MECHANISMS OF RESISTANCE TO THE MICROSPORIDIAN *LOMA SALMONAE* AND ITS LOCALIZATION IN GILLS FROM EXPERIMENTALLY EXPOSED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT

Loma salmonae is a microsporidian that infects salmonids of the genus *Oncorhynchus* and produces spore-filled xenomas in the gills. The resistance phenomenon of rainbow trout to *L. salmonae* has been documented, but the nature of this resistant state is unknown. The first objective examines how rapidly this resistance develops by using the exposure re-exposure model combined with a technique for measuring sphere-like structures. When fish received a primary oral challenge with live spores, partial resistance developed by week (wk) 2 postexposure (PE) and a complete resistance to re-challenge occurred at 8 wk PE. Fish which received their primary challenge in the form of non-viable spores administered intraperitoneally (IP), showed evidence of partial resistance by wk 3 and total resistance by wk 6 PE. Cell-mediated immunity has been postulated to be involved in the immune response of fish to *L. salmonae*; however, research in cell-mediated immunity to this parasite is still in its infancy. The second objective of this study investigates if head kidney lymphocytes from recovered, exposed, and unexposed fish could respond to antigens from *L. salmonae* when measured in an *in vitro* lymphocyte proliferation assay. Lymphocytes from fish orally exposed to live spores reached a maximum proliferation at wks 6 and 8 PE, when stimulated *in vitro* with the soluble and particulate antigens (Ag) respectively. When fish recovered from an initial infection were re-stimulated at wk 14 with live spores, a peak in the proliferative response to the particulate Ag occurred only at wk 15 PE. Lymphocytes from fish IP exposed to dead spores had a high lymphoproliferation at wk 2 PE when *in vitro* stimulated with the particulate Ag rather than with the soluble Ag. Finally, lymphocytes from fish IP exposed to dead spores and then orally re-exposed with fresh spores at wk 22 PE, had the highest proliferative response when stimulated *in vitro* with both Ag. Intraperitoneal inoculation with dead spores and subsequent exposure to live spores generated a stronger *in vitro* response to the particulate Ag and the parasitosis was considerably reduced in those fish. Studies of the events that occur during the gill localization and development of *L. salmonae* have not been made. The third objective of this study was to examine the ultrastructural features of gills from fish experimentally infected with *L. salmonae* to explain the mechanisms of infection in gills. At 3 wk PE, meronts were recognized within blood cells and pillar cells (no xenomas) associated with the capillary channels, lamellae and lamellar arteries and inflammation was absent. In lamellar arteries, infected cells were underneath endothelium. At wk 4, PE xenomas were observed isolated from gill lamella capillaries, beneath lamellar artery endothelium and within the interstitial tissue of the filament. Inflammatory reaction to parasites occurred at 4 wk PE. Peripheral distribution of spores inside some xenomas was observed at wk 5 and 6 PE. Degenerative changes in some meronts were observed at wk 7 PE. Round, flattened and irregular shaped xenomas were observed at wk 8 PE. Round xenomas showed inflammatory response accompanied by germination of spores and by the presence of two tubular appendages, types I and II tubules. Flattened xenomas were observed underneath the endothelium of gill lamella arteries. The irregular xenoma was located in the connective tissue of the gill filament and showed multiple projections occupied by spores. Both flattened and irregular xenomas showed no evidence of inflammatory reaction. Three hypotheses: Isolation, Internalization and Evasion, are proposed to explain the localization of *L. salmonae* in the gills.

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DEDICATION

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
°C	Degrees Celsius
ConA	Concanavalin A
cpm	Counts per minute
ER	Endoplasmic reticulum
h	Hours
H&E	Haematoxylin and eosin
HRLM	High resolution light microscopy
IFN-γ	Interferon gamma
IL	Interleukin.
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
LPS	Lipopolysaccharide
µCi	Microcurie
µg	Microgram
min	Minutes
ml	Milliliter
µl	Microlitre
µm	Micrometre
PCR	Polymerase chain reaction
PE	Postexposure
PHA	Phytohaemaglutinin
PMN	Neutrophil
PRE	Post re-exposure
PV	Parasitophorous vacuole
RER	Rough endoplasmic reticulum
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SD	Standard deviation
SE	Standard error
SI	Stimulation index
SPOV	Sporophorous vacuole
TEM	Transmission electron microscopy
TNF-α	Tumor necrosis factor alpha
Wk	Week
Xdiam	Xenoma diameter

1 GENERAL INTRODUCTION

1.1 MICROSPORIDIA: A GENERAL VIEW

Microsporidia are obligate intracellular parasites that can infect both invertebrates and vertebrates (Canning et al. 1986, Curry and Smith 1998). Microsporidia belong to the phylum Microspora and are characterized by the production of infective spores (Dyková 1992). Spores have a unique mode of infection through a complicated eversion apparatus by which the sporoplasm is injected into the host cell cytoplasm (Lom and Dyková 1992). These parasites were previously considered only as important pathogens for insects and fish. However, since the onset of AIDS epidemics in 1985, they have been diagnosed as opportunistic infections in immunocompromised individuals, and new species have been described (Curry and Smith 1998, Mathis 2000, Ambroise-Thomas 2001). In the aquaculture industry, microsporidia are common parasites causing diseases in fish in fresh water, estuarine and marine habitats (Dyková 1992). Infection with microsporidia occurs in salmonids and in other valuable fish, like flatfish, carp, and eels, causing diminished fish growth and reduced productivity (Dyková 1992, Constantine 1999, Shaw and Kent 1999).

