert pressure atrophy on the surrounding tissue as they grow. The host responds by local proliferation of connective tissue and collagen fibers, forming a concentric layer of connective tissue around the xenoma. The weakly reactive stage is also seen with fully developed xenomas.

Productive Stage.

Mature xenomas elicit prominent inflammation (Fig. 14). Inflammation includes influx of fibroblasts and macrophages. Proliferative inflammation accompanies changes in the xenoma wall, although it is not clear if this is a causal relationship (Dyková et al. 1980; Canning & Lom 1986). The wall of the xenoma swells, fibroblasts appear within it, and the wall ruptures and begins to disappear. In addition to fibroblasts, the formation of a capsule composed of eosinophilic granular cells around the xenoma was reported by Reimschuessel et al. (1987). *Ichthyosporidium* spp. elicit a slightly different inflammatory response characterized by epithelioid cells and histiocytes. Epithelioid cells encasing the xenoma are arranged with their long axis perpendicular to the xenoma wall. Destruction or rupture of the xenoma wall is accompanied by dystrophic changes that occur with the appearance of periodic acid-Schiff positive substances and Ca^{2+} within the mass of spores. The xenoma is then replaced by granulomatous tissue (Fig. 15).

Granuloma Involution Stage.

After the xenoma ruptures, spores are released and ingested by macrophages.

The granulation tissue matures and the granuloma gradually recedes. Fibrous connective tissue that has developed during the productive stage undergoes hyalinization, and the lesion is slowly resolved. Although the xenoma can be completely eliminated, functional restitution of heavily infected organs is not possible (Dyková et al. 1980).

Glugea spp. can cripple, disfigure, debilitate, and kill fishes (Sindermann, 1990; Dyková 1995). These microsporidia can also contribute to decreased fecundity and retarded growth (Chen & Power 1972; McVicar 1975). The large cysts of *G. anomala* create serious tissue injury due to pressure atrophy, although sticklebacks appear to survive even heavy infections (Canning & Lom 1986). Host response follows the stages described in this section under "pathology". *Glugea plecoglossi* has low organ specificity, although development and host tissue reactions are similar to those of *G. anomala* (Dyková et al. 1980). The prevalence of *G. hertwigi* in smelt can reach 100% in some lakes, and epizootics with intensities of 250 xenomas $fish^{-1}$ have occurred (Anenkova-Khlopina, 1920; Haley 1952, 1953, 1954; Bogdanova 1957; Petrushevski & Shulman 1958; Delisle 1965; Nepszy & Dechtiar 1972; Canning & Lom 1986). Delisle (1972) estimated that in Lake Erie alone, 10 million fish y^{-1} were lost due to *G. hertwigi* infections. The intestinal epithelium can disintegrate, resulting in general septicaemia, intoxication, and death (Canning & Lom 1986). The fecundity of smelt can also be severely affected by the parasite (Sindermann 1963). Infections by *G. stephani* occlude the intestinal lumen, disrupt its integrity, and contribute to emaciation or even death of the host (Cali et al. 1986). Xenomas replace the intestinal wall, forming a rigid layer up to 4 mm thick which has a chalk-white pebbled appearance (Fig. 11). Fish can die, even from low level infections, if xenomas form in the mucosa and rupture into the epithelial lining. In contrast, Cali et al. (1986) also showed that heavily infected fish may survive if

xenomas form in the serosal side of the intestinal tract. Olson (1976) reported collecting emaciated starry flounder *Platichthys stellatus* Pallas from Yaquina Bay, Oregon, infected with *G. stephani*. Cali et al. (1986) also observed *G. stephani* in moribund winter flounder of Sandy Hook Bay, New Jersey. Heavy infections may be fatal in plaice *Pleuronectes platessa* L. held in aquariums or at fish farms (Bückmann 1952; McVicar 1975). McVicar (1975) suggested *G. stephani* may be highly contagious at a farm site.

Transmission. *Glugea* spp., like most fish microsporidia, are transmitted directly via ingestion. These spp. have been transmitted by intraperitoneal injection (IP) or via a crustacean paratenic host (e.g. transport hosts such as *Daphnia* spp., brine shrimp, amphipods) (Weissenberg 1921, 1968; McVicar 1975; Olson 1976; Kim et al. 1996). Predation may disperse spores of *G. hertwigi*, or cannibalism may lead to infections in other smelt (Haley 1954; Delisle 1972). Olson (1976, 1981) found that spores of *G. stephani* pass directly through the crustacean digestive tract, and that infections heavier than IP injection result when a crustacean paratenic host was used. He suggested that amphipods may represent a natural route of transmission for *G. stephani*.

The target host cell of *Glugea* spp. can vary. *Glugea anomala* may target a migratory mesenchyme cell, such as a macrophage or histocyte (Weissenberg 1968), whereas *G. stephani* may infect neutrophils (Canning & Lom 1986). The formation of xenomas free within the body cavity by IP injection suggested to McVicar (1975) that *G. stephani* infected macrophages.

Seasonal and geographic variations in prevalence of *G. stephani* have been related to water temperature (Olson 1976, 1981; Takvorian & Cali 1981, 1984). Olson (1981) found that development of the parasite was arrested at 10°C and development resumed when water temperatures were raised to 15°C or higher.

LOMA

Loma is another important xenoma-forming genus of microsporidia. Xenomas of *Loma* spp., unlike those of *Glugea*, develop asynchronously with various developmental stages located throughout the xenoma. There are 9 described species of *Loma*, and this genus includes some species noted for infecting fishes of commercial importance (i.e., cod and salmon). *Loma branchialis* Nemeczek, 1911 (syn. *Nosema branchialis* Nemeczek, 1911, *Glugea branchialis* Nemeczek, 1911, and *L. morhua* Morrison & Sprague, 1981) have been described from several members of the family Gadidae: *Gadus aeglefinus* L., *G. callarias* L., *G. morhua marisalbi* L., *G. morhua kildinensis* L., *G. morhua* L., haddock *Melanogrammus aeglefinus* L., and rockling *Enchelyopus cimbrius* L. (Bazikalova 1932; Dogiel 1936; Fantham et al. 1941; Shulman & Shulman-Al-bova 1953; Morrison & Sprague 1981a,b; Morrison & Marryatt 1986). *Loma camerounensis* Fomena, Coste & Bouix, 1992 infects tilapia *Oreochromis niloticus* L. which are widely cultured in West Africa (Fomena et al. 1992). Morrison & Sprague (1981c; 1983) described *Loma fontinalis* Morrison & Sprague, 1983 from brook trout *Salvelinus fontinalus* Mitchill in Canada. *Loma salmonae* is well known as a serious pathogen in farmed salmonids (*Oncorhynchus* spp.) in the Pacific Northwest (Kent et al. 1989; Kent 1992; Shaw et al. 1997). This pathogen may have been introduced to Europe with rainbow trout and coho *Oncorhynchus kisutch* Walbaum transported from Californian stocks (Poynton 1986). Descriptions of *Loma* spp. from salmonids such as chinook, coho, and rainbow trout (Awakura et al. 1982; Hauck 1984, Mora 1988; Speare et al. 1989; Magor 1987; Gandhi et al. 1995) are likely *L. salmonae* based on host, parasite morphology, and site of infection.

All of these species except *L. camerounensis* infect endothelial cells causing formation of xenomas throughout vascularized organs (eg. heart, kidney, spleen, liver)

but primarily in the gills (Fig. 5). *Loma camerouensis*, forms xenomas in connective tissue of the gut submucosa.

Species descriptions for *Loma* have been problematic. Most earlier researchers designated new parasite species based on a new host, geographic location, and slight differences in spore morphology. Morrison & Sprague (1981a,b,c; 1983) described *L. morhua* and *L. fontinalis* recognizing that these species may be conspecific to *L. branchialis* and *L. salmonae*, respectively (Canning & Lom 1986). Analysis of the rDNA of *Loma* spp. and transmission studies, are beginning to clarify the taxonomy of this genus (Docker et al. 1997a; Shaw et al. 1997). Shaw et al. (1997) described *L. embiotocia* Shaw, Kent, Docker, Brown, Devlin and Adamson, 1997 in shiner perch *Cymatogaster aggregata* Gibbons with all these techniques. They found that the internal transcribed spacer (ITS) region of *L. embiotocia* differed significantly from the ITS region of *L. salmonae* but not within isolates of each species. Furthermore, they were unable to transmit *L. salmonae* to shiner perch and thus assigned the shiner perch parasite as a new species, *L. embiotocia*. I have been unable to transmit *L. embiotocia* to chinook, or *Loma* spp. isolated from Pacific cod *Gadus macrocephalus* Tilesius, or ling cod *Ophiodon elongatus* Girard to chinook. This suggests that *Loma* spp. being found in marine hosts (Kent et al. 1998) may be distinct species, or are all at least distinct from *L. salmonae*.

Wales & Wolf (1955) found 75% of wild yearling rainbow trout were infected with *L. salmonae* in California. I have observed *L. salmonae* to be widespread in wild Pacific salmon populations in both fresh and marine habitats in British Columbia, Canada (see Chapter VI). Epizootics of *L. salmonae* have been recorded in Japan, the Pacific Northwest, the eastern United States, and Europe (Wales & Wolf 1955; Hauck 1984; Bekhti & Bouix 1985; Canning & Lom 1986; Kent et al. 1989; Bruno et al. 1995). Wales

& Wolf (1955) first described transport and grow-out of farmed fish being more difficult because of *L. salmonae*. Since that time the parasite has continued to cause large economic losses to chinook farms in British Columbia. Recently, high mortality in wild spawning sockeye of the Babine Lake system, British Columbia occurred in association with heavy *L. salmonae* infections (Higgins MJ pers. comm.).

I have found that *L. salmonae* infects all 7 species of *Oncorhynchus*, and brook trout (see Chapter IV). Speare et al. (1998a) were unable to infect brook trout, although their fish may have developed resistance from a previous exposure to *L. salmonae* or *L. fontinalis* (DJ Speare pers. comm.). Atlantic salmon, Arctic char *Salvelinus alpinus* L. herring *Clupea pallasi* Valenciennes, prickly sculpin *Cottus asper* Richardson, three-spined sticklebacks, and guppy *Poecilia reticulata* are resistant to experimental infection by *L. salmonae* (Kent et al. 1995a; Chapter IV). *Loma salmonae*, therefore, displays host specificity with the main hosts being species of the genus *Oncorhynchus*.

Pathology. Cod infected with *L. branchialis* exhibit no obvious clinical signs (Morrison & Sprague 1981a). However, xenomas in the gills reach 1.2 mm in size, resulting in distortion and displacement of gill tissue and blood vessels (Kabata 1959; Morrison & Sprague 1981a). Pathological changes caused by these infections are similar to those described for *G. anomala*, except that Morrison (1983) found that spores remained in the center of granulomas with both phagocytes and fibroblasts undergoing coagulative necrosis. Impact on host mortality is unknown, although infections may affect metabolism negatively through decreased respiratory efficiency (Morrison & Sprague 1981a).

Gross clinical signs of *L. salmonae* infection may include: darkening of the tail or body, lethargy, gill pallor, petechial haemorrhaging in the gills, skin and fins, ascites, hemorrhagic pyloric caeca; and white cysts (xenomas) on the gills (Hauck 1984; Kent

1992; Markey et al. 1994; Bruno et al. 1995). Reduced growth, impaired swimming efficiency, and increased mortality of young chinook have also been noted (Hauck 1984). The growth rate in rainbow trout appears to be reduced during xenoma formation (DJ Speare pers. comm.). Hauck (1984) provides a comprehensive pathological description of a *Loma salmonae* systemic infection, that includes necrosis of cartilage and musculature, occlusion of arteries, pericarditis of the bulbus arteriosus, and hyperplasia of gill and heart tissues. The effects of occlusion are seen when xenomas extend through the tunicae media and adventitia of arteries. Xenomas and/or free spores in the heart can result in mural emboli, hyperplasia of ventricular and atrial tissue, and an oligocythaemic condition. Gill xenomas can cause subacute to chronic vasculitis, perivasculitis, vascular thrombosis, and hyperplasia of gill tissue (Kent et al. 1989; Speare et al. 1989). As with other xenoma-forming species, the inflammatory response and associated tissue damage is more severe after xenomas have ruptured (Kent et al. 1989).

Fomena et al. (1992) did not describe pathology of *L. camerounensis* except to note a high prevalence (94%) of the parasite and that large xenomas could protrude into the intestinal cavity. Negative impacts on fish health were also not described by Morrison & Sprague (1981c, 1983) for *L. fontinalis*. It is likely that the pathology is similar to that of *L. salmonae*.

Transmission. Spores of *Loma* spp. released from xenomas can be infectious when ingested. Spores may be liberated directly from the gills and urine into the external environment (Hauck 1984). Autoinfection may occur by spores released from ruptured xenomas being transported to other tissues. Morrison (1983) proposed this phenomenon for *L. branchialis.* Shaw et al. (1998) substantiated this for *L. salmonae* by experimentally inducing infection by injecting purified spores intravascularly. Hauck

(1984) suggested that infection may occur by direct phagocytic uptake of spores by the gills. However, placement of infectious spores directly on gills of chinook did not result in infection (Shaw et al. 1998). Salmon may also be infected experimentally by anal gavage, *per os*, intravascular (IV), intramuscular (IM), and IP injection (see Chapter II). Poynton (1986) and Kent et al. (1995a) suggested that *L. salmonae* is transmitted from fish to fish within a freshwater or marine netpen. Fish have been infected by cohabitation with carrier fish in a flow through system, suggesting that direct fish-to-fish transmission occurs within a net pen (Shaw et al. 1998). The infection is prevalent in wild ocean-caught salmonids (Kent et al. 1998), which may be a source of infection for marine fish farms. However, infections are probably maintained and perpetuated at these farms by stocking of parasite-free smolts at the same sites with older, infected fish. Chinook can remain infected for extended periods of time (i.e. > 80 d at 10°C) and purified spores of *L. salmonae* are viable for 85 d in both fresh and sea water (see Chapter III). Epizootics of *L. salmonae* have occurred at freshwater hatcheries (Hauck 1984; Magor 1987) indicating smolts may also be infected before they are transferred to seawater farms.

Development of immunity to *L. salmonae* occurs in rainbow trout. Speare et al. (1998b) found that fish resolved experimental infections by 10 wk after initial exposure and were resistant to experimental reinfection. My preliminary observations suggest that the same phenomenon can occur with chinook salmon.

The structure and development of xenomas formed by *Loma* spp. have been studied extensively. However, very little is known about the route the parasite takes after entering the gut of the host (see Chapter II). It is likely that sporoplasms from extruded spores are injected into epithelial cells of the alimentary canal. Shaw et al. (1998) observed intracellular structures, possible sporogonic stages, progressing

though intestinal epithelial cells and into the lamina propria. Markey et al. (1994) described unidentified intracellular structures preceeding xenoma formation in all infected tissues. These structures may have represented earlier stages of the parasite. Work is currently underway using *in-situ* hybridization with the rDNA probe (Docker et al. 1997a) to clarify the route of early *L. salmonae* infections (DJ Speare pers. comm.).

TETRAMICRA

Tetramicra brevifilum, another xenoma forming microsporidian, has been noted to cause significant disease in cultured and wild fish. The host fish, turbot, is becoming more widely cultured in countries such as Spain, and the parasite represents a significant threat to turbot culture (Figueras et al. 1992). *Tetramicra brevifilum* infects connective tissue of the skeletal musculature (Fig. 16), and in heavy infections xenomas can be found in the intestine, kidney, liver and spleen (Estévez et al. 1992; Figueras et al. 1992). Matthews & Matthews (1980) first described this parasite in wild turbot collected off the north coast of Cornwall, United Kingdom. Figueras et al. (1992) next isolated the parasite in turbot cultured in Galicia, Spain during 1990. Recently Maillo et al. (1998) found *T. brevifilum* in a new host, *Lophius budegassa* Spinola in the Northwest Mediterranean sea. Xenomas lack the multilaminate layer characterizing *Glugea* spp. However, xenomas of *T. brevifilum* may reach 1.5 mm in diameter with additional adhesions between xenomas creating composite cysts (Matthews & Matthews 1980; Dyková & Figueras 1994). Spores of this species contain a conspicuous inclusion in the sporoplasm and posterior vacuole, which is unique among fish-infecting microsporidia (Lom & Dyková 1992).

Pathology. Turbot with heavy infections of *T. brevifilum* exhibit erratic swimming behavior, general tissue swelling, dorsal darkening, overproduction of mucus, muscle

liquefaction, visible cysts, and chronic low mortality. A 50% reduction in growth rate and a jelly-like consistency of muscle in infected stocks contribute to decreased marketability (Figueras et al. 1992). The impairment of swimming ability could contribute to decreased feeding rates in cultured stocks and increased predation and starvation in wild stocks (Matthews & Matthews 1980).

The presence of such large xenomas creates pressure atrophy consistent with trauma in the perimysium (connective tissue of muscle fibers), leading to displacement of muscle fibers and loss of fiber attachment to myocommata or myomeres (Matthews & Matthews 1980). Canalization in connective tissue of the spinal column, and localized haemorrhaging, fibrosis, and necrosis of infected tissues occur with this infection (Matthews & Matthews 1980; Estévez et al. 1992). Rupture of xenomas results in general cellular infiltration and collagen deposition, and spores are phagocytized by macrophages and subsequently destroyed. Myodegeneration by vacuolization of the sarcoplasm and separation of myofibrils can create liquefaction of musculature. As in *Pleistophora*-type infections, this damage is thought to be caused by substances secreted by the parasite (Dyková & Lom 1980). Estévez et al. (1992) noted that degeneration of the muscularis mucosa occurred but to a lesser extent than in skeletal muscles. Fish that survive infection may recover fully (Estévez et al. 1992). Figueras et al. (1992) and Leiro et al. (1993) investigated the effects of *T. brevifilum* on the immune status of fish (see "Immunology" previously).

Transmission. Matthews & Matthews (1980) were able to transmit *T. brevifilum* by IM injection, but not by *per os*. No cross transmission to controls was observed by these researchers during the 7 wk fish were held at 15°C. They suggested that phagocytes may be involved in the life cycle of *T. brevifilum* by acting as a transport mechanism within the host, which could transport the parasite through the endothelium, or act as

sites for development themselves. Phagocytes might become directly infected by a sporoplasm or during phagocytosis of meronts in the lamina propria of the intestine. Estévez et al. (1992) observed free spores within the lamina propria of the intestine.

Figueras et al. (1992) were unable to infect fish by IP or by waterborne exposure. No microsporidia were found in the food fed to cultured turbot, leading them to conclude the fish were infected by aquatic crustaceans such as copepods, decapod larva, or mysids. In their study, infection corresponded with a drop in temperature, possibly reflecting stress induced in the fish. The death of the host and the release of spores directly into sea water and/or cannibalism of moribund fish may be important in transmission of *T. brevifilum*. Frequent mortality in the juvenile population could ensure transmission of the parasite to other fish (Matthews & Matthews 1980).

Non Xenoma-Forming Genera

PLEISTOPHORA

Pleistophora, like *Glugea*, causes disease in a wide variety of fish species. These parasites usually invade skeletal muscles, replacing the sarcoplasm and destroying the cells. In general, they are associated with musculature destruction or liquefaction, deformity, and production of tumour-like masses in fish (Pulsford & Matthews 1991). Some species, such as *P. mirandellae* and *P. ovariae*, invade and destroy oocytes. *Pleistophora* spp. are characterised by diffuse infections, with only a few species such as *P. senegalensis* Faye, Touguebaye & Bouix, 1990, and *P. hippoglossoideos* Bosanquet, 1910 forming xenomas (Morrison et al. 1984; Faye et al. 1990). More than 30 species of *Pleistophora* have been described. Of these, 8 infect fishes of economic importance and include: *P. ehrenbaumi* Reichenow, 1929 in wolffish *Anarhichas* spp.; *P. finisterrensis* Leiro, Ortega, Iglesias, Estévez & Sanmartin, 1996 in blue whiting *Micromesistius*

poutassou Risso; *P. hippoglossoideos* in plaice *Hippoglossoides platessoides* Fabricius; *P. hyphessobryconis* in ornamental fishes; *P. macrozoarcides* in ocean pout; *P. mirandellae* in European cyprinids; *P. ovariae* in bait fishes (family Cyprinidae); and *P. senegalensis* in sea bream. *Heterosporis anguillarum* (formerly *P. anguillarum*), is discussed with other species of *Heterosporis*.

Muscle fibers of wolffish *A. lupus* and *A. minor* are infected by *P. ehrenbaumi* and up to 10% of wolffish in the waters of Iceland may be infected (Meyer 1952). The parasite creates tumour-like swellings up to 8 x 15 x 4 cm, which can make the commercial catches unfit for consumption (Egidius & Soleim 1986). Wolffish are a promising species for aquaculture (Wiseman & Brown 1996), and thus this parasite may ultimately become a problem in the rearing of these fish.

Blue whiting collected off the coast of northwest Spain have been infected by *P. finisterrensis.* Leiro et al. (1996c) found 5% of fish harbored infections in the hypoaxial musculature, in which infective 3 to 6 mm foci were located. They did not associate any significant morbidity with the infection.

The type host for *P. hippoglossoideos* is *H. platessoides* (syn. *H. limandoides*, *Drepanopsetta hippoglossoides*) (Bosanquet 1910; Kabata 1959; Canning & Lom 1986). Canning & Lom (1986) and Lom & Dyková (1992) also included sole *Solea solea* Quensel as a host. Muscles of the fins, walls of the visceral cavity, and somatic musculature of fish may be infected (Fig. 4). Cystlike structures are visible externally and can be up to 2.5 x 10 mm, in some cases, making the fish unfit for eating (Canning & Lom 1986; Dyková 1995). Morrison et al. (1984) provide a redescription of this species.

Originally imported from wild fishes of the upper Amazon river, *P. hyphessobryconis* is now distributed world wide in many families of freshwater tropical fishes, infecting over 16 species (Lom & Dyková 1992; Dyková 1995). It invades skeletal

muscle (Fig. 1) and heavy infections can include connective tissue of the ovaries, intestinal epithelium, skin, and renal tubules. Large cysts (2 mm) can form, and spores can concentrate in the subcutaneous tissue and skin (Canning & Lom 1986). Heavy losses occur in culture ponds of ornamental species infected by *P. hyphessobryconis*, with few successful treatments available (see section 1.3).

Skeletal muscles of ocean pout infected with *P. macrozoarcides* can contain tumourlike masses up to 8 cm in size that exude a pus-like fluid when cut (Canning & Lom 1986). These pseudocysts increase with age and size of fish and have been blamed for the 1940's collapse of the ocean pout fishery (Fischthal 1944; Sandholzer et al. 1945) as a result of difficulties in marketing fish infected with *P. macrozoarcides* to consumers (Sheehy et al. 1974).

Pleistophora mirandellae (syn. *P. longifilis* Schuberg, 1910, *P. oolytica* Weiser, 1949) infects oocytes of common European cyprinids such as bleak *Alburnus alburnus* L., barbel *Barbus barbus* L., and roach *Rutilus rutilus* (Dyková 1995). It has been found also in common pike *Esox lucius* L. (Maurand et al. 1988). Macroscopic white lesions appear in the ovary, and an infection of 10 to 20% of follicles can significantly decrease fecundity of fish (Lom & Dyková 1992). Wilkund et al. (1996) reported that roach in the Archipelago Sea (Finland) infected with *P. mirandellae* likely experienced decreased fecundity as a result of ovary degeneration and destruction.

Golden shiner and fathead minnow *Pimephales promelas* Rafinesque are the main hosts of *P. ovariae* (Fig. 17). Summerfelt & Warner (1970) found an overall prevalence of 48% in shiner from U.S. bait-minnow hatcheries they surveyed. The lowest prevalence was found in young fish, with infection rate increasing up to 79% for older fish. The prevalence then dropped off, possibly as a result of selective mortality. Although this parasite was widespread in the golden shiner, hatcheries were able to obtain eggs from

young fish not yet damaged by *P. ovariae* (Nagel & Hoffman 1977). The parasite may cause a 40% decrease in fecundity (Summerfelt 1964).

Sea bream is an important species in markets of Senegal. Faye et al. (1990) described *P. senegalensis* from the muscularis of this host's intestinal wall, but no significant pathological changes were associated with infection.

Pathology. In non-xenoma-forming genera such as *Pleistophora*, infected cell contents are replaced without inducing prominent cell hypertrophy. These microsporidia infect muscle cells or oocytes, and the host reaction is often minimal. Minor inflammatory cell infiltration into the myosepta can take place as the parasite develops. Fusion of infected muscle fibers can also occur with some parasite species (eg. *Pleistophora ehrenbaumi* and *P. macrozoarcidis*). Eventually all the muscle cell contents are replaced and the cell destroyed, liberating mature spores. The host then responds with an influx of macrophages that phagocytize and digest the spores, but tissue regeneration is limited (Dyková & Lom 1980; Pulsford & Matthews 1991). Invaded muscle fibers are often not isolated by granuloma formation, as is the case in the xenoma-forming species. However, granulomas can develop as a result of heavy infections by certain species (eg. *P. macrozoarcidis, P. hyphessobryconis*). *Pleistophora hyphessobryconis* is unique in that it forms discrete islets of degenerated sarcoplasm in the muscle that directly abuts intact myofibrils. These islets result from substances secreted by the parasite (Dyková & Lom 1980).

Pathologic changes have not been described for *P. ehrenbaumi* infections. However, fish can become extremely emaciated, suggesting that such infections can be fatal (Egidius & Soleim 1986; Lom & Dyková 1992). Mortality due to *P. hippoglossoideos* in smaller individuals has been implied, but not confirmed (Morrison et al. 1984). Large cysts of *P. hippoglossoideos* can cause compression and distortion of tissues, but no great

damage results (Kabata 1959; Canning & Lom 1986). Morrison et al. (1984) observed influx of phagocytes and engulfment of spores in infected muscle, and some nodules were also encapsulated with fibrous tissue.

Fishes infected with *P. hyphessobryconis* show anomalous behaviour and movement, fading of color, and appearance of greyish-white patches in the muscle. Emaciation, scoliosis, kyphosis, and bristled scales are also characteristic of the infection (Canning & Lom 1986). Granulomas can form in the mesenteries or viscera with an envelope of connective tissue cells. Canning & Lom (1986) found connective tissue encapsulation was better at limiting spread of the parasite than phagocytosis by macrophages. Heavy infections can cause high mortality within 14 d (Thieme 1954, cited in Canning & Lom 1986). Destruction of the host muscle begins with a unique halo formation around the parasite, which consists of disrupted smooth endoplasmic reticulum, free ribosomes, and myofibrils (Canning & Lom 1986; Dyková 1995). Infections can lead to atrophy of testes (parasitic castration) and liver, although these organs do not have to be heavily infected (Lom & Dyková 1992).

Canning & Lom (1986) describe the pathology of *P. macrozoarcides* infections. The parasite can form small, whitish cylinders in muscle fibers during early stages of infection, but large tumour-like masses result when the host encapsulates several infected fibers with concentrically arranged connective tissue. The center of these "pseudocysts" contains free spores. The muscle turns brownish, and muscle fibers are hyalinized and destroyed. The host response follows that described earlier for other non-xenoma forming species.

Both *P. mirandellae* and *P. ovariae* infect oocytes of fishes, although the pathogenesis of these infections differs. *Pleistophora mirandellae* infects before the *zona radiata* forms, developing in the yolk and replacing it with spores. A significant proliferative granulomatous inflammatory response can occur, with destruction of infected oocytes and

spores (Canning & Lom 1986; Dyková 1995). Infected connective tissue of the seminiferous tubules can also create hypertrophy of local epithelial cells leading to decreased fecundity (Schuberg 1910). In contrast, inflammation and encapsulation of infected oocytes is not associated with *P. ovariae* infection. The inflammatory reaction has not been well described for this species, although Summerfelt & Warner (1970) provide a brief description of its pathogenesis. Infected ova become mottled with white spots and streaks, each representing a mass of ovarian stroma and spores. Atresia of heavily infected ova occurs, followed by spores coalescing in a stroma of *zona radiata* and yolk. Hyperplasia of the follicular epithelium occurs, and then a collapse of the *zona radiata*, and an influx of phagocytes that destroy the oocyte. Atretic follicles of the ovary are invaded by fibroblasts and some fibrosis can occur. Infected ova develop a stroma of connective tissue and increase in size, as reflected by postspawning fish having heavier ovaries compared to healthy fish. Parasitic castration can be pronounced, reducing fecundity by 37% or more (Canning & Lom 1986). Curiously, infected fish are often larger than healthy spawning fish, because of reduced commitment of nutrients to egg production (Summerfelt & Warner 1970).

Transmission. As with many microsporidia, death of the host may liberate free spores of *Pleistophora* spp. to infect the next host. Spores may also be released with urine, or directly from the skin, of fishes infected with *P. hyphessobryconis* (Canning & Lom 1986). Tetras and goldfish have been infected *per os*, and IM injections are helpful in maintaining *P. hyphessobryconis* in the laboratory (Lom 1969; Canning & Lom 1986). Autoinfection, in which spores produced within the host hatch and form secondary infections, has been proposed for *P. hyphessobryconis*. Nigrelli (1946) proposed that infection of large areas of the host by *P. macrozoarcides* could be explained by autoinfection. It is likely that autoinfection occurs in some *Pleistophora* spp., but this has yet to be confirmed.

Summerfelt (1972) infected golden shiners with *P. ovariae per os*, and suggested transovarial (vertical) transmission after finding the microsporidian in 5% of 38 blastulas he examined. Canning & Lom (1986) did not consider this observation definitive evidence for transovarial transmission.

Interestingly, Leiro et al (1994) infected turbot *per os* with *P. finisterrensis* from blue whiting. Outbreaks of *P. finisterrensis* may occur in turbot culture in the future as raw blue whiting is often used as feed.

HETEROSPORIS

There are 3 species infecting fishes: *Heterosporis finki* Schubert, 1969, *H. schuberti* Lom, Dykovà, Körting & Flinger, 1989, and *H. anguillarum*. The first two infect ornamental fishes, whereas the latter is an important pathogen in eel culture.

Heterosporis finki infects the musculature of angel fish *Pterophyllum scalare* (Schubert 1969; Michel et al. 1989), a popular aquarium fish. *Heterosporis schuberti* has been described from musculature of *Pseudocrenilabrus multicolor* (family Cichlidae) and *Ancistrus cirrhosus* Eigenmann (family Loricariidae) (Lom et al. 1989). Both species are characterized by formation of a sporophorocyst which is a thick, dense envelope of parasitic origin inside the sarcoplasm (Fig. 18) (Lom et al. 1993). The sporophorocyst contains only parasite stages, unlike a xenoma, which contains host cell cytoplasm and nuclei. *Heterosporis anguillarum* development resembles that of *Pleistophora* spp. (see species under this genus).

Pathology. Michel et al. (1989) noted that few fish exhibited clinical signs during an outbreak of *H. finki* at an angel fish farm in France. However, some fish were emaciated, and exhibited lesions up to 5 mm diameter. Liquefaction of infected striated muscle was also seen, although internal organs appeared normal. Lom et al. (1989)

described signs of distress, emaciation, and up to 95% mortality of fish infected with *H. schuberti*. Sporophorocysts provoked only moderate cellular infiltration. Rupture of these sporophorocysts however, resulted in the formation of aggregates of macrophages containing phagocytized spores in the mesenteries, intestine, and kidney. A more severe reaction in *B. splendens* infected by a *Heterosporis* sp. was described by Lom et al. (1993). In these fish granulomatous myositis was characterized by a central area of lesions containing spores. Recovery of muscle function was deemed impossible.

The growth of Japanese eels can be retarded and market value decreased by *H. anguillarum* infection. Eels mount an extensive inflammatory response when cysts rupture followed by a fibroblastic response. Deformities and muscle liquefaction can result from such infections (T'sui & Wang 1988).

Transmission. Little information is available on transmission of *Heterosporis* spp. Michel et al. (1989) hypothesized that clinically normal adult angel fish transferred *H. finki* to juveniles or that the infection was introduced with a food source. Infection in eels is possible by oral administration of *H. anguillarum* spores (Kano & Fukui 1982), or by water-borne exposure (T'sui et al. 1988).

MICROSPORIDIUM

The collective group *Microsporidium* contains many species that infect fishes, some of which may not even belong to the phylum Microsporidia. For example, *Microsporidium pseudotumefaciens* Pflugfelder, 1952, infects common freshwater aquarium fishes in the genera *Brachydanio, Colisa, Lebistes, Gambusia, Platypoecilus, Molliensia,* and *Xiphophorus*. It was thought to cause considerable mortality but has been reported only once (Pflugfelder 1952, cited in Canning & Lom 1986). Canning & Lom (1986) transferred this parasite to the group *Microsporidium* but pointed out that its

life cycle did not fit that of a microsporidian.

Both Beko disease in yellowtail *Seriola quinqueradiata* Temminck & Schlegal caused by *M. seriolae* Egusa, 1982 (syn. *Pleistophora* sp. Ghittino, 1974), and *M. takedai* Awakura, 1974 in freshwater salmonids infect muscle and can be pathogenic in commercially important fishes (Awakura 1974; Egusa 1982). *Microsporidium takedai* has been recorded from various salmonid hosts and geographic locations in Japan (Takeda 1933; Awakura et al. 1966; Kubota 1967; Funahashi et al. 1973; Awakura 1974,1978; Vialova 1984). Hosts for *M. takedai* include: chinook, chum *Oncorhynchus keta* Walbaum, pink *O. gorbuscha* Walbaum, kokanee *O. nerka* Walbaum, and masu *O. masou* Brevoort salmon, white-spotted char *Salvelinus leuconaenis* Pallas, brown trout *Salmo trutta* L., dolly varden *S. malma* Walbaum, and rainbow trout. Kokanee salmon, and rainbow trout are particularly susceptible to infection (Urawa & Awakura 1994). Urawa (1989) found that hatchery-reared masu salmon smolt were very susceptible to *M. takedai* infection when released into rivers. Susceptibility is age dependent, with young fish being highly susceptible (Awakura 1974). The prevalence of *M. takedai* is also seasonal, peaking during the months of highest water temperature. Urawa (1989) found prevalence ranged from 80 to 100% in wild and hatchery-reared salmon from 1982 to 1984.

Pathology. Beko disease is characterized by cyst formation in the muscle. Small and large depressions on the lateral surface of yellowtail can indicate the presence of *M. seriolae* cysts (Canning & Lom 1986). Spores can be found within cysts bounded by a fibrous host membrane (Egusa 1982). The parasite causes muscle liquefaction, which undoubtedly contributes to lower marketability of infected yellowtail.

Microsporidium takedai also forms cysts, which are located in the heart during chronic infections, and the trunk, fin, jaw, eye, throat, and gullet muscles during acute

infections (Figs. 19 & 20) (Awakura 1974). Cysts of *M. takedai* are spindle shaped (2 to 3 mm wide, 3 to 6 mm long) and lack a xenoma wall (Awakura 1974; Urawa & Awakura 1994). Both acute and chronic infections, with associated mortality, have been recorded from wild and hatchery-reared fishes. Chronic infections are characterized by cyst formation causing extreme hyptertophy, multiplication of connective tissue, vacuolization, and deformation of the heart (Fig. 19) (Urawa 1989; Urawa & Awakura 1994). Acute infections include cyst formation in general skeletal musculature that can result in considerable necrosis. Acute infections have also been associated with extremely high mortality in hatchery fishes (Awakura 1965; Urawa & Awakura 1994). Urawa (1989) observed a strong correlation between the condition of fish and the intensity of *M. takedai* infections. Hosts mount a typical inflammatory response to the parasite, and formations of granulomas are observed (Awakura 1974).

Transmission. Little information is available on transmission of *M. seriolae*. Awakura (1974) transmitted *M. takedai* by *per os*, and by water-borne infection and suggested that paratenic hosts such as rotifers *Euchlanis* spp., and glochidia larvae of mussels *Margaritifera* spp. may play a role in transmission. However, Canning & Lom (1986) noted the organisms he described in potential paratenic hosts were not microsporidia. Temperature plays a significant role in transmission and development of *M. takedai*. Development may be arrested at 8°C and retarded between 11 and 15°C (Awakura 1974). A successful control strategy has been to rear smolts below 15°C (Urawa & Awakura 1994), and fish that have recovered from infection can develop immunity for up to 1 y (Awakura & Kurahashi 1967).

NUCLEOSPORA

Nucleospora salmonis is a remarkable microsporidium that causes disease in

salmonid fishes. It infects the nuclei of hemoblasts, particularly lymphoblasts or plasmablasts. Infected cells exhibit massive proliferation and are immature, suggestive of neoplasia. This microsporidium was first observed in pen-reared chinook in Washington State, where it was associated with anemia (Elston et al. 1987). The parasite has also been reported in freshwater-reared chinook, kokanee, and steelhead trout (Morrison et al. 1990; Hedrick et al. 1990; 1991b). The infection is common in caged-reared chinook salmon in British Columbia (Kent 1998), and in Atlantic salmon in Chile (Bravo 1996). Intranuclear microsporidia very similar to *N. salmonis* have been observed in Atlantic lumpfish *Cyclopterus lumpus* L. (Mullins et al. 1994), and Atlantic halibut *Hippoglossus hippoglossus* L. (Nilsen et al. 1995), and these organisms were tentatively assigned to the genus *Enterocytozoon.*

The intranuclear microsporidian of salmonids was originally described as *N. salmonis* by Hedrick et al. (1991a), but was described shortly thereafter as *Enterocytozoon salmonis* by Chilmonczyk et al. (1991). Ribosomal DNA sequence comparison of *N. salmonis* and *E. bieneusi* Desportes, Charpentier, Galian, Bernard, Cochand-Priolett, Lavergne, Ravisse, Modigliani, 1985 does not provide compelling evidence to suppress the nominal genus *Nucleospora.* Although intrageneric sequence comparisons among microsporidia are limited, *N. salmonis* and *E. bieneusi* (at 20.1% genetic divergence in the 16S and 28S genes) show greater differences than congeneric species examined to date (Docker et al. 1997b).

Differences in host and site of infection also support the separation of *Nucleospora* from *Enterocytozoon. Nucleospora* develops within the nuclei of fishes, whereas *Enterocytozoon* infects the cytoplasm of enterocytes in humans. *Nucleospora* spp. from fishes differ from *E. bieneusi* in that they possess spores with 8 to 12 turns in the polar filament, whereas those of *E. bieneusi* have only 5 or 6. On the basis of these

distinctions and the morphological characteristics available to date, the intranuclear microsporidian found in both Atlantic lumpfish and Atlantic halibut should also be transferred to the genus *Nucleospora*.

Despite the reestablishment of *Nucleospora* and *Enterocytozoon* as separate genera, they are more closely related to each other than they are to other microsporidian genera. Docker et al. (1997b), therefore, proposed that *Nucleospora* be retained in the family Enterocytozooidae. *Nucleospora salmonis* exhibits many of the distinctive characteristics of the family Enterocytozooidae (Cali & Owne 1990; Desportes-Livage et al. 1996). In other microsporidia, polar tubes do not form until sporonts divide and the plasmalemma has thickened, whereas *Nucleospora* and *Enterocytozoon* form polar tube precursors (i.e., electron dense discs) prior to plasmodial division and thickening of the sporoginal plasmalemma. In addition, both *Nucleospora* and *Enterocytozoon* are polysporous but do not form sporophorous vesicles or pansporoblastic membranes, and do not possess diplokarya in any stage of development. Nevertheless, after more detailed comparisons, Desportes-Livage et al. (1996) concluded that the morphology and development of these microsporidia are less closely related than originally supposed.

Nucleospora infections are usually associated with a concurrent neoplastic condition involving massive lymphoproliferation, known as plasmacytoid leukemia (PL) in chinook salmon in British Columbia (Kent et al. 1990). The actual cause of PL is controversial. Laboratory transmission studies indicated that *N. salmonis* may not be the primary cause of all cases of PL ; i.e., PL was transmitted with tissue homogenates or filtrates in the absence of the microsporidian (Kent & Dawe 1990; Newbound & Kent 1991; Kent & Dawe 1993). Furthermore, Eaton & Kent (1992) described a retrovirus from fish with PL.

The most convincing studies suggesting a viral etiology for PL was conducted with tissues from fish with no sign of *N. salmonis* infection, which was often the case in pen-reared salmon that were examined in the late 1980's and early 1990's (Kent ML pers. comm.). However, in essentially all cases that were investigated in recent years, and in studies from other countries, *N. salmonis* is consistently observed in the proliferating plasmablasts. Therefore, it is possible that these morphologically similar lymphoproliferative disorders are actually different diseases caused by two different parasites. Studies with fumagillin (Hedrick et al. 1991b) and its analog TNP-470 (Higgins et al. 1998) support the microsporidian hypothesis. In these studies, both the parasite and the lesions were prevented by treatment with these antimicrosporidian compounds, which is in contrast to earlier experiments with fumagillin in the control of PL (Kent & Dawe 1993). The microsporidian has been maintained in lymphocyte cultures, and soluble fractions of these cultures stimulate uninfected cells to proliferate (Wongtavatchai et al. 1995a).