1.2 GENERAL FEATURES OF MICROSPORIDIA

1.2.1 Mature spore

Although microsporidia are considered true eukaryotes, they lack a typical Golgi complex, peroxisomes and mitochondria (Vávra and Ronny-Larson 1999). The taxonomic position of this group of parasites is still controversial, since molecular studies indicate that is more closely associated to fungi (Keeling and McFadden 1998, Keeling et al. 2000, Van

de et al. 2000). Basically the only parasitic stage that survives outside the host is the mature spore (Infective Phase). Spores are very complex structures comprised of a thick wall, an extrusion apparatus and genetic material (Figure 1.1). The spore wall is composed of 2 layers: the electron-lucent inner layer (endospore), which is made of chitin, and the proteinaceous outer electron-dense layer (exospore), which provides resistance to extreme conditions (Amigo et al. 1996, Cali and Takvorian 1999, Bigliardi and Sacchi 2001). A plasma membrane outlines the internal part of the endospore and encloses the cytoplasm and the internal structures of the spore, such as the extrusion apparatus, the infective agent (sporoplasm), and the posterior vacuole. The extrusion apparatus is a unique system for the infection of the host cell (Canning et al. 1986, Cali and Takvorian 1999), and works like a harpoon system (Vivarès and Méténier 2001). It has three parts: the posterior vacuole, the polar tube, and the polaroplast. The posterior vacuole resides in the last third of the spore and consists of a series of membrane-bound vesicles that are considered part of the Golgi apparatus (Bigliardi and Sacchi 2001). During the spore germination the posterior vacuole swells increasing the internal pressure of the spore. The polar tube is a long structure similar to a thread and apparently starts in an electron-dense mushroom-like structure (polar sac) situated in the apical part of the spore. The polar tube extends toward the posterior end of the spore forming several coils that are useful for species identification (Hazard et al. 1981). At the ultrastructural level, the polar tube is covered by a membrane that is believed to be part of the polaroplast membrane system (Chioralia et al. 1998, Vávra and Ronny-Larson 1999). Cross-sections of the polar tube show concentric areas of different densities (Bigliardi and Sacchi 2001).

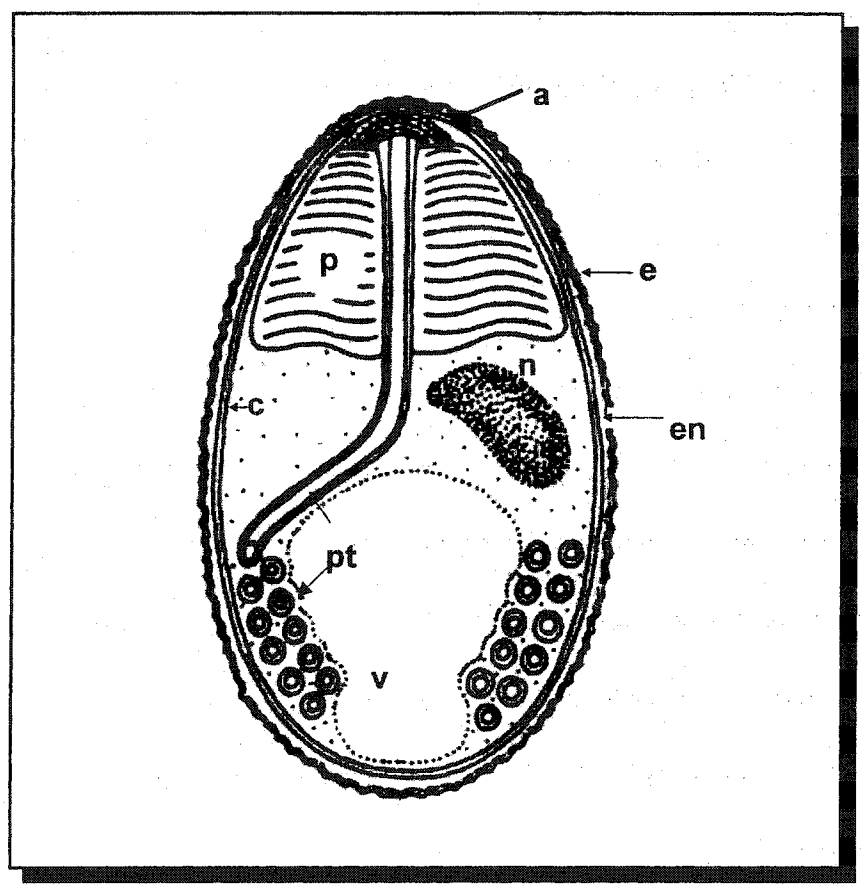

Figure 1.1. The basic structure of a microsporidian spore. a = anchoring disk; e = exospore; en = endospore; c = cell membrane; p = polaroplast; n = nucleus; pt = coiled polar tube; v = posterior vacuole (From Woo, P.T.K. 1995. Fish Diseases and Disorders. Vol 1. Protozoan and Metazoan Infections. CAB International).

The eversion of the polar tube has been compared to a tube sliding within another tube (Chioralia et al. 1998, Keohane and Weiss 1999). The extrusion of the tube follows a screw-like movement which is suggested to accelerate the expulsion of the sporoplasm from the spore (Chioralia et al. 1998). Consequently, the uncoiling of the polar tube causes a counter-rotating motion of the spore (Vivarès and Méténier 2001).

The polaroplast is located in the first third of the spore and consists of a series of vesicles and flattened membranes arranged perpendicularly to the polar tube. This membrane system may supply the membrane that surrounds the sporoplasm when it is internalized into the host cell (Weidner et al. 1984). During the eversion of the polar tube, the polaroplast swells due to sudden osmotic changes in its matrix (Undeen 1990, Undeen and Solter 1996, Bigliardi and Sacchi 2001). The space between the polaroplast and the posterior vacuole is occupied by cytoplasm and the nucleus (sporoplasm) of the spore (Canning et al. 1986). The germination of the spore occurs by osmotic changes and swelling of both the posterior vacuole and the polaroplast, increasing the spore internal pressure and impelling the sporoplasm through the polar tube (Vávra and Ronny-Larson 1999) which is everted in a few seconds (Chioralia et al. 1998). Two theories have been proposed to explain the mechanism for increasing the osmotic pressure in the spore. One establishes the development of an osmotic event generated by the conversion of trehalose into glucose with ATP production (Undeen 1990, Undeen and Solter 1996, Méténier and Vivarès 2001). The subsequent flow of water into the spore increases the spore internal pressure and polar tube discharge (Keohane and Weiss, 1999). The other mechanism proposes the entry of water into the spore after the entrance of monovalent ions, like calcium (Ca+). This last process involves the

participation of protein receptors, aquaporins, that are activated by a proton gradient generating an increase in the internal hydrostatic pressure of the spore (Dall 1983).