Sensitive and specific polymerase chain reaction (PCR) tests have been developed for the detection of *N. salmonis* based on rDNA sequence from the small subunit region (Barlough et al. 1995) or ITS region (Docker et al. 1997b).

Pathology. Heavily infected fish are anemic, with a packed blood cell volume as low as 5%. Typical of severe anemia in fish, affected salmon exhibit prominent pallor of the gills. Histological examination of affected fish reveals a proliferation of plasmablasts in essentially every organ, including the kidney, spleen, liver, intestine, pancreas and associated mesenteric fat, meninges, heart, skeletal muscle, skin, and eye (Hedrick et al. 1990; Morrison et al. 1990). In histological sections, the plasmablasts contain large, often deeply clefted or lobated nuclei, and prominent nucleoli. They have a moderate amount of finely granular, eosinophilic or amphophilic cytoplasm, and many of these

cells are mitotically active. The eye, spleen, and kidney are the primary organs affected (Fig. 21).

In the eye, there is massive infiltration of plasmablasts into the periorbital connective tissue and ocular muscles. Kidneys exhibit prominent hyperplasia of the interstitium due to proliferation of the plasmablasts. Thickened basement membranes in the capillaries of the glomeruli are often observed. In severely affected fish, there is a perivascular infiltration of the plasmablasts within the liver, and the cells often proliferate within the sinusoids.

The pericardium of the ventricle, atrium, and bulbus arteriosus of the heart may be infiltrated by plasmablasts, which form a thick cellular capsule that surrounds the heart. When the lower intestine is affected, there is massive proliferation of the plasmablasts in the lamina propria and in the submucosa, resulting in expansion of the intestinal villi.

The microsporidian is very small and is identified by careful examination of nuclei of hemoblasts in histological sections (Fig. 22), in Gram-stained imprints (Fig. 23), or by electron microscopy (Fig. 24). In tissue sections stained with hematoxylin and eosin, the parasites appear as eosinophilic spherical bodies (2 to 4 µm) in host cell nuclei, surrounded by a rim of basophilic host cell chromatin. The Warthin-Starry stain combined with hematoxylin and eosin enhances the detection of the parasite in tissue sections (Kent et al. 1995b). In these preparations, prespore stages stain brown or black, and spores stain dark black. In one outbreak, the infection in pen-reared Atlantic salmon was associated with multiple focal lesions appearing mainly on the head. Histologically the lesions were characterized by massive fibroplasia in which the nuclei of proliferating fibrocytes were infected by the parasite.

Transmission. *Nucleospora salmonis* is transmitted by cohabitation or feeding infected

tissues to fish in fresh water (Baxa-Antonio et al. 1992). Kent ML (pers. comm.) repeated these findings in the laboratory, but was unable to transmit the infection by cohabitation in sea water.

SUMMARY AND THESIS OVERVIEW

In recent years there has been an increased research interest in fish microsporidia along with an increase in the economic importance of microsporidia in fish culture. This has lead to a better understanding of the pathogenesis and immune response of the host to the parasite. Furthermore, advances are being made in the development of effective chemotherapeutants for these microsporidia. In the past, fish microsporidia were assigned to specific taxa based mostly on morphological characteristics. Unfortunately, few useful morphological characteristics are present in fish-infecting microsporidia. The use of molecular systematics, along with interhost transmission experiments, has provided a better understanding of the taxonomic relationships of fish microsporidia, and I suggest that these tools be employed (when possible) as new taxa are proposed.

Aquaculture of salmonids in British Columbia has continued to expand since the early 1980's. As the industry grows successful diagnosis and treatment of disease outbreaks is vital (Kent & Poppe 1998). Very little is known about the basic biology of *Loma salmonae* an important pathogen of salmonids (*Oncorhynchus* spp.) from the Pacific Northwest. Yearly, Pacific salmon farmers incur significant economic losses due to epizootics of *L. salmonae*. This thesis was undertaken to contribute to knowledge on *L. salmonae* transmission and viability, host susceptibility and immunology. In addition, wild salmonid and nonsalmonid hosts of the parasite were examined. Findings in these areas will be presented in subsequent chapters.

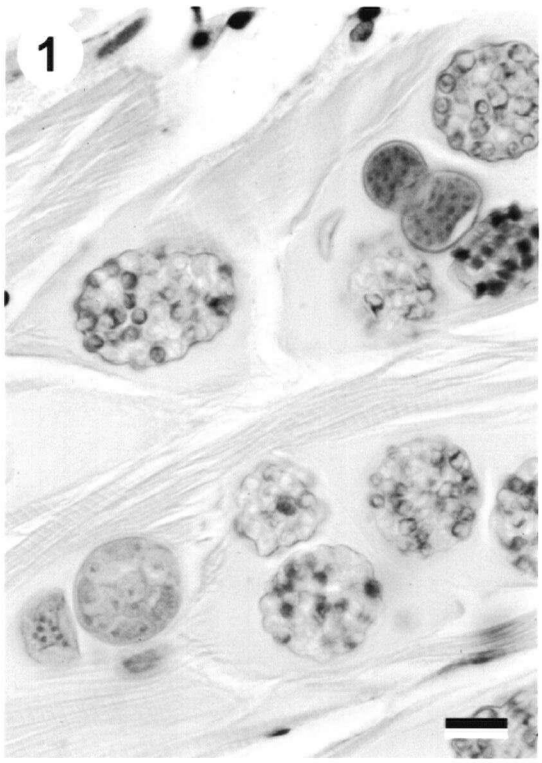


Fig. 1. *Pleistophora hyphessobryconis* infection in skeletal muscle of neon tetra *Paracheirodon inessi*. Scale bar = 14 μm

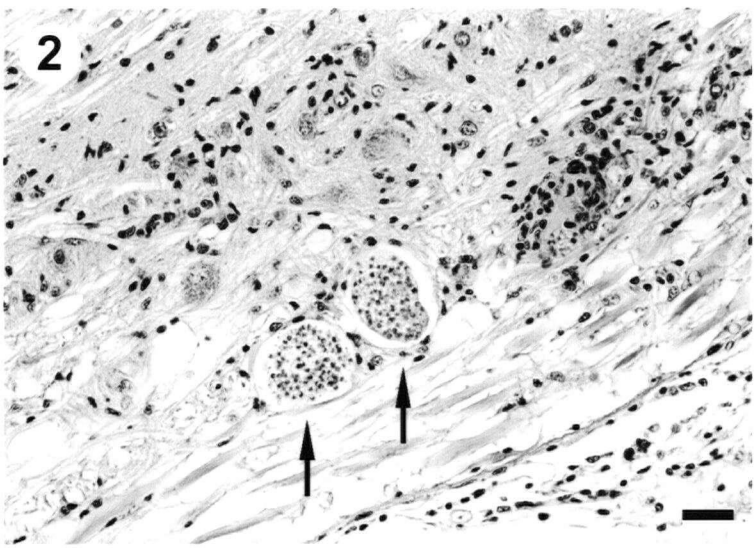


Fig. 2. *Microsporidium* sp. infecting the central nervous system of zebrafish *Brachydanio rerio*. Arrows denote xenomas. Scale bar = 35 μm

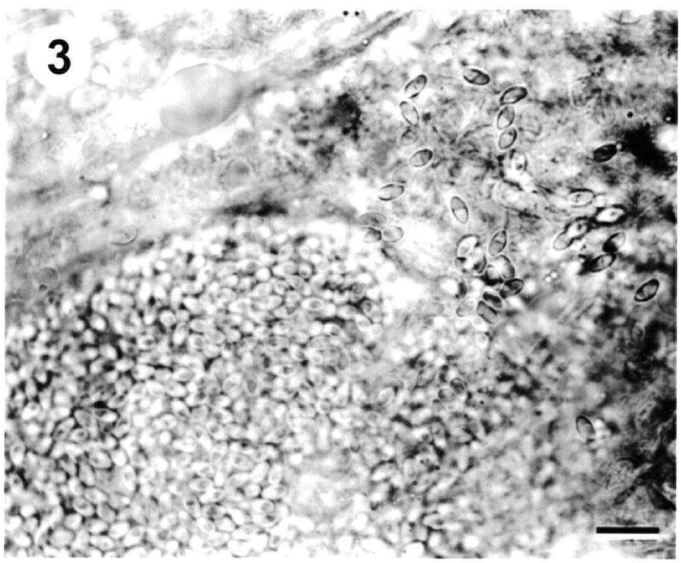


Fig. 3. Wet mount of a ruptured xenoma filled with spores of undescribed *Loma* sp. from the gill of ling cod *Ophiodon elongatus*. Scale bar = 12 μm

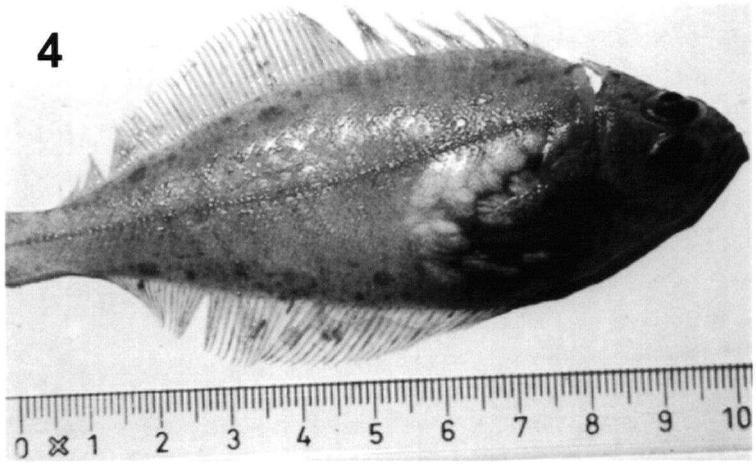


Fig. 4. *Pleistophora hippoglossoideos* in the musculature of plaice *Hippoglossoides platessoides*. Reprinted with permission of the author and publisher from Möller & Möller (1986)

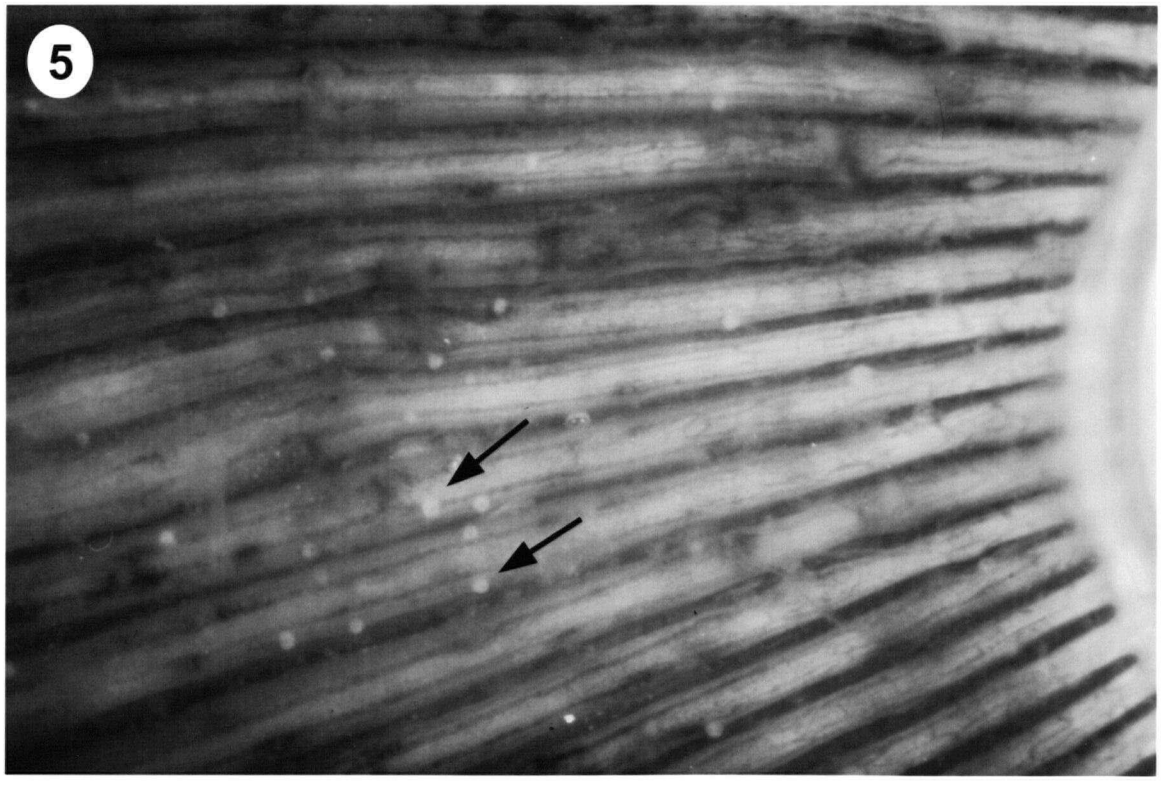


Fig. 5. Heavy infection of *Loma salmonae* (white xenomas) in fresh gill of wild spawning sockeye *Oncorhynchus nerka* salmon from Babine lake, British Columbia, Canada. Reprinted with permission from M. Higgins

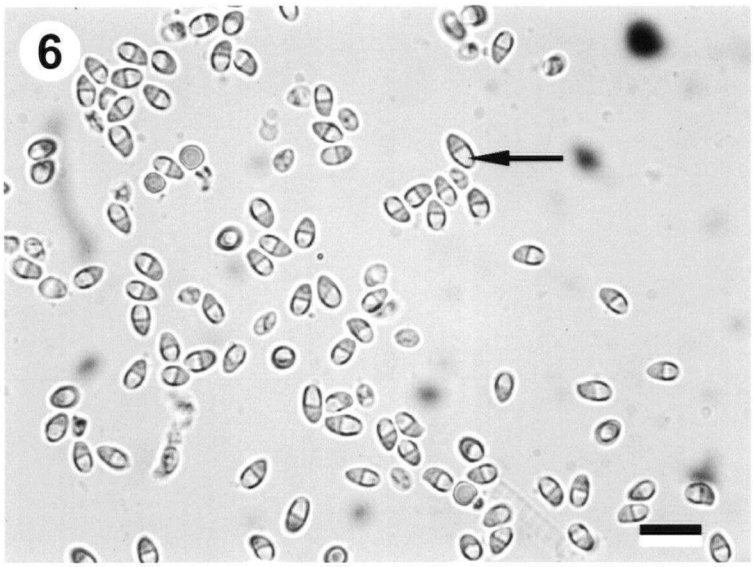


Fig. 6. Wet mount of spores from an undescribed microsporidian from mountain whitefish *Prosopium williamsoni*. Arrow indicates large posterior vacuole. Scale bar = 10 μm

Fig. 7. Electron micrograph of phagocytic cell with prominent nucleus (N) and remnants of *Pleistophora ovariae* spore walls within a food vacuole (FV). Scale bar = 2 μm. Reprinted with permission of the author and publisher from Canning & Lom (1986)

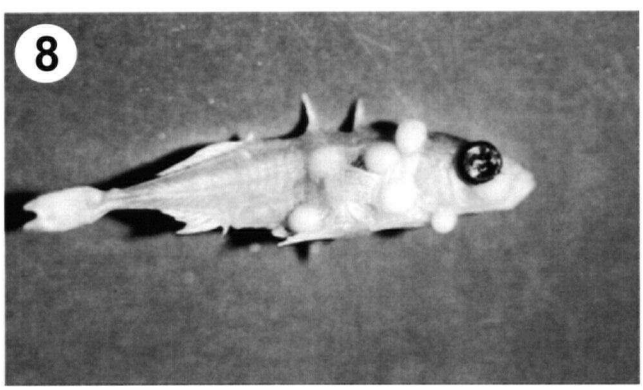


Fig. 8. Large cysts of *Glugea anomala* in a stickleback *Gasterosteus aculeatus* Reprinted with permission of the author and publisher from Möller & Möller (1986)

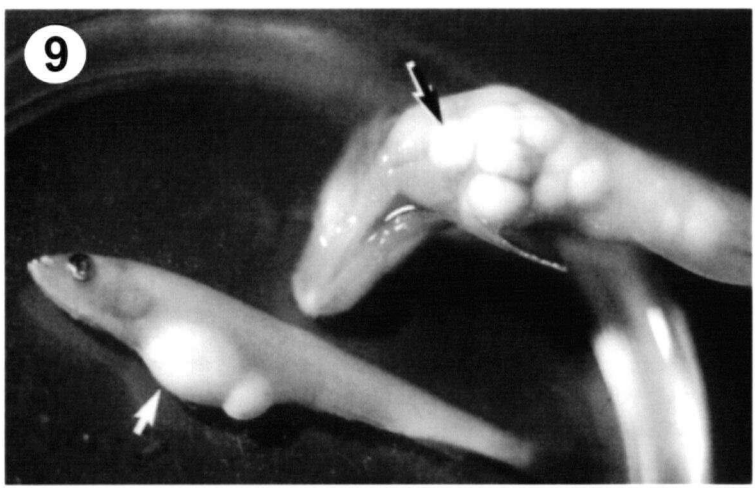
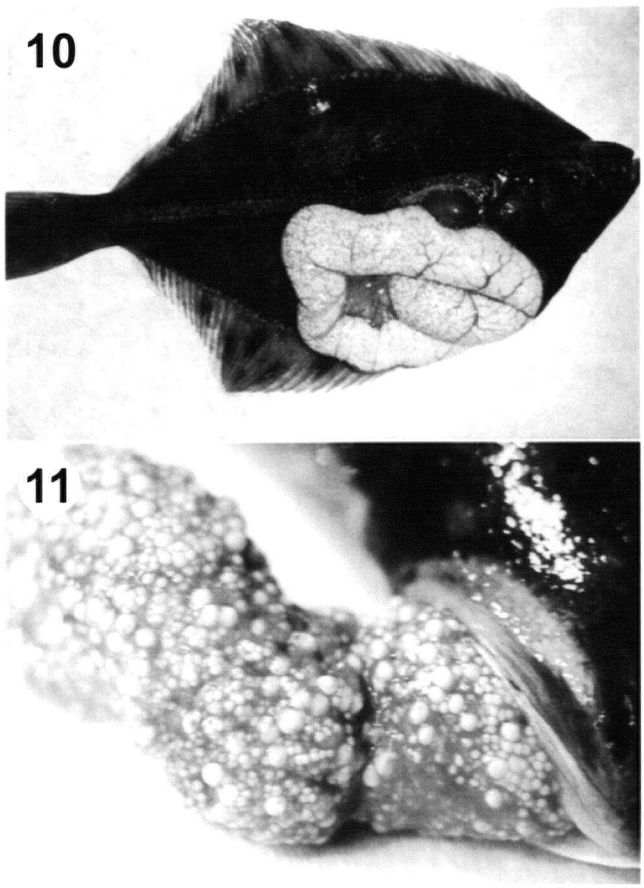
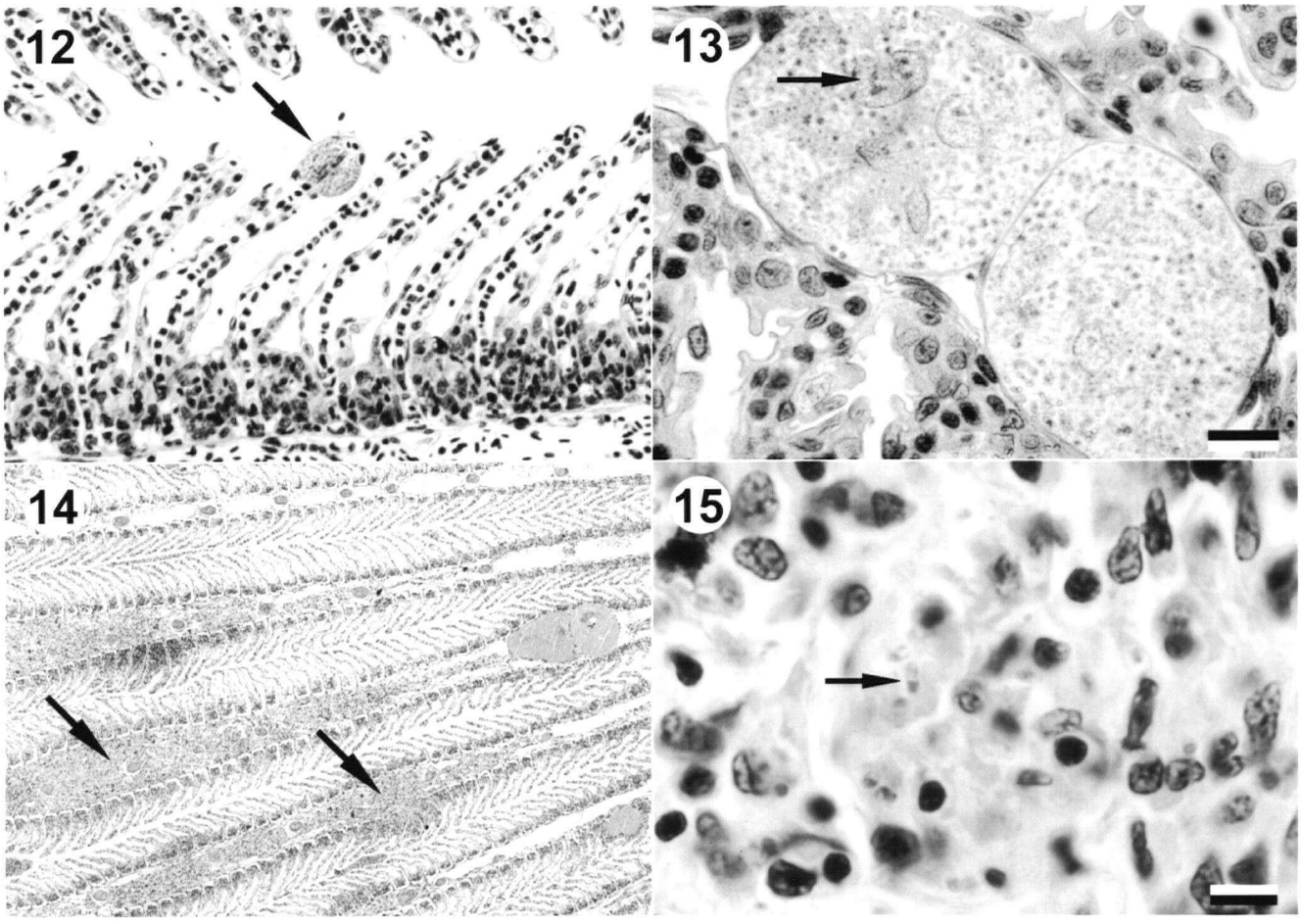


Fig. 9. Abdominal cysts of *Glugea hertwigi* denoted by arrows in rainbow smelt *Osmerus mordax*. Reprinted with permission of the author and publisher from Noga (1996)



Figs. 10 & 11. *Glugea stephani* in winter flounder *Pleuronectes americanus*. Fig. 10. Heavy infection of cysts in the intestine. Fig. 11. Close up to show pebbled chalky-white appearance of intestine in the same individual. Reprinted with permission of the author and publisher from Möller & Möller (1986)



Figs. 12 to 15. Various stages of microsporidian development represented by histological sections of *Loma salmonae* in coho salmon *Oncorhynchus kisutch* gills. Fig. 12. Young xenoma in secondary lamellae denoted by arrow. Fig. 13. Mature xenoma with few remnants of host cell nuclei denoted by arrow. Scale bar = 45 μm. Fig. 14. Proliferative inflammatory response denoted by arrows associated with infection in the primary lamellae. Fig. 15. Granuloma resulting from a ruptured xenoma with a few remaining spores denoted by arrow in macrophages. Scale bar = 10 μm

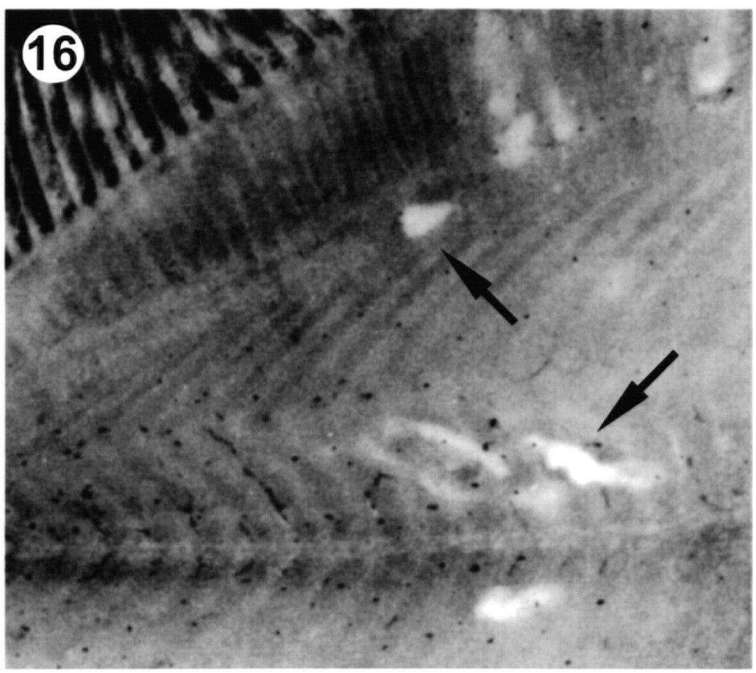


Fig. 16. *Tetramicra brevifilum* denoted by arrows in skeletal musculature of turbot *Scophthalmus maximus*. Reprinted with permission of the author and publisher from Matthews & Matthews (1980)

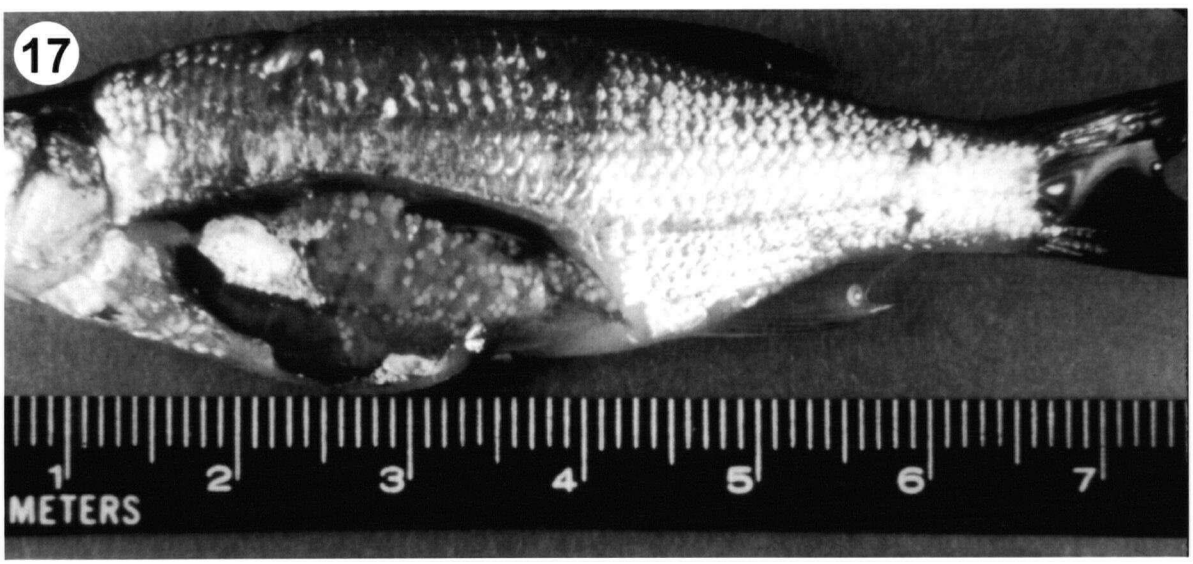


Fig. 17. *Pleistophora ovariae* in the golden shiner *Notemigonus chrysoleucas*. Reprinted with permission from G. Hoffman

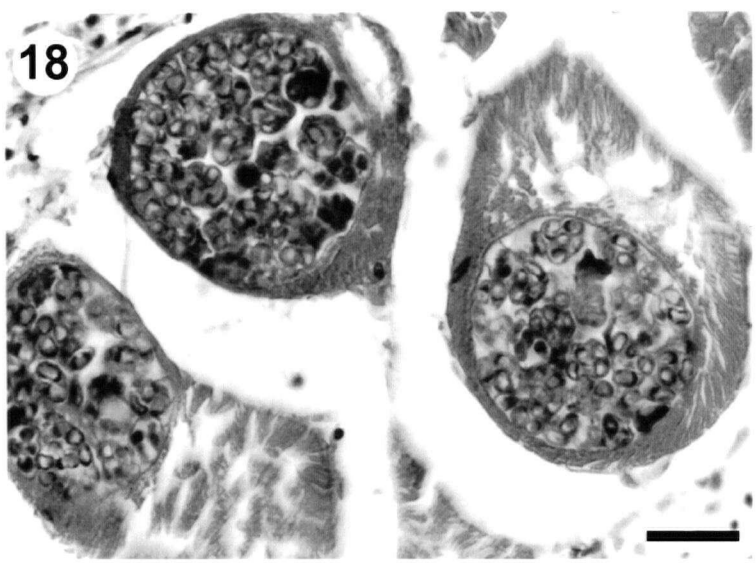
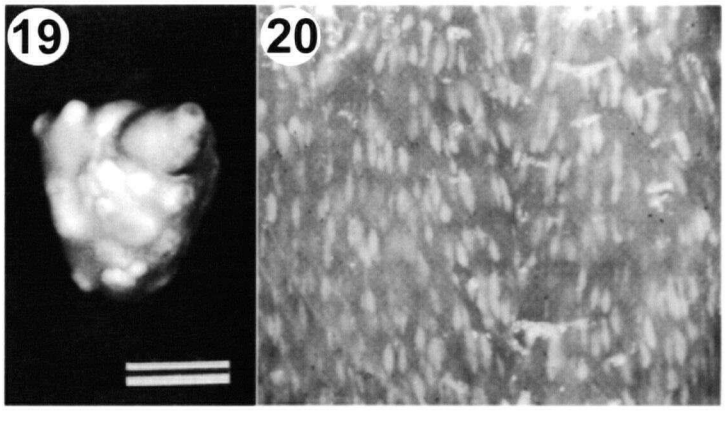


Fig. 18. Sporophorocyts of *Heterosporis finiki* in the musculature of angelfish *Pterophyllum scalare*. Scale bar = 20 μm. Reprinted with permission of the publisher from Michel et al. (1989)



Figs. 19 & 20. Fig. 19. *Micrsporidium takedai* in heart of sockeye salmon *Oncorhynchus nerka*. Scale bar = 4 mm. Reprinted with permission of the author and publisher from Canning & Lom (1986). Fig. 20. *M. takedai* in musculature of rainbow trout *Oncorhynchus mykiss*. Reproduced with permission of the authors and publisher from Lom & Dyková (1992)

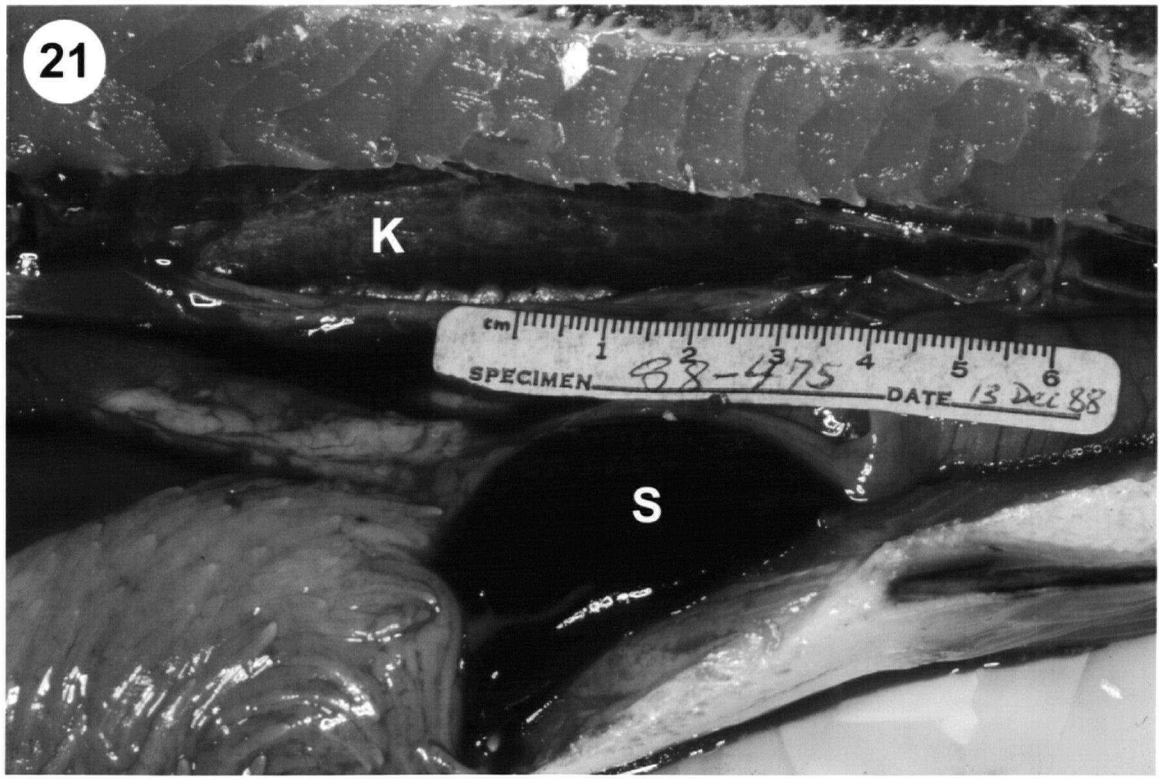
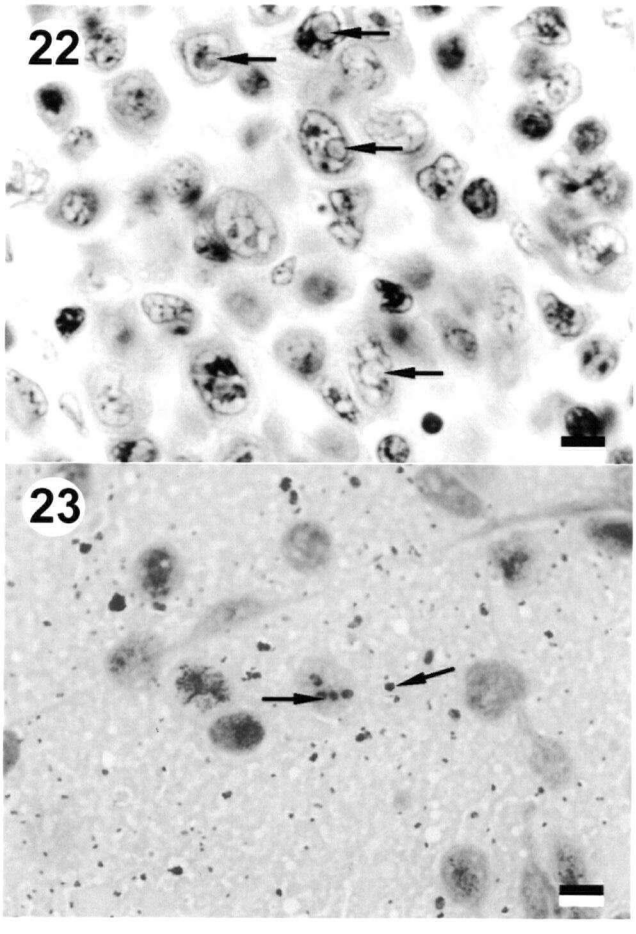


Fig. 21. Reno-splenomegaly in chinook salmon *Oncorhynchus tshawytscha* infected with *Nucleospora salmonis*. K: kidney; S: spleen



Figs. 22 & 23. Fig. 22. *Nucleospora salmonis* denoted by arrows in the nuclei of hemoblasts from the retrobulbar tissue of the eye of chinook salmon *Oncorhynchus tshawytscha* in histological section. Scale bar = 10 μm. Fig. 23. Gram stain of the same tissue, shows spores in nucleus remnant and free denoted by arrows. Scale bar = 10 μm

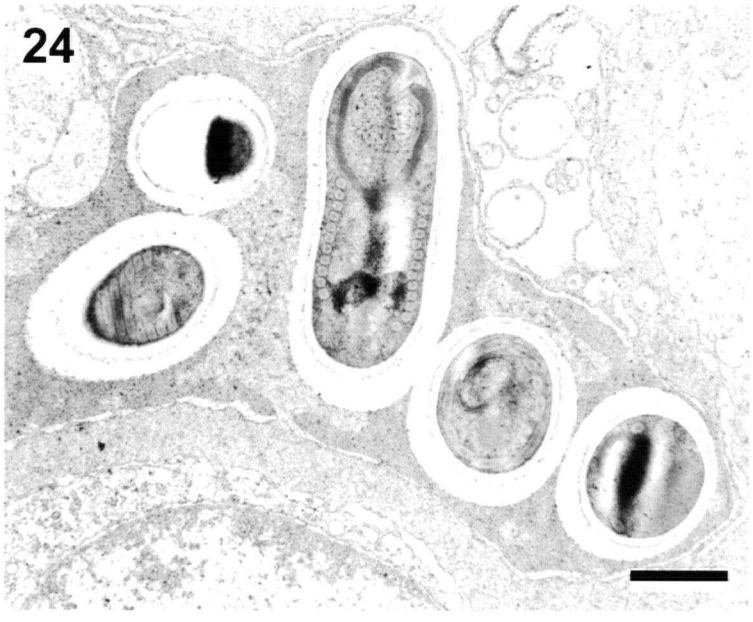


Fig. 24. Electron micrograph of *Nucleospora salmonis* in the nucleus of a hemoblast. Scale bar = 0.25 μm

CHAPTER II

Modes of Transmission

INTRODUCTION

Loma salmonae, is a microsporidian parasite infecting endothelial cells of salmonids. Fish infected with *L. salmonae* often exhibit pale gills with petechial hemorrhages, inflammation, hyperplasia, and white cysts termed xenomas (Wales & Wolf 1955; Hauck 1984; Kent 1992). Outbreaks of *L. salmonae* in Pacific salmon *Oncorhynchus* spp. and rainbow trout have occurred in the Pacific Northwest (Hauck 1984; Kent et al. 1989), eastern United States (Markey et al. 1994), and Scotland (Bruno et al. 1995).

Kent et al. (1995a) demonstrated that fish can be infected by *Loma salmonae* when ingesting infected tissues. Whereas details on the early development of *Loma* species are unknown, in microsporidia that have been studied in this respect, the spore extrudes a polar filament that pierces a gut epithelial cell and injects the parasite's sporoplasm. For xenoma forming genera such as *Loma* and *Glugea*, the sporoplasm eventually divides forming numerous spores within a hypertrophied cell (i.e. a xenoma) (Canning & Lom 1986).

Loma salmonae has been transmitted experimentally both in fresh and sea water by feeding fish macerated gills containing spores (Kent et al. 1995a). Hauck (1984) suggested that gills may be infected directly by phagocytic uptake of pillar cells. However, the method by which *Loma* spp. spread within the host is unknown. It may be by infected macrophages, or by being free in body fluids (Hauck 1984). In the present study, experimental transmission of *L. salmonae* to chinook and coho was attempted by various routes: *per os*, intraperitoneal (IP), intramuscular (IM), and intravascular (IV) injection, cohabitation of infected live and dead fish with naive fish, and placement of *L.*

salmonae spores directly on the gills.

MATERIALS AND METHODS

Fish husbandry. Fish were obtained from Rosewall Creek Hatchery, Fisheries and Oceans Canada, Vancouver Island, Canada. Strains of chinook were donated by the industry from Sea Springs Hatchery in Duncan, Vancouver Island, Canada. These hatcheries receive only well water and have no history of *L. salmonae*. During experiments all fish were held in flow-through pot tanks (41 L), mid-ovals (204 L) or deep-ovals (726 L) on fresh or seawater at the Pacific Biological Station (PBS) in Nanaimo, British Columbia. This station receives residential freshwater, which is then dechlorinated. Seawater is pumped in from a depth of 30 m, filtered to 25μm, and provided for use. Temperatures, unless specified otherwise, were kept at 14 to 16°C. All effluent from experimental tanks was chlorinated (10 ppm) before discharge to city sewage.

Preparation of infective *Loma salmonae* spores. All suspensions were kept at 4°C during preparation of infective material. Gills were first obtained from infected chinook at a seawater netpen site on the west coast of Vancouver Island. Gill tissue was scraped from each arch using a scalpel and suspended in dechlorinated fresh water. This solution was ground (level 1, 20 s at 4°C) using a Polytron tissue homogeniser (Brinkmann Instruments Co., Rexdale, Ontario, Canada) to create a slurry. The slurry was centrifuged at 2000 x *g* for 10 min at 4°C. The pellet was resuspended in water and filtered through a wire mesh and then a 50 μm nylon screen. The filtrate was either centrifuged at 800 x *g* for 45 min on a layered 34%/51% Percol (Sigma) gradient or centrifuged at 2000 x *g* for 10 min on a 51% layer both at 4°C. The latter method required a 1:10 dilution of filtrate, but rapidly produced a pure product. Pure spores

were collected from the pellet and Percol layers. Spores were stored at 4°C until used. Number of spores were determined using a standard haemocytometer.