The nucleus can be a single unit (monokaryon), seen in *Loma, Enterocytozoon, or Encephalitozoon*, or it can be divided in two apposed units (diplokaryon) as in *Nosema* (Canning et al. 1986, Lom and Dyková 1992). When released as a minute cell into the host, the sporoplasm appears to contain abundant membranes and is accompanied by ribosomes (Canning et al. 1986, Vávra and Ronny-Larson 1999, Bigliardi and Sacchi 2001).

1.2.2 Life cycle

For most microsporidia, natural infection begins when a susceptible host ingests infective spores, although experimentally other means of transmission have been reported, including intravenous (IV), intramuscular (IM) and anal gavage routes (Shaw and Kent 1999, Dunn and Smith 2001). Normally, the spores germinate in the gut under appropriate stimuli (Dyková 1992), which could involve sudden changes in pH or enzymatic events in the spore (Cali and Takvorian 1999). High pressure is created inside the spore causing the eversion of the polar tube through the thinnest part of the apical spore wall. Experimentally, germination of the spore can be induced *in vitro* by chemical or physical changes, such as using monovalent ions (Fl^-, Cl^-), hydrogen peroxide, and manual pressure of the spores between two cover slides (Undeen 1990). The polar tube turns outward with enough force to perforate a host cell membrane (epithelial cells or intraepithelial leucocytes), injecting the sporoplasm directly into its cytoplasm and avoiding any interaction with the extracellular milieu. During

its transit through the polar tube, the sporoplasm acquires its external membrane, apparently originating from the polaroplast membrane system (Weidner 1972, Weidner et al. 1984).

Recently, some microsporidians, particularly *Encephalitozoon intestinalis*, were shown to enter epithelial cells and macrophages by a phagocytic mechanism (Magaud et al. 1997, Foucault and Drancourt 2000). In addition, the sporoplasm is liberated only when the spore has established specific interaction between a microsporidia ligand with a receptor on the host cell (Foucault and Drancourt 2000). In the host cell cytoplasm, the sporoplasm matures into a meront (Vávra and Ronny-Larson 1999), which can either multiply, creating a primary infection in that tissue or can be transported to other sites for development. However, little is known about the mechanisms of transportation of the early stages of the parasite. Body fluids, i.e. blood and lymph, or cells including macrophages, undifferentiated mesenchymal cells, and leucocytes are suggested to contribute in the parasite distribution inside the host (Weissenberg 1968, Bekhti and Bouix 1985b, Canning et al. 1986, Dunn and Smith 2001).

For most microsporidia, the development of the parasite within the host cell cytoplasm starts immediately after infection. Two stages of development are recognized: merogony or proliferative phase, which produces meronts, and sporogony or spore developing phase (Dyková 1992, Cali and Takvorian 1999), which involves the conversion of meronts into sporonts, the cells that produce the sporoblasts and subsequently the mature spores (Bigliardi and Sacchi 2001). During merogony, the meront divides many times by binary or multiple fission, forming uni- or binucleate meronts or multinucleate plasmodia, or both depending on the genera (Dyková 1992). Meronts are rounded cells that can multiply

in direct contact with the host cell cytoplasm or be isolated from it inside an envelope (Cali and Takvorian 1999). In general the definition for the host-parasite interface during the parasite development can be classified into two types: parasitophorous vacuole (PV), when the envelope originates from the host, and sporophorous vesicle (SPOV), when the envelope originates from the parasite (Cali and Takvorian 1999). The SPOV is associated with other intracellular microorganisms as well (Suss-Toby et al. 1996, Sinai et al. 1997). However, this classification is not so simple: depending on the genera, the relationship between the meront plasmalemma and the host cell cytoplasm can be further divided into 4 types. Type I, the plasmalemma of the parasite is in direct contact with the host cell cytoplasm (hyaloplasm) *(Enterocytozoon, Nosema)*. Type II, a parasitophorous vacuole, which contains the developing meronts, is surrounded by a single membrane produced by the host (*Encephalitozoon*). Type III, an amorphous coat is secreted by the parasite during its development (*Pleistophora*), and Type IV, proliferating parasites are surrounded by host endoplasmic reticulum (ER) (*Loma, Vittaforma*) (Bigliardi and Sacchi 2001). This classification is not yet definitive, evolving as new morphological features are discovered.

Meronts have a large nucleus covered by a nuclear envelope with the typical double membrane arrangement and a prominent nucleolus. Electron-dense spindle plaques are localized in invaginations of the nuclear envelope. The cytoplasm contains a few elements of rough endoplasmic reticulum (RER), abundant ribosomes and multiple vesicles that are from the Golgi apparatus (Morrison and Sprague 1981c).

Sporogony is the culmination of microsporidial development and involves the transitional development of sporonts into sporoblasts and finally into spores. The first

indication that meronts are transforming into sporonts is the formation of patches of an electron-dense material next to the plasmalemma, which eventually will become the exospore of the parasite (Méténier and Vivarès 2001). In addition, the cytoplasm of the developing sporonts increases in density due to increases in the amounts of RER and ribosomes (Lom and Dyková 1992, Cali and Takvorian 1999). After the electron-dense coat is completely formed, the number of cell divisions that occur in the sporont is modified depending on the genera (Bigliardi and Sacchi 2001). They can divide by binary fission forming two sporoblasts, but if nuclear division is not accompanied by cell division, then multinucleate structures (sporogonial plasmodia) are formed and eventually undergo metamorphosis into sporoblasts (Canning et al. 1986).