Fish handling procedures. Fish were fed commercial salmon feed every other day, and anesthetized with tricaine methanesulfonate (MS-222) before handling. Fish were infected *per os* with a 16g needle tipped with 2.0 mm inner diameter tubing by either a gill tissue slurry (0.25 ml) or by pure spores (0.1 ml). A reservoir of fish infected with *L. salmonae* was maintained at PBS from Aug 1995-1998. During sampling fish were killed with an overdose of MS-222, and the first left gill arch of each fish was examined by wet mount for *L. salmonae*. Remaining gills were placed in Davidson's solution (Humason 1979), processed using standard histological techniques, and examined.

Sample collection. Fish heart, stomach, intestine, kidney, liver, spleen, and a section of dorsal muscle were also preserved for histology. Experiments were terminated at 56 d unless otherwise noted, to allow sufficient time for development of visible xenomas (Kent et al. 1995a).

Exposure protocol. Various experiments, termed trials, were conducted as outlined in Tables 1 & 2. *Loma salmonae* spores were introduced directly into the stomach as described above (fish hangling procedures). For anal gavage, a 22 g needle tipped with 0.58 mm inner diameter tubing was used. Various numbers of pure spores (Tables 1 & 2) in 0.1 ml innoculum were used for anal gavage, IP, IM, and IV injections. Intravascular injections were given using the ventral gill sinus or dorsal aorta.

Trials were conducted using the above exposure methods as outlined in Tables 1 & 2. Controls had their left ventral fin clipped and were held in the same tank as exposed fish. In trial 8 (outlined in Table 2) controls were given Earle's Buffered Saline Solution (EBSS) and blood smears were collected at all sampling times from fish receiving IV injections. In trial 3, blood smears from 3 IV injected fish were made within

30 min of injection.

In trial 4, pure spores (0.1 ml) were placed directly on the left gill arch of 10 chinook held out of water for 1 min. After 1 min, each chinook was dipped for 5 s in a rinse bucket before being placed in the holding tank. Controls were passed through the rinse bucket before being placed in the same tank. The same innoculum was used here as in trial 1.

For trial 5 donor chinook were infected *per os* and held for 72 d at 12 to 14°C. Ten donor fish with clinical signs of *L. salmonae* (anemia and petechial hemorrhages on gills) were placed in a 204 L tank with 45 naïve chinook (differentiated by size from infected fish). After exposure periods, sets of 10 recipient fish were removed (Table 1). Following 7 d cohabitation (i.e. 79 d post-exposure of donor fish), all donor fish were examined for *L. salmonae*. Intensity of infection in donors was measured from the average of 3 counts of the number of xenomas per x100 field of view (1.5 mm diameter) converted to mm^2.

For trial 6 donor chinook were infected as in trial 5, selected for clinical signs of *L. salmonae*, and killed with MS-222. The same protocol was then followed as in trial 5.

For trial 7 donor chinook were again infected as in trail 5 except 12 donor chinook were killed, and placed in 4 acrylic containers (62 x 32.3 x 11.7 cm) at 3 $container^{-1}$. Water was pumped into these containers at 1.7 L min^{-1}. Two 8.5 cm high baffles kept dead fish from blocking outflow of each container that fed into a smaller pot tank containing 10 naïve chinook. Four tanks of recipient chinook were exposed to outflow from the containers for 24 h, 3, 5, and 7 d after which they were placed on uncontaminated flow for 56 d before being examined for *L. salmonae*. All donor fish were examined for *L. salmonae* as in trial 5.

Detection of spores in blood. Blood smears from trial 3 were air dried, heat and methanol fixed, and an indirect fluorescent antibody test (IFAT) was conducted to locate spores. Monoclonal antibody (MAB) for *L. salmonae* developed using standard techniques (Schots et al. 1992) was provided by F. Markham, Department of Pathology and Microbiology, Atlantic Veterinary School, Charlottetown, Prince Edward Island, Canada. The MAB (225 µl) was applied directly to blood smears and allowed to incubate at room temperature for 30 min. The smears were washed 3 times and incubated in phosphate buffered saline (PBS) at pH 7.2 for 10 min. Fluorescein isothiocynate (FITC) conjugated goat anti-mouse IgG antibody diluted in PBS (1:128) was counterstained with 1% Evan's blue (1:156) and applied to the smears. Smears were incubated for 30 min, washed 3 times and incubated for 10 min in PBS. IFAT slides were examined with a fluorescent microscope at x1000 using pH 9 mounting fluid (Difco).

RESULTS

All exposure methods, except placement of *L. salmonae* spores directly on the gill (trial 4, Table 1), resulted in infection (Tables 1 & 2). When administered *per os*, spores were found within 6.5 h in histological sections of stomach in free material of the lumen and in association with epithelial cells (Figs. 25 & 26). Between 2.5 and 24 h possible sporoplasms were found in epithelial cells of the stomach and pyloric caeca. These stages consisted of a dark staining body (probably nuclear material) surrounded by a lighter-staining area. At 24 h epithelial cells of the anterior intestine contained these sporoplasms, and some sporoplasms were also associated with the lamina propria (Figs. 27-29). These structures were not found in controls or in fish sampled at 35 or 56 d.

Loma salmonae spores were detected in the blood by IFAT 30 min after fish received IV injections via the dorsal aorta and up to 24 h after injections via the ventral gill sinus (Fig. 30). By 56 d all fish given IP injections had swollen kidneys and spleens, petechial hemorrhaging, and bloody ascites. This was not seen in fish infected *per os* or in controls. One fish given an IV injection had edema and hemorrhaging. Fish receiving IM injections of spores exhibited edema and a pronounced inflammatory response in muscle tissue at the site of injection by 35 d (infection shown after 56 d in Fig. 31). No inflammation was seen in controls. In trial 8 (Table 2) xenomas were first detected 35 d post infection in gill, heart and spleen. Xenomas were found most often in the gills followed by heart and spleen (Table 3). Xenomas were not found in intestine, liver, muscle, or stomach.

Xenomas in donor fish (Table 1; trials 5 to 7) were completely opaque and filled with spores. Intensity mm^{-2} in donor fish for trials 5 to 7 respectively was: 0.5 (range 0.2-1.3); 2.4 (range 0.2-3.7); 2.6 (range 0.2-4.0) per x100 field of view. Few fish became infected during cohabitation with dead donor fish, and no fish became infected when receiving outflow from tanks containing dead donor fish (Table 1; trials 6 & 7).

DISCUSSION

Oncorhynchus spp. were susceptible to *L. salmonae* administered by *per os*, anal gavage, IP, IM, and IV injection, and by cohabitation with infected fish. Microsporidia such as *Glugea*, *Heterosporis*, *Microsporidium*, and *Pleistophora* that infect fish species, have been successfully experimentally transmitted *per os*, by IP, and IM injection, and by water-borne exposure (Lom 1969; Awakura 1974; McVicar 1975; Leiro et al. 1994). Water-borne infections can result from direct immersion in a spore solution (Awakura 1974; T'sui et al. 1988), or by feeding a crustacean, previously exposed to spores, to fish (Awakura 1974; Olson 1976, 1981). *Loma salmonae* has

been transmitted *per os* to chinook (Kent & Dawe 1994) and rainbow trout (Speare et al. 1998a). In addition Speare et al. (1998b) transmitted *L. salmonae per os* and by IP injection of semi-purified spores to rainbow trout. Hauck (1984) suggested phagocytic uptake of *Loma* sp. by gill pillar cells as water passes through the gills. I was unsuccessful in infecting fish by placing spores directly on gills for 1 min.

Association of *L. salmonae* spores with stomach epithelial cells, and possible sporoplasms released from spores located within epithelial cells of the stomach, pyloric caeca, and anterior intestine, most likely indicate initial sites of infection. Spores discharging polar filaments may pierce gut epithelial cells, injecting infective sporoplasms. These sporoplasms might move through epithelial cells and enter the lamina propria, which is richly supplied with blood capillaries. It is generally believed that microsporidia that infect fish enter gut epithelial cells, where they develop or are then transported to their preferred site of development (Canning & Lom 1986). These microsporidia might infect migratory cells such as histiocytes, neutrophils or macrophages (Weissenberg 1968; Canning & Lom 1986; McVicar 1975). Matthews & Matthews (1980) proposed that macrophages act as a transport mechanism for *Tetramicra brevifilum* through the endothelium of turbot, or become infected themselves during phagocytosis of the parasite in the lamina propria of the intestine. However, to my knowledge none of these hypotheses have been previously demonstrated experimentally. Further studies using *in situ* hybridization would demonstrate the site of initial infection and how subsequent spread of *L. salmonae* occurs within the fish host. Docker et al. (1997a) developed a PCR test for *L. salmonae*, which would be useful for this purpose.

Autoinfection within fish-infecting microsporidia has been proposed but not verified (Lom & Dyková 1992). The systemic distribution of *Loma* sp. xenomas and free

spores suggests that, as xenomas rupture, infective stages are liberated and move throughout the fish via the blood (Hauck 1984; Markey et al. 1994). My experiments, in which free spores injected into the blood resulted in xenoma formation in the gills support the hypothesis that autoinfection may occur in *L. salmonae*, i.e. in natural infections, xenomas may rupture in blood vessels and free spores may circulate and establish new xenomas. The high number of xenomas in the gills compared to other organs, regardless of the route of exposure, suggests that the gill endothelium is the preferred site of development of *L. salmonae*, rather than a coincidental site due to route of exposure or circulatory patterns.

Transmission of *L. salmonae* from infected to cohabitating naive fish in a flow-through system indicates how readily the parasite may be transmitted. My results also suggest the parasite is more easily transmitted from infected live fish than from infected dead fish. Kano et al. (1982) noted that *Heterosporis anguillarum* spread to healthy eels from infected eels in the same aquarium. During cohabitation in my study, naive fish may have ingested spores from ruptured or whole xenomas released from the gills (i.e. the secondary lamellae) of infected fish. Spores could also be liberated in fish urine when fish have kidney infections (Hauck 1984).

My results demonstrate that *L. salmonae* is transmissible by experimental (e.g. IP, IM) and natural (e.g. *per os*, cohabitation) exposure routes. Infection by IV injection, and distribution of xenomas throughout vascular tissue suggests that once a fish is infected, *L. salmonae* may spread within the host by autoinfection. Kent et al. (1995a) proposed *L. salmonae* spreads within a seawater netpen site by spores released from decomposing fish or by other fish feeding on the remains of mortalities. Salmonid farmers should be aware of this and the possibility of infected fish transmitting the parasite to naive fish when formulating management strategies to control the infection.

Table 1. *Loma salmonae* in *Oncorhynchus tshawytscha* and *O. kisutch*. Infection of *L. salmonae* in chinook and coho salmon exposed by various routes. Fish were examined at 56 d post-exposure and considered positive upon detection of the parasite in wet mounts or histological sections

Trial	No. fish exposed	No. positive/No. examined
Trial 1		
Chinook (Avg. 16.6 cm; 38.4 g)		
Spore dosage (except controls) = 2.8×10^6		
Per os	10	9/10
Anal gavage	10	10/10
Control	4	0/4
Trial 2		
Coho (Avg. 18.8 cm; 60.1 g)		
Spore dosage = 1.2×10^6		
Per os	10	9/9
Intraperitoneal	10	8/8
Intravascular[a]	10	10/10
Trial 3		
Chinook (Avg. 23.2 cm; 119.2 g)		
Spore dosage (except controls) = 5.9×10^5		
Per os	4	4/4
Intravascular[b,c]	13	10/10
Control	4	0/4
Trial 4		
Spore dosage (except controls) = 2.8×10^6		
Chinook (Avg. 11.2 cm; 12.9 g)		
Coho (Avg. 20.4 cm; 80.1 g)		
Gill arch	10	0/10
Control	2	0/2

Table 1, continued.

Trial 5		
Cohabitation exposure (live donors)		
Donor chinook (Avg. 19.8 cm; 66.4 g)		
Recipient chinook (Avg. 12.4 cm; 17.4 g)		
Donor fish (spore dosage = 1.3×10^6)	10	10/10
Recipient fish, 24 h exposure[d]	45	3/10
Recipient fish, 3 d exposure[d]	35	7/10
Recipient fish, 5 d exposure[d]	25	6/10
Recipient fish, 7 d exposure[d]	15	10/10
Trial 6		
Cohabitation exposure (dead donors)		
Donor chinook (Avg. 22.4 cm; 119.5 g)		
Recipient chinook (Avg. 18.5 cm; 56.3 g)		
Donor fish (spore dosage = 1.3×10^6)	10	10/10
Recipient fish 24 h exposure[d]	45	0/10
Recipient fish 3 d exposure[d]	35	2/10
Recipient fish 5 d exposure[d]	25	0/10
Recipient fish 7 d exposure[d]	15	1/10
Trial 7		
Outflow exposure (dead donors)		
Donor chinook (Avg. 22.3 cm; 115.6 g)		
Recipient chinook (Avg. 18.4 cm; 53.0 g)		
Donor fish (spore dosage = 1.3×10^6)	12	12/12
Recipient fish 24 h exposure[d]	10	0/10
Recipient fish 3 d exposure[d]	10	0/10
Recipient fish 5 d exposure[d]	10	0/10
Recipient fish 7 d exposure[d]	10	0/10

[a] Injection by ventral gill sinus [b] Injection by dorsal aorta
[c] Spores detected in blood by an indirect fluorescent antibody test (IFAT)
[d] Fish removed at time indicated and raised separately for 56 d before examination

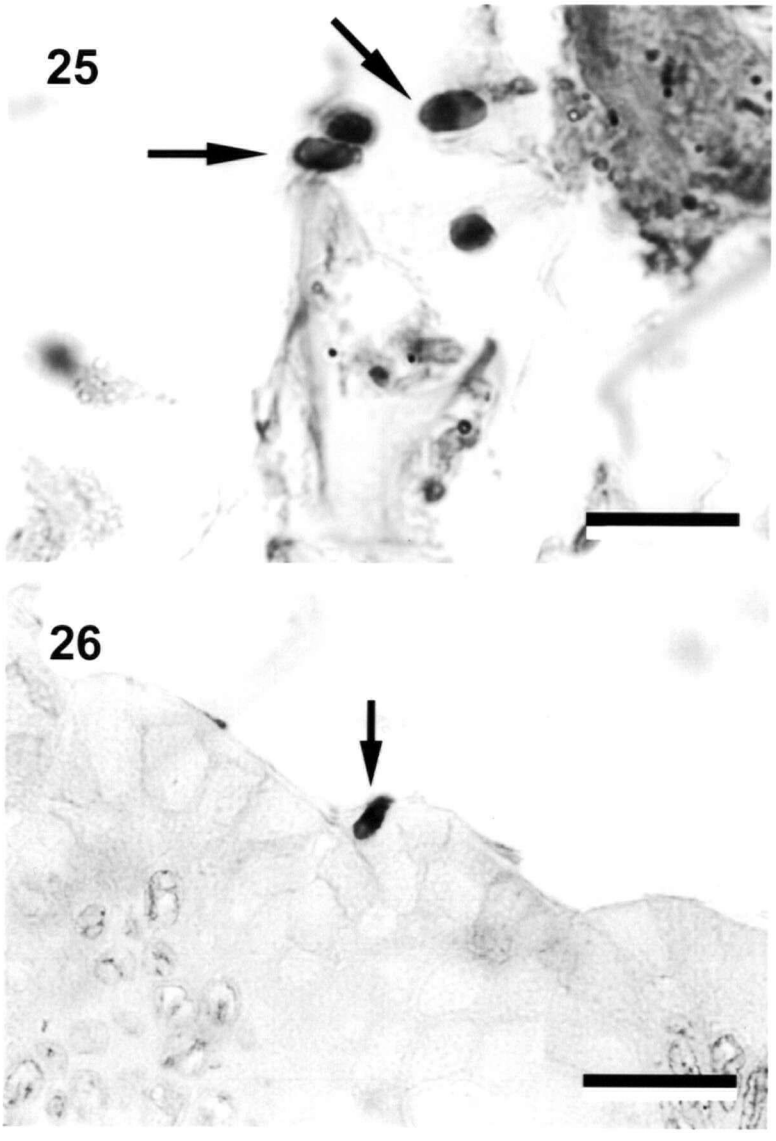
Table 2. *Loma salmonae* in *Oncorhynchus kisutch*. Infection of *L. salmonae* in coho from trial 8 exposed by various routes to 1.2 x 10^6 spores $fish^{-1}$ (except controls). Fish were considered positive upon detection of the parasite in wet mounts or histological sections

Mode of exposure	No. of fish	Time examined	No. positive/ No. examined
Per os	16	2.5 h[a]	0/2
		6.5 h[ab]	0/2
		24 h[a]	0/2
		35 d	1/2
		56 d	7/8
Control	8	2.5 h - 56 d	0/8
Intraperitoneal	16	2.5 h	0/2
		6.5 h	0/2
		24 h	0/2
		35 d	0/2
		56 d	3/4
Control	4	2.5 h - 56 d	0/4
Intravascular[c]	16		
		2.5 h[d]	0/2
		6.5 h[d]	0/2
		24 h[d]	0/2
		35 d	1/2
		56 d	6/6
Control	8	2.5 h - 56 d	0/8
Intramuscular	16	2.5 h	0/2
		6.5 h	0/2
		24 h	0/2
		35 d	1/2
		56 d	6/7
Control	8	2.5 h – 56 d	0/7

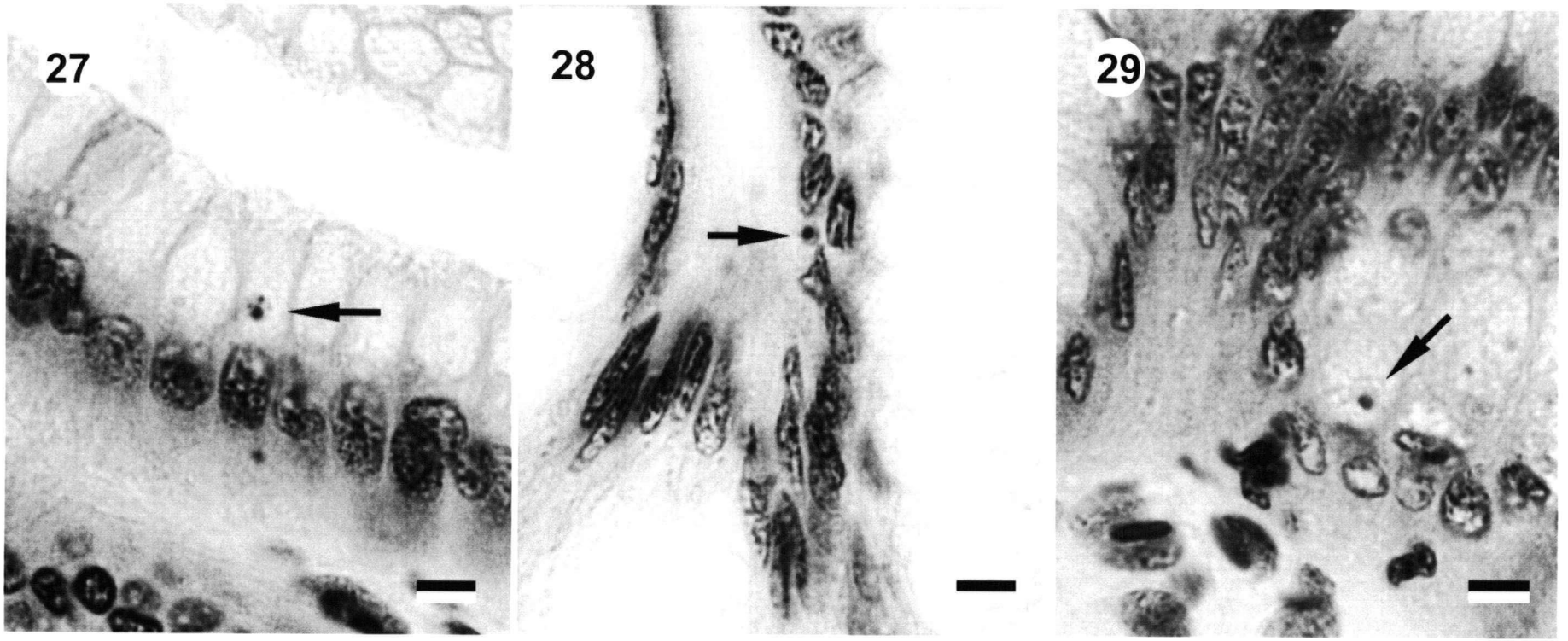
[a] Possible sporoplasms detected in alimentary canal
[b] Spores detected in histological sections of stomach
[c] Injection by ventral gill sinus
[d] Spores detected in blood by IFAT

Table 3. *Loma salmonae* in *Oncorhynchus kisutch*. Distribution of *L. salmonae* in organs of coho salmon from trials 2 and 8 combined, at 56 d after exposure by various routes. Values for tissue are a percentage of fish showing infection in organ indicated

Infection Method	No. Fish	Tissue			
		Gill	Heart	Spleen	Kidney
Per os	17	100.0	22.2	17.6	0.0
Intraperitoneal	12	92.2	41.7	41.7	25.0
Intravascular	16	100.0	43.8	41.2	6.3
Intramuscular	7	85.7	14.3	0.0	0.0



Figs. 25 & 26. ***Loma salmonae*** **in** ***Oncorhynchus kisutch.*** **Hematoxylin & eosin sections of** ***L. salmonae*** **spores in coho salmon infected** ***per os*** **and sampled within 6.5 h. Fig. 25. Spores in stomach material denoted by arrows. Scale bar = 12 μm. Fig. 26. Single spore in association with stomach epithelial cells. Scale bar = 12 μm**



Figs. 27 to 29. *Loma salmonae* in *Oncorhynchus kisutch*. Hematoxylin & eosin sections of possible sporoplasms (denoted by arrows) of *L. salmonae* in anterior intestinal epithelial cells of coho salmon infected *per os* and sampled within 24 h. Fig. 27. Sporoplasm in intestinal cell. Scale bar = 6 μm. Figs. 28 & 29. Sporoplasms in association with lamina propria of intestinal cells. Scale bar = 6 μm

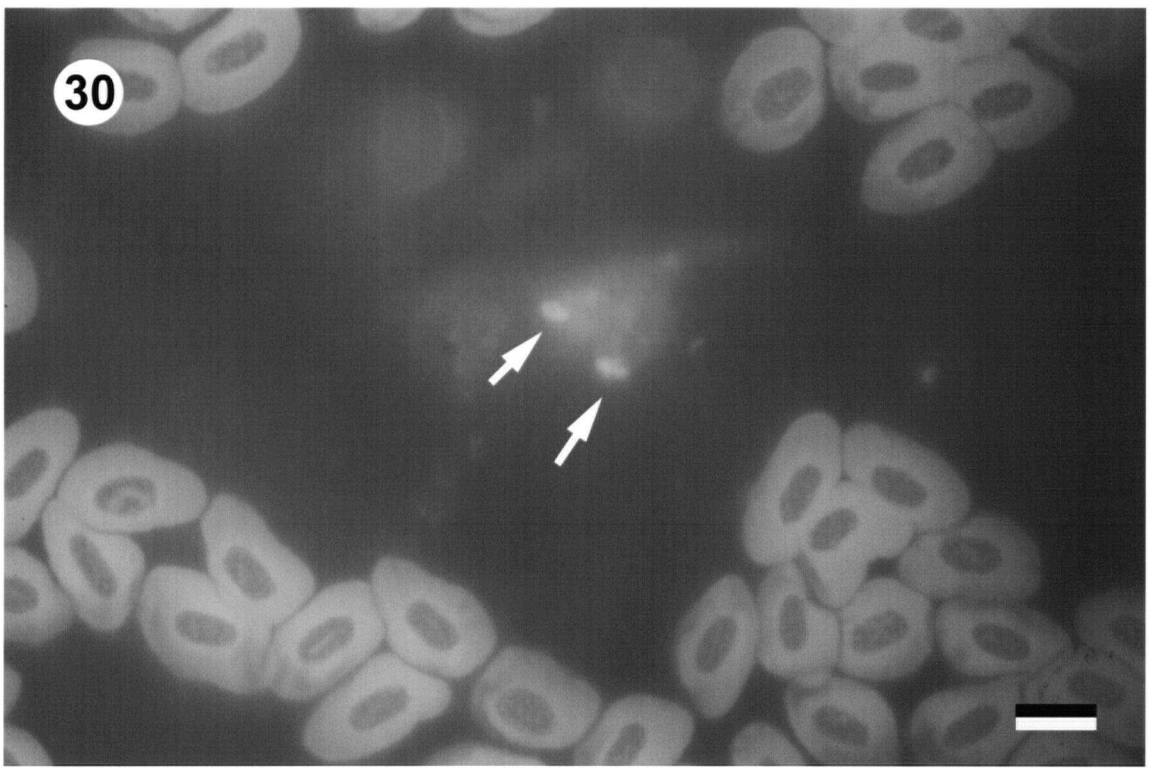


Fig. 30. *Loma salmonae* in *Oncorhynchus kisutch*. *L. salmonae* spores detected by IFAT in the blood of coho salmon within 24 h of fish being injected with pure spores via the ventral gill sinus. Scale bar = 12 μm

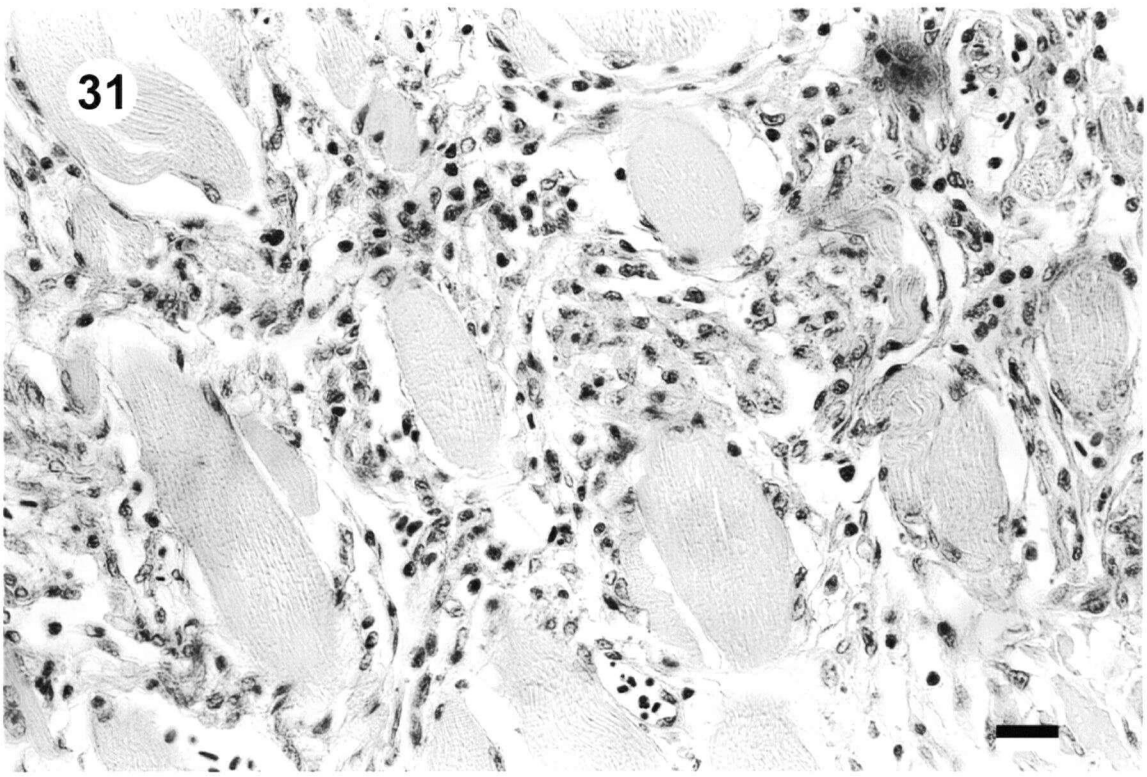


Fig. 31. *Loma salmonae* in *Oncorhynchus kisutch*. Pronounced inflammatory response in coho salmon muscle after 56 d in association with intramuscular injection of *L. salmonae* spores. Hematoxylin & eosin. Scale bar = 6 μm

CHAPTER III

Viability Under Various Laboratory Conditions

INTRODUCTION

Storage and longevity of insect-infecting microsporidia has been researched extensively (Oshima 1964; Henry & Oma 1974; Pilley 1978; Fuxa & Brooks 1979; Teetor-Barsch & Kramer 1979). Some species can be stored under refrigeration, in frozen water, lyophilized, or vacuum-dried quantities, and even long term (>20 y) in liquid nitrogen (Maddox & Solter 1996). These species retain viability for a long time, and have been documented surviving for years in only 4°C Ringer's solution (Revell 1960; Kramer 1970).

In contrast, very little is known about storage and longevity of fish-infecting microsporidia. Lom (1975) reported capillary tubes as a convenient method for storing spores, although he did not address viability. Lom & Dyková (1992) reported microsporidia surviving up to 1 y in water at 4°C. Amigó et al. (1996) studied viability of *Glugea stephani* from commercial flatfishes (family Pleuronectidae) using extrusion rates of spore polar filaments, and flow cytometry. Flow cytometry indirectly represents viability by measuring membrane integrity. Amigó et al. (1996) found that *G. stephani* spores were able to resist heating (60°C for 30 min) and freezing (-19°C for 24 h), and retained extrusion rates (28.6%) and viability (67.3%) up to 17 mon. However, no studies to my knowledge, have examined the effect of disinfectants on fish-infecting microsporidia. Iodophor disinfectants (1.0% iodine) are commonly used in the aquaculture industry to disinfect equipment, surfaces, and fish eggs. Docker et al. (1997a) found *L. salmonae* spores in the ovaries of sexually-mature chinook salmon. Although it has not been demonstrated, spores on the outside or inside of eggs might infect newly emerging fry.

No studies have examined viability of *L. salmonae* stored under various conditions. Viability of spores was determined by exclusion of the dye methylene blue and percent extrusion of spore polar filaments. Viability was also measured by ability of spores to infect fish. To address the risk of *L. salmonae* spores infecting fry, I examined the efficacy of an iodophor to kill spores at various concentrations.

MATERIALS AND METHODS

Assays of spore viability. Spores were isolated as described in chapter II. Exclusion of methylene blue was based upon protocol described by Hoffman & Markiw (1977). Their assumption was that live myxosporean spores did not absorb methylene blue. I tested this assumption with *L. salmonae* spores by incubating spores in various treatments (Tables 4 & 5), and then combining 37 µl of spore suspension with 18 µl of 0.25 % methylene blue on a slide. A total of 300 spores counted at x1000 within 10 min were categorized as clear (viable) or blue (non-viable). Spores were exposed to arbitrarily chosen temperatures (Table 4).

Extrusion counts were done by combining 30 µl of the spore solution with 30 µl of 3.0% H_2O_2 on a slide. The solution was allowed to incubate 15 min at room temperature and 300 spores were counted and categorized as extruded (visible polar filaments) or not extruded.

Viability was measured directly by infecting fish *per os* (see Chapter II) after spores were exposed to various treatments (Tables 5 & 6). Aged dechlorinated aquarium water (fresh water) and sea water were filtered through 20 µm nylon mesh before use. All incubation times were at 4°C unless otherwise indicated.

Viability in fresh or sea water

Trials 1 & 2

Spores were kept in EBSS (1% penicillin-streptomycin antibiotic, GibcoBRL 10

000 units ml^{-1} penicillin G; 10 000 µg ml^{-1} streptomycin sulfate) 24 h and 5 d respectively before being resuspended in various treatments (Table 4; Fig. 32). Artificial sea water (1.025 specific gravity) was created using Forty Fathoms salt (Marine Enterprises International Inc, Baltimore, MA). Antibiotic (1.0% penicillin-streptomycin) was added to treatments of distilled water and artificial sea water. At 7, 37, 99, and 190 d 10 fish $treatment^{-1}$ (Fig. 32) were infected at a dose of 1.3×10^6 spores. Fish were kept on sea water for 56 d before being examined for *L. salmonae*. Spore suspensions for trial 2 (Fig. 32) were changed only on days of methylene blue viability measurements.

Trial 3

Spore dosage was 1.0×10^6 spores ml^{-1} with 2 fish $treatment^{-1}$ given 0.5 ml. Fish were fin clipped to differentiate treatment (Table 5), and placed in the same tank on freshwater (10 to 14°C). Spore suspensions were not changed, and total spore number ml^{-1} was measured from a duplicate tube.

Trial 4

Per os dosage was 6.3×10^5 spores ml^{-1} with 5 fish $treatment^{-1}$ given 0.25 ml (Table 6). Fish were kept in separate freshwater tanks. Spore suspensions were changed once $week^{-1}$. Trials 5 & 6 followed the same protocol as trial 4 but 10 fish $treatment^{-1}$ were used.

Freezing treatment. Purified spores and infected gills were frozen in various solutions, thawed, and given to fish *per os* as outlined in Table 7. Gill arches were removed from infected chinook obtained from the reservoir tank (see Chapter II) and examined at x100. Gills with a high number of xenomas (> 2.1 mm^{-2}) were selected for freezing. Gills were quick frozen by placement directly in the freezer (-20°C & -70 °C), and slow frozen using a Nalgene Cryo 1°C freezing container (Gibco) for at least 24 h at

-20°C. Material was thawed gradually on ice and either filtered with 40 μm Nytex screen (whole gills) or centrifuged at x 2000 *g* for 10 min (pure spores) to remove test solution. Gills were resuspended in dechlorinated water and prepared for *per os* infection of fish (see Chapter II).

Iodophor treatment. Fish were experimentally infected following the protocol of chapter II with modifications described herein. Gill tissue suspended in dechlorinated fresh water was ground using the Polytron tissue homogeniser to create a homogenate (speed 1, 30 s at 4 °C). This homogenate contained only free spores, disrupted cells, and cartilage debris while intact xenomas or host cells were absent. The homogenate was divided among 3 centrifuge tubes, centrifuged at 2000 x *g* for 10 min at 4°C, and resuspended in either dechlorinated fresh water (control), 100, 150, or 200 ppm iodine (Ovadine; Syndel International Inc.) for 15 min at 4°C. The suspensions were centrifuged again at 2000 x *g* for 10 min and resuspended in dechlorinated water. Total iodophor treatment time was 25 min. Two trials were run as outlined in Table 8, with 20 fish treatment^{-1}. Total length and weight of fish were recorded at the termination of the experiment, and abundance of xenomas was determined from wet mounts as described in chapter II. In addition, gills from fish that were negative for the infection by this method were preserved in Davidson's fixative, and examined using standard histological techniques.

RESULTS

Viability, as measured by methylene blue exclusion, decreased in spores subjected to extremes in temperature or long term storage (Table 4; Fig. 32). All chinook (avg. 8.1 cm; 4.2 g) infected *per os* in trial 2 were negative. By 190 d all spores incubated in ambient sea water were dead (Fig. 32). Spores stored at 4°C retained a high percent viability as indicated by methylene blue exclusion and infectivity tests

(chinook avg. 21.2 cm; 106.2 g), but began to die by 10 d in fresh water and 5 d in sea water (Table 5).

Extrusion rates of recently isolated spores was as high as 51.0% decreasing over time to 0.0% at 100 d and 95 d, in fresh and seawater respectively. (Fig. 33). Prevalence of infection in chinook (avg. 15.2 x cm; 33.7 g) remained high (Table 6) while intensity decreased over length of time spores were stored in fresh or seawater (Figs. 34 & 35). *Loma salmonae* spores stored in either fresh or seawater were still able to infect fish after 95 d (Table 6).

Attempts to infect chinook (avg. 12.8 cm; 17.1 g) with spores that had been frozen were unsuccessful (Table 7).

All groups of fish receiving iodophor treatment, as well as the controls, developed *L. salmonae* (Table 8). Fish that received parasites treated at the highest concentration (200 ppm) showed reduced prevalence and intensity of infection, and detection of parasites was delayed compared to other groups.

DISCUSSION

My results suggest that methylene blue exclusion overestimates viability and is not an accurate measurement of *L. salmonae* viability. Fish did not become infected by exposure to spores held 7 d at 10°C, although approximately 80.0% of spores excluded methylene blue (Fig. 32). Keeping spores under these conditions, without weekly changing of solutions, might have led to acidification and inactivation of spores.

Microsporidian spore extrusion requires an extracellular pH shift that triggers an influx of monovalent ions into the spore (Pleshinger & Weidner 1985; Frixione *et al.* 1992). Calcium may also be involved: it is concentrated in the polaroplast and Ca^{2+} mobilization is associated with swelling (Pleshinger & Weidner 1985; Leitch et al. 1993). Polar filament extrusion has been successfully stimulated by monovalent cations

(Frixione et al. 1994) and inhibited by removal of Ca^{2+}, raising osmolarity, and use of D_2O, ethanol, and Hg^{2+} (Leitch et al. 1993; Frixione et a. 1997). Extrusion rate also overestimates viability as not all spores that extrude a polar filament also eject the sporoplasm (Amigó et al. 1996).

I found *L. salmonae* spore extrusion rates decreased over time reaching 0.0% after 100 d storage (Fig. 33). Amigó et al. (1996) also reported decreased extrusion rates for *G. stephani*, although rates did not reach 0.0%. I was still able to produce infections in fish using spores that exhibited no extrusion in hydrogen peroxide. The osmolarity of the host alimentary canal may induce *L. salmonae* spores to extrude when hydrogen peroxide does not. *Loma salmonae* spores lasted longest in fresh water although I did not determine the maximum time that spores remain viable. It is possible that spores may be viable for longer than 100 d, however, I did not detect infections in 1 trial at 100 d. The rapid decrease in intensity of infections produced by spores stored for 95 d or longer indicates a lower number of viable spores, and it is unlikely *L. salmonae* spores would be viable for a year as described for some fish-infecting microsporidia (Lom & Dyková 1992). Spores of *L. salmonae* are also killed by rapid or slow freezing in fresh or sea water, limiting the methods of long-term storage available to a researcher.

Although *L. salmonae* occurs in ovaries of sexually-mature salmon, I do not know whether the parasite can be transmitted through eggs. Even if spores are not transmitted within eggs, eggs are often contaminated with blood and fecal material during spawning, and may become exposed to spores through contaminated ovarian fluid. Oral treatments with fumagillin (Kent & Dawe 1994; Higgins et al. 1998) and quinine hydrochloride (Speare et al. 1998c) may reduce *L. salmonae* infections in fish, but currently there are no approved drugs available to the fish farming industry. Iodine is an effective disinfectant for most egg pathogens and has been widely adopted by the

aquaculture industry (Evelyn et al. 1986). For salmonid eggs, the normal treatment is 100 ppm for 10 min at pH 7.0.

I found that iodine at 100 ppm reduced *L. salmonae* infections and was most effective at 200 ppm. Although infections still developed, treatment decreased intensity of infections, and delayed their detection. Kumagai et al. (1998) found iodophor treatment of *Cytophaga psychrophila* reduced live bacteria numbers from salmonid eggs, but failed to kill all bacteria even at 1000 ppm iodophor. My treatment was applied to very heavily infected gill homogenates, and use of iodophores on eggs and ovarian fluid in a field situation might be more effective as number of spores and reactive organic matter would be lower. Fertilized salmonid eggs can tolerate concentrations as high as 200 ppm iodine, although they require a pH > 6.0 (Alderman 1984). Fish farmers will have to determine what concentration is acceptable for each species and stock as mortality of eggs due to iodophor toxicity varies with species, parental stock, pH, egg condition, and state of development (Alderman 1984; Fowler & Banks 1990, 1991).

My results indicate that exclusion of methylene blue and extrusion rates of *L. salmonae* spores decrease when subjected to extremes in temperature or long term storage. The most practical method for evaluating spore viability is their infectivity in fish. Vital staining with methylene blue overestimates spore viability. I was unable to determine the maximum viability of *L. salmonae* spores but my findings suggest it may be close to 100 d in fresh or seawater at 4°C. To prevent transmission of the parasite to progeny, I recommend that farmers avoid contamination of gametes with tissue products (e.g., ovarian tissue or blood), and continue to use standard iodophor disinfection protocols for eggs, using the highest concentrations that are non-destructive to eggs or embryos.

Table 4. *Loma salmonae* spore viability as measured by methylene blue exclusion for various treatments in dechlorinated (aquarium) fresh water in trial 1

Treatment	Length of treatment	Percent excluding methylene blue
24 °C	24 h	68.0
24 °C	7 d	70.8
92 °C	10 min	4.5
97 °C	10 min	0.0
-20 °C	1 h	26.5
-20 °C	48 h	7.3
-70 °C	24 h	38.3
-70 °C[a]	24 h	3.0

[a]Solution was frozen, thawed, and refrozen

Table 5. Percent viability of *Loma salmonae* spores incubated in fresh water (aquarium) or sea water (ambient) for various times as measured by methylene blue exclusion, *per os* infection of chinook salmon *Oncorhynchus tshawytscha*, and changes in spore number in trial 3

Solution	Incubation time	Percent viable (Methylene blue)	No. positive/No. examined	Spore number ml^{-1}
Fresh water	1 h	85.0	2/2	1.0×10^6
	24 h	84.0	2/2	1.0×10^6
	3 d	82.0	2/2	1.0×10^6
	5 d	82.0	0/2	1.0×10^6
	7 d	83.0	2/2	1.0×10^6
	10 d	84.0	2/2	7.0×10^5
Sea water	1 h	83.0	2/2	1.0×10^6
	24 h	83.0	1/2	1.0×10^6
	3 d	83.0	2/2	1.0×10^6
	5 d	78.0	2/2	8.0×10^5
	7 d	80.0	2/2	7.0×10^5
	10 d	82.0	1/2	7.0×10^5

Table 6. Prevalence of *Loma salmonae* in chinook salmon *Oncorhynchus tshawytscha* infected *per os* with spores previously incubated in fresh water (aquarium), sea water (ambient), or Earl's Balanced Salt Solution (EBSS) over time for 3 separate trials

Solution	Incubation time	No. positive/No. examined		
		Trial 4	Trial 5	Trial 6
Fresh water	24 h	4/5	10/10	9/10
	12 d	3/3	-	-
	18 d	5/5	10/10	-
	50 d	5/5	0/10	9/10
	78 d	-	-	10/10
	95 d	-	-	8/10
	100 d	-	0/10	-
Sea water	24 h	-	10/10	10/10
	12 d	-	-	-
	18 d	-	10/10	-
	50 d	-	10/10	10/10
	78 d	-	-	10/10
	95 d	-	-	10/10
	100 d	-	NA	-
EBSS	24 h	5/5	-	-
	12 d	3/3	-	-
	18 d	5/5	-	-
	50 d	4/5	-	-

- = Not determined
NA = All spores dead

Table 7. Gill material infected with *Loma salmonae* was stored under various conditions and times before *per os* exposure to chinook salmon *Oncorhynchus tshawytscha* at 4.1 x 10^6 spores $fish^{-1}$

Solution	Temperature	Time	No. fish positive/No. fish exposed
DMSO[a]	-20 °C	15 d	0/12
DMSO	-20 °C	13 d	0/20
Dechlorinated freshwater	-20 °C	13 d	0/20
EBSS	-20 °C & -70 °C	24 h & 48 h	0/5
Dechlorinated freshwater	-20 °C & -70 °C	24 h & 48 h	0/5
DMSO	-20 °C	24 h	0/3[b]
Dechlorinated freshwater	-20 °C	24 h	0/3[b]

[a]8% dimethyl sulphoxide
[b]This trial used purified spores

Table 8. Prevalence and intensity of *L. salmonae* in chinook salmon *Oncorhynchus tshawytscha* infected *per os* by a gill homogenate previously exposed to various concentrations of iodine. Fish were examined 56 and 70 d after exposure

Trial	Calculated iodine concentration (ppm)	No. positive/No. examined		Intensity of infection (avg. xenomas mm^{-2})	
		56 d	70 d	56 d	70 d
Trial 1 Chinook (avg. 11.9 cm; 13.7 g) Spore dosage = 4.1×10^5	100	5/10	9/10	-	-
	Control	8/10	10/10	-	-
Trial 2 Chinook (avg. 14.2 cm; 22.4 g) Spore dosage = 5.8×10^5	150	1/1	9/10	1.64	0.36
	200	4/10	6/10	0.06	0.13
	Control	10/10	10/10	1.26	0.77

ND = Not determined

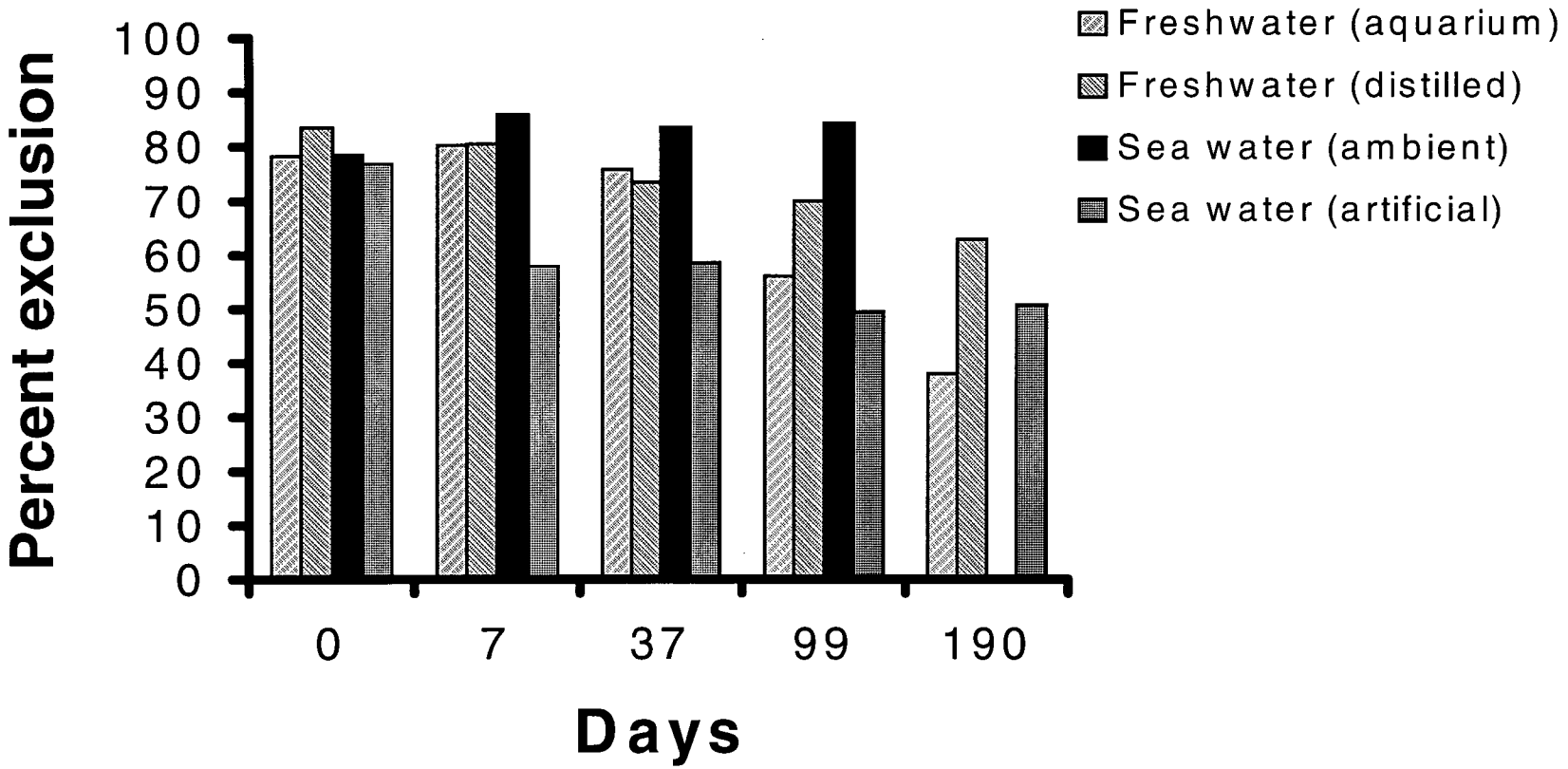


Fig. 32. Viability of *Loma salmonae* spores held in various solutions at 10°C over time as measured by methylene blue exclusion in trial 2

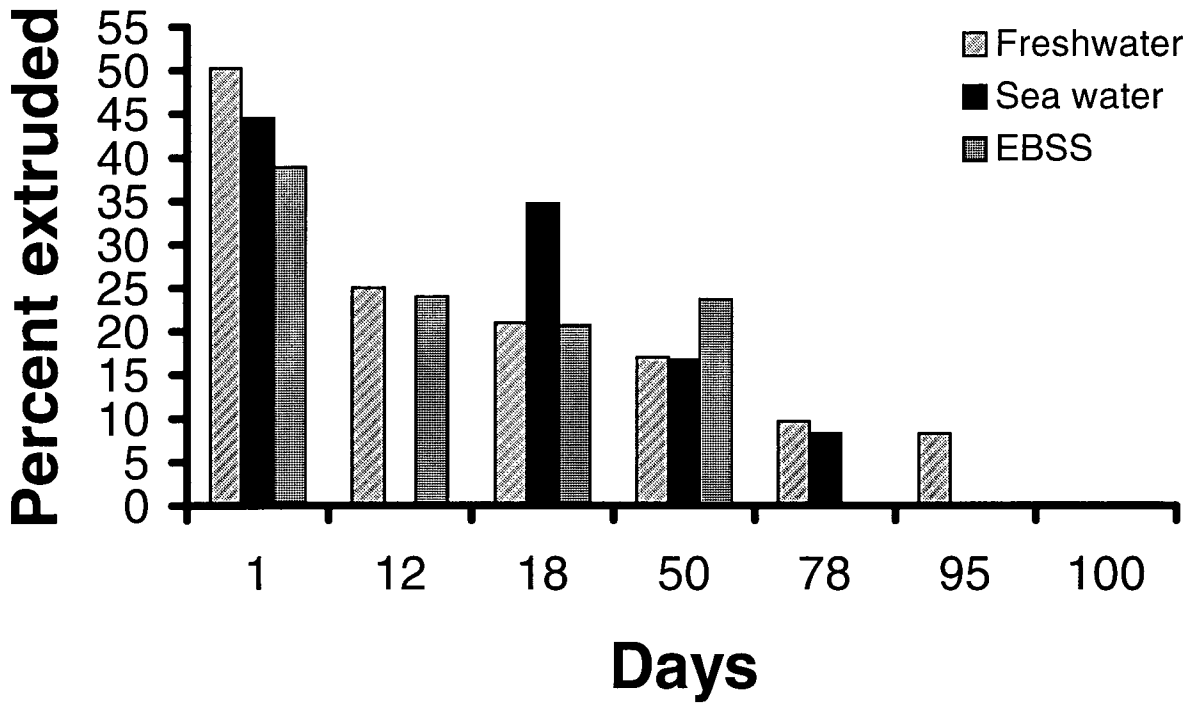


Fig. 33. Average percent extrusion of *Loma salmonae* spores in a 3% H_2O_2 solution from trial 4-6, after storage at 4°C in fresh water (aquarium), sea water (ambient), or Earl's balanced salt solution (EBSS) over time

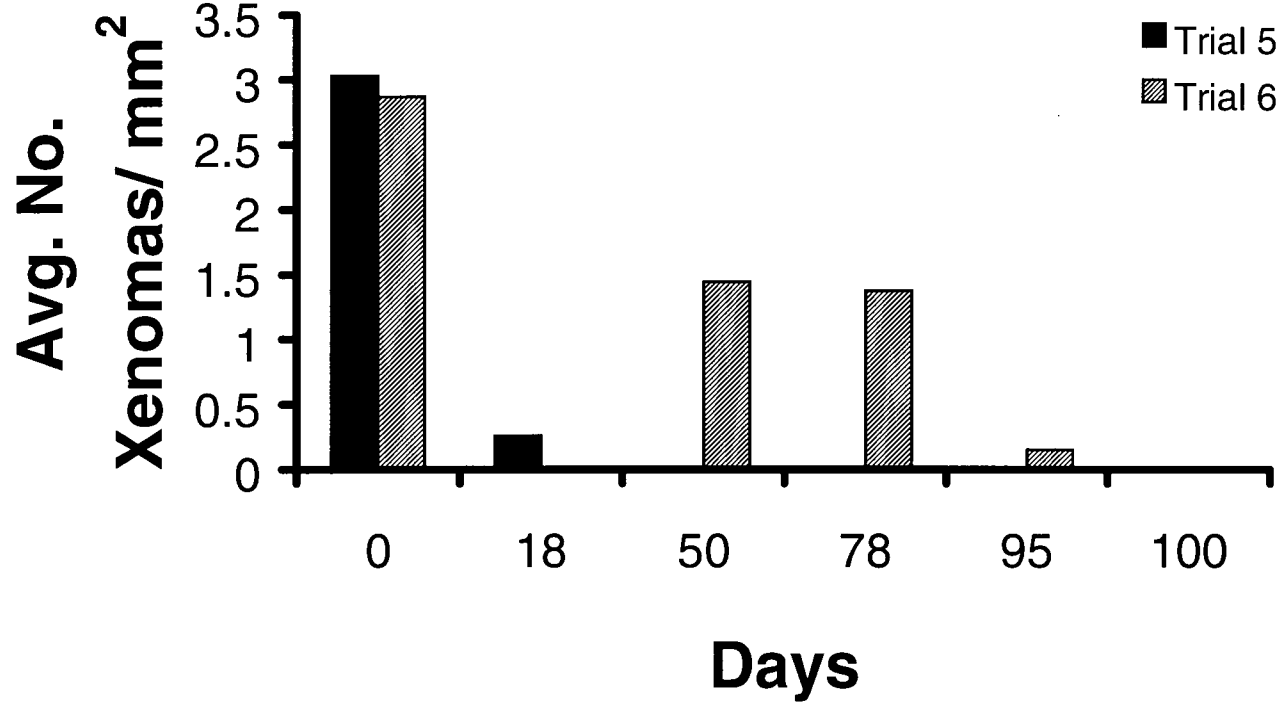


Fig. 34. Average xenoma intensity of *Loma salmonae* in chinook salmon *Oncorhynchus tshawytscha* from trial 5 & 6 infected *per os* with spores stored at 4°C in fresh water (aquarium) for increasing periods of time

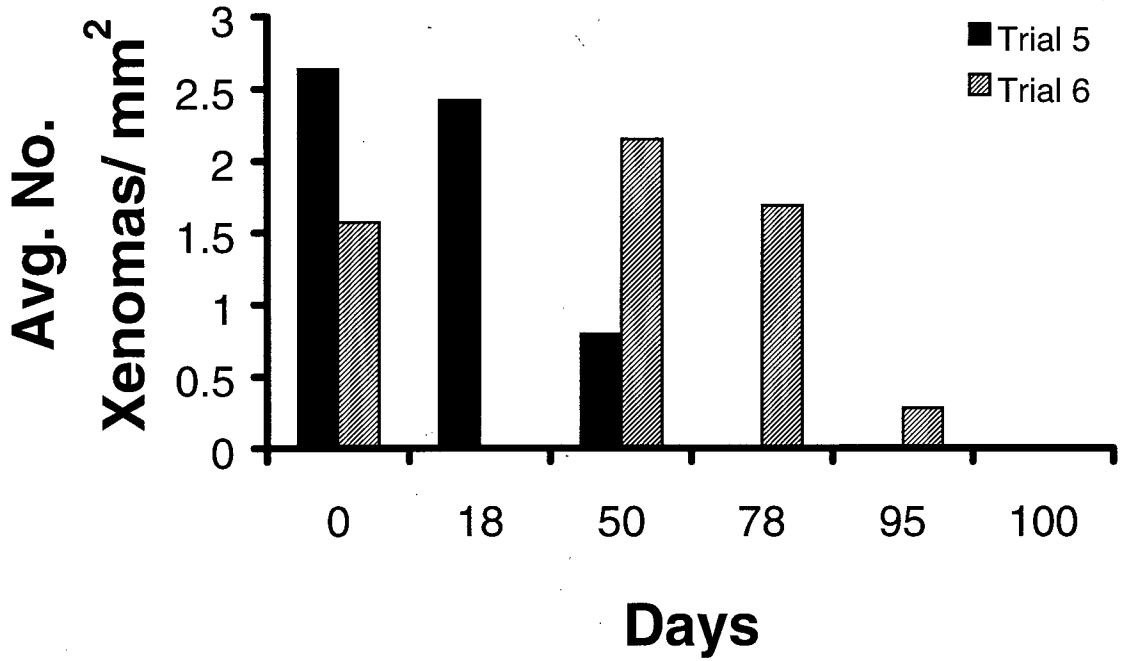


Fig. 35. Average xenoma intensity of *Loma salmonae* in chinook salmon *Oncorhynchus tshawytscha* from trials 5 & 6 infected *per os* with spores stored at 4°C in sea water (ambient) for increasing periods of time

CHAPTER IV

Host Susceptibility

INTRODUCTION

Loma salmonae occurs mainly in species of Pacific salmon, although Poynton (1986) described it in brown trout and Wales & Wolf (1995) in an unidentified *Cottus* sp. Recently Shaw et al. (1997) described *L. embiotocia* in shiner perch, and new *Loma* spp. are being found in numerous marine fishes (Kent et al. 1998). Host specificity of *L. salmonae* was first examined by Kent et al. (1995a) who unsuccessfully attempted to infect Atlantic salmon, shiner perch, and Pacific herring. Further research has been limited to documenting natural infections of *L. salmonae*.

Genetic variation between strains of fish from different geographic areas has been demonstrated in viral, bacterial, and protist infections. LaPatra et al. (1996) found hybrid strains of brown and lake trout *Salvelinus namaycush* Walbaum were more resistant to infectious hematopoietic necrosis virus (IHNV) than were rainbow trout. Carp *Cyprinus carpio* L. exhibit variable resistance to furunculosis *Aermonas salmonicida* (Houghton et al. 1991), and Atlantic salmon, chinook, coho, and chum salmon show variation in resistance to vibriosis *Vibrio salmonicida*, bacterial kidney disease *Renibacterium salmoninarum*, and furunculosis (Withler & Evelyn 1990; Beacham & Evelyn 1992; Gjedrem & Gjøen 1995). Clayton & Prince (1992) noted both inter and intraspecific variation in resistance to the ciliate *Ichthyophthirius multifiliis* Fouquet, 1876. Numerous studies have examined differential susceptibility of salmonid strains to the myxosporean *Ceratomyxa shasta* Noble, 1950 and haemoflagellate *Cryptobia salmositica* Katz, 1951 (Bower & Evelyn 1988; Ibarra et al. 1991, 1992, 1994; Bower 1995; Bower et al. 1995; Forward et al. 1995; Forward & Woo 1996). Chevassus & Dorson (1990) and Wiegertjes et al. (1996) provide extensive reviews of genetic

variation in resistance to disease in fish.

No studies have examined host specificity of *L. salmonae* or strain differences within chinook salmon. Therefore, I assessed host specificity of *L. salmonae* with selected salmonid species, and unrelated fresh and seawater fish species. A fish farming company had anecdotally reported different susceptibility of 3 chinook strains to *L. salmonae*. This was investigated by infecting these strains under controlled conditions.

MATERIALS AND METHODS

Species susceptibility. Fishes were tested as outlined in Table 9, and all were tested using the following methodology except where noted. Twenty fish from a test species were placed in a tank (fresh or seawater dependent on fish species) with 10 positive control fish (chinook or coho), and taken off food for 2 d. Only 10 goldfish *Carassius auratus* L. and 10 guppies *Poecilia reticulata* were tested. Fish were fed macerated gill tissue over 3 d. During feeding, water flow was turned off for 2 h to facilitate ingestion of gill tissue. On the fourth day 10 experimental fish and 5 controls were removed and infected *per os* with a gill slurry (see Chapter II), fin clipped, and placed back in the tank. Using *per os* exposure ensured at least 10 fish were exposed to the parasite. All fish were examined 56 d later by wet mount and histology for *L. salmonae*.

When test species were non-salmonids and smaller than controls, the test species were kept in a separate tank during the feeding of macerated gill tissue. On day 4 after exposure all fish were placed in the same tank.

Strain susceptibility. Susceptibility of 3 strains (named after egg source) of chinook was examined in 2 separate trials. Southern Coastal (SC) is named after wild stocks of Big Qualicum River, Vancouver, Island, Canada. Northern Stream (NS) is named after

the Yukon River, Yukon, and a hybrid (H) of these 2 strains. All fish were obtained from Sea Springs Hatchery, Duncan, Vancouver, Island, Canada. Before trials, 30 fish from each strain were screened by wet mount, and histology for infection by *L. salmonae*.

Trial 1

Seventy fish strain^{-1} were fin-clipped, placed in separate deep-oval tanks (fresh water, 18°C), taken off food for 2 d, and then fed 23.8 g of macerated *L. salmonae* infected gill tissue tank^{-1} over 3 d. On day 4 all fish were placed in a single tank to which 17 controls strain^{-1} were added. Length and weight were recorded 27 d later when fish were examined for prevalence and intensity of *L. salmonae* by histology only. Prevalence and intensity of *L. salmonae* was also recorded for dead fish by wet mount. Viscera from 20 fish strain^{-1} were preserved for histological examination.

Trial 2

A group of 120 fish strain^{-1} were pit tagged (BioSonics Inc., Seattle, WA), and held in tanks for 4 wk to heal from tagging. One hundred fish strain^{-1} were given *L. salmonae per os* at 4.5 x 10^6 spores ml^{-1}, and placed in 1 deep-oval tank. A day later 10 controls strain^{-1} were added. Length and weight of fish, and prevalence and intensity of *L. salmonae*, was recorded in dead fish, and all fish after 43 d (50% mortality overall). At 43 d all fish were examined for the parasite by histology and wet mounts.

Statistics. Prevalence data were tested using a Chi-square test for goodness of fit. Intensity data was log transformed X'=log(X+1), then tested with an analysis of variance (ANOVA), followed by a Tukey Honestly Significantly Different (HSD) test (Zar 1984). Histological and wet mount enumeration were compared as an estimate of abundance in trial 2. Time to death and survival was also tested for trial 2 first by ANOVA, and then by THSD test.

RESULTS

Host susceptibility. All 5 non-salmonid species tested were negative, although controls became infected (Tables 9 & 10). All salmonid species tested became infected except Atlantic salmon and Arctic char. In most cases, a higher number of positives were obtained using the *per os* method of infection rather than feeding macerated tissue.

Strain susceptibility. All pre-screened fish were negative. Average size of fish in trial 1 was: SC 11.0 cm, 11.6 g; NS 9.3 cm, 6.9 g; H 12.9 cm, 21.0 g. Average size for trial 2 was: SC 12.3 cm, 22.0 g; NS 9.5 cm, 10.0 g; H 11.6 cm, 18.0 g. There were no significant differences between strains for prevalence of infection, however, intensity was consistently significantly higher ($p<0.001$) in the NS fish (Table 11). One outlier was omitted in trial 1. In trial 2 the NS strain had significantly higher numbers of xenomas when screened by either histology or wet mount (Table 12). This was also true for mortalities (Table 13), although days to death did not differ significantly ($p=0.152$) between strains of chinook (Fig. 36). Survival in strain NS was significantly lower ($p=0.0$).

Loma salmonae was distributed in all organs sampled (Table 14; Figs. 37-40).

DISCUSSION

The ability to infect chum and pink salmon verifies that *L. salmonae* can infect all 7 species of Pacific salmon. Anonymous (1984) recorded *L. salmonae* in gills and somatic muscle of pink, chum, and coho from Kemano river, and rainbow trout from Kenny Dam area British Columbia, Canada. They identified spores from stained smears of somatic muscle suggesting fish were heavily infected. I rarely detected xenomas in sections of muscle (Table 14) and only in the dermis of heavily infected fish (Fig. 39). In

addition I was able to infect brook trout, which is in contrast to findings of Speare et al. (1998a). Brook trout used by Speare et al. (1998a) may have been previously exposed to *L. salmonae* or *L. fontinalis*, and may thus have developed resistance to re-infection (Speare DJ pers. comm.). In light of my ability to infect brook trout, *L. fontinalis* described by Morrison & Sprague (1983) may well have been *L. salmonae*. This should be investigated further using PCR primers for *L. salmonae* developed by Docker et al. (1997a) on samples of *L. fontinalis*. *Loma* spp. that are distinct tend to differ in their ribosomal DNA sequence (rDNA). For example, Shaw et al. (1997) found that the internal transcribed spacer region of *L. embiotocia* differed significantly from that of *L. salmonae* (see Chapter VI).

Usually most fish are susceptible to initial infection but differ in their ability to limit or destroy the pathogen (Wiegertjes et al. 1996). The terminology "resistant" and "susceptible" therefore, is always relative. A reproducible method of exposure is vital in demonstrating differences between strains. My first trial involved feeding macerated gill tissue and larger fish may have consumed more gill tissue leading to higher intensity of infections. Hence, trial 2 where equal doses were given to fish, will be discussed herein. Intensity of infection was significantly higher and survival significantly lower in the Northern Stream (NS) strain compared to the other 2 strains. In addition, samples of wild Y fish screened for *L. salmonae* (see Chapter VI) were negative suggesting this strain may represent a naïve strain. The northern distribution of NS chinook may exceed the natural distribution of *L. salmonae*, or NS fish may be exposed to lower numbers of parasite. Bower et al. (1995) found that coho salmon from the Kitimat River were highly susceptible to *C. salmositica* compared with coho from Big Qualicum River. They postulated this may be because the parasite and it's vector are not enzootic to the Kitimat River.

Natural disease resistance in fish is a complex interaction of genetic and non-genetic factors that may have multiple components (Chevassus & Dorson 1990). Price (1985) discusses genetic factors that include: barriers (i.e. skin and mucous); effects on pathogens of the host environment (i.e. gastric secretions, pH of blood, deficiencies in compounds required by the pathogen, natural antitoxins); and humoral and/or cellular immune responses. Non-genetic factors may include pathogen abundance, virulence, and temperature effects on pathogen development (Ibarra et al. 1994). Differences in disease resistance between strains of fish has been linked to variation in levels of mucus precipitin activity (Cipriano et al. 1994), plasma lipid components (Maita et al. 1998), antibody production (Strønsheim et al. 1994a,b), complement (Røed et al. 1990, 1992, 1993), and respiratory burst activity in head kidney macrophages (Balfry et al. 1994).

I found that significantly fewer Nothern Stream chinook survived than SC or H and that there were no differences in time to death. Survivability is considered the best criterion for evaluating resistance in strains of fish as this represents the sum of all host-parasite interactions (Wassom & Kelly 1990). Ibarra et al. (1991) noted only 13% of the *C. shasta* resistant strain of rainbow trout died compared with 90% of the susceptible strain, although they did not find any difference in time to death (Ibarra et al. 1994). Studies of *C. shasta* suggest two interacting mechanisms: initial invasion by the parasite and the ability of the fish to mount an effective immune response (Ibarra et al. 1994). This may also apply to *L. salmonae*, and possible immunological mechanisms accounting for strain and species differences will be discussed in chapter V. The SC strain has been farmed for a much longer period of time than the NS strain and therefore, may be more susceptible to the stress of captive conditions.

The host range specificity of *L. salmonae* now includes all 7 species of Pacific

salmon (*Oncorhynchus*), and brook trout. Atlantic salmon are resistant and brown trout are susceptible indicating that *L. salmonae*'s host range cannot be strictly determined by the generic status of the host. My findings of a naïve strain of chinook likely demonstrate both genetic and non-genetic factors are involved in *L. salmonae* resistance. Further investigations should include a more extensive genetic analysis, and attempt to validate the abundance of *L. salmonae* in the Yukon River.

Table 9. Species of fish tested for susceptibility to *Loma salmonae* listed by source, and dosage of parasite. Chinook salmon *Oncorhynchus tshawytscha* were used as positive controls

Test species	Source	Macerated gill fed (g)		*Per os* spore dosage
		Test species	Controls	
Carassius auratus	Nanaimo Pet Store, FW	15.0	15.0	8.8×10^5
Cottus asper	Nanaimo River, FW	24.0	24.0	2.5×10^6
Cymatogaster aggregata	Departure Bay, M	15.0	15.0	2.5×10^5
Gasterosteus aculeatus	Departure Bay, M	25.0	15.0	1.0×10^5
Oncorhynchus gorbuscha	Rosewall Creek Hatchery, FW	24.0	24.0	2.3×10^6
Oncorhynchus keta	Rosewall Creek Hatchery, FW	24.0	24.0	4.5×10^6
Poecilia reticulata	Trinidad, FW	0.5[a]	15.0	1.0×10^5
Salmo salar	Rosewall Creek Hatchery, FW	34.0	34.0	1.3×10^6
Salmo trutta	Sea Springs Hatchery, FW	24.0	24.0	3.5×10^5
Salvelinus alpinus	Sun Valley Trout Farm[b], FW	13.0	13.0	1.9×10^6
Salvelinus fontinalis	Summerland Hatchery[b], FW	24.0	24.0	1.9×10^6

FW = Freshwater
M = Marine
[a]Gill lamellae only
[b]Located on mainland British Columbia, all other sources are on Vancouver Island, British Columbia

Table 10. Prevalence of *Loma salmonae* in species tested for susceptibility by feeding infected macerated gill tissue and by *per os* administration of an infected gill slurry. Chinook salmon *Oncorhynchus tshawytscha* were used as positive controls

Test Species	No. positive/ No. examined					
	Test species			Controls		
	Fed gill	*Per os*	Total	Fed gill	*Per os*	Total
Carassius auratus (avg. 6.0 cm; 3.5 g)	0/5	0/5	0/10	5/5	5/5	10/10
Cottus asper (avg. 8.8 cm; 8.8 g)	0/10	0/10	0/20	5/5	5/5	10/10
Cymatogaster aggregata (avg. 8.0 cm; 5.0 g)	0/10	0/10	0/20	5/5	5/5	10/10
Gasterosteus aculeatus (avg. 6.6 cm; 1.7 g)	0/10	0/10	0/20	5/5	5/5	10/10
Oncorhynchus gorbuscha (avg. 17.5 cm; 41.1 g)	3/10	4/10	7/20	3/5	5/5	8/10
Oncorhynchus keta (avg. 16.8 cm; 36.6 g)	3/10	8/9	11/19	4/4	3/5	7/9
Poecilia reticulata (avg. 2.7 cm; 0.2 g)	0/5	0/5	0/10	5/5	5/5	10/10
Salmo salar (avg. 277.8 cm; 193.7 g)	0/10	0/10	0/20	2/5	5/5	7/10
Salmo trutta (avg. 19.8 cm; 80.3 g)	10/10	10/10	20/20	5/5	3/5	8/10
Salvelinus alpinus (avg. 13.5 cm; 25.0 g)	0/10	0/10	0/20	2/5	5/5	7/10
Salvelinus fontinalis (avg. 20.3 cm; 97.2 g)	2/10	3/10	5/20	5/5	5/5	10/10

Table 11. Prevalence and intensity of *Loma salmonae* infection in 3 strains of chinook salmon *Oncorhynchus tshawytscha* after exposure by feeding infected gill tissue (trial 1) or administration *per os* (trial 2) of an infected gill slurry

Screening Method	No. positive/No. examined				Average xenoma intensity mm^{-2} (range)			
	Southern Coastal	Northern Stream	Hybrid	Control	Southern Coastal	Northern Stream	Hybrid	Control
Histology (trial 1)	54/55	43/43	56/62	0/51	1.7 (0.0-4.5)	1.9 (1.0-6.4)	0.9 (0.0-10.2)	0.0 (0.0-0.0)
Histology (trial 2)	59/59	51/51	35/36	0/30	1.0 (0.1-6.0)	4.4 (0.4-20.9)	1.0 (0.0-5.0)	0.0 (0.0-0.0)
Wet mount (trial 2)	58/59	51/51	36/36	0/30	6.1 (0.0-17.0)	18.1 (6.4-38.5)	5.1 (1.2-13.9)	0.0 (0.0-0.0)

Table 12. Significant differences in *Loma salmonae* xenoma intensity between various comparisons of 3 strains of chinook salmon *Oncorhynchus tshawytscha* in 2 trials

Trial (method of screening)	Tukey HSD[a] Comparison		
	Southern Coastal X Northern Stream	Southern Coastal X Hybrid	Hybrid X Northern Stream
Trial 1 (histological)		*	*
Trial 2 (histological)	*		*
(wet mount)	*		*

[a]Honestly Significantly Different
*Statistically different ($p<0.001$)

Table 13. Average xenoma intensity in mortalities of 3 strains of chinook salmon *Oncorhynchus tshawytscha* infected by *per os* with *Loma salmonae* in trial 2

Strain (No. of fish)	Avg. xenoma intensity mm^{-2} (range) in mortalities
Southern Coastal (19)	6.6 (2.3-13.1)
Northern Stream (19)	16.2 (2.4-42.1)
Hybrid (8)	7.3 (3.5-11.9)

Table 14. Distribution of *Loma salmonae* in organs of 3 strains of chinook salmon *Oncorhynchus tshawytscha* after exposure by feeding infected gill tissue (trial 1) or administration *per os* (trial 2) of an infected gill slurry. Values for tissue are percentage of fish showing infection in organ indicated

Trial	Strain	No. Fish	Tissue								
			Gill	Heart	Kidney	Spleen	Liver	Intestine	Pyloric caeca	Dermis	Mesentery
One	Southern Coastal	20	100.0	20.0	0.0	30.0	1.0	-	-	-	-
	Northern Stream	20	100.0	10.0	0.0	40.0	0.0	-	-	-	-
	Hybrid	20	100.0	20.0	35.0	30.0	0.0	-	-	-	-
Two	Southern Coastal	58	100.0	39.7	31.0	32.8	1.7	1.7	5.2	1.7	0.0
	Northern Stream	51	100.0	68.6	96.1	49.0	1.7	5.9	9.8	7.8	9.8
	Hybrid	36	97.2	16.7	58.3	8.3	0.0	8.3	5.5	2.8	5.5

- = Not determined

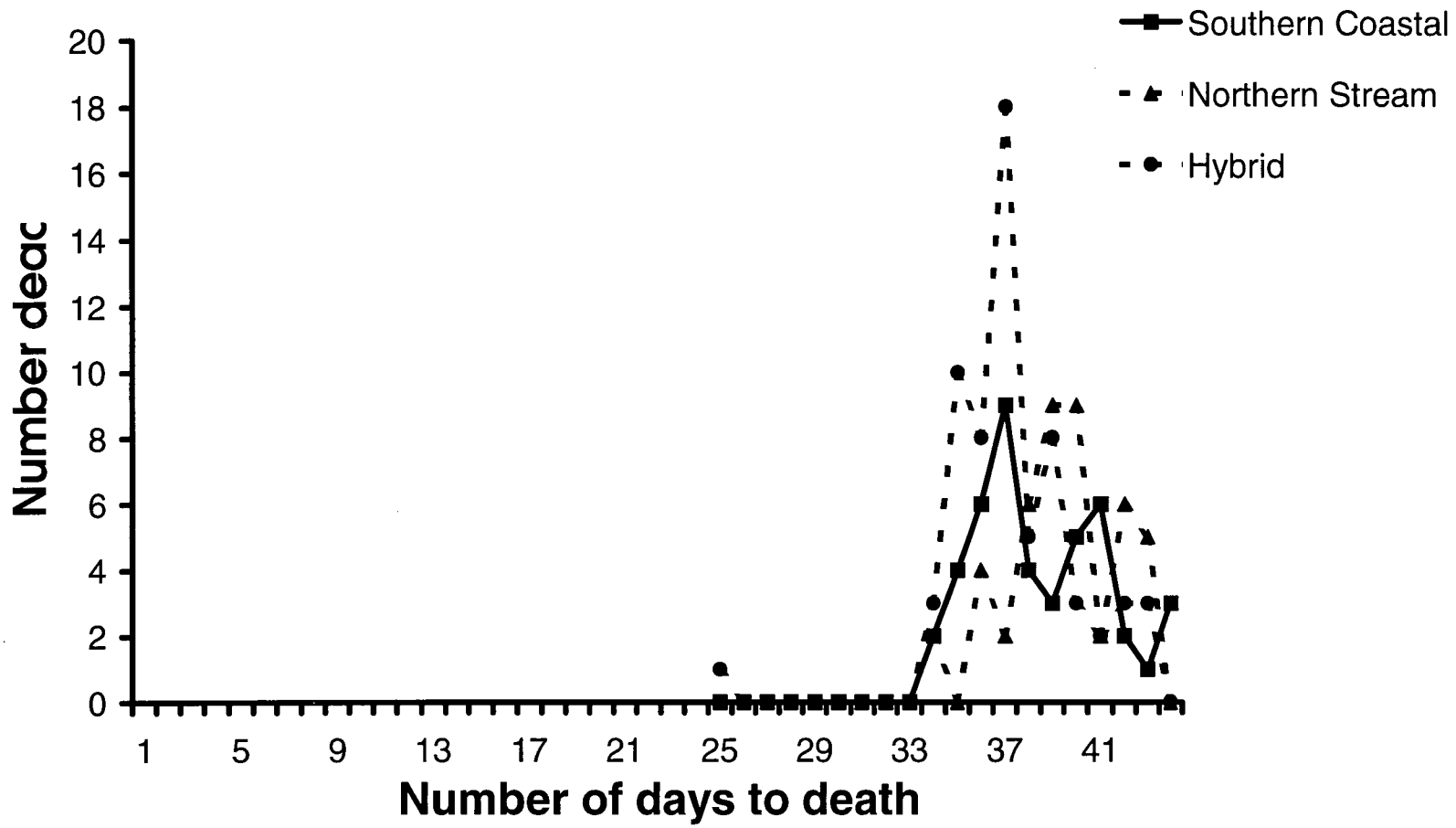


Fig. 36. Mortality in 3 strains of chinook salmon *Oncorhynchus tshawytscha* over time after *per os* infection with *Loma salmonae*

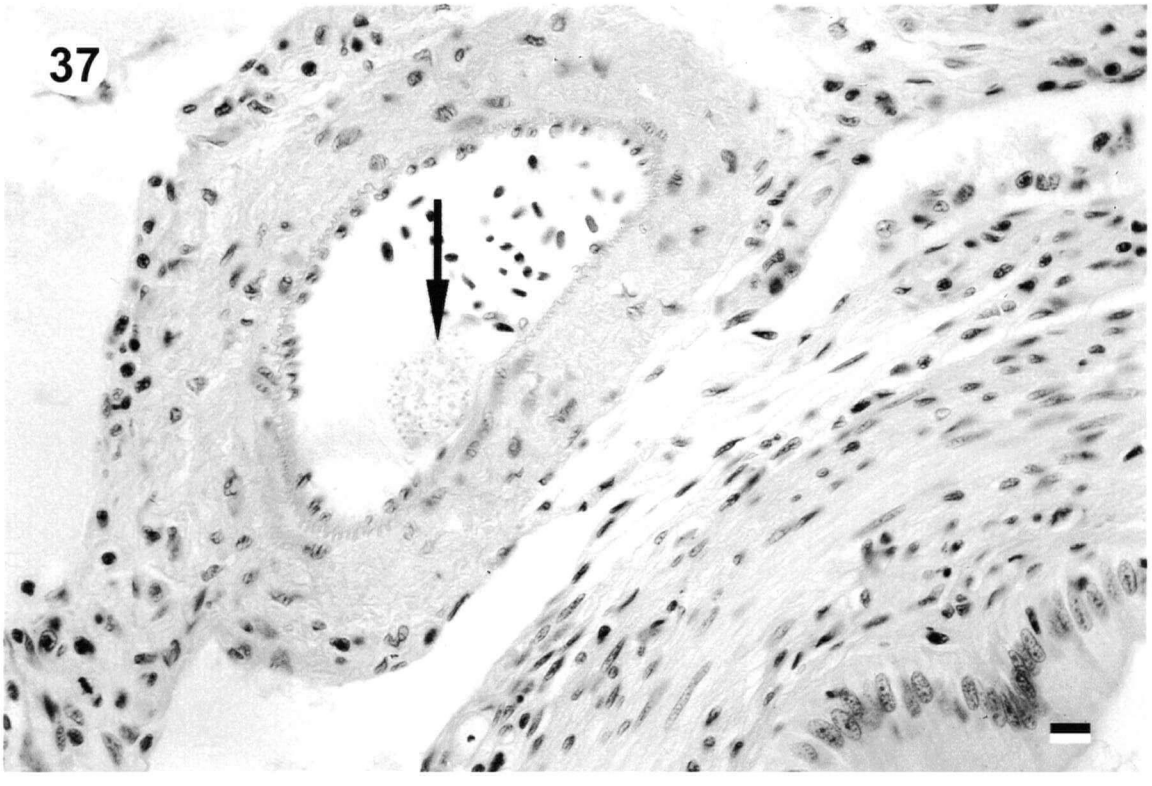
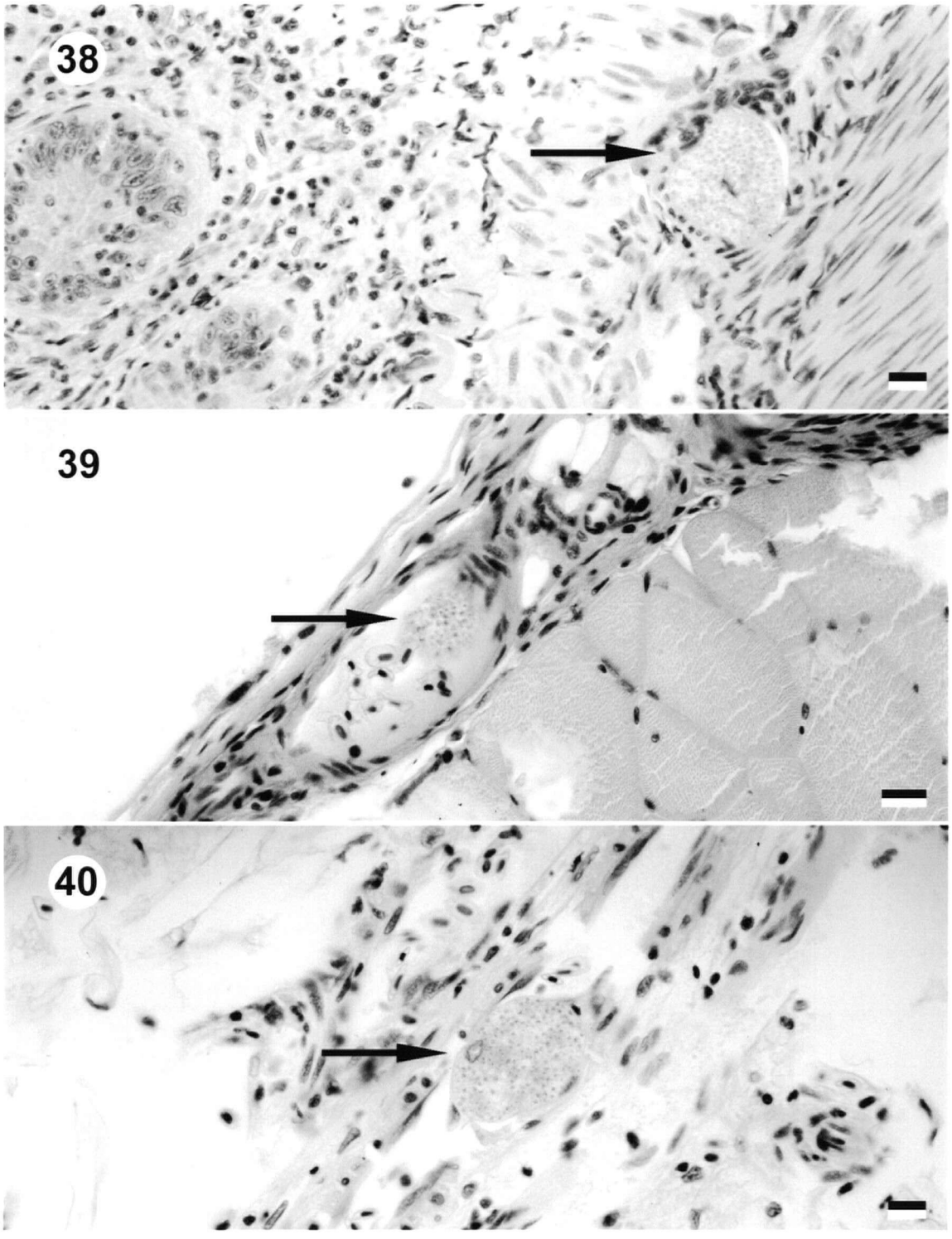


Fig. 37. Histological section of a *Loma salmonae* xenoma denoted by arrow in a chinook salmon *Oncorhynchus tshawytscha* artery adjacent to pyloric caeca. Scale bar = 14 μm



Figs. 38 to 40. Histological sections of *Loma salmonae* xenomas in chinook salmon *Oncorhynchus tshawytscha* tissue. Fig. 38. Xenoma denoted by arrow in a section of intestine. Scale bar = 20 μm. Fig. 39. Xenoma denoted by arrow in dermis of dorsal muscle section. Scale bar = 20 μm. Fig. 40. Xenoma denoted by arrow in mesentery. Scale bar = 20 μm

CHAPTER V

Immunology

INTRODUCTION

Although they are generally poorly understood, humoral and cellular responses to microsporidian infections occur in fish (Kim et al. 1996). Specific humoral immune responses have been reported in fish serum (Leiro et al. 1993, 1996a,b; Kim et al. 1996; Hung et al. 1997), and mucosal secretions (Hung et al. 1996). A first line of defense includes proteases, lysins, and agglutinins in mucosal secretions, followed by mucosal cells, blood cells, and finally the reticuloendothelial system, comprised of endocytically active cells such as endothelial cells, granulocytes, and macrophages (Dalmo et al. 1997).