The next step before spore development is the formation of the sporoblast. During this stage the exospore is complete and acquires a crenate aspect. In addition, the extrusion apparatus is observed in different stages of differentiation, and the endospore is formed by the continuous deposition of chitin between the electron-dense exospore and the plasmalemma. At this stage the sporoblasts become reduced in size and the density of the parasite cytoplasm increases (Bigliardi and Sacchi 2001, Méténier and Vivarès 2001). The sporoblast is considered a mature spore after all its internal structures are arranged in their typical position.

1.3 Microsporidia in mammals

Microsporidia are ubiquitous parasites and important pathogens of arthropods (Avtalion 1981), and fish (Dyková and Lom 1980, Dyková 1992, Lom and Dyková 1992,

Shaw and Kent 1999). They are well known as destructive parasites of the honey bee and silkworm industry, and in fisheries they are a constant threat (Kent 2000). In mammals, they naturally infect several laboratory animals, such as guinea pigs, rats, mice and hamsters (Shadduck and Orenstein 1993). Domestic large animals are not as affected as carnivores, like dogs (McCully et al. 1978, Botha et al. 1986), cats (Pang and Shadduck 1985), and blue foxes (Mathis et al. 1996). The most common microsporidia isolated from these mammals is *Encephalitozoon cuniculi*, which can also infect non-human primates and humans (Khan et al. 2001, Vivarès and Méténier 2001). Microsporidiosis caught attention as an emergent disease since the onset of AIDS as a pathogen causing persistent diarrhea in immunosuppressed people (Curry and Smith 1998, Ambroise-Thomas 2000, Ambroise-Thomas 2001, Franzen and Muller 2001). The current classification for species identification of most microsporidia in mammals is based on transmission electron microscopy analysis (Hazard et al. 1981, Fedorko and Hijazi 1996, Franzen and Muller 1999, Schottelius et al. 2000). Presently, there are 12 microsporidial species in 7 genera described in humans: *Encephalitozoon, Enterocytozoon, Vittaforma, Nosema, Microsporidium, Trachipleistophora, and Brachiola* (Didier et al. 2000, Mathis 2000). Research in microsporidia has been intensified recently since infections that were originally detected in animals are now being recognized in humans (Mathis 2000), increasing the risk of zoonoses (Curry and Smith 1998, Conway and Roper 2000). For instance, *E. cuniculi* has a broad host range and strains I and III, which normally infect rabbits and dogs respectively, have also been identified in humans (Snowden et al. 1998). *Enterocytozoon bieneusi*, one of the most important microsporidian infections in humans, is the natural parasite of pigs and certain non-human primates

(Chalifoux et al. 1998). This parasite infects epithelial cells of the small intestine in AIDS patients causing in severe diarrhea and wasting (Cali and Owen 1990, Shadduck and Orenstein 1993, Weber et al. 1994, Dunn and Smith 2001). Recently it was hypothesized that fish could be a reservoir for microsporidia infections, but after ultrastructural and biochemical studies this supposition was rejected (Desportes-Livage et al. 1996). *Encephalitozoon intestinalis* is a microsporidian that has been identified in AIDS patients and immunosuppressed mice presenting persistent diarrhea, and has now been detected, by using PCR techniques, in the excrement of immunocompetent ruminants, donkeys, pigs, and dogs (Bornay-Llinares et al. 1998). The common denominator of all the microsporidial infections in humans is the presence of an immunosuppressive status. Nevertheless, microsporidiosis has been recognised in immunocompetent hosts with a few or no clinical signs, which increases the potential development of reservoirs of infection (Didier and Bessinger 1999, Ambroise-Thomas 2001).

1.4 MICROSPORIDIA IN FISH

Microsporidial infections are a constant threat for aquaculture; these parasites are widely distributed in seawater, fresh water and estuaries (Dyková 1992). Prevalence is especially elevated when the fish population density is extremely high, increasing the morbidity and the mortality rates in young fish (Canning et al. 1986). Wild salmonids can disseminate spores and are a constant threat to salmonid species reared in netpens (Kent 2000). Infections caused by microsporidia reduce the growth rate of fish and decrease the productivity in fish farms (Kinkelin 1980, Constantine 1999). Microsporidia affect valuable

fish species like salmonids (Kent 2000), flatfish (Matthews and Matthews 1980), and eels (Leiro et al. 1999), and also ornamental fish, including zebra fish (*Brachydanio rerio*) (Kinkelin 1980), and killifish (Family *Cyprinodontidae*) (Lom et al. 1995).

Microsporidia in fish can be classified according to their ability to form xenomas, like *Glugea, Tetramicra, Loma, Ichthyosporidium, Jirovecia, Microsfilum, Microgemma* and *Nosemoides*, and those that do not form xenomas, such as *Nucleospora, Pleistophora, Heterosporis*, and *Thelohania* (Shaw and Kent 1999). This chapter will focus only on the xenoma-forming parasites.

The xenoma is a complex cyst-like structure comprised of a hypertrophic host cell harboring the microsporidia in multiple stages of development (Weissenberg 1968, Weidner 1976, Sprague and Hussey 1980). The infected host cell increases in size not only because of the proliferating parasites, but by hypertrophy of the infected cell, including the nucleus (Canning et al. 1986, Lom and Dyková 1992). The parasite modifies the cytoskeleton of the host cell to support its development (Leitch et al. 1999). The early developmental stages of the parasite, meronts, are observed closely associated with host mitochondria (and sometimes RER), suggesting control of energy and protein metabolism in the host cell (Weidner 1970, Weidner 1976). The xenoma (hypertrophic cell fully integrated with the parasite) is considered as a complete separate entity from the rest of the host (Weidner 1976). Xenoma formation has a dual purpose: 1) protects the parasite from the host attack, and 2) it confines the parasite in one site preventing its dissemination through the host body (Sprague and Hussey 1980).