Phagocytes such as granulocytes, monocytes, and macrophages are believed to be important in microsporidian infections of fish (Secombes & Fletcher 1992). Macrophages are present throughout the animal kingdom and play a central role in non-specific defense (Dalmo et al. 1997). Although macrophages recognize and attack pathogens, they may also act as a route of infection. In mice infected with *Encephalitozoon cuniculi*, spores are engulfed by macrophages but not destroyed due to prevention of phagosome-lysosome fusion, and the microsporidia *E. cuniculi* replicates within the macrophage (Weidner 1975; Schmidt & Shadduck 1984). A similar mechanism was discovered in *Glugea hertwigi* by Weidner & Sibley (1985) and it has been suggested macrophages may act as a transport mechanism for microsporidia in fish (Matthews & Matthews 1980; Canning & Lom 1986). Thus, examining the phagocytic response in fish may lead to a better understanding of interactions between microsporidia and fish (Secombes & Fletcher 1992).

Tissue reactions to *L. salmonae* have been well described (Hauck 1984; Kent et

al. 1989; Speare et al. 1989), but immune responses of the host are unknown. I investigated the *in vitro* phagocytosis of *L. salmonae* spores by a resistant species Atlantic salmon and two susceptible strains of chinook (Southern Coastal and Nothern Stream). The effects of complement activity on phagocytosis were quantified and compared to uptake of Baker's yeast *Sarccharomyces cerevisiae*.

MATERIALS AND METHODS

Macrophage isolation. Macrophages were isolated following protocol of Secombes (1990) with modifications described herein. All blood was removed from fish using heparinized vacutaners (VWR) and centrifuged at 2000 x *g* for 10 min at 18°C. Plasma was collected and frozen at -86°C until use. Base media was RPMI-1640 (Sigma) with phenol red, and 80 u ml^{-1} heparin (Sigma) unless indicated otherwise. All solutions and tissues were kept at 4°C. Total length and weight of fish was recorded before the head kidney was aseptically removed and placed in 25 ml media. Head kidney was macerated and pushed through a wire mesh tissue separator (Sigma) while being rinsed with media. The filtered suspension was centrifuged at 500 x *g* for 15 min and resuspended in 30 ml media using a pipette and 5 s of vortexing. This was layered in 3 ml aliquots on 34%/51% Percol (Sigma) gradients, centrifuged at 800 x *g* for 30 min at 4°C and macrophages recovered from the interface. Macrophages were washed twice (500 x *g* 10 min), counted using 0.25% Trypan blue (Sigma), and settled for 90 min (18°C) at 1.0 x 10^7 cells ml^{-1} in Leighton tubes (GibcoBRL) containing 9x24 mm glass coverslips. Macrophages were then washed twice with media (18°C) and incubated for 48 h and media changed every 24 h before phagocytic assays. Adherence to glass and preincubation for at least 48 h before an assay results in > 95.0% macrophage purity (Secombes 1996).

Spore treatments. *Loma salmonae* spores were purified (see Chapter II), kept at 4°C, and used within 1 wk. All centrifugation was at 2000 x *g* for 10 min at 4°C and incubation during an assay was done at 18°C for 90 min. Plasma collected from fishes was thawed on ice and subdivided into 500 µl aliquots. Four treatments were prepared: normal plasma, no plasma (media only), and heat and EDTA inactivated plasma. Two replicates were run for each treatment. Plasma was heat inactivated at 50 °C for 30 min. A final concentration of 0.04 molar EDTA was used to inactivate plasma. Spore number was adjusted to 2.5 x 10^6 ml^{-1}, 1 ml aliquots centrifuged, resuspended, and incubated in individual treatments.

Phagocytic assays. After incubation, spores were centrifuged, resuspended in 1 ml media, and incubated in Leighton tubes with macrophages. Macrophages were then washed twice with the basal medium (18°C), and fixed in 70% methanol for 24 h, dried, and used in IFAT assays.

IFAT assays. The same protocol as described for blood smears (see Chapter II) was used on coverslips with fixed macrophages. A final wash with distilled water was added to the protocol, and slides read 24 h later with a fluorescent microscope.

Percent phagocytosis (PP) was defined as the percent of spores phagocytosed, and enumerated by counting 300 random spores as either phagocytosed or not (bound to surface of macrophages). The average of two replicate counts was recorded. Phagocytic index (PI) was defined as the average number of spores engulfed per macrophage, and was enumerated by counting the number of spores in 100 randomly selected macrophages that had engulfed spores.

Yeast assays. Baker's yeast *Sarccharomyces cerevisiae* (Sigma) was inactivated as described by (Matsuyama et al. 1992) with modifications described herein. Yeast was heated at 100°C for 1 h in PBS, washed 3 times, filtered through 50 um Nytex, and

stored at -86°C until use. Macrophages were purified as described previously, and yeast cells were incubated in the same treatments as described for *L. salmonae* spores. Fixed macrophages were stained with Wright's Modified Leukostat Stain (Sigma) and examined at x1000.

Fish types. Atlantic salmon, and 2 strains of chinook Southern Coastal and Nothern Stream (see Chapter IV) were examined for PP and PI of *L. salmonae* spores and yeast. Ten fish of each strain were used for *L. salmonae* spores, and 5 of each strain for yeast assays.

Statistics. All prevalence data was tested using a Chi-square test for goodness of fit. Intensity data was log transformed X'=log(X+1), and then tested with an ANOVA. Percent phagocytosis was examined with a 3 x 4 contingency table with 2 factors: fish type, and treatment. A Chi-square test when applicable, was used to test for differences between levels of the factors as outlined in Table 16. Phagocytic index was square root transformed $X' = \sqrt{X}+.05$, and tested with an ANOVA, followed by a Tukey HSD test.

RESULTS

Phagocytic assays. Macrophages used in assays were purified from large fish (Table 15). *Loma salmonae* spores were easily differentiated as bound or engulfed (Figs. 41 & 42) by macrophages. Bound or engulfed yeast cells were also easily separated by phagosome formation around engulfed yeast cells (Fig. 43).

Atlantic salmon macrophages had significantly ($p<0.001$) higher PP and PI than chinook (Figs. 44 & 46; Tables 16 & 17). Incubating spores in normal plasma produced significantly ($p<0.001$) higher uptake than in the other treatments for all fish types. No plasma or heat treated plasma resulted in equally low uptake however, EDTA treated plasma resulted in significantly ($p<0.05$) higher PP of spores compared to the former

treatments. There were no significant differences between fish strains or species in PP and PI of yeast cells (Figs. 45 & 47).

DISCUSSION

Percent phagocytosis is usually defined as the percentage of cells containing target particles (Leiro et al. 1995). This definition was not useful in my study as greater than 95.0% of cells did not contain target particles therefore, I enumerated the number of target particles phagocytosed. Leiro et al. (1995) found that phagocytic uptake is affected by temperature, time, and pathogen to phagocyte ratio (R). For turbot spleen cells ingesting *Glugea caulleryi* spores R had the greatest impact on phagocytosis. R was optimal at 68:1 for PP and 85:1 for PI of *G. caulleryi* spores. Secombes & Fletcher (1992) reported optimal killing of bacteria by macrophages usually occurred at an R of 1:2 or 1:10. It is difficult to estimate the R value in my study as a final cell count was not done (i.e., not all cells settled at 1.0×10^7 ml^{-1} adhered to the glass nor survived the initial 48 h before assays were conducted). After pilot experiments with various numbers of spores, I chose a value that was easily read by IFAT, and resulted in PI values of 1 or more. As PP is considered less sensitive than PI (Leiro et al. 1995), my discussion herein will focus on the latter.

I found significantly higher uptake by macrophages of spores preincubated in normal plasma versus heated, no plasma, or EDTA inactivated plasma. My results also suggested a higher uptake of yeast cells preincubated in normal plasma, but this was not statistically significant. Engstad & Robertsen (1993) also found this relationship with yeast particles engulfed by Atlantic salmon macrophages. Higher uptake of particles by fish macrophages in normal serum is well established (Sakai 1984; Johnson & Smith 1984; Matsuyama et al. 1992; Rose & Levine 1992; Sakai 1992; Secombes & Fletcher

1992). Normal serum contains complement (C') which increases phagocytosis through opsonization. Heat inactivation of serum inhibits C' activity due to the presence of heat labile C' components in serum (Sakai 1992). In addition, incubation of opsonized particles with antisera to fish C3 negates any opsonizing effect (Secombes 1996). Divalent cation Ca^{2+} is essential for phagocytosis (Secombes 1996) and EDTA binds such free cations. Conflicting reports as to the effectiveness of C' as an opsonin are a result of variation in pathogens and species examined (Secombes & Fletcher 1992). Bower & Evelyn (1988) demonstrated the importance of C' in acquired and innate resistance of sockeye salmon stocks to *Cryptobia salmositica*. Lysis of the parasite was mediated by C' and triggered by activation of the alternative pathway. Although reports certainly suggest the presence of C' receptors on salmonid macrophages, no receptors have been isolated or cloned (Dalmo et al. 1997).

In my study, Atlantic salmon macrophages had significantly higher spore uptake than macrophages of chinook. Atlantic salmon are resistant to *L. salmonae* (Kent et al. 1995a) and higher phagocytic ability might be one of many mechanisms contributing to resistance. The main sites of phagocytic activity in fish include the lymphoid organs (kidney and spleen), peritoneum, atrium of the heart, and gills (Leiro et al. 1995). Mucosal intraepithelial and free lymphocytic aggregations have been identified in fish (Kaattari & Piganelli 1996; Steinhagen & Jendrysek 1994), and macrophages also exist free in the blood. Therefore, spores are likely recognized when initially entering the alimentary canal and later during autoinfection (see Chapter II). Microsporidia spore surface antigens are first recognized by fish immune cells (Leiro et al. 1993; Kim et al. 1996), and spores also have recognition domains for antibody (Pomport-Castillon et al. 1997). Kim et al. (1996) proposed that spores of *G. plecoglossi* in ayu gut epithelium result in delivery of antigens by intestinal phagocytes eliciting the immune response.

Atlantic salmon may also have a higher C' activity or use another non-specific defense such as C-reactive protein, which can act as an opsonin (Dalmo et al. 1997). Alternatively, *L. salmonae* may have evolved a mechanism to evade or suppress initial binding by macrophages of chinook. Bacteria have many mechanisms for avoiding phagocytosis. They can avoid opsonization with C' by intracellular infection, or by producing capsules (Matsuyama et al. 1992; Sakai 1992). Leukocytolysins produced by bacteria extracellularly can prevent ingestion by macrophages, and bacteria can resist intracellular killing within phagolysosomes (Goren et al. 1976; Sakai 1992). Leiro et al. (1996a) suggested that microsporidia have developed mechanisms for avoidance or suppression of the host immune response. Little information exists on these mechanisms in fish beyond immunological suppression (see Immunology Chapter I). Leiro et al. (1996b) proposed the phagocytic response of turbot is modulated by *G. caulleryi* to the extent that serum antibody responses are decreased.

Although I did not find differences between the 2 strains of chinook in phagocytic uptake of spores, there may be differences in respiratory burst activity. Respiratory burst activity is believed to be phylogenetically conserved (Dalmo et al. 1997), and this might account for strain susceptibility differences I found (see Chapter IV). In addition, *L. salmonae* may prevent killing by macrophages in *G. hertwigi* (Weidner & Sibley 1985) through prevention of phagosome-lysosome fusion. Further investigations should examine this possibility at the ultrastructural level.

In contrast to my findings with *L. salmonae*, Leiro et al. (1996b) found no significant differences in PP or PI of turbot spleen phagocytes (macrophages) given C' or antibody treated *G. caulleryi* spores. They suggested turbot serum might contain enzymes with chitinolytic activity degrading the number of spore surface binding sites. In addition to activity of C' varying by species and target pathogen, macrophage activity

may vary with tissue type and species assayed (Secombes & Fletcher 1992; Neumann & Belosevic 1996; Dalmo et al. 1997). My results demonstrate that generalizations about C' activity cannot be made about *in vitro* phagocytosis of fish-infecting microsporidia.

Southern Coastal chinook had significantly higher phagocytosis of EDTA treated spores than Atlantic salmon indicating EDTA treatment of plasma may have been ineffective. Alternatively, non-specific binding might have occurred via lectins on the surface of macrophages or in the plasma to carbohydrates on the spore surface. Lectinophagocytosis is used to describe this process (Ofek & Sharon 1988). Saggers & Gould (1989) reported "lectin like" receptors in macrophages from tilapia aided in the phagocytosis of bacteria. The authors inhibited phagocytosis by blocking with various sugars, and proposed a dual function CR3 receptor similar to that described on human macrophages (Ross et al. 1985). This receptor contains a lectin and C' part that act independently. My results show that macrophages from both chinook and Atlantic salmon phagocytosed yeast cells quite well regardless of treatment. The yeast cell wall is composed of β-glucans which stimulate the nonspecific defense of animals. Jørgensen et al. (1991) reported 50-60% of salmon macrophages phagocytosed glucan particles in a serum free medium. Engstad & Robertsen (1993) demonstrated that Atlantic salmon macrophages recognize β-glucan through either a specific receptor or indirectly as the glucan binds C' or other serum opsonins: i.e., fibronectin is an important component of salmonid serum. In addition, their heat and EDTA treatments caused marked, although not statistically significant, reduction in opsonizing ability of serum. This may have been an artifact of their small sample size. Leiro et al. (1996b) also found a significant difference in sodium *m*-periodate (a mild oxidizing agent) treated spores and suggested lectinophagocytosis may play an important role in phagocytosis

of microsporidian spores.

Although chinook salmon (ML Kent pers. comm.) and rainbow trout (Speare et al. 1998b) develop resistance to *L. salmonae* when re-exposed, the mechanism of resistance is unknown. It may involve phagocytosis by macrophages and expression of antigens and/or aspects of the humoral defense system. Neutralization and C' fixation antibody and cell mediated immunity all play an important part in acquired immunity (Hung et al. 1996). Kim et al. (1996, 1997) found that antibodies to intact *G. plecoglossi* spores are produced by ayu but do not play a protective role against infection.

In conclusion, my results show that C' opsonization of *L. salmonae* spores leads to a significant increase in *in vitro* uptake by macrophages of Atlantic salmon and chinook salmon. Uptake by resistant Atlantic salmon macrophages was significantly higher than chinook macrophages indicating a possible mechanism of resistance. Simple differences in C' activity between species might account for this or more complex mechanisms may be involved such as suppression or evasion of phagocytosis by *L. salmonae* in chinook salmon. To investigate this further ultrastructural studies and respiratory burst activity should be examined. My results also indicate that lectinophagocytosis may be involved in binding of *L. salmonae* spores by chinook. This is not surprising, as such nonspecific binding has been proposed for other fish-infecting microsporidia.

Table 15. Average length and weight of Atlantic salmon *Salmo salar* and 2 strains of chinook salmon *Oncorhynchus tshawytscha* that macrophages were purified from for immunoflurescent (IFAT) and yeast assays

Fish type	IFAT assay		Yeast assay	
	Length (cm)	Weight (g)	Length (cm)	Weight (g)
Atlantic	39.6	668.6	35.2	546.0
Southern Coastal	26.1	214.7	27.0	253.5
Northern Stream	32.8	393.7	34.9	480.7

Table 16. Macrophage percent phagocytosis (percent of spores engulfed) of *Loma salmonae* spores between various comparisons of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Spores were preincubated in the plasma treatments

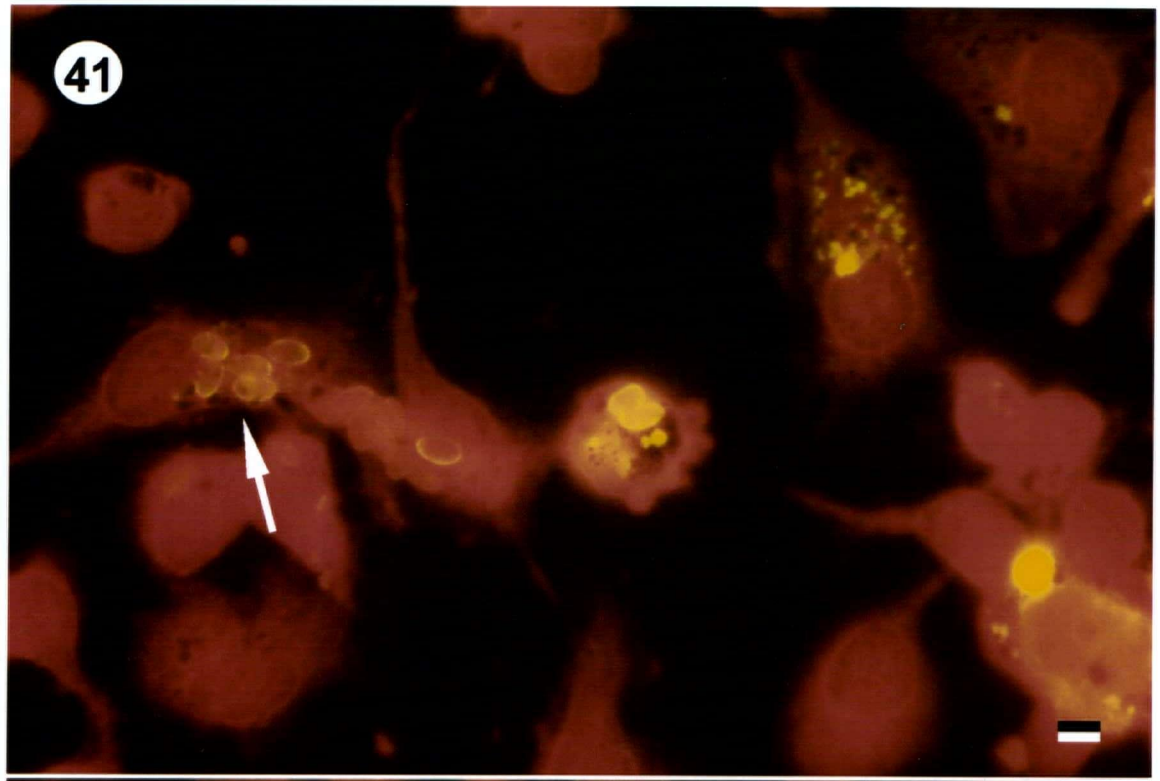
Plasma Treatment	Interaction by fish type		
	Atlantic X Southern Coastal	Atlantic X Northern Stream	Southern Coastal X Northern Stream
Normal	96.7 X 80.5*	96.7 X 78.4*	80.5 X 78.4
None	10.9 X 7.7	10.9 X 9.5	7.7 X 9.5
Heated	7.2 X 6.7	7.2 X 6.7	6.7 X 6.7
EDTA	19.3 X 34.0*	19.3 X 31.2*	34.0 X 31.2

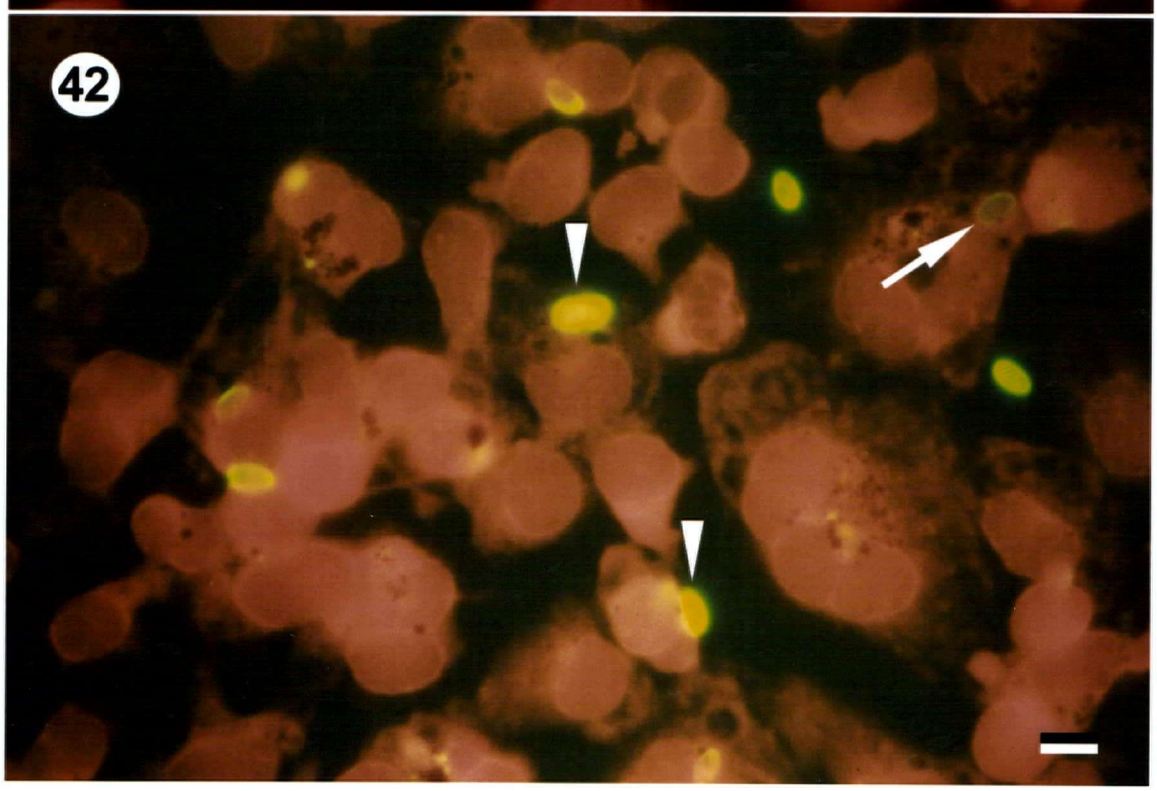
*Statistically different ($p<0.05$) by Chi-square test

Table 17. Macrophage phagocytic index (average number engulfed) of *Loma salmonae* spores between various comparisons of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Spores were preincubated in the plasma treatments

Plasma Treatment	Interaction by fish type		
	Atlantic X Southern Coastal	Atlantic X Northern Stream	Southern Coastal X Northern Stream
Normal	2.8 X 1.5*	2.8 X 1.6*	1.5 X 1.6
None	1.0 X 1.0	1.0 X 1.0	1.0 X 1.0
Heated	1.0 X 1.0	1.0 X 1.0	1.0 X 1.0
EDTA	1.0 X 1.2*	1.0 X 1.0	1.2 X 1.0*

*Statistically different ($p<0.001$) by Tukey HSD





Figs. 41 & 42. ***Loma salmonae*** **phagocytosed by macrophages of** ***Salmo salar*** **and** ***Oncorhychus tshawytscha*****. Immunofluorescent (IFAT) fields with green** ***L. salmonae*** **spores and counterstained red macrophages. Spores were preincubated in normal plasma (Atlantic salmon) or no plasma (chinook salmon). Fig. 41. Spores phagocytosed by Atlantic macrophages denoted by arrow. Scale bar = 6 μm. Fig. 42. Spores phagocytosed by chinook macrophages denoted by arrow and unphagocytosed denoted by arrowheads. Scale bar = 6 μm**

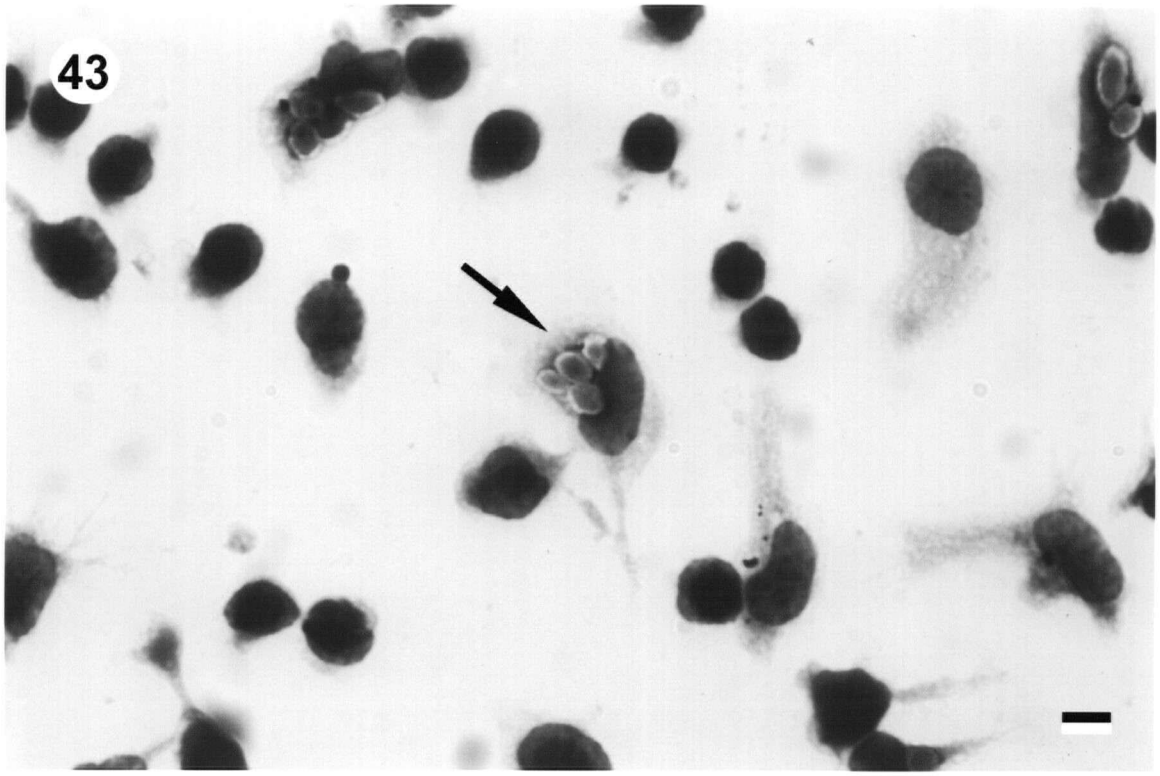


Fig. 43. *Saccharomyces cerevisiae* (arrow) phagocytosed by macrophages of *Oncorhynchus tshawytscha* stained with Wright's Modified Leukostat Stain. Scale bar = 6 μm

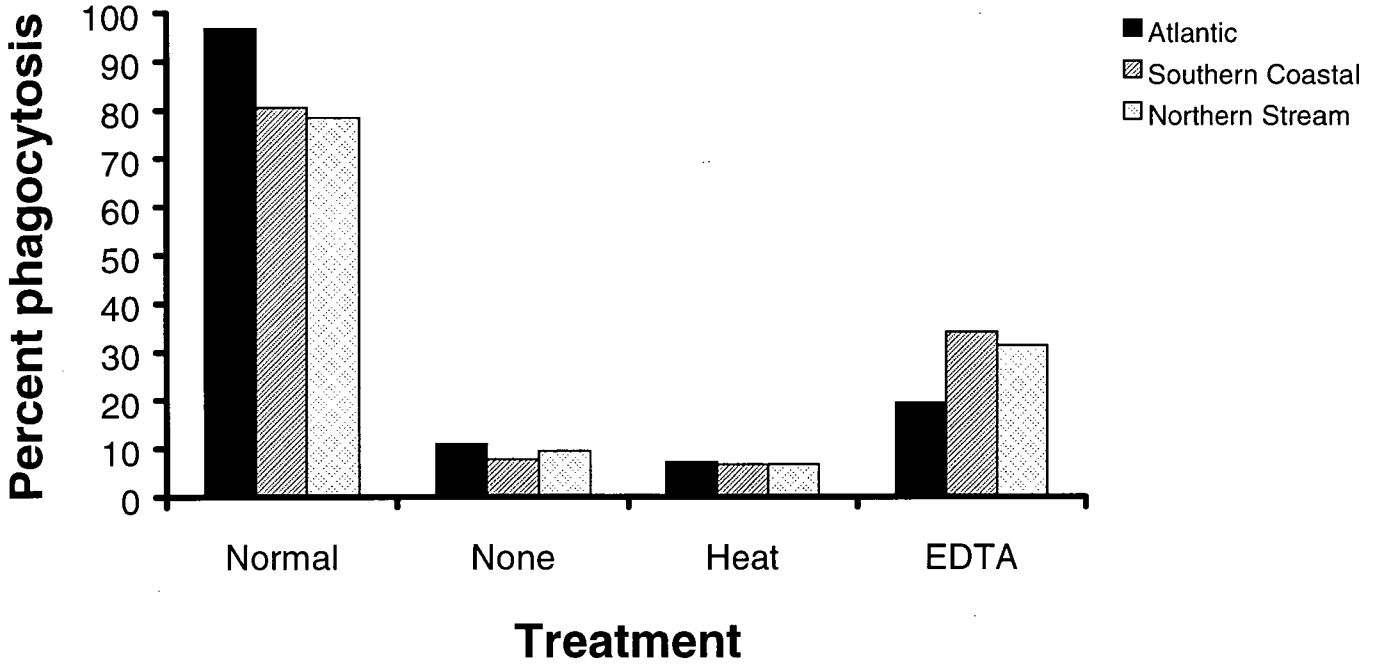


Fig. 44. Percent of *Loma salmonae* spores engulfed by macrophages (percent phagocytosis) of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Spores were previously incubated in plasma treatments

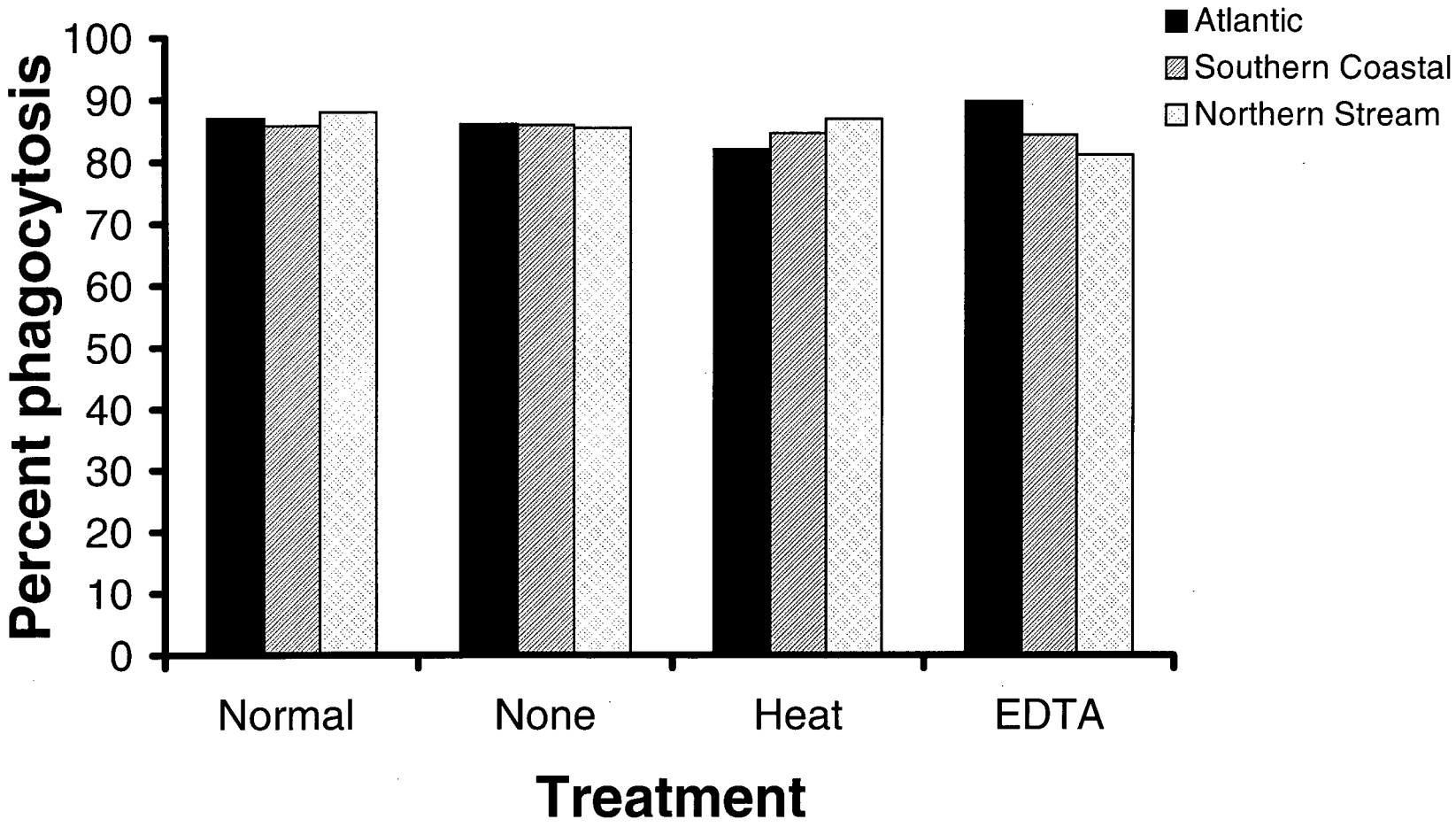


Fig. 45. Percent of baker's yeast *Sarccharomyces cerevisiae* cells engulfed by macrophages (percent phagocytosis) of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Yeast cells were previously incubated in plasma treatments

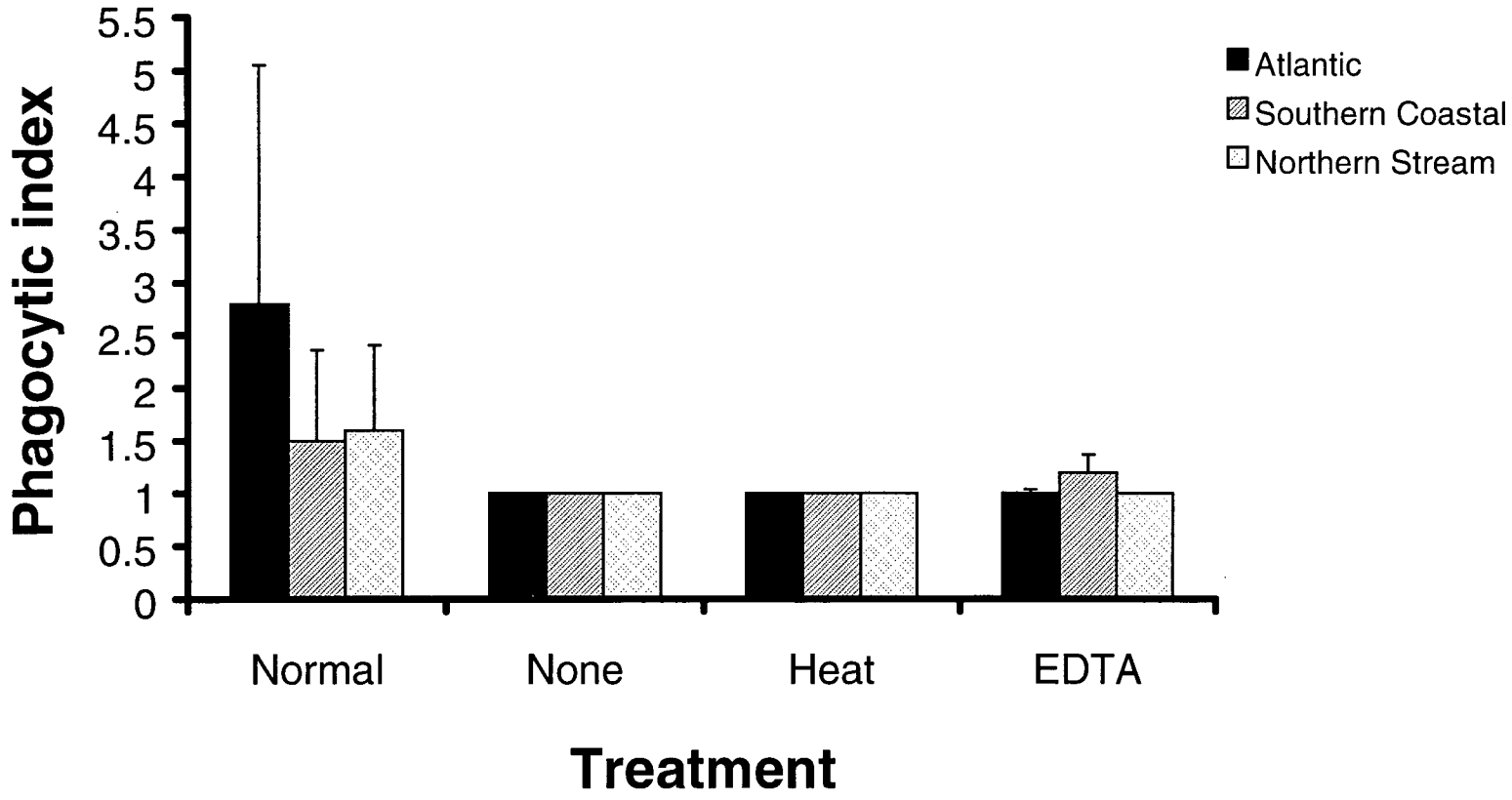


Fig. 46. Average number of *Loma salmonae* spores engulfed by macrophages (phagocytic index) of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Spores were previously incubated in plasma treatments. Error bars represent standard deviations

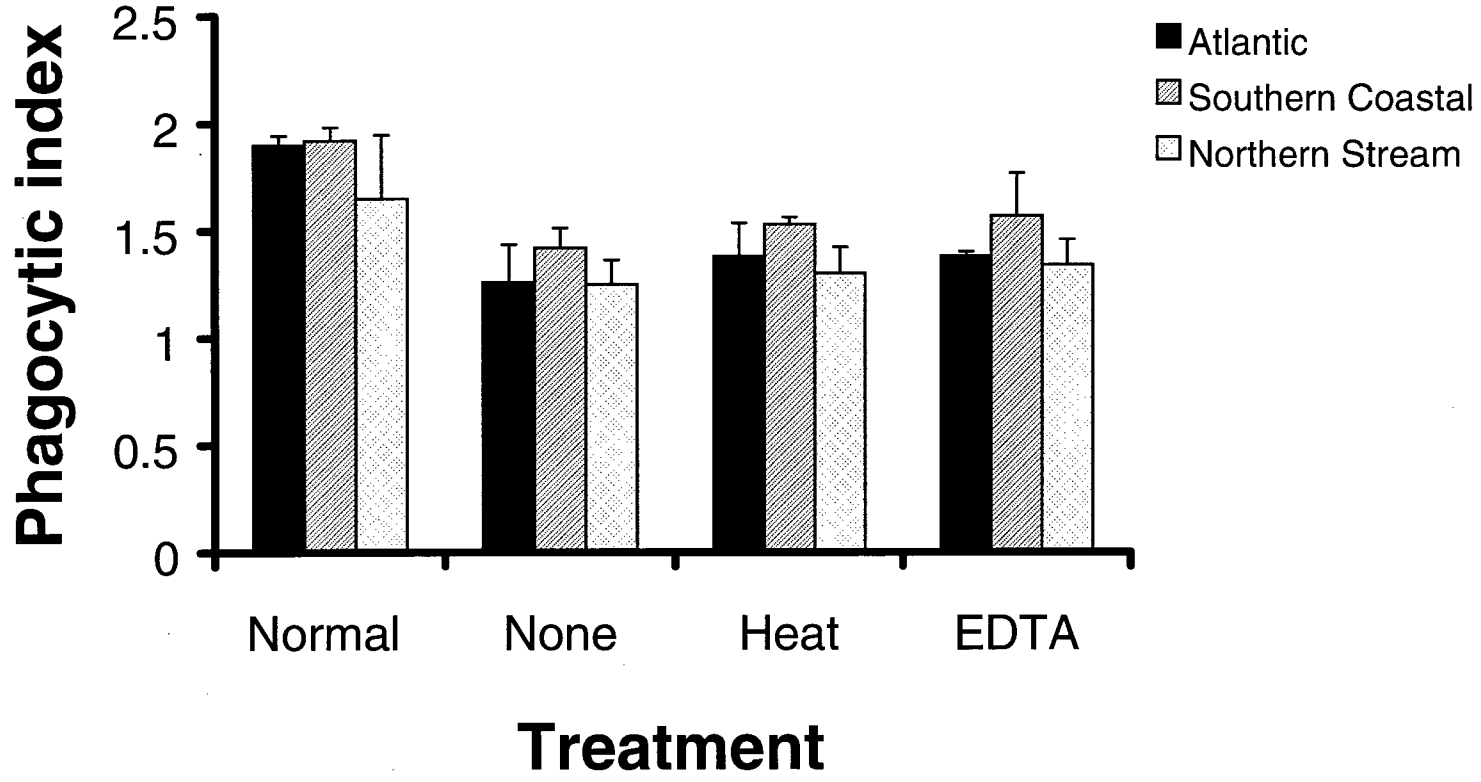


Fig 47. Average number of baker's yeast *Sarccharomyces cerevisiae* cells engulfed by macrophages (phagocytic index) of Atlantic salmon *Salmo salar*, and 2 strains of chinook salmon *Oncorhynchus tshawytscha*. Yeast cells were previously incubated in plasma treatments. Error bars represent standard deviations

CHAPTER VI

Wild Salmonid and Nonsalmonid Hosts

INTRODUCTION

Species of *Loma* have been described from freshwater and marine fishes (Morrison & Sprague 1981a, 1983) and *Loma salmonae* is an important pathogen of salmonid fishes reared in fresh and sea water (Wales & Wolf 1955; Kent et al. 1989).