The size of the xenoma, implantation site and tissue reaction to the xenoma depends on the genera. For example, *Glugea*, one of the most studied fish microsporidia, causes impressive xenomas and injury in the submucosa intestinal cells of wild and cultured fish (Weidner 1976, Shaw and Kent 1999). An important classification regarding the progression of the host response to xenoma (weak, productive and granuloma formation stages) was created based on histopathological observations in fish infected with several species of *Glugea* (Dyková and Lom 1980).

The only species of the genus *Tetramicra*, *T. brevifilum*, affects turbot (*Scophthalmus maximus*), and produces visible xenomas in connective tissue of muscles and viscera. Sometimes there is adherence between xenomas creating composite cysts of variable size. The rupture of the xenomas and the release of spores cause muscular degeneration, necrosis and infiltration by mononuclear cells, such as macrophages and lymphocytes. Fish die because of decreased mobility leading to predation or starvation (Matthews and Matthews 1980).

As the aquaculture industry continues to grow worldwide, microsporidial infections continue to inflict large losses (Constantine 1999), yet there is still a considerable lack of information regarding the biology, pathogenesis, control and immune response of fish to microsporidia (Leiro et al. 1993a). The development of animal models for studying fish microsporidial infections under controlled conditions has been advocated to study the pathogenesis of microsporidia (Leiro et al. 1999, Speare et al. 1998a). New genera (Lom et al. 1999) and species of microsporidia are appearing in cultured fish (Shaw et al. 1997). Some of these microsporidial parasites have been redefined and transferred to a new genus, like *G. caulleryi,* a parasite of the sand-eel (*Hyperoplus lanceolatus*), which has been relocated to the

genus *Microgemma* (Leiro et al. 1999). However, there are microsporidia that still have not been assigned a defined species and have been assigned to the collective group *Microsporidium* (Brocklebank et al. 1995, Kent et al. 1999b).

1.5 RESISTANCE MECHANISMS TO MICROSPORIDIA IN FISH

Teleosts have developed an immune system comparable to mammals (van Muiswinkel 1995, Iwama and Nakanishi 1996, Ellis 2001), and although research in host resistance to bacteria, viruses (Ellis 1999), and large parasites (Hoffman 1999, Jones 2001) is fairly extensive, the study of the immune response against fish microsporidia is still unexplored (Dyková 1992, Leiro et al. 1993b, Shaw and Kent 1999).

1.5.1 Innate immunity to fish microsporidia

1.5.1.1 Soluble factors

Recently, complement and lectins have been implicated in the destruction of *L. salmonae* spores by macrophages from the Atlantic salmon (*Salmo salar*), a naturally resistant salmonid (Shaw et al. 2001). Previous treatment of *L. salmonae* spores with plasma from naive fish significantly increase the phagocytic activity of Atlantic salmon macrophages when compared to that observed with macrophages from the chinook salmon, which is a susceptible fish. Complement, acting as an opsonin, and the presence of lectins on the spore surface could have facilitated the uptake and intracellular destruction of the spores (Shaw et al. 2001). Other plasma substances, like chitinases have been proposed in the elimination of the parasite and increase resistance to fish microsporidia (Leiro et al. 1997). Additionally, chitin itself seems

to stimulate the innate immune response when either included in the fish diet (Esteban et al. 2001), or when injected via IP (Esteban et al. 2000).

1.5.1.2 Leucocytes

One of the most recognized mechanisms for the elimination of microsporidia from the host tissue has been through the phagocytic process (Canning et al. 1986). After phagocytes arrive at the xenoma site, they ingest and destroy the spores. The signal that triggers this event has not been characterized, but the polysaccharide chitin is thought to be involved in the process (Speare et al. 1998b, Esteban et al. 2000). During the phagocytic event, there is complete dissolution of the internal structures of the spore, followed by distortion and complete collapse of the inner and outer layers (Matthews and Matthews 1980).

Some microsporidia have evolved mechanisms that allow them to resist destruction by phagocytes. When spores of *G. hertwigi* are ingested and contained in a phagosome, they prevent the normal fusion of lysosomes with the phagosome by the formation of anionic bonds. These bonds increase the rigidity of the phagosome membrane preventing its acidification and the subsequent phagolysosome formation (Weidner and Sibley 1985). The escape of the parasite from the phagocytic mechanisms was studied in turbot macrophages exposed to viable and non-viable spores of *M. caulleryi*. Viable spores were ingested in higher proportion than non-viable spores. However, macrophages ingesting viable spores produced less reactive oxygen (ROI) and nitrogen intermediates (RNI), than those that ingested non-viable spores. Apparently, live spores were able to induce their own phagocytosis by macrophages, but they were able to block the intracellular killing mechanisms of the

phagocytes (Leiro et al. 2000). A similar inhibitory mechanism of the respiratory burst of leucocytes was observed in the macrophages from ayu (*Plecoglossus altivelis*) exposed to spores of *G. plecoglossi* (Kim et al. 1998).

1.5.2 Specific immunity to fish microsporidia

1.5.2.1 Humoral response

The study of the humoral response to fish microsporidia is relatively new and controversial. In some cases antibody responses are well established, while in other situations the humoral response is not protective or is depressed (Kim et al. 1996). Specific antibodies (Ab) to spores of *G. plecoglossi* have been detected in experimentally and naturally infected ayu, but they were not protective as fish still developed xenomas (Kim et al. 1996). Similar results were found in turbot experimentally exposed to soluble and particulate Ag from *T. brevifilum* (Leiro et al. 1993b). Likewise, levels of Ab in the Japanese eel (*Anguilla japonica*) recognizing soluble protein from *P. anguillarum*, were higher in exposed fish than in naive individuals (Hung et al. 1996). In contrast, a decrease in the total IgM levels was observed in experimental infections with *G. stephani* in the winter flounder, *Pleuronectes americanus*. Subsequent challenges with the same Ag provoked even lower Ab levels. This immunosuppression was caused by prostaglandins released from the infected host (Laudan et al. 1986a, b, Laudan et al. 1989, Dyková 1992).

1.5.2.2 Cellular response

Study of the cellular response against fish microsporidia is still in its infancy. Although the nature of the microsporidial infection suggests participation of cellular mechanisms, few investigations have focused on the cellular immune response in infected fish. The arguments suggesting participation of cellular immunity to microsporidia infections are: 1) microsporidia are intracellular parasites (Bigliardi and Sacchi 2001, Dunn and Smith 2001); 2) apparently the humoral response is not protective (Laudan et al. 1987, Sanchez et al. 2001a); 3) cells observed around the xenoma after its rupture are macrophages and lymphocytes (Dyková and Lom 1980, Shaw and Kent 1999); 4) it causes granuloma formation (Canning et al. 1986, Dyková 1992), 5) it can cause a chronic disease; and 6) it induces a strong protection in recovered fish that is not conferred by the humoral response (Speare et al. 1998b, Kent et al. 1999a).

Research on the cellular response to microsporidia is far more advanced in mammals (Didier 2000, Didier et al. 2000, Khan et al. 2001, Franzen and Muller 2001) where distinct populations of T lymphocytes have been identified as the protective element. Recently, the effect of *Enterocytozoon salmonis*, an intranuclear microsporidia causing lymphoproliferative disease in salmonids, was studied in experimentally infected chinook salmon. The proliferative response of lymphocytes was depressed in infected fish when their cells were exposed to mitogens *in vitro* (Wongtavatchai et al. 1995a). In contrast, a mitogenic effect was observed when lymphocytes from naive fish were cultured with supernatants obtained from cells infected with *E. salmonis* (Wongtavatchai et al. 1995b). It was suggested that the mitogenic soluble factor involved in the proliferation of naive lymphocytes was some kind of cytokine-like molecule released into the culture medium (Wongtavatchai et al. 1995a).

1.6 *LOMA*

Parasites belonging to the genus *Loma* are characterized by the production of small xenomas found mainly in the gills, viscera or digestive tract of infected, apparently healthy fish (Shaw and Kent 1999). Based on the sporogonic sequences, they are classified within the suborder Pansporoblastina: their development is inside a membrane or envelope originating from the parasite, called sporophorous vesicle (SPOV) (Cali and Takvorian 1999). The SPOV contains 1 to 8 spores, and sometimes up to 16 (Canning et al. 1986), depending if 2 or more of the SPOV coalesce (Morrison and Sprague 1983). Characteristic tubular appendages (Tubules type II) (Cali and Takvorian 1999) surround the spores and are more evident during the middle stage of spore development, disappearing at the end of sporogony (Morrison and Sprague 1981a). Tubules are believed to be metabolic products of the parasite (Moore and Brooks 1992), or a mechanism for transporting nutrients into the xenoma (Morrison and Sprague 1981a, Takvorian and Cali 1983, Cali and Takvorian 1999). Meronts divide by binary fission into uninucleate cells, although multinucleate cells (plasmodia) can be generated. There are 7 reported *Loma* species infecting fish: *L. salmonae, L. branchialis, L. morhua, L. fontinalis, L. embiotocia, L. dimorpha,* and *L. camerounensis*. The first 5 affect mainly the gills, although some are distributed along the vascularized organs, and the last 2 cause diverse lesions in digestive tract and muscular tissue.

Loma branchialis infects the gills of haddock, *Melanogrammus aeglefinus* (Morrison and Sprague 1981c, Bekhti and Bouix 1985a). It is very similar to *L. morhua*, but xenomas generated by *L. branchialis* in haddock gills are larger and more numerous (Morrison and Sprague 1981b, Morrison and Sprague 1983). *Loma morhua* produces xenomas in the gills

of several species of the family Gadidae including haddock (Morrison and Sprague 1981b, c, Morrison and Marryatt 1986). *Loma fontinalis* infects the gills of brook trout, *Salvelinus fontinalis* and although the xenomas produced are similar to those of *L. salmonae*, which has also been reported to infect brook trout as well (Bader et al. 1998), there is enough ultrastructural evidence suggesting these are two distinct *Loma* species (Morrison and Sprague 1983). *Loma embiotocia* was found in shiner perch (*Cymatogaster aggregata*) collected in 3 different locations from Vancouver Island. It forms small xenomas in the gills of infected fish and although it is morphologically similar to *L. salmonae*, both species can be distinguished by PCR and by the geographical distribution of the affected fish (Shaw et al. 1997). Experimentally, *L. embiotocia* cannot be transmitted to chinook salmon (Shaw and Kent 1999). *Loma dimorpha* is a microsporidian that causes xenomas in the connective tissue of the digestive tract from fish of the family Gobiidae (Loubes et al. 1984). However, this parasite was discovered in a new host, shanny (*Lypophrys pholis* family Blenniidae), generating xenomas in the intestine and the muscle (Arias et al. 1999). *Loma camerounensis* was reported in the connective tissue of the digestive tract (mainly duodenum) of the cichlid, *Oreochromis niloticus*. Xenomas were variable in size and inflammatory response was absent (Fomena et al. 1992).