Nonsalmonid fishes have also been suggested as potential reservoirs for infections of *L. salmonae* acquired by seawater-reared salmon (Kent et al. 1995a). Shiner perch are common in and around salmon netpen farms in British Columbia, some of which have experienced severe outbreaks of *L. salmonae*. A species of *Loma* was found in the gills of shiner perch that were being examined as part of a study of potential reservoir hosts for *L. salmonae*. This species of *Loma* can be distinguished from *L. salmonae* by host and by geographic, morphological, and molecular (rDNA) characters. I, therefore, describe this microsporidian as a new species (published previously by Shaw et al. 1997; refer to preface).

Wales & Wolf (1955) first described *L. salmonae* from yearling rainbow trout in California. Kent et al. (1998) described *Loma* spp. from 5 species of Pacific salmon caught during their seawater phase. Little information exists on abundance of *L. salmonae* in Pacific salmon during their freshwater phase. I surveyed prespawning, spawning, and smolts migrating to sea from 5 rivers and 1 lake in British Columbia, Canada for *L. salmonae*.

MATERIALS AND METHODS

Nonsalmonid collection. *Cymatogaster aggregata* were collected from 3 localities off Vancouver Island, British Columbia, a commercial dock at Tofino and a netpen salmon

farm at Eagle Bay (both in Clayoquot Sound 13 km apart), and a beach at Departure Bay, Nanaimo. Fish were collected by seine in Nanaimo, by angling at the Clayoquot Sound localities, and by dipnet at the fish farm.

Fish were killed with MS-222 then stored on ice or frozen. Fish were weighed and measured (total length), and the first 3 gill arches on the left side of each fish were examined for the presence of the parasite.

Gill material for spore and xenoma measurements and abundance was collected at all sites. Material for histology was collected in July for the Eagle Bay sample, whereas material for electron microscopy was collected from the Tofino dock sample. Material for molecular biology was collected at all sites and frozen at -70°C.

Salmonid collection. Salmonids were collected as outlined in Table 20. Fish were killed with a blow to the head or overdosed with MS-222. The first left gill arch was removed and examined by wet mount. Total length and weight was recorded for smolts only. Prevalence was determined for prespawning and spawning fish at x50 using a dissecting microscope. Intensity was examined as described in chapter II except a subsample of the first left gill arch was taken for large (spawning) fish. Sockeye smolts sampled from Great Central Lake were also screened by PCR (as described by Docker et al. 1997a). Smolts left over after examination on 10/22/97 were held in ambient seawater for 1 y and re-examined for *L. salmonae*. Smolts examined on 5/8/98 were sorted into a 1997 y class (< 3.0 g) and fish 2 y or older (> 8 g).

Measurements and abundance. Xenomas and spores were measured from wet mounts of fresh material. For size comparison, 30 spores of *L. salmonae* from seawater-reared chinook salmon (British Columbia) were measured under identical conditions (fresh). Prevalence was determined by examination of gills in wet mount preparations, except for the July sample from Eagle Bay, in which prevalence was

determined from histological sections. Intensity was determined by counting the total number of xenomas in all gill arches of the infected fish collected from Eagle Bay in September. Wet mount preparations for prevalence and intensity were examined at x100. Fish were separated into underyearlings (<= 1-y-old) and adults (> 1-y-old) based on frequency plots and biology (Hart 1973).

Histology. Gills were removed from fish and placed in Davidson's solution for histology. Histological sections were prepared from these gills and stained with hematoxylin and eosin.

Electron microscopy. Infected lamellae were fixed for 24 hr in 4% glutaraldehyde, then transferred to Millonig's solution for an additional 24 hr. Lamellae were postfixed in 1% osmium tetraoxide, embedded in epoxy resin, sectioned, stained with lead citrate and uranyl acetate, and examined with a transmission electron microscope.

Polymerase chain reaction (PCR) and DNA sequencing. DNA was extracted from infected shiner perch gill tissue from all 3 sites by proteinase K digestion at 37 °C for 6 h (10 mM Tris, pH 8.0; 10 mM EDTA; 1% SDS; 150 mM NaCl; 200 μg ml^{-1} proteinase K). The suspension was extracted twice with a 50:50:1 ratio of phenol to chloroform to isoamyl alcohol (pci), and the DNA precipitated on ice with ethanol. The DNA was resuspended in TE, and stored at 4°C.

Fragments of the above DNA were amplified by PCR with 2 sets of primers developed from the rDNA sequence of *L. salmonae* in chinook salmon (Docker et al. 1997a). The first set, the forward primer Loma f (5'-ATTAGTGAGACCTCAGCC-3') and the reverse primer LS-2 (5'-ATGACATCTCACATAATTGTG-3'), are located in the small subunit (SSU) and large subunit (LSU) rDNA genes, respectively; they amplify a 627-base pair (bp) fragment in *L. salmonae* which spans the internal transcribed spacer (ITS) region. The second PCR assay used the reverse primer LS-2 and a second

forward primer, LS-1 (5'-CTGGATCAGACCGATTTATAT-3'), which was developed from the hypervariable ITS region in *L. salmonae* and amplifies a 272-bp fragment in this species. Docker et al. (1997a) found the second PCR assay (LS-1/LS-2) to be highly sensitive, capable of reliably detecting as few as 0.01 *L. salmonae* spores per 50 µl PCR reaction (or 40 spores g^{-1} of chinook salmon gill tissue). The first set of primers, Loma f and LS-2, was 3 orders of magnitude less sensitive for *L. salmonae* under these conditions.

Gene amplification was accomplished using standard PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, and 0.2 mM dNTP (Saiki 1990). Each 50 µl reaction contained 25 pmol of each primer, 1.25 units of Taq DNA polymerase, and 0.6 µg of genomic DNA. The reactions were run in a PTC-200 Thermocycler (MJ Research, Watertown, MA) for 35 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 90 sec; these were preceded by a 3 min denaturation at 95°C and followed by a 10 min 72°C extension.

The PCR product generated by primers Loma f and LS-2 in the Tofino shiner perch sample was sequenced using the Sequenase PCR Product Sequencing Kit (United States Biochemical, Cleveland, OH), according to the manufacturer's instructions, and in the other 2 samples by Taq terminator sequencing using fluorescent dye-labeled terminators on the 373 DNA automated sequencer. Internal primers also generated from *L. salmonae* sequence were used to sequence 47 to 97% of the product in both directions.

The sockeye smolt samples from Great Central Lake (10/22/97 & 5/8/98) were screened by PCR using the same protocol as described for nonsalmonids except for differences described herein. Gill arches from the 10/22/97 sample were lumped into groups of 5 before digestion and DNA amplification. The samples from 5/8/98 were separated by year class and individual samples digested for 24 hr at 37°C. Amplification

reactions were run at 45 cycles of 95°C for 35 sec, 54°C for 20 sec, and 72°C for 1 min; these were preceded by a 1 min denaturation at 95°C and followed by a 7 min 72°C extension.

RESULTS

Nonsalmonid host.

DESCRIPTION

Loma embiotocia

(Figs. 48-53)

Spores (n=30) are 4.8 μm long (4.0-5.0 μm) x 2.6 μm wide (2.0-3.0 μm) and xenomas (n=10) 0.13 mm (0.06-0.16 mm). Xenomas are found in endothelial cells of secondary gill lamellae. Spores (n=20) have 14-18 coils of the polar filament. Earlier developmental stages (meronts, sporoblasts) and small tubules are present. Sporoblasts are not highly vacuolated and the sporophorous vesicle formed before sporogony. The xenoma wall is smooth lacking complex invaginations into xenoma cytoplasm.

Taxonomic summary.

Type host: *Cymatogaster aggregata.*

Type locality: Eagle Bay, Clayoquot Sound, Vancouver Island, British Columbia.

Site of infection: Secondary lamellae of gills.

Distinguishing characters: Round xenomas in gill, spore size, polar filament count and rDNA sequence.

Material deposited: Hapantotypes in histological sections, Canadian Museum of

Nature, Invertebrate Collection (Parasites), Ottawa, Canada; sequence information as GenBank accession number U78815.

Etymology: Named after family name for shiner perch, Embiotocidae.

Ecology. *Loma embiotocia* was detected in the gills of shiner perch from all 3 locations (Table 18). Shiner perch examined ranged from 6.1 to 15.5 cm in length and 2.8 to 58.1 g in weight, which represented 2 y classes (underyearlings, 6.1 to 9.4 cm; adults, 10.5 to 15.5 cm). Both year classes were infected with *L. embiotocia*. Prevalence was as high as 15.2% in underyearlings and 13.5% in adults (Table 18). Intensity of *L. embiotocia* in underyearling fish (n=12) from Eagle Bay (September), ranged from 4 to 583 xenomas $fish^{-1}$.

Remarks. Spore size, the formation of xenomas of the cell-hypertrophy type, location in the gill, and mixture of developmental stages randomly throughout xenomas place this parasite within the genus *Loma* (Canning & Lom 1986). Species of *Loma* show extreme overlap in spore size, number of polar filament turns, and other characters (Table 19). *Loma embiotocia* resembles *Loma diplodae* Bekhti and Bouix, 1985 and *L. salmonae* in having a smooth xenoma wall lacking invaginations of the wall (interdigitated) into the cytoplasm, a low level of sporoblast vacuolation, and in the developmental timing of the sporophorous vesicle. Note that *L. branchialis* is insufficiently known for comparison (Table 19). *Loma embiotocia* is distinguished from *L. diplodae* by host and geographic location. Host and rDNA sequence separate *L. embiotocia* from *L. salmonae*.

The PCR primers Loma f and LS-2, both developed from the relatively conserved regions of rDNA sequence of *L. salmonae*, yielded positive assays for the shiner perch infected with *L. embiotocia* from all 3 sites; the size of the fragment was approximately the same as that from *L. salmonae* (Fig. 54). Repeated assays with primers LS-1,

designed from the hypervariable ITS region of *L. salmonae*, and LS-2 consistently failed to amplify DNA from any of the infected shiner perch (Fig. 54).

Of the 635 bp amplified by primers Loma f and LS-2 in the 3 localities of shiner perch, 564 bp were sequenced and consisted of 334 bp of SSU rDNA sequence, 45 bp of ITS, and 185 bp of LSU rDNA, although the exact boundaries of the ITS are not known. As the PCR results suggested, the SSU rDNA and LSU rDNA regions in *L. embiotocia* were similar to those of *L. salmonae*, differing by only 4 and 2 base substitutions, respectively. All 6 substitutions were transitions, and all were found in the 3 different *L. embiotocia* isolates. There were no differences between these isolates in the SSU and LSU gene fragments, although multiple nucleotide signals were observed at 1 SSU rDNA site in the Nanaimo (position 101, Fig. 55) and Eagle Bay (position 317, Fig. 55) samples. These sites may indicate that several slightly different copies were present, either due to the presence of different *L. embiotocia* isolates in the pooled gill samples or to differences among dispersed members of the ribosomal gene array. To determine this, cloning and sequencing of individual repeats is required.

In contrast to the SSU and LSU gene fragments, the short ITS region (45 and 37 bp, respectively) differed significantly between *L. embiotocia* and *L. salmonae* by having 3 indels, 4 transitions, and 1 transversion (Fig. 55). The lack of amplification with primer LS-1 in infected shiner perch from all 3 sites reflects these sequence differences, many of which are at the 3' end of the LS-1 primer site. The ITS region was identical among *L. embiotocia* from different localities, except for 2 sites each with 2 nucleotide signals in the Eagle Bay sample (Fig. 55).

Salmonid hosts. *Loma salmonae* was detected in wild salmonids sampled from 4 rivers and 1 lake (Table 20). A prevalence of 30.0% occurred in prespawning sockeye which had recently entered freshwater during their migration to reproduce. Intensity of

infection was quite high in some fish with large xenomas clearly visible to the naked eye. None of the sockeye smolts held on ambient sea water for 1 y were positive by wet mount examination. I was unable to detect *L. salmonae* in newly migrating smolts except by PCR (Table 20; Figs. 56-58).

DISCUSSION

In species of *Loma* that infect gills, most descriptions were based on spore measurements taken from formalin fixed spores, or from sections of resin-embedded material (Table 19). This makes comparison of the size of my spores with those of other species difficult. The number of polar filament coils overlaps, and developmentally there are only minor differences between most species of *Loma* (Table 19). Morrison & Sprague (1983) realized that the structure of spores of *L. salmonae* and *Loma morhua* contained no differences of taxonomic value, and these species were separated based on differences in hosts and habitats. These species may be valid, as transmission studies by Kent et al. (1995a) suggest that *L. salmonae* is host-specific, i.e., marine fishes, including shiner perch, were not susceptible to experimental infection by *L. salmonae*.

The ribosomal DNA sequence comparisons presented here (Fig. 55) further demonstrate that *L. embiotocia*, is distinct from *L. salmonae*, although the 2 species appear to be very closely related. Intraspecific differences in SSU-rDNA sequence have been studied in a number of microsporidia and have been shown to range from 0.2 to 11.4% within the genera *Encephalitozoon*, *Variomorpha*, and *Nosema* (Baker et al. 1995). The 1.2% difference (4 base pairs in 334) found between *L. embiotocia* and *L. salmonae* SSU rDNA fragments approaches the lower end of the observed range and thus reflects a close relationship between these species. The relatively low level of

variation nevertheless is greater than expected due to intraspecific variation (Didier et al. 1995). Although comparison between *Enterocytozoon bieneusi* SSU rDNA sequence generated by 2 different studies (Zhu et al. 1994; Hartskeerl et al. 1993) yielded differences as high as 1.73%, intraspecific variation in the SSU gene of microsporidia is generally believed to be negligible (Didier et al. 1995; Docker et al. 1997b). Similarly, the more substantial differences in the ITS region of *L. embiotocia* and *L. salmonae* also surpass that expected due to intraspecific variation. ITS sequence differences between the 3 isolates of *L. embiotocia* were minimal, and intraspecific variation in the second ITS region of mosquitos, flukes, and lake trout have also been shown to be negligible (Porter & Collins 1991; Adlard et al. 1993; Zhuo et al. 1994). Even where intraspecific variation has been shown to be more substantial (e.g., Fritz et al. 1994; Vogler & DeSalle 1994; Didier et al. 1995; Docker et al. 1997b), it was not of the magnitude noted in this study between *L.embiotocia* and *L. salmonae.*

PCR assays used in the present study were useful both in detecting species of *Loma* infections that were not readily observed in wet mount preparations (primers Loma f and LS-2) and in distinguishing between *L. salmonae* and *L. embiotocia* (LS-1 and LS-2). In chinook salmon infected with *L. salmonae* however, the assay using primers Loma f and LS-2 was found to be several orders of magnitude less sensitive than that using LS-1 and LS-2. A more sensitive PCR assay for the detection of *L. embiotocia* in shiner perch could be developed by replacing Loma f with an *L. embiotocia* specific primer in the region of the LS-1 site in *L. salmonae.* Such an assay could speed transmission and cross-infection studies of potential reservoir hosts.

Kent et al. (1998) identified other potential reservoir hosts for *Loma* spp. which include Pacific cod, tomcod *Microgadus proximus* Girard, walleye pollock *Theragra chalcogramma* Pallas, lingcod, and sablefish *Anoplopoma fimbria* Pallas. Description of

the *Loma* spp. from these hosts is ongoing by AMV Brown at the University of British Columbia.

I found a low prevalence of *L. salmonae* in 4 Pacific salmon species examined, although intensity was quite high in some fish. Kent et al. (1998) reported a maximum prevalence of 41.7% for *Loma* spp. in wild Pacific salmon sampled from seawater. Anonymous (1984) noted *L. salmonae* in gills and somatic musculature of coho, chum, and pink salmon of the Kemano river and rainbow trout of the Kenny Dam area, British Columbia, Canada. They reported a maximum prevalence of 17% and considered the parasite to be typical of post-spawned fish in the Kemano river. Mortalities in spawning sockeye salmon from the Fulton river, British Columbia, Canada have been associated with *L. salmonae* (MJ Higgins pers. comm.).

I detected fully developed infections in fish that had recently entered freshwater, and in spawning fish. *Loma salmonae* was also present in smolts migrating out from Great Central lake. It seems likely that smolts became infected by conspecific freshwater salmonids or by spores deposited with eggs (see Chapter III). I was unable to detect *L. salmonae* xenomas by wet mount preparations after holding sockeye smolts with subclinical infections (i.e. positive by PCR) for 1 y on ambient seawater. An appealing hypothesis is that fish first become infected as aelvins by spores deposited with eggs. Infected fish may maintain the infection subclinically as they mature during their seawater phase. Shortly before beginning their return migration, and when entering warmer water, fish may begin to develop clinical signs of *L. salmonae*.

In conclusion, based on host, geographic location, spore morphology, and rDNA sequence, the microsporidian of shiner perch described here is a new species within the genus *Loma*. To clarify the relationships and validity of other described species of *Loma*, further comparisons should be done of inter- and intraspecific differences in

rDNA combined with transmission studies for members of the genus *Loma*. In addition I found *L. salmonae* to be prevalent in prespawning, spawning, and young (smolt) salmon from rivers and lakes of British Columbia. This suggests that *L. salmonae* may be present throughout the life cycle of Pacific salmon maintaining itself subclinically until salmon mature at sea.

Table 18. Infection statistics for *Loma embiotocia* found in shiner perch *Cymatogaster aggregata* collected at 3 sites around Vancouver Island, British Columbia in 1995

Site	Month	Number of fish	Prevalence (%)		
			Underyearlings	Adults[a]	Total
	Jul	23			2/23 (8.7)
Eagle Bay	Aug	54	1/40 (2.5)	1/14 (7.1)	2/54 (3.7)
	Sep	80	12/79 (15.2)	0/1 (0.0)	12/80 (15.0)
Tofino Dock	Sep	38	0/1 (0.0)	5/37 (13.5)	5/38 (13.2)
Nanaimo	Sep	60	0/60 (0.0)	0/0 (0.0)	0/60 (0.0)
	Oct	60	1/60 (1.7)	0/0 (0.0)	1/60 (1.7)

[a]Adults are greater than 1 y of age.

Table 19. Comparison of *Loma embiotocia* host, morphological, and developmental characteristics with *Loma* species infecting the gills of various fishes. Characteristics of turns in filament, xenoma wall, sporoblast vacuolation, and that of sporophorous vesicle (SPV) are ultrastructural. SPV formation is summarized as occurring before, during sporogony, or after spore formation

	Host	Distribution[a]	Spore		Xenoma wall	Sporoblast vacuolation	SPV
			Size (μm)[b]	Turns in filament			
L. embiotocia (Shaw et al. 1997)	*Cymatogaster aggregata*	Vancouver Island, British Columbia, M	4.8 x 2.6 c	14-18	Smooth	Low	Before
L. branchialis (Morrison & Sprague 1981a)	*Melanogrammus aeglefinus*	Nova Scotia, M	6.3 x 3.5 d				
L. diplodae (Bekhti & Bouix 1985)	*Diplodus sargus* L.	France, M	4.2 x 2.2 d	17-18	Smooth	Low	Before
L. fontinalis (Morrison & Sprague 1983)	*Salvelinus fontinalis*	Nova Scotia, FW	3.7 x 2.2 d	14-15	Smooth	High	After
L. morhua (Morrison & Sprague 1981a)	*Gadus morhua*	Nova Scotia, M	4.2 x 2.0 d	16-17	Inter-digitated	Low	Before
	Melanogrammus aeglefinus	Nova Scotia, M	4.4 x 2.1 d	16-18			

Table 19, continued.

L. salmonae (Putz et al. 1965)	*Salmo gairdneri* Richardson	California, FW	4.5 x 2.2 e	14-17	Smooth	Low	Before
(Kent et al. 1989)	*Oncorhynchus kisutch*	Washington, M	4.4 x 2.3 e	14-17			
(this study)	*Oncorhynchus tshawytscha*	British Columbia, FW	5.5 x 2.7 c				

[a]FW = freshwater; M = marine
[b]Measurement given as a mean of length x thickness; spores measured were fresh (c), from resin sections (d), or preserved in formalin (e)

Table 20. Prevalence and intensity of *Loma salmonae* in 4 species of salmonid sampled from various locations on Vancouver Island, and 2 mainland locations in British Columbia, Canada

Species	Location	Date	Life history stage of fish	No. fish examined	Prevalence (%)	Intensity (avg. xenomas mm^{-2})
Oncorhynchus nerka	Great Central Lake	11/26/96	Spawning	66	15.2	0.3
(avg. 16.8 cm; 42.2 g)		3/22/97	Smolts	65	8.3 to 41.7[a,b]	NA
(avg. 9.7 cm; 7.5 g)		5/8/98	Smolts	48	6.3[b]	NA
	Stamp River	7/24/97	Prespawn	33	30.0[c]	0.7
	Sproat River	7/24/97	Prespawn	61	26.2[c]	0.2
	Fulton River	10/7/97	Spawning	63	20.6	0.5
Oncorhynchus kisutch	Big Qualicum River	12/12/96	Spawning	66	3.0	0.4
		12/4/97	Spawning	65	12.3	0.2
Oncorhynchus keta	Big Qualicum River	12/4/97	Spawning	60	1.0	0.2
Oncorhynchus tshawytscha	Yukon River	8/20/97	Spawning	60	0.0	0.0

[a]Prevalence given as a range as 5 fish were lumped per sample
[b]Positives detected by PCR
[c]These fish were in fresh water less than 3 d
NA:= Prevalence negative by wet mount but positive by PCR

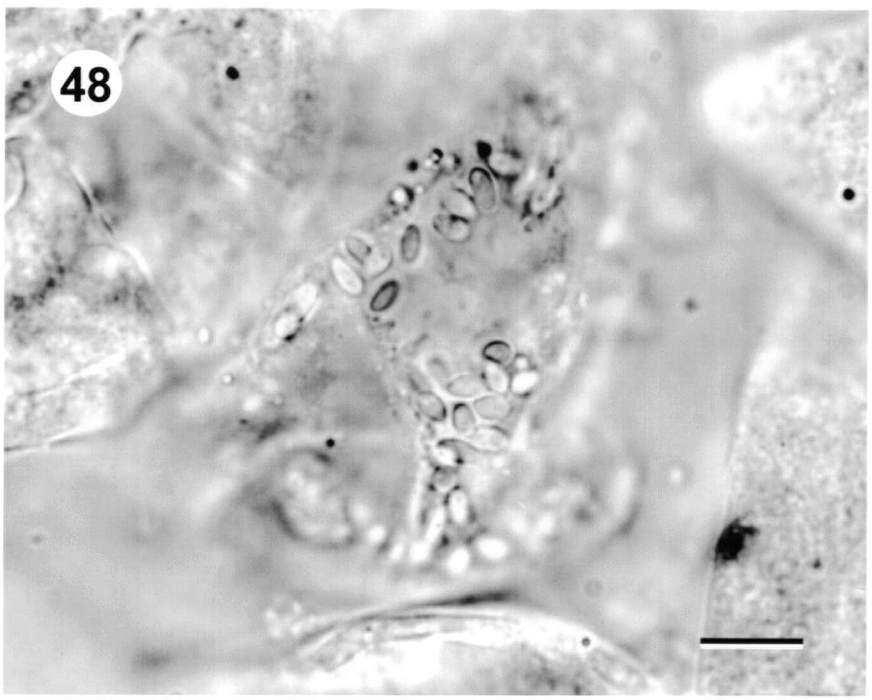
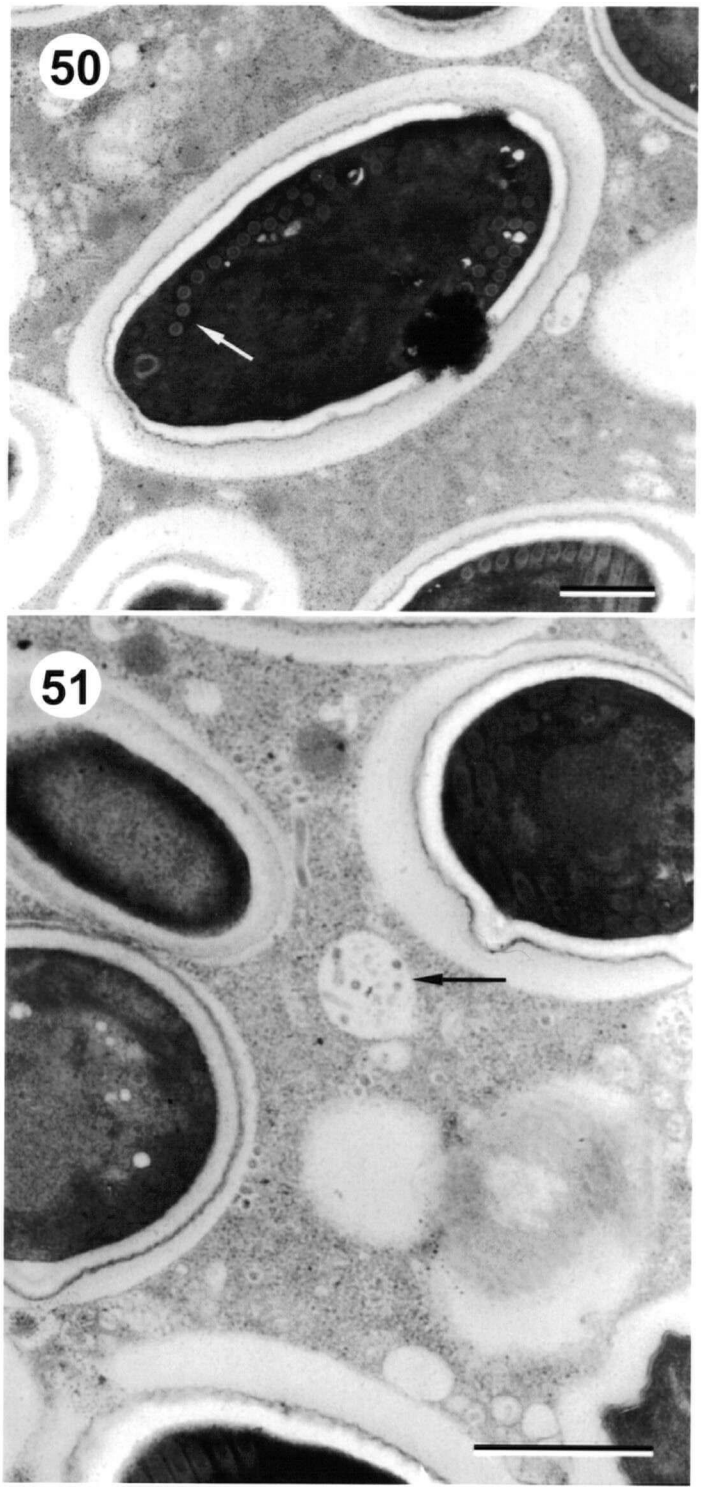
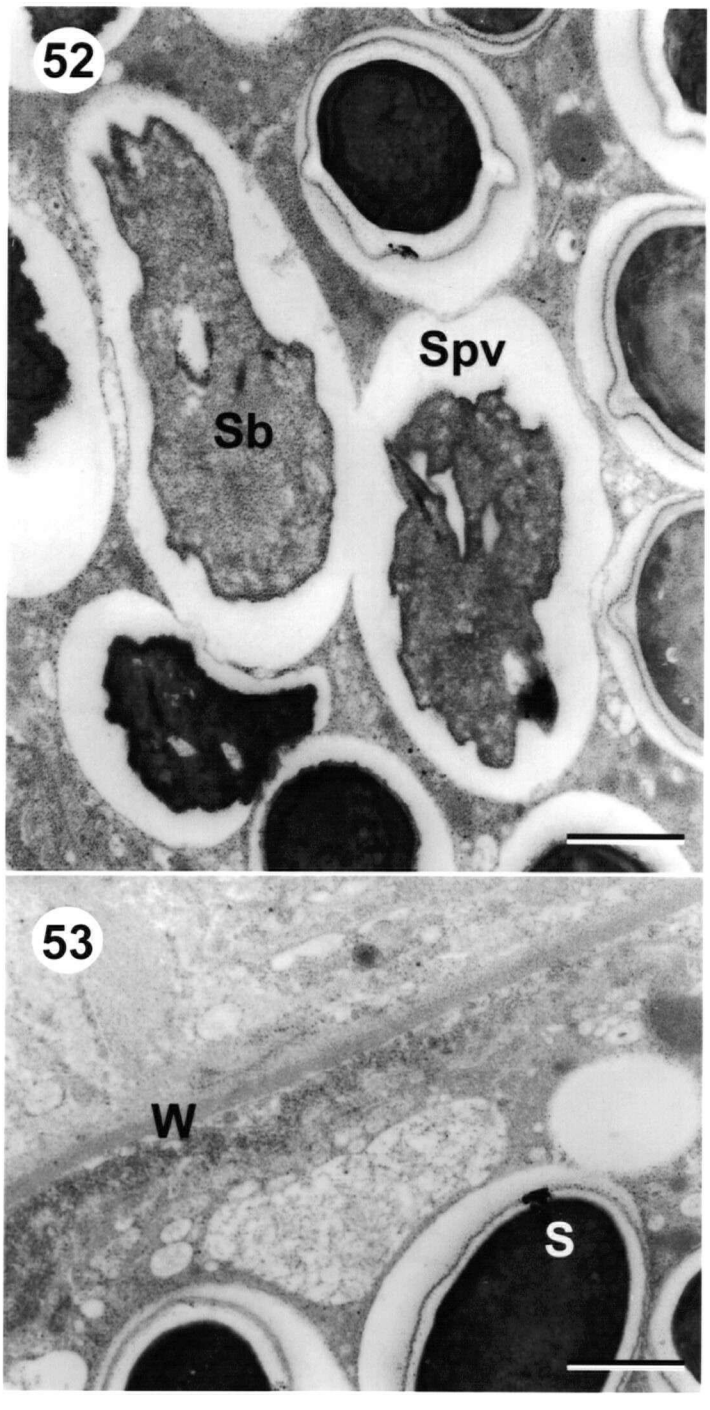


Fig. 48. Fresh spores of *Loma embiotocia* observed under oil immersion. Scale bar = 10 μm

Fig. 49. Xenoma of the hypertrophic cell type formed by *Loma embiotocia* associated with shiner perch *Cymatogaster aggregata* gill secondary lamellae. N; host cell nucleus remnants. Scale bar = 10 μm



Figs. 50 & 51. Transmission electron micrographs of *Loma embiotocia* spores and xenoma structure. Fig. 50. Mature spore with 17 coils of the polar filament denoted by arrow. Scale bar = 1 μm. Fig. 51. Small tubules denoted by arrow within xenoma cytoplasm. Scale bar = 1 μm



Figs. 52 & 53. Transmission electron micrographs of *Loma embiotocia* spores and xenoma structure. Fig. 52. Sporoblasts (Sb) with minimal vacuolation within sporophorous vesicles (Spv). Scale bar =1 μm. Fig. 53. Smooth xenoma wall (W) containing spores (S). Scale bar =1 μm

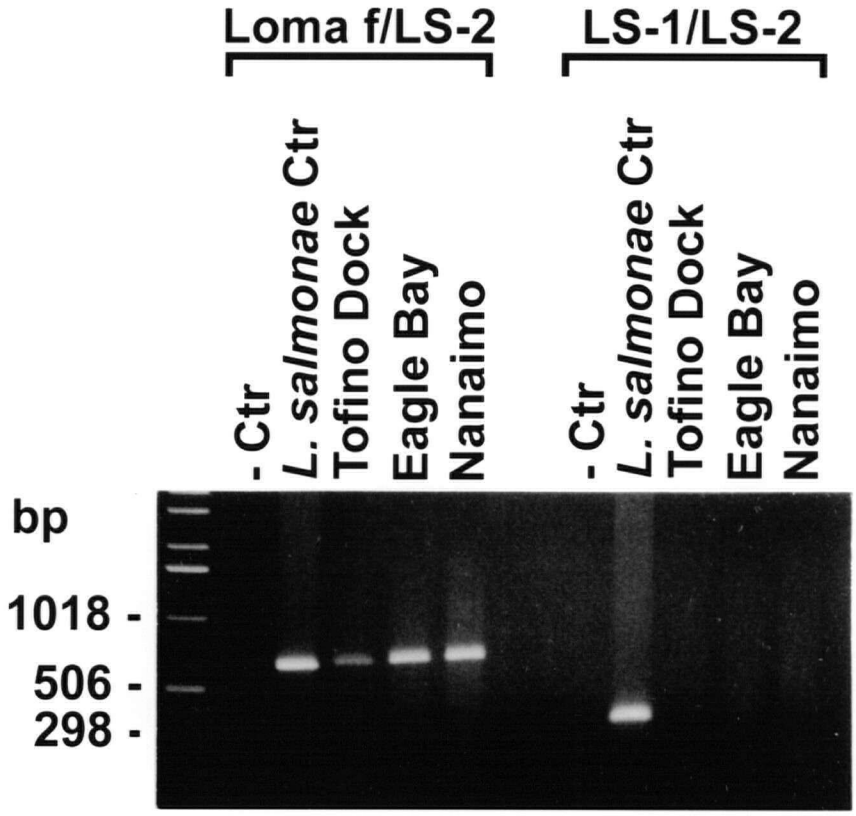


Fig. 54. PCR of DNA from chinook salmon *Oncorhynchus tshawytscha* gill infected with *Loma salmonae* and from gills of shiner perch *Cymatogaster aggregata* infected with *L. embiotocia*. Shiner perch were collected at Eagle Bay, Tofino Dock, and Nanaimo sites, and parasite DNA amplified with primers Loma f and LS-2 or primers LS-1 and LS-2 (1% agarose gel, ethidium-bromide stained). Negative control used distilled water instead of template DNA. Molecular weight markers (bp) are shown in the left lane

```
L. salmonae      CAGGTGCGCA AAGCACAGGA AGGATGGGTC AAGGACAGGT CAGTGATGCC
L. embio Tofino CAGGTGCGCA AAGCACAGGA AGGATGGGTC AAGGACAGGT CAGTGATGCC
L. embio EagleB CAGGTGCGCA AAGCACAGGA AGGATGGGTC AAGGACAGGT CAGTGATGCC
L. embio Nanaim CAGGTGCGCA AAGCACAGGA AGGATGGGTC AAGGACAGGT CAGTGATGCC

                                                        *    *
    L. salmonae CTTAGATGGT CCGGGCTGCA CGCGCACTAC AGTGGTCGCC GAAATTACCT
L. embio Tofino CTTAGATGGT CCGGGCTGCA CGCGCACTAC AGTGGTCGCC GGAATTRCCT
L. embio EagleB CTTAGATGGT CCGGGCTGCA CGCGCACTAC AGTGGTCGCC GGAATTRCCT
L. embio Nanaim CTTAGATGGT CCGGGCTGCA CGCGCACTAC AGTGGTCGCC GGAATTRCCT

                +
    L. salmonae GATAATTATA AAGGCGATCG AGAGGGAATG AGCTTTGTAA GAGGCTCAGG
L. embio Tofino GATAATTATA AAGGCGATCG AGAGGGAATG AGCTTTGTAA GAGGCTCAGG
L. embio EagleB GATAATTATA AAGGCGATCG AGAGGGAATG AGCTTTGTAA GAGGCTCAGG
L. embio Nanaim RATAATTATA AAGGCGATCG AGAGGGAATG AGCTTTGTAA GAGGCTCAGG

    L. salmonae AACGAGGAAT TGCTAGTAAT CGCGGACTCA TTAAGACGCG ATGAATACGT
L. embio Tofino AACGAGGAAT TGCTAGTAAT CGCGGACTCA TTAAGACGCG ATGAATACGT
L. embio EagleB AACGAGGAAT TGCTAGTAAT CGCGGACTCA TTAAGACGCG ATGAATACGT
L. embio Nanaim AACGAGGAAT TGCTAGTAAT CGCGGACTCA TTAAGACGCG ATGAATACGT

                                                        *
    L. salmonae CCCTGTTCTT TGTACACACC GCCCGTCGTT ATCGAAGATG AAGATAGGCG
L. embio Tofino CCCTGTTCTT TGTACACACC GCCCGTCGTT ATCGAAGATG AAGACAGGCG
L. embio EagleB CCCTGTTCTT TGTACACACC GCCCGTCGTT ATCGAAGATG AAGACAGGCG
L. embio Nanaim CCCTGTTCTT TGTACACACC GCCCGTCGTT ATCGAAGATG AAGACAGGCG

                                                    *
    L. salmonae CGAACGATCT ACCAGAAAGT GAGCGCAGGT TTTTAGATCT GATACAAGTC
L. embio Tofino CGAACGATCT ACCAGAAAGT GAGCGCAGGT CTTTAGATCT GATACAAGTC
L. embio EagleB CGAACGATCT ACCAGAAAGT GAGCGCAGGT CTTTAGATCT GATACAAGTC
L. embio Nanaim CGAACGATCT ACCAGAAAGT GAGCGCAGGT CTTTAGATCT GATACAAGTC

                                  +                             +
    L. salmonae GTAACAAGGT AGCTGTAGGA GAACCTGTAG CTGGatcaga ccga------
L. embio Tofino GTAACAAGGT AGCTGTAGGA GAACCTGTAG CTGGatcaga ccgacaaaaa
L. embio EagleB GTAACAAGGT AGCTGTWGGA GAACCTGTAG CTGGatcaga ccgacmaaaa
L. embio Nanaim GTAACAAGGT AGCTGTAGGA GAACCTGTAG CTGGatcaga ccgacaaaaa

                     * *  *   + *   *
    L. salmonae ----tttata taatctttgt atgaatgtaa tCTCTGCGCA AGGGATCTTT
L. embio Tofino gaaactcatg t-atatt-at atgaatgtaa tCTCTGCGCA AGGGATCTTT
L. embio EagleB gaaactcatg t-wtatt-at atgaatgtaa tCTCTGCGCA AGGGATCTTT
L. embio Nanaim gaaactcatg t-atatt-at atgaatgtaa tCTCTGCGCA AGGGATCTTT

    L. salmonae TGGTTCGCTA GACGAAGAAG GGCGCAGCGG AATGCGAAAT GTGCAGGAGT
L. embio Tofino TGGTTCGCTA GACGAAGAAG GGCGCAGCGG AATGCGAAAT GTGCAGGAGT
L. embio EagleB TGGTTCGCTA GACGAAGAAG GGCGCAGCGG AATGCGAAAT GTGCAGGAGT
L. embio Nanaim TGGTTCGCTA GACGAAGAAG GGCGCAGCGG AATGCGAAAT GTGCAGGAGT

                          *
    L. salmonae CGCAGCGAAG ATAGCACATG CCTGAAATCA CGAGAGTGAG ACTACCCCTT
L. embio Tofino CGCAGCGAAG ACAGCACATG CCTGAAATCA CGAGAGTGAG ACTACCCCTT
L. embio EagleB CGCAGCGAAG ACAGCACATG CCTGAAATCA CGAGAGTGAG ACTACCCCTT
L. embio Nanaim CGCAGCGAAG ACAGCACATG CCTGAAATCA CGAGAGTGAG ACTACCCCTT

                                                    *
    L. salmonae TGAATTAAGC ATATGAGTAA AGGGAGGAAA AGAAACTAAC AAGGATTCCT
L. embio Tofino TGAATTAAGC ATATGAGTAA AGGGAGGAAA AAAAACTAAC AAGGATTCCT
L. embio EagleB TGAATTAAGC ATATGAGTAA AGGGAGGAAA AAAAACTAAC AAGGATTCCT
L. embio Nanaim TGAATTAAGC ATATGAGTAA AGGGAGGAAA AAAAACTAAC AAGGATTCCT

    L. salmonae GTAGTAGCGG CGAGCG
L. embio Tofino GTAGTAGCGG CGAGCG
L. embio EagleB GTAGTAGCGG CGAGCG
L. embio Nanaim GTAGTAGCGG CGAGCG
```

Fig. 55. Sequence alignment of the gene fragments sequenced here, including a portion (334 bp) of the small subunit ribosomal DNA (SSU rDNA) and part (185 bp) of the large subunit DNA (LSU rDNA), and the internal transcribed spacer (ITS), for *Loma salmonae* and *Loma embiotocia* (GenBank U78815). Lowercase letters denote the presumptive ITS. Differences between *L. salmonae* and *L. embiotocia* are marked with "*". Differences between *L. embiotocia* from different localities are marked with +. R indicates both G and A, W indicates both A and T, and M indicates both C and A

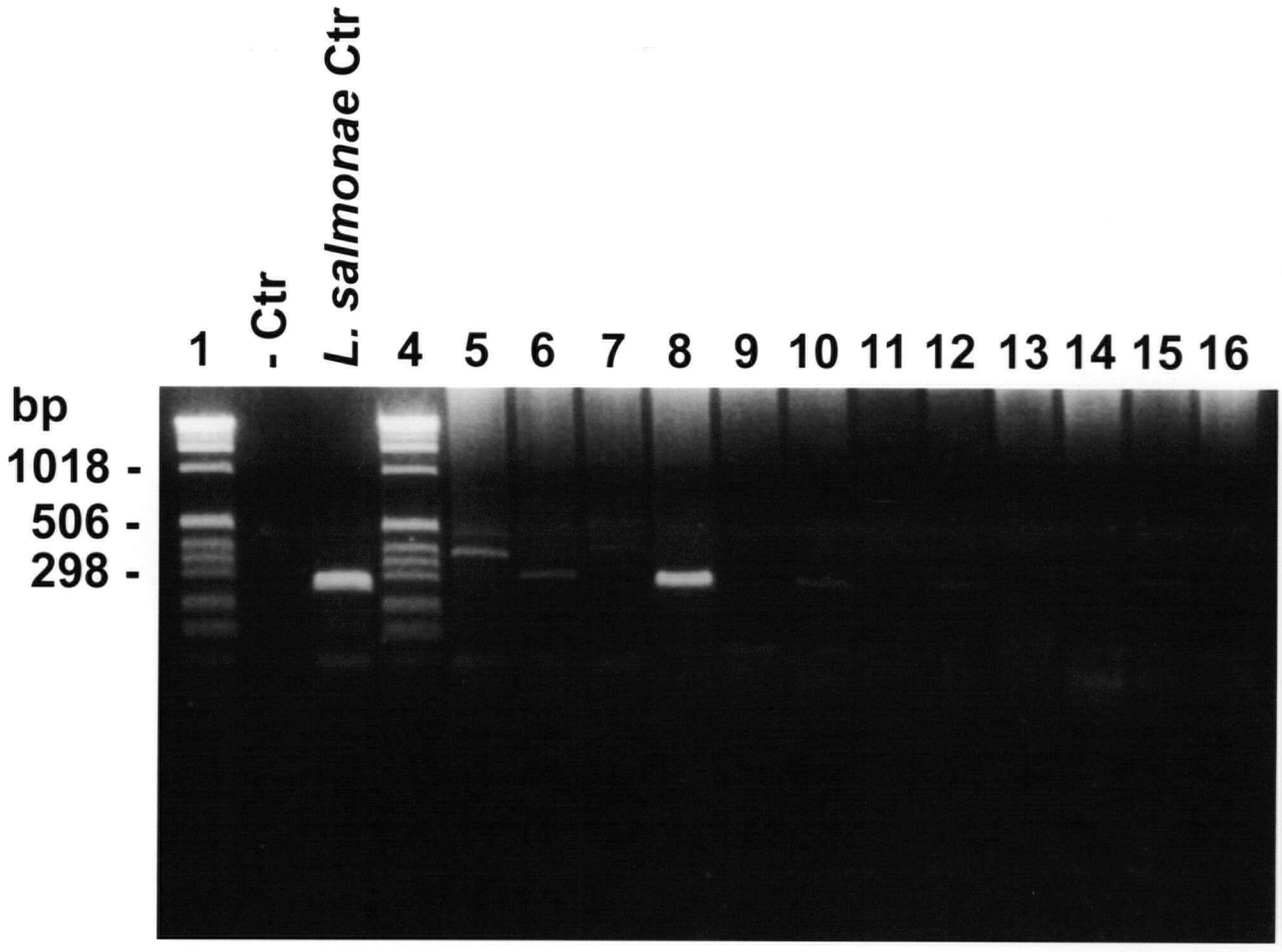


Fig. 56. PCR of DNA from sockeye salmon (*Oncorhynchus nerka*) gill infected with *Loma salmonae*. Parasite DNA amplified with primers LS-1 and LS-2 (2% agarose gell, ethidium-bromide stained). The negative and positive controls (Ctr), respectively, used distilled water and DNA from purified *L. salmonae* spores. Lanes 5 to 16 are grouped samples of 5 fish per lane. Fish were collected at Great Central Lake, Vancouver Island, British Columbia, Canada as migrating smolts (1996 y class). Note positives in lanes 6, 8, 10, 12 & 15. Molecular weight markers (bp) are shown in lane 1 & 4

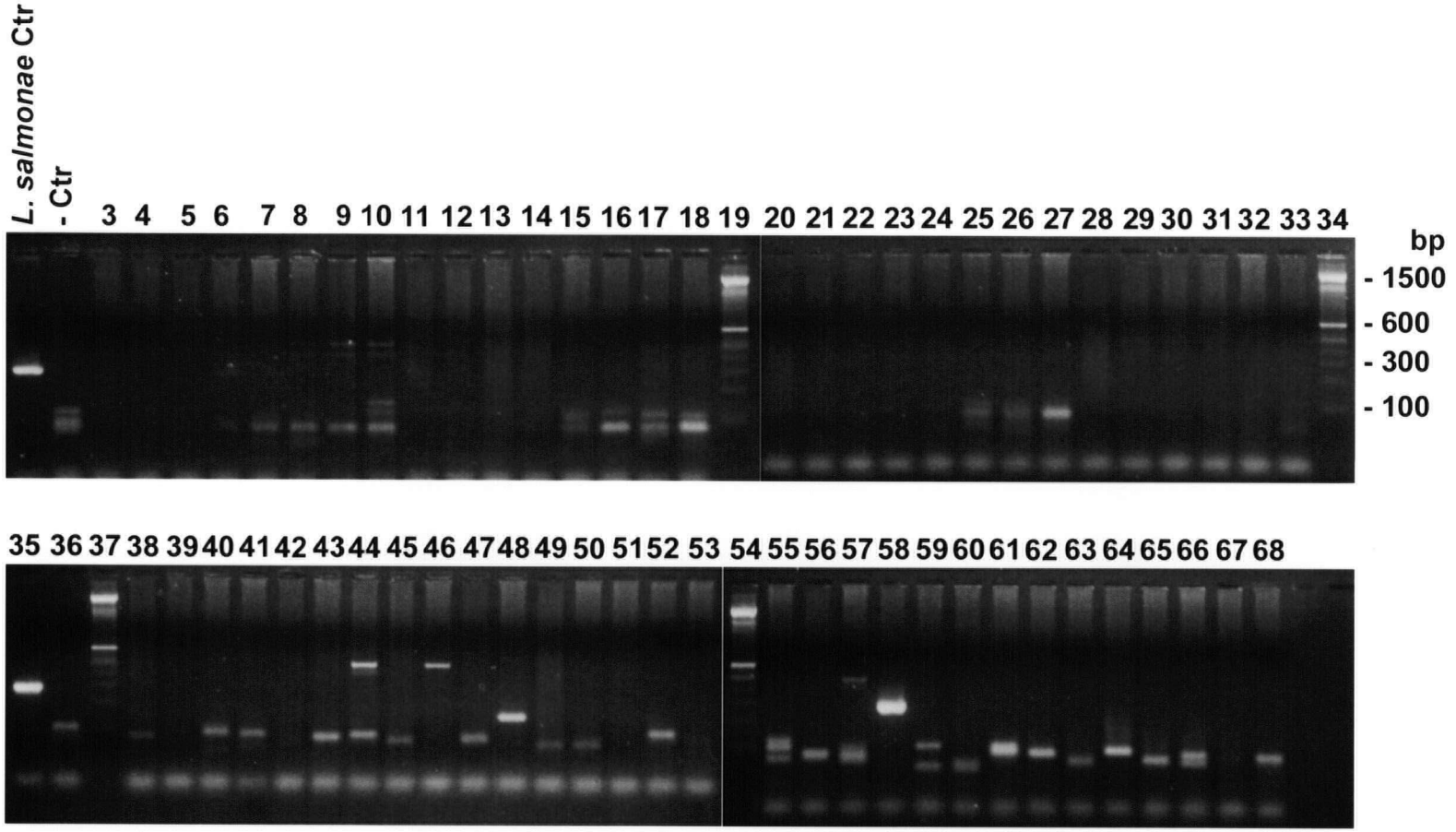


Fig. 57. PCR of DNA from sockeye salmon *Oncorhynchus nerka* gill infected with *Loma salmonae*. Parasite DNA amplified with primers LS-1 and LS-2 (1% agarose gell, ethidium-bromide stained). The negative and positive controls (lanes 1, 35 & 2, 36) respectively, used distilled water and DNA from purified *L. salmonae* spores. Fish were collected at Great Central Lake, Vancouver Island, British Columbia, Canada as migrating smolts (1997 y class). Note nonspecific bands in lanes 44, 46, 48 & 57 and 1 positive in lane 58. Molecular weight markers (bp) are shown in lanes 19, 34, 37 & 54

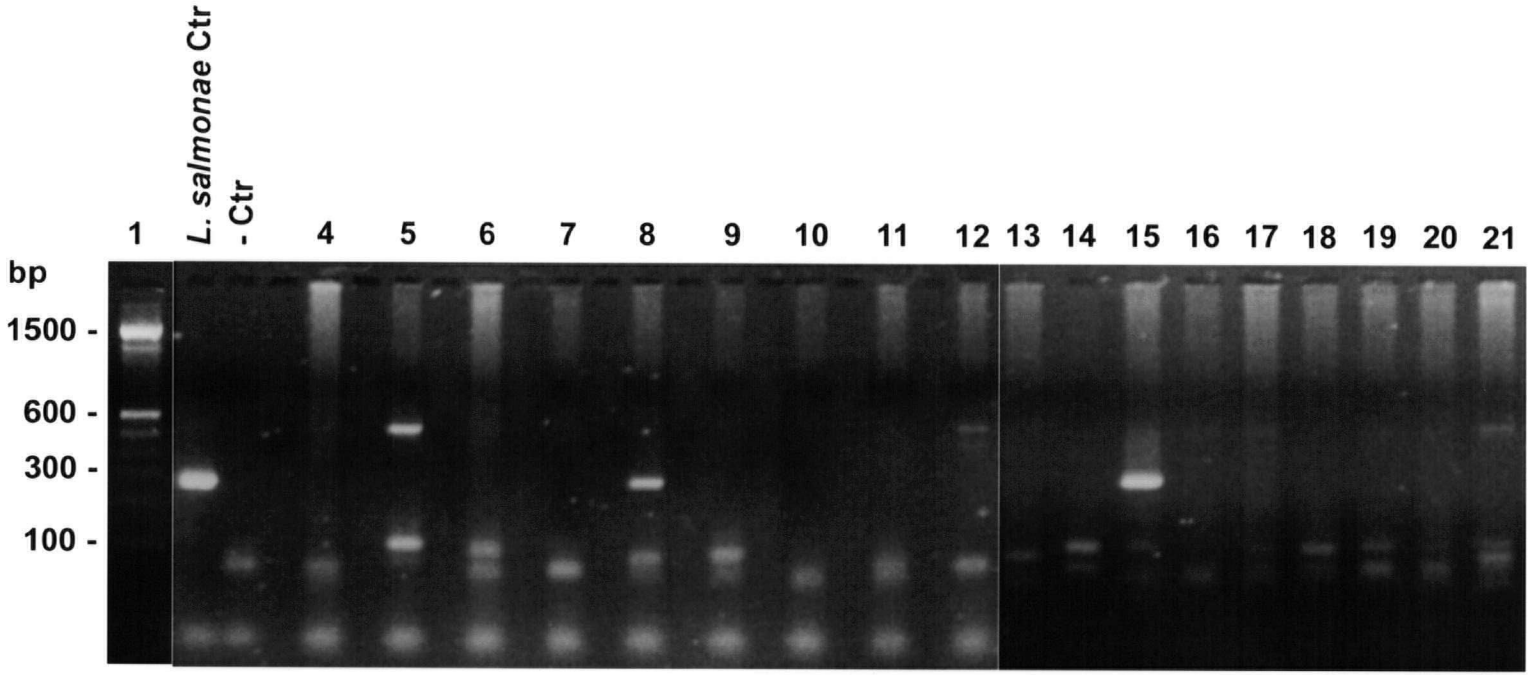


Fig. 58. PCR of DNA from sockeye salmon *Oncorhynchus nerka* gill infected with *Loma salmonae*. Parasite DNA amplified with primers LS-1 and LS-2 (1% agarose gell, ethidium-bromide stained). The negative and positive controls (Ctr), respectively, used distilled water and DNA from purified *L. salmonae* spores. Fish were collected at Great Central Lake, Vancouver Island, British Columbia, Canada as migrating smolts (≧2 y old). Note nonspecific bands in lanes 5, 12 & 21. Molecular weight markers (bp) are shown in lane 1

CHAPTER VII

Conclusions and Future Recommendations

Microsporidia of fish can cause morbidity (e.g., clinical disease, decreased fecundity, swimming ability), mortality, and reduced marketability in wild and cultured fishes. Cultured fishes are particularly susceptible to infection because of high stocking densities used during rearing. Fish-infecting microsporidia can be broadly classified into two morphological groups: xenoma and non-xenoma forming. Both groups cause significant pathological changes and result in a host immune response. However, microsporidia can evade detection by the host's immune system due to intracellular sequestration. These parasites can also immunosuppress fish as seen with *Glugea* spp. and although antibodies are produced in some cases, it is unclear if they play a protective role. Presently, fumagillin and its analogue TNP-470 are the most promising drugs available for treatment. Although not currently licensed for use in fish-farming, both drugs control fish-infecting microsporidia, such as *L. salmonae*, at low doses (2-4 or 0.1 mg kg^{-1} $fish^{-1}$ day^{-1} respectively). If licensed these drugs may provide a treatment to fish-farmers in the Pacific Northwest. In the mean time, a natural treatment for *L. salmonae* and other fish-infecting microsporidia may be manipulation of water temperature. Growth of these parasites can be accelerated or retarded with raising or lowering of water temperature respectively.

This thesis examined the basic biology of *L. salmonae* in Pacific salmon and related salmonids, and elucidates: mechanisms of parasite transmission; viability under laboratory conditions; host susceptibility; immunology; and potential wild and nonsalmonid hosts.

Modes of Transmission. *Loma salmonae* is transmissible by exposing *Oncorhynchus*

spp. *per os* to macerated gill or a gill slurry and by IP, IM, or IV injection of purified spores. Cohabitation of naive fish with infected dead or live fish in a flow through system will also transmit the parasite. Placement of infective spores directly on the gills of fish does not lead to infection. Other fish-infecting microsporidia have been transmitted by *per os*, IP, IM, and waterborne (i.e. food, spore solution) exposure. The route of *L. salmonae* development within the host after natural *per os* exposure is still unclear. After injecting a sporoplasm into an epithelial cell of the alimentary canal *L. salmonae* may enter the lamina propria and gain access to the blood stream. Once in the blood it may reach its preferred site of development, the gills. Because *L. salmonae* infects endothelial cells it may also be found in other vascularized areas of the host. Some have suggested that fish-infecting microsporidia use macrophages or neutrophils as a transport mechanism. Whereas this may be possible, it has not been experimentally demonstrated. Macrophages ingest and destroy spores and IV injection of purified spores can infect fish. The latter suggests that *L. salmonae* may reinfect the host when spores are released from xenomas. Future studies should examine the route of *L. salmonae* development within the host using *in situ* hybridization and the PCR probes that have been developed. Some of this work is currently underway by GJ Sanchez at the Atlantic Veterinary College in Prince Edward Island, Canada.

The importance of natural (*per os*, cohabitation) transmission routes used by *L. salmonae* should not be overlooked. Fish-farmers often experience yearly epizootics of *L. salmonae* and this may be due to maintaining multiple fish year classes at one site. Stocking of naive smolts next to infected market fish could result in infection of the smolts maintaining *L. salmonae* at farm sites over time. Fish may also be infected at the hatchery, especially if the hatchery uses surface water with wild salmonids.

Viability Under Various Laboratory Conditions. Viability was measured indirectly by

percent of spores excluding the dye methylene blue, and percent extruding a polar filament when placed in a hydrogen peroxide solution. Viability was also measured directly by infectivity of spores to fish using *per os* administration of spores. Methylene blue overestimated spore viability while extrusion rates underestimated spore viability when compared with infectivity of estimates from experimental infections. Spore viability decreased over time and when spores were exposed to extreme conditions (i.e., heating, freezing). Spores were killed by rapid or slow freezing and use of 100 and 200 ppm iodophor disinfectant. Use of the iodophor on heavily-infected gill tissue reduced intensity of infections and delayed detection of them but did not completely kill all spores. Future work should examine what iodophor concentration is needed to kill all spores in heavily-infected gill homogenates. Pacific salmon farmers should continue to use iodophor to disinfect salmon eggs especially in handling of eggs from broodstock infected with *L. salmonae*. Although vertical transmission has not been demonstrated, it is a possibility because the parasite has been found in association with ovarian tissue.

Loma salmonae spores stored at 4^0C were viable for 95 d in fresh water and sea water. The maximum viability of spores under those conditions should be determined and alternative methods of laboratory storage (e.g. cell culture) should be explored.

Host Susceptibility. *Loma salmonae* is specific to salmonids and infects mainly species of *Oncorhynchus*. It did not infect Atlantic salmon or Arctic char, nor any nonsalmonid tested, although it did infect brown trout. The parasite can also infect brook trout, and thus *L. fontinalis* may be a synonym of *L. salmonae*. This should be investigated using sequencing of rDNA from both as differentiation of *Loma* spp. based on host, geography, and morphological criteria is of limited value in distinguishing species.

Genetic and non-genetic factors interact to produce variations in susceptibility of

fish strains to a pathogen. Three strains of chinook were examined and the Northern Stream (NS) strain was consistently more susceptible and had a lower survivability when exposed to the parasite. *Loma salmonae* may not be enzootic to the Yukon river where this strain originates. Alternatively, other unknown genetic or non-genetic factors may play a role. Further study is necessary to quantify strain differences through breeding experiments and determine the factors that contribute to it. This information might be used by the aquaculture industry if they continue to select fish of the Southern Coastal (SC) strain, especially those that have been exposed to, and survived, infection by *L. salmonae*.

Immunology. Fish respond humorally and cellularly to infections by microsporidia. Phagocytes such as macrophages may be especially important in innate or non-specific defense mechanisms of fish to microsporidia. This area is poorly understood and I investigated the ability of Atlantic salmon and chinook salmon macrophages to phagocytose *L. salmonae* spores. Atlantic salmon exhibited a significantly higher percent phagocytosis (PP) and phagocytic index (PI) than either Y or SC strains of chinook. This superior phagocytic ability may explain resistance of Atlantic salmon to *L. salmonae*. Spores incubated in normal plasma were phagocytosed significantly more than those incubated in heat inactivated plasma, no plasma, or EDTA inactivated plasma. Atlantic salmon may have a higher C` titres, or other non-specific defense activity than chinooks. Alternatively, *L. salmonae* may have evolved some mechanism to avoid or suppress binding by macrophages of chinook. Prevention of phagosome-lysosome fusion or stimulation of prostaglandin and/or leukotrien secretion has been noted in other fish-infecting microsporidia (Weidner & Sibley 1985; Laudan et al. 1989). Lectinophagocytosis of *L. salmonae* spores may also be important in chinook. This has been suggested for a related *Glugea* sp. and fish-infecting microsporidia in general.

I did not determine a phagocyte to *L. salmonae* spore ratio (R), and an optimal ratio exists for each fish and pathogen species combination. I used a practical, approximate R value, based on purification time and ease at which phagocytosed spores could be enumerated. Future work should calculate an exact *L. salmonae* R value for the fish species being investigated. The R value might be used in assays requiring optimal phagocytic uptake of *L. salmonae* spores. In addition to phagocytic ability, other mechanisms may be involved in the response of a resistant species to a *L. salmonae* challenge. No significant differences in PP or PI were found between the Y and SC strain of chinook. Other mechanisms that must be investigated include respiratory burst activity of macrophages, and ultrastructural interactions between immune cells and spores. Finally, no information currently exists as to the mechanisms of resistance in *Oncorhynchus* spp. to *L. salmonae*.

Wild Salmonid and Nonsalmonid Hosts. *Loma salmonae* was detected in wild freshwater stocks of sockeye, coho, and chum salmon. The parasite was found in adult fish recently entering fresh water and smolts migrating seaward. Clinical signs were not present in smolts and the parasite was only detected using PCR. *Loma salmonae* may contaminate the gravel so that fish become infected when they hatch. The infection might remain subclinical until fish mature at sea and begin their return migration. More extensive sampling of wild fish at different life history stages, and holding subclinically infected fish for greater than 1 y could provide data to support or refute this hypothesis.

Loma embiotocia, was described in shiner perch based on host, geography, morphology, and molecular (rDNA) characteristics. Previous *Loma* spp. descriptions show extreme overlap in morphological characters and *L. embiotocia* was differentiated from *L. salmonae* based mainly on differences in the ITS region of it's rDNA. Transmission studies further support this and in light of the many *Loma* spp. found

recently in marine nonsalmonids, it is recommended species be differentiated based on host, transmission studies, and molecular sequencing when possible. Currently, such work is ongoing by AMV Brown at the University of British Columbia, Vancouver, British Columbia, Canada.

BIBLIOGRAPHY

Abela M, Brinch-Iversen J, Tanti J, Breton AL (1996) Occurrence of a new histozoic microsporidian (Protozoa, Microspora) in cultured gilt head sea bream *Sparus aurata* L. Bull Eur Ass Fish Pathol 16:196-199

Adlard RD, Barker SC, Blair D, Cribbs TH (1993) Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). Internat J Parasitol 23:423-425

Alderman DJ (1984) The toxicity of iodophors to salmonid eggs. Aquaculture 40:7-16

Amigó JM, Gracia MP, Rius M, Salvadó H, Maillo PA, Vivarés CP (1996) Longevity and effects of temperature on the viability and polar-tube extrusion of spores of *Glugea stephani*, a microsporidian parasite of commercial flatfish. Parasitol Res 82:211-214

Anenkova-Khlopina NP (1920) Contribution to the study of parasitic diseases of *Osmerus eperlanus*. Izv Otd Rybovad Nauch-prom Issled Vol. 1 &2

Anonymous (1984) Environmental studies associated with the proposed Kemano completion hydroelectric development, Vol 7, Fish diseases and parasites baseline information. Prepared by Envirocon Ltd. for Aluminum Co. of Canada, Vancouver, BC

Awakura T (1965) Studies on the *Plistophora* disease of salmonid fishes - I. Observation on the state of the occurrence and the cause in Chitose River in Hokkaido. Sci Rep Hokkaido Fish Hatchery 20:1-27

Awakura T (1974) Studies on the microsporidian infection in salmonid fishes. Sci Rep Hokkaido Fish Hatchery 29:1-96

Awakura T (1978) A new epizootic of a microsporidiosis of salmonids in Hokkaido, Japan. Fish Pathol 13:17-18

Awakura T, Kurashashi S (1967) Studies on the Plistophora disease of salmonid fish. III. On prevention and control of the disease. Sci Rep Hokkaido Fish Hatchery 22:51-68

Awakura T, Kurashashi S, Matsumoto H (1966) Studies on the *Plistophora* disease of salmonid fishes - II. Occurrence of the microsporidian disease in a new district. Sci Rep Hokkaido Fish Hatchery 21:1-12

Awakura T, Tanaka M, Yoshimiz M (1982) Studies of masu salmon, *Oncorhynchus masou* -IV *Loma* sp. (Protozoa:Microsporea) found in the gills. Sci Rep Hokkaido Fish Hatchery 37:49-54

Baker MD, Vossbrinck CR, Didier ES, Maddox JV, Shadduck JA (1995) Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. J Eukaryot Microbiol 42:564-570

Balfry SK, Iwama, GK, Evelyn, TPT (1994) Components of the non-specific immune system in coho salmon associated with strain differences in innate disease resistance. Dev Comp Immunol 18:S82

Barker DE, Khan RA, Hooper R (1994) Bioindicators of stress in winter flounder, *Pleuronectes americanus*, captured adjacent to a pulp and paper mill in St. George's Bay, Newfoundland. Can J Fish Aquat Sci 51:2203-2209

Barlough JE, McDowell TS, Milani A, Bigornia L, Slemenda SB, Pieniazek NJ, and Hedrick RP (1995). Nested polymerase chain reaction for detection of *Enterocytozoon salmonis* genomic DNA in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 23:17-23

Barton BA, Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. In: Faisal M, Hetrick FH (eds) Annual review of fish diseases. Pergamon Press, New York, NY, p 3-26

Baxa-Antonio D, Groff JM, Hedrick RP (1992) Experimental horizontal transmission of *Enterocytozoon salmonis* to chinook salmon, *Oncorhynchus tshawytscha*. J Protozool 39:699-702

Bazikalova A (1932) Additions to parasitology of Murmansk fishes. In: Mittel'mana S Ia (ed) Sbor nauchno-prom Rab Murman, CNIRC, Moscow, USSR, p 136-153

Beacham TD, Evelyn TPT (1992) Genetic variation in disease resistance and growth of chinook, coho, and chum salmon with respect to vibriosis, furunculosis, and bacterial kidney disease. Trans Amer Fish Soc 121:456-485

Bekhti M, Bouix G (1985) *Loma salmonae* (Putz, Hoffman et Dunbar, 1965) et *Loma diplodae* n. sp., microsporidies parasites de branchies de poissons téléostéens: implantation et données ultrastructurales. Protistologica 21:47-59

Bogdanova EA (1957) The microsporidian *Glugea hertwigi* Weissenberg in the stint (*Osmerus eperlanus* m. *spirinchus*) from Lake Ylyna-yarvi. In: Petrushevskii GK (ed) Parasites and diseases of fish. Izvestiya Vsesoyuznoga Nauchno-Issledovatelskogo Instituta Ozernogo I Rechnogo Rybnogo Khozyaistva, Leningrad, Russia, p1-328

Bosanquet WC (1910) Brief notes on two Myxosporidian organisms (*Pleistophora hippoglossoideos*, n. sp. and *Myxidium mackiei* n. sp.). Zool Ans 35:434-439

Bower SM (1995) Differences in acquired and innate resistance among Pacific salmon (*Oncorhynchus* spp.) against the haemoflagellate *Cryptobia salmositica* and numbers delivered by the leech vector *Piscicola salmositica*. Can J Fish Aquat Sci 52 (Suppl. 1):1-6

Bower SM, Evelyn TPT (1988) Acquired and innate resistance to the haemoflagellate *Cryptobia salmonsitica* in sockeye salmon (*Oncorhynchus nerka*). Dev Comp Immunol 12:749-760

Bower SM, Withler RE, Ridell BE (1995) Genetic variation in resistance to the haemoflagellate *Cryptobia salmositica* in coho and sockeye salmon. J Aquat Anim Health 7:185-194

Bravo S (1996) *Enterocytozoon salmonis* in Chile. Fish Health Section/Am Fish Soc Newsl 24(1):12-13

Bruno DW, Collins RO, Morrison CM (1995) The occurrence of *Loma salmonae* (Protozoa: Microspora) in farmed rainbow trout, *Oncorhynchus mykiss* Walbaum, in Scotland Aquaculture 133:341-344

Buchmann K, Ogawa K, Lo CF (1992) Immune reponse of the Japanese eel (*Anguilla japonica*) against major antigens from the microsporean *Pleistophora anguillarum* Hoshina, 1951. Gyobyo Kenkyu 27:157-161

Bückmann A (1952) Infektionen mit *Glugea stephani* und mit *Vibrio anguillarum* bei Schollen (*Pleuronectes platessa* L.). Kurze Mitteilung. Fishbiol Abt Max Planck Inst Meeresbiol 1:1-7

Cali AM, Takvorian PM (1999) Developmental morphology and life cycles of the microsporidia. In: Wittner M, Weiss LM (eds) The microsporidia and microsporidiosis. ASM Press, Washington, DC, p 85-128

Cali A, Owen RL (1990) Intracellular development of *Enterocytozoon*, a unique microsporidian found in the intestine of AIDS patients. J Protozool 37:145-155

Cali A, Takvorian PM, Ziskowski JJ, Sawyer TK (1986) Experimental infection of American winter flounder (*Pseudopleuronectes americanus*) with *Glugea stephani* (Microsporida). J Fish Biol 28:199-206

Canning EU, Lom J (1986) The Microsporidia of vertebrates. Academic Press, New York, p 1-39

Chatton E (1920) Un complexe xéno-parasitaire morphologique et physiologique *Neresheimeria paradoxa* chez *Fritillaria pellucida*. CR Acad Sci Paris 171:55-57

Chen M, Power G (1972) Infection of the American smelt in Lake Ontario and Lake Erie with the microsporidian parasite *Glugea hertwigi* (Weissenberg). Can J Zool 50:1183-1188

Chevassus B, Dorson M (1990) Genetics of resistance to disease in fishes. Aquaculture 85:83-107

Chilmonczyk S, Cox WT, Hedrick RP (1991) *Enterocytozoon salmonis* n. sp.: An intranuclear microsporidium from salmonid fish. J Protozool 38:264-269

Cipriano RC, Ford LA, Jones TE (1994) Relationship between resistance of salmonids to furunculosis and recovery of *Aeromonas salmonicida* from external mucus. J Wildl Dis 30:577-580

Clayton GM, Prince DJ (1992) Interspecific and intraspecific variation in resistance to ichthyophthiriasis among poeciliid and goodeid fishes. J Fish Biol 40:445-453

Dalmo RA, Ingebrigsten K, Bøgwald J (1997) Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J Fish Dis 20:241-273

Debaisieux P (1920) Etudes sure les microsporidies IV. *Glugea anomala* Monz. Cellule 30:215-245

Delisle CE (1965) A study in the mass mortality of the stunted smelt population, *Osmerus eperlanus mordax* at Heney Lake, Gatineau Co. Quebec. In: Proceedings of the 10th Technical Session of the Ontario Research Foundation, Kingston, Ontario, Canada, p 25-27

Delisle CE (1972) Variations mensuelles de *Glugea hertwigi* (Sporozoa:Microsporidia) chez différents tissues et organes de l'éperlan adulte dulcicole et consequences de cette infection sur une mortalité massive annuelle de ce poisson. Can J Zool 50:1589-1600

Desportes-Livage I, Chilmonczyk S, Hedrick R, Ombrouck C, Monge D, Maiga I, Gentilini M (1996) Comparative development of two microsporidian species: *Enterocytozoon bieneusi* and *Enterocytozoon salmonis*, reported from AIDS patients and salmonid fish, respectively. J Eukaryot Microbiol 43:49-60

Didier ES, Vossbrinck CR, Baker MD, Rogers LB, Bertucci DC, Shadduck JA (1995) Identification and characterization of three *Encephalitozoon cuniculi* strains. Parasitology 111:411-421

Docker MF, Devlin RH, Richard J, Kent ML (1997a) Sensitive and specific polymerase chain reaction assay for detection of *Loma salmonae* (Microsporea). Dis Aquat Org 29:41-48

Docker, MF, Kent ML, Hervio DML, Weiss L, Cali A, Devlin RH (1997b) Ribosomal DNA sequence of *Nucleospora salmonis* Hedrick, Groff, and Baxa, 1991 (Microsporea: Enterocytozooidae): implications for phylogeny and nomenclature. J Eukaryot Microbiol 44:55-60

Dogiel VA (1936) Parasites of cod from the relic lake Mogilny. Tr Leningrad Univ Biol 7:123-133

Drew GH (1910) Some notes on parasitic and other diseases of fish, 2nd series. Parasitology 3:54-62

Dyková I (1995) Phylum Microspora. In: Woo PTK (ed) Fish diseases and disorders volume 1 protozoan and metazoan infections, CAB International, Oxon, UK, p149-179

Dyková I, Figueras A (1994) Histopathological changes in turbot *Scophthalmus maximus* due to a histophagous ciliate. Dis Aquat Org 18:5-9

Dyková I, Lom J (1978) Tissue reaction of the three-spined stickleback *Gasterosteus aculeatus* L. to infection with *Glugea anomala* (Moniez, 1887). J Fish Dis 1:83-90

Dyková I, Lom J (1980) Tissue reactions to microsporidian infections in fish. J Fish Dis 3:265-283

Dyková I, Lom J, Egusa S (1980) Tissue reaction to *Glugea plecoglossi* infection in its natural host, *Plecoglossus altivelis*. Folia Parasitol 27:213-216

Eaton WD, Kent ML (1992) A retrovirus in chinook salmon (*Oncorhynchus tshawytscha*) with plasmacytoid leukemia and evidence for the etiology of the disease. Cancer Res 52:6496-6500

El-Matbouli M, Hoffmann RW (1991) Prevention of experimentally induced whirling disease in rainbow trout *Oncorhynchus mykiss* by Fumagillin. Dis Aquat Org 10:109-113

Egidius, E, Soleim Ø (1986) *Pleistophora ehrenbaumi*, a microsporidian parasite in wolffish; *Anarhichas lupus*. Bull Eur Ass Fish Pathol 6:13-15

Egusa S (1982) A microsporidian species from yellowtail juveniles, *Seriola quinqueradiata*, with "Beko" disease. Fish Pathol. 16:187-192

Elston RA, Kent ML, Harrell LH (1987) An intranuclear microsporidium associated with acute anemia in chinook salmon, *Oncorhynchus tshawytscha*. J Protozool 34:274-277

Engstad RE, Robertsen B (1993) Recognition of yeast cell wall glucan by Atlantic salmon (*Salmo salar* L.) macrophages. Dev Comp Immunol 17:319-330

Estévez J, Iglesias R, Leiro J, Ubeira FM, Sanmartin ML (1992) An unusual site of infection by a microsporean in the turbot *Scophthalmus maximus*. Dis Aquat Org 13:139-142

Evelyn TPT, Posperi-Porta L, Ketcheson JE (1986) Persistence of the kidney-disease bacterium, *Renibacterium salmoninarum*, in coho salmon, *Oncorhynchus kisutch* (Walbaum), eggs treated during and after water-hardening with povidone-iodine. J Fish Dis 9:461-464

Fantham HB, Porter A, Richardson LR (1941) Some microsporidia found in certain fishes and insects in eastern Canada. Parasitology 33:186-208

Faye N, Touguebaye BS, Boix G (1990) Ultrastructure and development of *Pleistophora senegalensis* sp. nov. (Protozoa, Microspora) from the gilt-head sea bream, *Sparus aurata* L. (Teleost, Sparidae) from the coast of Senegal. J Fish Dis 13:179-192

Figueras A, Novoa B, Santarém M, Martínez E, Alvarez JM, Toranzo AE, Dyková I (1992) *Tetramicra brevifilum*, a potential threat to farmed turbot *Scophthalmus maximus*. Dis Aquat Org 14:127-135

Finn JP, Nielson NO (1971) The effect of temperature variation on the inflammatory response of rainbow trout. J Pathol 105:257-268

Fischthal JH (1944) Observations on a sporozoan parasite of the eelpout, *Zoarces anguillaris*, with an evaluation of candling methods for its detection. J Parasitol 30:35-36

Fomena A, Coste F, Bouix G (1992) *Loma camerounensis* sp. nov. (Protozoa:Microsporida) a parasite of *Oreochromis niloticus* Linnaeus, 1757 (Teleost:Cichlidae) in fish-rearing ponds in Melen, Yaoundé, Cameroon. Parasitol Res 78:201-208

Forward GM, Ferguson MM, Woo PKT (1995) Susceptibility of brook charr, *Salvelinus fontinalis* to the pathogenic haemoflagellate, *Cryptobia salmositica*, and the inheritance of innate resistance by progenies of resistant fish. Parasitology 111:337-345

Forward GM, Woo PKT (1996) An in vitro study on the mechanism of innate immunity in *Cryptobia*-resistant brook char (*Salvelinus fontinalis*) against *Cryptobia salmositica*. Parasitol Res 82:238-241

Fritz GN, Conn J, Cockburn A, Seawright J (1994) Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovaria* (Diptera:Culicidae). Mol Biol Evol 11:406-416

Fowler LG, Banks JL (1990) Iodophor toxicity to eggs and fry of fall chinook salmon. Prog Fish Cult 52:176-178

Fowler LG, Banks JL (1991) A safe level of iodophor for treating eggs of fall chinook salmon during water hardening. Prog Fish Cult 53:250-251

Frixione E, Ruiz L, Santillán M, Vargas LV, Tejero JM, Undeen AH (1992) Dynamics of polar filament discharge and sporoplasm expulsion by microsporidian spores. Cell Mot Cytoskel 22:38-50

Frixione E, Ruiz L, Undeen AH (1994) Monovalent cations induce microsporidian spore germination in vitro. J Eukaryot Microbiol 41:464-468

Frixione E, Ruiz L, Cerbón J, Undeen AH (1997) Germination of *Nosema algerae* (Microspora) spores: conditional inhibition by D_2O, ethanol, and Hg^{2+} suggests dependence of water influx upon membrane hydration and specific transmembrane pathways. J Eukaryot Microbiol 44:109-116