1.6.1 *Loma salmonae*: Important features

Loma salmonae is an important parasite for the fish industry since it causes economic loss mainly in chinook salmon (Kent et al. 1995, Constantine 1999) and coho salmon in the Pacific coast of Canada (Kent et al. 1986, Speare et al. 1989). As a result of one of the first

ultrastructural analysis of gills from infected rainbow trout (*Oncorhynchus mykiss*), the parasite, formerly *Pleistophora salmonae*, was placed into the genus *Loma* (Morrison and Sprague 1981c, Morrison and Sprague 1983). Subsequently, the parasite has been recognized worldwide in fish and has been described in the United States causing disease of moderate severity in hatcheries that received fresh water from local rivers (Markey et al. 1994, Bader et al. 1998). Also, it has been reported in Scotland (Bruno et al. 1995) and in England (Poynton 1986), where low mortalities in rainbow trout reared in fresh water were observed. In addition, *L. salmonae* has been reported in France and Japan (Bekhti and Bouix 1985a). The first report of *L. salmonae* in Canada occurred in a hatchery in Vancouver (Magor 1987), and currently, the disease is widely distributed in the Pacific Northwest among the feral salmonid population, which appear not as greatly affected as the salmonids maintained in seawater net-pens (Kent 2000). Apparently, infections caused by *L. salmonae* affect salmonid species only from the genus *Oncorhynchus*, whereas Atlantic salmon and Arctic charr (*Salvelinus alpinus*) are resistant to the experimental infection (Speare et al. 1998a, Shaw et al. 2001).

Loma salmonae causes white cysts of variable size, called xenomas, mainly in the gills of infected fish (Kent et al. 1986, Kent et al. 1995), and also in the heart, spleen and kidney, and other viscera (Hauck 1984, Kent et al. 1986). The presence of xenomas in the gills have been associated with high mortalities in susceptible fish (Bader et al. 1998). Under experimental conditions, the rupture of xenomas with the subsequent release of the spores occurs by week (wk) 7 or 8 postexposure (PE), generating a severe branchitis with

accumulation of inflammatory cells and fibroblasts, granuloma formation and fish death due to asphyxia (Speare et al. 1998c).

1.6.2 Pathogenesis of *Loma salmonae*

The life cycle of *L. salmonae* is not fully understood, but significant advance in this field has elucidated part of this enigma. Generally, infection by most microsporidia in vertebrates occurs after oral ingestion of infective spores (Kreier and Baker 1987, Didier et al. 2000, Dunn and Smith 2001), and this observation has recently been confirmed in *L. salmonae* infections (Shaw et al. 1998, Sanchez et al. 2001d). In these experiments, *L. salmonae* spores were observed adjacent to the gut epithelium during the first hours of experimental oral infection.

For microsporidia in general, after spores reach the digestive tract they germinate under an appropriated stimulus, which could be the sudden change in the pH within the gut environment (Cali and Takvorian 1999), although other factors could be involved (Undeen 1990, Bigliardi and Sacchi 2001). Spores evert their polar tubes and inject the infective sporoplasms directly into the target cell cytoplasm (enterocytes or intraepithelial leucocytes) (Bigliardi and Sacchi 2001). The mechanism by which these cells are infected is unknown (Dyková 1992); however, it was recently suggested that the host cells are being infected through a mechanism that involves an actin-dependant phagocytic activity from the host cell itself (Magaud et al. 1997, Foucault and Drancourt 2000). In most microsporidia infecting the gut, like *E. bieneusi*, the complete life cycle occurs in the epithelium and the pathogenesis is clearly understood. However, with *L. salmonae* a different situation occurs which seems to

require transport from the gut to the gills, its final site of development (Figure 1.2). Using *in situ* hybridization to detect *L. salmonae*-DNA in paraffin sections from experimentally infected rainbow trout (Sanchez et al. 2000, Sanchez et al. 2001a), it was found that the parasite moved from lamina propria of the gut to the heart, where its DNA was detected as early as 2 days postexposure (PE). After an apparent stasis in the heart endocardium, *L. salmonae* arrived in the gills at wk 2 PE (Sanchez et al. 2000). This is important since the first morphological evidence of the parasite in gills can only be detected at wk 4 PE using conventional light microscopy, when small xenomas can be seen in wet mounts and in histological (paraffin) sections of the infected gills (Speare et al. 1998b). In other studies of exposed rainbow trout, xenomas were visible around wk 5 PE and the rupture of the xenomas occurred by wk 8 PE (Speare et al. 1998a), accompanied by a severe branchitis (Hauck 1984, Kent et al. 1986, Speare et al. 1998a).

1.6.3 Transmission

Under natural conditions, *L. salmonae* infection is thought to be acquired in fresh water, and persists after fish are moved to seawater (Kent and Dawe 1994, Kent 2000). The infection can be transmitted from fish to fish by either the ingestion of fresh spores or tissue contaminated with the parasite, and by cohabitation with infected fish (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998a, b). Experimentally, *L. salmonae* has been transmitted through routes other than oral, such as anal gavage, and intravenous and intramuscular inoculation (Shaw et al. 1998). Recently, it was reported that fish that had