Funahashi N, Miyazaki T, Kubota SS (1973) Histopathological studies on microsporidian infection in young rainbow trout. Fish Pathol 8:64-67

Fuxa JR, Brooks WM (1979) Mass production and storage of *Varirimorpha necatrix* (Protozoa: Microsporida). J Invertebr Pathol 33:86-94

Gandhi S, Locatelli L, Feist SW (1995) Occurrence of *Loma* sp. (Microsporidia) in farmed rainbow trout (*Oncorhynchus mykiss*) at a site in South West England. Bull Eur Ass Fish Pathol 15:58-60

Gjedrem T, Gjøen HM (1995) Genetic variation in susceptibility of Atlantic salmon, *Salmo salar* L., to furunculosis, BKD and cold water vibriosis. Aquacult Res 26:129-134

Goren MB, Hart PD, Young M, Armstrong JA (1976). Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides by *Mycobacterium tuberculosis*. Proc Natl Acad Sci 73:2510-2514

Grabda J (1978) Studies on parasitic infestation of blue whiting (*Micromesistius* sp.) with respect to fish utilization for consumption. Acta Ichthologica et Piscatoria 8:29-38

Griffin BR (1983) Opsonic effect of rainbow trout (*Salmo gairdneri*) antibody on phagocytosis of *Yersinia ruckeri* by trout leukocytes. Dev Comp Immunol 7:253-255

Haley AJ (1952) Preliminary observations on a severe epidemic of microsporidiosis in the smelt, *Osmerus mordax* (Mitchell). J Parasitol 38:183

Haley AJ (1953) Observations on a protozoan infection in the freshwater smelt. Proc 32nd Ann Sess New Hampshire. Acad Sci p 7

Haley AJ (1954) Microsporidian parasite, *Glugea hertwigi*, in American smelt from the Great Bay region, New Hampshire Trans Am Microsc Soc 83:84-90

Hart JL (1973) Pacific fishes of Canada. Bull Fish Res Board Can 180:304-305

Hartskeerl RA, Schuitema AR, Van Gool T, Terpstra WJ (1993) Genetic evidence for the occurrence of extra-intestinal *Enterocytozoon bieneusi* infections. Nucleic Acid Res 21:4150

Hauck AK (1984) A mortality and associated tissue reactions of chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), caused by the microsporidan *Loma* sp. J Fish Dis 7:217-229

Hedrick RP, Groff JM, Baxa DV (1991a) Experimental infections with *Nucleospora salmonis* n.g. n.sp.: An intranuclear microsporidium from chinook salmon (*Oncorhynchus tshawytscha*). Fish Health Section/Am Fish Soc Newsletter 19:5

Hedrick RP, Groff JM, Baxa DV (1991b) Experimental infections with *Enterocytozoon salmonis* Chilmonczyk, Cox, Hedrick (Microsporea): an intranuclear microsporidium from chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 10:103-108

Hedrick RP, Groff JM, Foley P, McDowell T (1988) Oral adminsitration fumagillin DCH protects chinook salmon *Oncorhynchus tshawytscha* from experimentally-induced proliferative kidney disease. Dis Aquat Org 4:165-168

Hedrick RP, Groff JM, McDowell TS, Willis M, Cox WT (1990) Hematopoietic intranuclear microsporidian infections with features of leukemia in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 8:189-197

Henry JE, Oma, EA (1974) Effect of prolonged storage of spores on field applications of *Nosema locustae* (Microsporidia: Nosematidae) against grasshoppers. J Invertebr Pathol 23:371-377

Higgins MJ, Kent ML (1996) Field trials with Fumagillin for the control of proliferative kidney disease in coho salmon Prog Fish Cult 58:268-272

Higgins MJ, Kent ML (1998) TNP-470, the analogue of fumagillin-DCH, controls PKX in naturally infected sockeye salmon, *Oncorhynchus nerka* (Walbaum), underyearlings. J Fish Dis 21:455-457

Higgins MJ, Kent ML, Moran JDW, Weiss LM, Dawe SC (1998) Efficacy of the Fumagillin analog TNP-470 for *Nucleospora salmonis* and *Loma salmonae* infections in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 34:45-49

Hoffman GL, Markiw ME (1977) Control of whirling disease (*Myxosoma cerebralis*): use of methylene blue staining as a possible indicator of effect of heat on spores. J Fish Biol 10:181-183

Houghton G, Wiegertjes GF, Groeneveld A, Muiswinkel VWB (1991) Differences in resistance of carp, *Cyprinus carpio* L., to atypical *Aeromonas salmonicida*. J Fish Dis 14: 333-341

Humason GL (1979) Animal tissue techniques, 4th ed. W.H. Freeman and Company, San Francisco, Ca

Hung HW, Lo CF, Tseng CC, Kou GH (1996) Humoral immune response of Japanese eel *Anguilla japonica* Temminck & Schlegel, to *Pleistophora anguillarum* Hoshina, 1951 (Microspora). J Fish Dis 19:243-250

Hung HW, Lo CF, Tseng CC, Kou GH (1997) Antibody response of glass eels, *Anguilla japonica* Temminck and Schlegel, to *Pleistophora anguillarum* Hosina (Microspora) infection. J Fish Dis 20:237-239

Ibarra AM, Gall GAE, Hedrick RP (1991) Susceptibility of two strains of rainbow trout *Oncorhynchus mykiss* to experimentally induced infections with the myxosporean *Ceratomyxa shasta*. Dis Aquat Org 10:191-194

Ibarra AM, Hedrick RP, Gall GAE (1992) Inheritance of susceptibility to *Ceratomyxa shasta* (Myxozoa) in rainbow trout and the effect of length of exposure on the liability to develop ceratomyxosis. Aquaculture 104:217-229

Ibarra AM, Hedrick RP, Gall GAE (1994) Genetic analysis of rainbow trout susceptibility to the myxosporean, *Ceratomyxa shasta*. Aquaculture 120:239-262

Jaronski ST (1972) Cytochemical evidence for RNA synthesis inhibition by Fumagillin. J Antibiot 6:327-331

Johnson E, Smith P (1984) Attachment and phagocytosis by salmon macrophages. Dev Comp Immunol 8: 623-630

Jones TO, Hirsch HG (1972) The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J Exp Med 136:1173-1194

Jørgensen J, Robertsen B, Engstad R (1991) Phagocytosis of yeast glucan particles by Atlantic salmon macrophages. Dev Comp Immunol 15 (Supp. 1): S85

Kaattari SL, Piganelli JD (1996) The specific immune system:humoral defense. In: Iwama G, Nakanishi T (eds), The fish immune system: organism, pathogen, and environment. Academic Press, San Diego, Ca, p 207-254

Kabata Z (1959) On two little-known microsporidia of marine fishes. Parasitology 49:309-315

Kano T, Fukui H (1982) Studies on *Pleistophora* infection in eel, *Anguilla japonica*. I. Experimental induction of microsporidiosis and Fumagillin efficacy. Fish Pathol 16:193-200

Kano T, Okauchi T, Fukui H (1982) Studies on *Pleistophora* infection in eel, *Anguilla japonica* - II. Preliminary tests for application of Fumagillin. Fish Pathol 17:107-114

Kent ML (1992) Diseases of seawater netpen-reared salmonid fishes in the Pacific Northwest. Can Spec Publ Fish Aquat Sci 116:39-42

Kent ML (1998) Protozoan and Myxozoa. In: Kent ML, Poppe TT (eds), Diseases of Seawater Netpen-reared Salmonid Fishes. Pacific Biological Station, Nanaimo, British Columbia, Canada, p 49-67

Kent ML, Dawe SC (1990) Experimental transmission of a plasmacytoid leukemia of chinook salmon *Oncorhynchus tshawytscha*. Cancer Res. (suppl) 50:5679s-5681s

Kent ML, Dawe SC (1993) Further evidence for a viral etiology in plasmacytoid leukemia of chinook salmon. Dis Aquat Org 15:115-121

Kent ML, Dawe SC (1994) Efficacy of Fumagillin DCH against experimentally induced *Loma salmonae* (Microsporea) infections in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 20:231-233

Kent ML, Fournie JW (1993) Importance of marine fish diseases - an overview. In: Couch JA, Fournie JW (eds), Advances in fisheries science pathobiology of marine and estuarine organisms. CRC Press, Boca Raton, FL, p 1-24

Kent ML, Poppe TT (1998) Diseases of seawater netpen-reared salmonid fishes. Pacific Biological Station, Nanaimo, British Columbia, Canada.

Kent ML, Dawe SC, Speare DJ (1995a) Transmission of *Loma salmonae* (Microsporea) to chinook salmon in sea water. Can Vet J 36:98-101

Kent ML, Elliott DG, Groff JM, Hedrick RP (1989) *Loma salmonae* (Protozoa:Microspora) infections in seawater reared coho salmon *Oncorhynchus kisutch.* Aquaculture 80:211-222

Kent ML, Rantis V, Bagshaw JW, Dawe SC (1995b) Enhanced detection of *Enterocytozoon salmonis* (Microspora), an intranuclear microsporean of salmonid fishes, with the Warthin-Starry stain combined with hematoxylin and eosin. Dis Aquat Org 23:235-237

Kent ML, Groff JM, Traxler GS, Zinkl JG, Bagshaw JW (1990) Plasmacytoid leukemia in seawater reared chinook salmon *Oncorhynchus tshawytscha.* Dis Aquat Org 8:199-209

Kent ML, Traxler GS, Kieser D, Richard J, Dawe SC, Shaw RW, Prosperi-Porta G, Ketchenson J, Evelyn TPT (1998) Survey of salmonid pathogens in ocean-caught fishes in British Columbia, Canada. J Aquat Anim Health 10:211-219

Kim JH, Yokoyama H, Ogawa K, Takahashi S, Wakabayashi H (1996) Humoral immune response of ayu, *Plecoglossus altivelis* to *Glugea plecoglossi* (Protozoa:Microspora). Fish Pathol 31:215-220

Kim JH, Yokoyama H, Ogawa K, Takahashi S, Wakabayashi H (1997) Elevated water temperature treatment of ayu infected with *Glugea plecoglossi*: apparent lack of involvement of antibody in the effect of the treatment. Fish Pathol 32:199-204

Kinkelin PD (1980) Occurrence of a microsporidian infection in zebra danio *Brachydanio rerio* (Hamilton-Buchanan). J Fish Dis 3:71-73

Kramer JP (1970) Longevity of microsporidian spores with special reference to *Octosporea muscadomesticae* Flu. Acta Protozool 8:217-222

Kubota SS (1967) Pathological studies on the regressive processes of cardiac muscle found on the X-disease, and the additional discussion on the etiology of the disease. J Fac Fish Prefectural Univ of Mie 7:199-209

Kumagai A, Takahashi K, Yamaoka S, Wakabayashi H (1998). Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila.* Fish Pathol 33:123-128

Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, Ingber D, Folkman J (1994) Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). Br J Cancer 69:212-216

LaPatra SE, Lauda KA, Jones GR, Shewmaker WD, Groff JM, Routledge D (1996) Susceptibility and humoral response of brown trout X lake trout hybrids to infectious hematopoietic necrosis virus: a model for examining disease resistance mechanisms. Aquaculture 146:179-188

Laudan R, Stolen JS, Cali A (1986a) Immunoglobulin levels of the winter flounder *Pseudopleuronectes americanus* and summer flounder *Paralichthys dentarus* injected with the microsporidian parasite *Glugea stephani*. Dev Comp Immunol 10:331-340

Laudan R, Stolen JS, Cali A (1986b) The immune response of a marine teleost, *Pseudopleuronectes americanus*, (winter flounder) to the protozoan parasite *Glugea stephani*. Vet Immunol Immunopathol 12:403-412

Laudan R, Stolen JS, Cali A (1987) The immunomodulating effect of the microsporidan *Glugea stephani* on the humoral response and immunoglobulin levels in winter flounder, *Pseudopleuronectes americanus*. J Fish Biol 31:155-160

Laudan R, Stolen JS, Cali A (1989) The effect of the microsporidia *Glugea stephani* on the immunoglobulin levels of juvenile and adult winter flounder (*Pseudopleuronectes americanus*). Dev Comp Immunol 13:35-41

Laurén DJ, Wishkovsky A, Groff JM, Hedrick RP, Hinton DE (1989) Toxicity and pharmocokinetics of the antibiotic Fumagillin in yearling rainbow trout (*Salmo gairdneri*). Toxicol Appl Pharmacol 98:444-453

Leiro J, Estévez J, Santamarina MT, Sanmartin ML, Ubeira FM (1993) Humoral immune response of turbot, *Scophthalmus maximus* (L.), to antigens from *Tetramicra brevifilum* Matthews & Matthews, 1980 (Microspora). J Fish Dis 16:577-584

Leiro J, Estévez J, Ubeira FM, Santamarina MT, Sanmartin ML (1994) Serological relationships between two microsporidian parasites of fish. Aquaculture 125:1-9

Leiro J, Ortega M, Estévez J, Santamarina MT, Sanmartin ML, Ubeira FM (1996a) The humoral immune response of turbot, *Scophthalmus maximus* L., to spore-surface antigens of microsporidian parasites. Vet Immunol Immunopathol 55:235-242

Leiro J, Ortega M, Estévez J, Ubeira FM, Sanmartin ML (1996b) The role of opzonization by antibody and complement in *in vitro* phagocytosis of microsporidian parasites by turbot spleen cells. Vet Immunol Immunopathol 51:201-210

Leiro J, Ortega M, Iglesias R, Estévez J, Sanmartin ML (1996c) *Pleistophora finisterrensis* n. sp., a microsporidian parasite of blue whiting *Micromesistius poutassou*. Sys Parasitol 34:163-170

Leiro J, Siso MIG, Ortega M, Santamarina MT, Sanmartin ML (1995) A factorial experimental design for investigation of the effects of temperature, incubation time, and pathogen-to-phagocyte ratio on *in vitro* phagocytosis by turbot adherent cells. Comp Biochem Physiol 112C:215-220

Leitch GJ, Visvesvara GS, He Q (1993) Inhibition of microsporidian spore germination. Parasitol Today 9:422-424

Liu S, Windom J, Kemp CW, Crews CM, Clardy J (1998) Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science 282:1324-1327

Lom J (1969) Experimental transmission of a microsporidian, *Plistophora hyphessobryconis*, by intramuscular transplantation. J Protozool (suppl.)16:17

Lom J (1975) A capillary tube method for storage of myxosporidian and microsporidian spores. Folia Parasitol (Praha) 22:275-277

Lom J, Dyková I (1992) Developments in aquaculture and fisheries science, Vol 26. Protozoan parasites of fishes. Elsevier Science, Amsterdam, p 125-157

Lom J, Dyková I, Körting W, Klinger H (1989) *Heterosporis schuberti* n.sp., a new microsporidian parasite of aquarium fish. Europ J Protistol 25:129-135

Lom J, Dyková I, Tonguthai K, Chinabut S (1993) Muscle infection due to *Heterosporis* sp. in the Siamese fighting fish, *Betta splendens* Regan. J Fish Dis 16:513-516

Lom J, Noga EJ, Dyková I (1995) Occurrence of a microsporean with characteristics of *Glugea anomala* in ornamental fish of the family Cyprinodontidae. Dis Aquat Org 21:239-242

Maddox JV, Solter LF (1996) Long-term storage of infective microsporidian spores in liquid nitrogen. J Euk Microbiol 43:221-225

Maillo PA, Amigó JM, Baena R, Salvadó H, Gracia MP (1998) *Tetramicra brevifilum* (Matthews & Matthews, 1980) (Microsporida: Tetramicriidae) in a new fish host, *Lophius budegassa* (Spinola, 1807) in Spain. Parasitol Res 84:208-212

Maita M, Satoh K, Fukuda Y, Lee H, Winton JR, Okamoto N (1998) Correlation between plasma component levels of cultured fish and resistance to bacterial infection. Fish Path 33:129-133

Magor BG (1987) First report of *Loma* sp. (Microsporida) in juvenile coho salmon (*Oncorhynchus kisutch*) from Vancouver Island, British Columbia. Can J Zool 65:751-752

Mann H (1954) Die wirtschaftliche Bedeutung von Krankheiten bei Seefischen. Fischwirtschaft Bremerhaven 6:38-39

Markey PT, Blazer VS, Ewing MS, Kocan KM (1994) *Loma* sp. in salmonids from the Eastern United States: associated lesions in rainbow trout. J Aquat Anim Health 6:318-328

Matsuyama H, Yano T, Yamakawa T, Nakao M (1992) Opsonic effect of the third complement component (C3) of carp (*Cyprinus carpio*) on phagocytosis by neutrophils. Fish Shellfish Immunol 2:68-78

Matthews RA, Matthews BF (1980) Cell and tissue reactions of turbot *Scophthalmus maximus* (L.) to *Tetramicra brevifilum* gen. N., sp. n. (Microspora). J Fish Dis 3:495-515
Maurand J, Loubés C, Gasc C, Pelletier J, Barral J (1988) *Pleistophora mirandellae* Vaney and Conte, 1901, a microsporidian parasite in cyprinid fish of rivers in Hérault: taxonomy and histopathology. J Fish Dis 11:251-259

McVicar AH (1975) Infection of plaice *Pleuronectes platessa* L. with *Glugea* (*Nosema*) *stephani* (Hagenmüller 1899) (Protozoa: Microsporidia) in a fish farm and under experimental conditions. J Fish Biol 7:611-619

Meyer A (1952) Veränderung des Fleisches beim Katfish. Fischereiwelt 57-58

Michel C, Maurand J, Loubès C, Chilmonczyk S, Kinkelin P (1989) *Heterosporis finki*, a microsporidian parasite of the angel fish *Pterophyllum scalare*: pathology and ultrastructure. Dis Aquat Org 7:103-109

Möller H, Möller K (1986) Diseases and parasites of marine fishes, Verlag Möller, Kiel, Germany

Molnár K, Baska F, Székely C (1987) Fumagillin, an efficacious drug against renal sphaerosporosis of the common carp *Cyprinus carpio*. Dis Aquat Org 2:187-190

Mora JAL (1988) Prospeccion de los principales generos parasitarios que se encuentran en salmonidos provenientes de tress pisciculturas de agua dulce de la provincia de Llanquihue (X region) Chile, Vet. Thesis, Universidad Austral de Chile, Valdivia, Chile, p 21-25

Morrison CM (1983) The distribution of the microsporidian *Loma morhua* in the tissues of the cod *Gadus morhua* L. Can J Zool 61:2155-2161

Morrison CM, Marryatt V (1986) Further observations on *Loma morhua* Morrison & Sprague, 1981. J Fish Dis 9:63-67

Morrison CM, Marryatt V, Gray B (1984) Structure of *Pleistophora hippoglossoideos* Bosanquet in the American plaice *Hippoglossoides platessoides* (Fabricius). J Parasitol 70:412-421

Morrison CM, Sprague V (1981a) Electron microscopical study of a new genus and new species of microsporida in the gills of Atlantic cod *Gadus morhua* L. J Fish Dis 4:15-32

Morrison CM, Sprague V (1981b) Light and electron microscope study of microsporida in the gill of haddock, *Melanogrammus aeglefinus* (L.). J Fish Dis 4:179-184

Morrison CM, Sprague V (1981c) Microsporidian parasites in the gills of salmonid fishes. J Fish Dis 4:371-386

Morrison CM, Sprague V (1983) *Loma salmonae* (Putz, Hoffman and Dunbar, 1965) in the rainbow trout, *Salmo gairdneri* Richardson, and *L. fontinalis* sp. nov. (Microsporida) in the brook trout, *Salvelinus fontinalis* (Mitchill). J Fish Dis 6:345-353

Morrison JK, MacConnell E, Chapman PF, Westgard RL (1990) A microsporidium-induced lymphoblastosis in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 8:99-104

Mullins JE, Powell M, Speare DJ, Cawthorn R (1994) An intranuclear microsporidian in lumpfish *Cyclopterus lumpus*. Dis Aquat Org 20:7-13

Nagel ML, Hoffman GL (1977) A new host for *Pleistophora ovariae* (Microsporida). J Parasitol 63:160-162

Nagel ML, Summerfelt RC (1977) Nitrofurazone for control of the microsporidan parasite *Pleistophora ovariae* in golden shiners. Prog Fish Cult 39:18-23

Nemecsek A (1911) Beiträge zur kenntnis der myxo and microsporidien der fische. Archiv für Protisten 22:143-169

Nepszy SJ, Budd J, Dechtiar AO (1978) Mortality of young-of-the-year rainbow smelt (*Osmerus mordax*) in Lake Erie associated with the occurrence of *Glugea hertwigi*. J Wildl Dis 14:233-239

Nepszy SJ, Dechtiar AO (1972) Occurrence of *Glugea hertwigi* in Lake Erie rainbow smelt (*Psmerus mordax*) and associated mortality of adult smelt. J Fish Res Board Can 29:1639-1641

Neumann NF, Belosevic M (1996) Antimicrobial mechanisms of activated macrophages and their induction by cytokines. Advan Struct Biol 4: 233-254

Newbound GC, Kent ML (1991) Experimental interspecies transmission of plasmacytoid leukemia in salmonid fishes. Dis Aquat Org 10:159-166

Nilsen F, Ness A, Nylund A (1995) Observations on an intranuclear microsporidian in lymphoblasts from farmed Atlantic halibut larvae (*Hippoglossus hippoglossus* L.). J Eukaryot Microbiol 42:131-135

Nigrelli RF (1946) Studies on the marine resources of southern New England. V. Parasites and diseases of the ocean pout, *Macrozoarces americanus*. Bull Bingham Oceanogr College 9:187-221

Noga EJ (1996) Fish disease diagnosis and treatment. Mosby-Year Book Inc., St. Louis, MO

Ofek I, Sharon N (1988) Lectinophagocytosis: A molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect Immunol 56:539-547

Olivier G, Eaton CA, Campbell N (1986) Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). Vet Immunol Immunopathol 12:223-234

Olson RE (1976) Laboratory and field studies on *Glugea stephani* (Hagenmuller), a microsporidan parasite of Pleuronectid flatfishes. J Protozool 23:158-164

Olson RE (1981) The effect of low temperature on the development of microsporidan *Glugea stephani* in English sole (*Parophrys vetulus*). J Wildl Dis 17:559-562

Oshima K (1964) Method of gathering and purifying active spores of *Nosema bombycis* and preserving them in good condition. Annot Zool Japan 37:94-101

Overstreet RM (1993) Parasitic diseases of fishes and their relationship with toxicants and other environmental factors. In: Couch JA, Fournie JW (eds) Advances in fisheries science pathobiology of marine and estuarine organisms. CRC Press, Boca Raton, FL, p 111-156

Pedrera IM, Rodríguez AB, Salido GM, Barriga C (1993) Phagocytic process of head kidney granulocytes of tench (*Tinca tinca*, L.). Fish Shellfish Immunol 3:411-421

Petrushevski GK, Shulman SS (1958) Parasitic diseases in fish in water reservoirs of the USSR. In: Petrushevski GK, Polyanski YI (eds) Parasitology of fishes. Leningrad University Press, Leningrad, Russia, p 301-320

Pilley BM (1978) The storage of infectious spores of *Varimorpha necatrix* (Protozoa:Microsporida) in antibiotic solution at 4°C. J Invertebr Pathol 31:341-344

Pleshinger J, Weidner E (1985) The microsporidian spore invasion tube. IV. Discharge activation begins with pH-triggered Ca2+ influx. J Cell Biol 100:1834-1838

Pomport-Castillon C, Gasc C, Romestand B (1997) Development, characterization and future prospects of monoclonal antibodies against spores of *Glugea atherinae* (Protozoa-Microsporidia-fish parasites). J Eukaryot Microbiol 44: 643-648

Porter CH, Collins FH (1991) Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). Am J Trop Med Hygiene 45:271-279

Poynton SL (1986) Distribution of the flagellate *Hexamita salmonis*, Moore, 1922 and the microsporidian *Loma salmonae* Putz, Hoffman and Dunbar, 1965 in brown trout, *Salmo trutta* L., and the rainbow trout, *Salmo gairdner* Richardson, in the River Itchen (U.K.) and three of its fish farms. J Fish Biol 29:417-429

Price RL (1982) Incidence of *Pleistophora cepedianae* (Microsporida) in gizzard shad (*Dorosoma cepedianum*) of Carlyle Lake, Illinois. J Parasitol 68:1167-1168

Price DJ (1985) Genetics of susceptibility and resistance to disease in fishes. J Fish Biol 26:509-519

Pulsford A, Matthews RA (1991) Macrophages and giant cells associated with a microsporidian parasite causing liquefaction of the skeletal muscle of the Norway pout, *Trisopterus esmarkii* (Nilsson). J Fish Dis 14:67-78

Putz RE (1969) Parasites of freshwater fishes; II. Protozoa 1. Microsporidia of fishes. US Fish Wildl Fish Dis Leaf 20

Putz RE, Hoffman GL, Dunbar CE (1965) Two new species of *Pleistophora* (Microsporidea) from North American fish with a synopsis of microsporidea of freshwater and euryhaline fishes. J Protozool 12:228-236

Ralphs JR, Matthews RA (1986) Hepatic microsporidiosis of juvenile grey mullet, *Chelon labrosus* (Risso), due to *Microgemma hepaticus* gen. nov. sp. nov. J Fish Dis 9:225-242

Reimschuessel R, Bennett RO, May EB, Lipsky MM (1987) Eosinophilic granular cell response to a microsporidian infection in a sergeant major fish, *Abudefduf saxatilis* (L.). J Fish Dis 10:319-322

Revell IL (1960) Longevity of refrigerated Nosema spores - *Nosema apis*, a parasite of honey bees. J Econ Entom 53:1132-1133

Røed KH, Brun E, Larsen HJ, Refstie T (1990) The genetic influence on serum haemolytic activity in rainbow trout. Aquaculture 85:109-117

Røed KH, Fjalestad KT, Laersen HJ, Midthjel L (1992) Genetic variation in haemolytic activity in Atlantic salmon (*Salmo salar*). J Fish Biol 40:739-750

Røed KH, Fjalestad KT, Stønsheim A (1993) Genetic variation in lysozyme activity and spontaneous haemolytic activity in Atlantic salmon (*Salmo salar*). Aquaculture 114:19-31

Rose AS, Levine RP (1992) Complement-mediated opsonization and phagocytosis of *Renibacterium salmoninarum*. Fish Shellfish Immunol 2:223-240

Ross GD, Cain JA, Lachmann PJ (1985) Membrane complement receptor type 3 (Cr3) has lectin-like properties analagous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J Immunol 134: 3307-3315

Saggers BA, Gould ML (1989) The attachment of microorganisms to macrophages isolated from tilapia *Oreochromis spilurus* Gunther. J Fish Biol 35:287-294

Saiki RS (1990) Amplification of genomic DNA. In: Innis MA, Gelfand D H, Sninsky JJ, White TJ (eds) PCR protocols. Academic Press, San Diego, Ca, p 13-20

Sakai DK (1984) Opzonization by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fishes. J Fish Dis 7:29-38

Sakai DK (1992) Repertoire of complement in immunological defense mechanisms of fish. Annu Rev Fish Dis 2:223-247

Sandholzer LA, Nostrand T, Young L (1945) Studies on an Ichthyosporidian-like parasite of ocean pout (*Zoarces anguillaris*). US Fish Wildl Serv Spec Sci Rep 31:1-12

Schmahl G, Mehlhorn H (1989) Treatment of fish parasites. 6. Effects of sym. Triazinone (Toltrazuril) on developmental stages of Glugea anomala Moniez, 1887 (Microsporidia): A light and electron microscopic study. Europ J Protistol 24:252-259

Schmahl G, Toukhy AE, Ghaffer FA (1990) Transmission electron microscopic studies on the effects of toltrazuril on *Glugea anomala*, Moniez, 1887 (Microsporidia) infecting the three-spinded stickleback *Gasterosteus aculeatus*. Parasitol Res 76:700-706

Schmidt EC, Shadduck JA (1984) Mechanism of resistance to the intracellular protozoan *Encephalitozoon cuniculi* in mice. J Immunol 133:2712-2719

Schots A, Pomp R, Muiswinkel WB (1992) Production of monoclonal antibodies. In: Stolen JA, Fletcher TC, Anderson DP, Kaattari SL, Rowley AF (eds) Techniques in fish immunology, Vol 2. SOS Publications, Fair Haven, NJ, p 1-18

Schuberg A (1910) Über Mikrosporidien aus dem Hoden der Barbe und durch sie verursachte Hypertrophie der Kerne. Arb Kaiserl Gesundheitsamte Berlin 33:401-434

Schubert G (1969) Ultracytologische Untersuchungen an der Spore der Mikrosporidienart, *Heterpsoris finki* gen. n., sp.n. Z Parasitenk 32:59-79

Scott AL, Rogers WA, Klesius PH (1985) Chemiluminescence by peripheral blood phagocytes from channel catfish: function of opsonin and temperature. Dev Comp Immunol 9:241-250

Secombes CJ (1990) Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, Muiswinkel WB van (eds) Techniques in fish immunology, Vol 1, SOS Publications, Fair Haven, NJ, p 137-144

Secombes CJ (1996) The nonspecific immune system: cellular defenses. In: Iwama G, Nakanishi T (eds) The fish immune system: organism, pathogen, and environment. Academic Press, San Diego, Ca, p 63-103

Secombes CJ, Fletcher TC (1992) The role of phagocytes in the protective mechanism of fish. Annu Rev Fish Dis 2:53-71

Shaw RW, Kent ML, Adamson ML (1996) Variation in susceptibility of three chinook salmon (*Oncorhynchus tshawytscha*) strains to *Loma salmonae* (Protozoa: Microspora). Joint meeting of the American Society of Parasitologists and the Society of Protozoologists, Tucson, AZ, 11-15 June 1996. Abstract book, p 109

Shaw RW, Kent ML, Adamson ML (1998) Modes of transmission of *Loma salmonae* (Microsporidia). Dis Aquat Org 33:151-156

Shaw RW, Kent ML, Docker MF, Brown AMV, Devlin RH, Adamson ML (1997) A new species of *Loma* (Microsporea) in shiner perch (*Cymatogaster aggregata*). J Parasitol 83:296-301

Sheehy DJ, Sissenwine MP, Saila SB (1974) Ocean pout parasites. Mar Fish Rev 36:29-33

Shulman SS, Shulman-Albova RE (1953) Parasites of White Sea fishes. Academy of Sciences Publishing House, USSR, p 1-199

Sindermann CJ (1963) Disease in marine populations. Trans N Am Wildl Nat Res Conf 28:336-356

Sindermann CJ (1966) Diseases of marine fishes. Adv Mar Biol 4:1-89

Sindermann CJ (1990) Principle diseases of marine fish and shellfish, 2nd ed. vol. 1 Academic Press, San Diego, Ca, p 79-105

Sin N, Meng L, Wang MQW, Wen JJ, Bornmann WG, Crews CM (1997) The antiangiogeneic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, Met AP-2. Proc Natl Acad Sci USA 94:6009-6103

Sitjà-Bobadilla A, Alvarez-Pellitero P (1992) Effect of Fumagillin treatment on sea bass *Dicentrarchus labrax* parasitized by *Sphaerospora testicularis* (Myxosporea: Bivalvulida). Dis Aquat Org 14:171-178

Speare DJ, Brackett J, Ferguson HW (1989) Sequential pathology of the gills of coho salmon with a combined diatom and microsporidian gill infection. Can Vet J 30:571-575

Speare DJ, Arsenault GJ, Boute MA (1998a) Evaluation of rainbow trout as a model species for studying the pathogenesis of the branchial microsporidian *Loma salmonae*. Contemp Top Lab Anim Sci 37:55-58

Speare DJ, Beaman HJ, Jones SRM, Markham RJF, Arsenault GJ (1998b) Induced resistance of rainbow trout to gill disease associated with the microsporidian gill parasite *Loma salmonae*. J Fish Dis 21:93-100

Speare DJ, Ritter G, Schmidt H (1998c). Quinine hydrochloride treatment delays xenoma formation and dissolution in rainbow trout challenged with *Loma salmonae*. J Comp Pathol 119:459-465

Sprengel G, Lüchtenberg H (1991) Infection by endoparasites reduces maximum swimming speed of European smelt *Osmerus eperlanus* and European eel *Anguilla anguilla*. Dis Aquat Org 11:31-35

Steinhagen D, Jendrysek S (1994) Phagocytosis by carp granulocytes: *in vivo* and *in vitro* observations. Fish Shellfish Immunol 4:521-525

Stønsheim A, Eide DM, Fjalestad KT, Larsen HJS, Røed, KH (1994a) Genetic variation in the humoral immune response in Atlantic salmon (*Salmo salar*) against *Aeromonas salmonicida* A-layer. Vet Immunol Immunopathol 41:341-352

Stønsheim A, Eide DM, Hofgaard PO, Larsen HJS, Refstie T, Røed KH (1994b) Genetic variation in the humoral immune response against *Vibrio salmonicida* and in antibody titre against *Vibrio anguillarum* and total IgM in Atlantic salmon (*Salmo salar*). Vet Immunol Immunopathol 44:85-95

Summerfelt RC (1964) A new microsporidian parasite from the golden shiner, *Notemigonus crysoleucas*. Trans Am Fish Soc 93:6-10

Summerfelt RC (1972) Studies on the transmission of *Pleistophora ovariae*, the ovary parasite of the golden shiner (*Notemigonus crysoleucas*). In: Final Report, Project No.4-66-R Nat Mar Fish Serv US Dept of Commerce MIS, p 1-19

Summerfelt RC, Warner MC (1970) Geographical distribution and host parasite relationships of *Plistophora ovariae* (Microsporida, Nosematidae) in *Notemigonus crysoleucas*. J Wildl Dis 6:457-465

Székely CS, Molnár K, Baska F (1988) Efficacy of Fumagillin against *Myxidium giardi* Cépéde, 1906 infection of the European eel (*Anguilla anguilla*): New observations of imported class eels. Acta Vet Hung 36:239-246

Takeda S (1933) A new disease of rainbow trout. Keison Iho 5:1-9

Takahashi S, Ogawa K (1997) Efficacy of elevated water temperature treatment of ayu infected with the microsporidian *Glugea plecoglossi*. Fish Pathol 32:193-198

Takvorian PM, Cali A (1981) The occurrence of *Glugea stephani* (Hagenmuller, 1899) in American winter flounder, *Pseudopleuronectes americanus* (Walbaum) from the New York-New Jersey lower bay complex. J Fish Biol 18:491-501

Takvorian PM, Cali A (1984) Seasonal prevalence of the microsporidan, *Glugea stephani* (Hagenmuller), in winter flounder, *Pseudopleuronectes americanus* (Walbaum), from the New York-New Jersey lower bay complex. J Fish Biol 24:655-663

Teetor-Barsch GE, Kramer JP (1979) The preservation of infective spores of *Octosporea muscaedomesticae* in *Phormia regina*, of *Nosema algerae* in *Anopheles stephensi*, and of *Nosema whitei* in *Tribolium castaneum* by lyophilization. J Invertbr Pathol 33:300-306

T'sui WH, Wang CH (1988) On the *Pleistophora* infection in eel I. Histopathology, ultrastructure and development of *Pleistophora anguillarum* in eel, *Anguilla japonica*. Bull Inst Zool Acad Sin 27:159-166

T'sui WH, Wang CH, Lo CF (1988) On the *Pleistophora* infection in eel II. The development of *Plistophora anguillarum* in experimentally infected elvers, *Anguilla japonica*. Bull Inst Zool Acad Sin 27:249-258

Urawa S (1989) Seasonal occurrence of *Microsporidium takedai* (Microsporida) infection in masu salmon, *Oncorhynchus masou*, from the Chitose river. Physiol Ecol Japan Spec 1:587-598

Urawa S, Awakura T (1994) Protozoan diseases of freshwater fishes in Hokkaido. Sci Rep Hokkaido Fish Hatchery 48:47-58

Vogler AP, DeSalle R (1994) Evolution and phylogenetic information content in the ITS-1 region in the tiger beetle *Cicindela dorsalis*. Mol Biol Evol 11:393-405

Vialova GP (1984) Microsporidian infection in pink salmon. Fish Res Inland Waters 10:9-11

Wales JH, Wolf H (1955) Three protozoan diseases of trout in California. Calif Fish Game 41:183-187

Wassom DL, Kelly EAB (1990) The role of the major histocompatibility complex in resistance to parasitic infections. Crit Rev Immunol 10:31-52

Waterstrat PR, Ainsworth AJ, Capley G (1991) In vitro responses of channel catfish, *Ictalurus punctatus*, neutrophils to *Edwardsiella ictaluri*. Dev Comp Immunol 15:53-63

Weidner E (1975) Interactions between *Encephalitozoon cuniculi* and macrophages. Parasitophorous vacuole growth and the absence of lysosomal fusion. Zeitschrift fur Parasit 47:1-9

Weidner E, Sibley LD (1985) Phagocytized intracellular microsporidian blocks phagosome acidification and phagosome-lysosome fusion. J Protozool 32:311-317

Weissenberg R (1921) Zür Wirtsgewebsableitung des Plasmakorpers der *Glugea anomala* Cysten. Arch Protistenk 42:400-421

Weissenberg R (1922) Mikrosporidien und Chlamydozoen als Zellparasiten von Fischen. Verh Deutsch Zool Ges 27:41-43

Weissenberg R (1968) Intracellular development of the microsporidian *Glugea anomala* Moniez in hypertrophying migratory cells of the fish *Gasterosteus aculeatus* L., an example of the formation of "xenoma tumors". J Protozool 15:44-57

Wiegertjes GF, Stet RJM, Parmentier HK, Muiswinkel WB van (1996) Immunogenetics of disease resistance in fish: a comparative approach. Dev Comp Immunol 20:365-381

Wilkund T, Lounasheimo L, Lom J, Bylund G (1996) Gonadal impairment in roach *Rutilus rutilus* from Finnish coastal areas of the northern Baltic sea. Dis Aquat Org 26:163-171

Wiseman DL, Brown JA (1996) Early growth and survival of larval striped wolffish (*Anarhichas lupus*): a behavioural approach. Bull Aquacult Assoc Can 1:12-13

Wishkovsky A, Groff JM, Lauren DJ, Toth RJ, Hedrick RP (1990) Efficacy of Fumagillin against proliferative kidney disease and its side effects in rainbow trout (*Oncorhynchus mykiss*) fingerlings. Fish Pathol 25:141-146

Withler RE, Evelyn TPT (1990) Genetic variation in resistance to bacterial kidney disease within and between two strains of coho salmon from British Columbia. Trans Am Fish Soc 119:1003-1009

Wongtavatchai J, Conrad PA, Hedrick RP (1995a) In vitro characteristics of the microsporidian: *Enterocytozoon salmonis*. J Eukaryot Microbiol 42:401-405

Wongtavatchai J, Conrad PA, Hedrick RP (1995b) Effect of the microsporidian *Enterocytozoon salmonis* on the immune response of chinook salmon. Vet Immunol Immunopathol 48:367-374

Wyrick RB, Brownridge EA (1978) Growth of *Chlamydia psittaci* in macrophages. Infect Immun 19:1054-1060

Yokoyama H, Ogawa K, Wakabayashi H (1990) Chemotherapy with Fumagillin and toltrazuril against kidney enlargement disease of goldfish caused by the myxosporean *Hoferellus carassii*. Fish Pathol 25:157-163

Zar JH (1984) Biostatistical analysis, 2nd edn. Prentice Hall, New Jersey, p 185-243

Zhu X, Wittner M, Tanowitz HB, Cali A, Weiss LM (1994) Ribosomal RNA sequences of *Enterocytozoon bieneusis*, *Septata intestinalis* and *Ameson michaelis*: Phylogenetic construction and structural correspondence. J Eukarot Microbiol 41:204-209

Zhuo LS, Sajdak L, Phillips RB (1994) Minimal intraspecific variation in the sequence of the transcribed spacer regions of the ribosomal DNA of lake trout (*Salvelinus namaycush*). Genome 37:664-671

APPENDIX I

Peer Reviewed Publications

Shaw RW, Kent ML (1999) Fish microsporidia. In: Wittner M, Weiss LM (eds) The microsporidia and microsporidiosis. ASM Press, Washington, DC, p 418-446

Shaw RW, Kent ML, Adamson ML (1998) Modes of transmission of *Loma salmonae* (Microsporidia). Dis Aquat Org 33:151-156

Shaw RW, Kent ML, Adamson ML (1999) Iodophor treatment is not completely efficacious in preventing *Loma salmonae* (Microsporidia) transmission in experimentally challenged chinook *Oncorhynchus tshawytscha* (Walbaum) salmon. J Fish Dis 22:1-3

Shaw RW, Kent ML, Docker MF, Brown AMV, Devlin RH, Adamson ML (1997) A new species of *Loma* (Microsporea) in shiner perch (*Cymatogaster aggregata*). J Parasitol 83:296-301