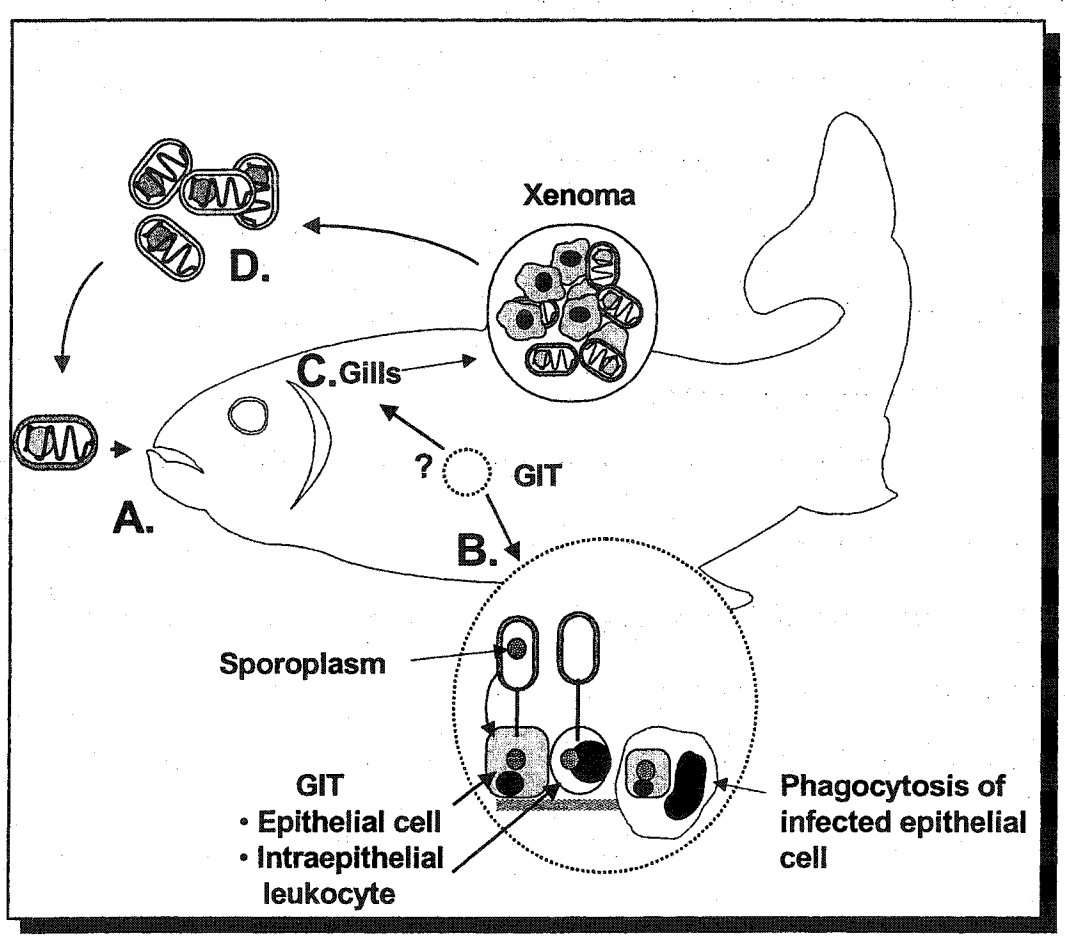

Figure 1.2. Diagram of the probable life cycle of *Loma salmonae* based on the results from this research and that of Speare et al. (1998a) and Sanchez et al. (2001d). **A.** Infective spores are ingested by a susceptible host, rainbow trout (*Oncorhynchus mykiss*). **B.** Spores hatch in the gastrointestinal tract (GIT). The polar tube is everted by sudden pH changes in the gut environment. The polar tube pierces a host cell membrane (lymphocyte or macrophage or epithelial cell, which is ingested by a leucocyte). From here the host cell migrates into a blood vessel while the parasite undergoes merogonic divisions. Eventually the infected cell localizes in the gills. **C.** The parasite arrives in the gills at week (wk) 2 postexposure (PE). Merogony continues until wk 3 PE and by wk 4 PE a complete xenoma is formed. Xenoma rupture occurs at wk 8 PE accompanied by a severe inflammatory reaction. **D.** Spores are released into the aquatic environment.

recovered from disease showed no evidence of xenoma formation in the gills after wk 12 PE. However, when viscera from those fish were taken 15 or 20 wk PE and were fed to naive fish, xenomas did develop in the naive fish (Ramsay et al. 2001). This suggests that clinically normal fish may harbor the parasite and can act as a source of the infection. In the same study, water samples taken from the tanks of the infected fish were positive for the presence of *L. salmonae* using PCR analysis.

1.6.4 Resistance to *Loma salmonae*

Little is known about the immune response of fish to *L. salmonae* and just a few studies have investigated the nature of resistance to the parasite. After spores are released at wk 8 PE, a severe inflammatory response is generated in the gills (Speare et al. 1998a). The exact biological and biochemical mechanisms by which spores are released are unknown and the signal that triggers the recruitment of leucocytes around and into the xenoma with subsequent parasite destruction is undetermined. Chitin, an abundant component of the spore wall (Méténier and Vivarès 2001), is thought to act as one inflammatory mediator (Speare et al. 1998b, Speare et al. 1998c), and also be involved in the resistance to *L. salmonae*. Chitin apparently has immunomodulator effects on fish leucocytes and on innate immunity (Kajita et al. 1990, Esteban et al. 2000). Chitin enhances the immune response through the non-specific modulation of the macrophage respiratory burst activity and it is mediated through the production of γ -interferon (Esteban et al. 2001).

Presently there is no approved drug or vaccine in Canada for the treatment and prevention of *L. salmonae*. Some experimental drugs control or delay the formation of

xenomas (Kent and Dawe 1994, Schmahl and Benini 1998, Speare et al. 1998d, Shaw and Kent 1999, Shaw et al. 1999, Speare et al. 2000). Yet, there is still inadequate knowledge on the control of the infection and the nature of the natural resistance to *L. salmonae*. Innate immunity to *L. salmonae* has been studied in Atlantic and chinook salmon (Shaw et al. 2001). Macrophages from Atlantic salmon had a higher capacity to ingest spores from *L. salmonae* than the phagocytes from chinook salmon. This heightened capacity was increased when spores were pre-incubated with homologous plasma (Shaw et al. 2001). Apparently some plasma factors in Atlantic salmon, like complement or lectins, are involved in the natural resistance to *L. salmonae*. Although adaptive immunity in teleosts has been intensively investigated and all the requirements to mount an adequate response are present in fish (Nakanishi et al. 1999, Watts et al. 2001), presently little is known about the humoral and cellular immune responses to *L. salmonae*. Only one experiment has described the role of passive immunity during the establishment and development of the parasite in the host (Sanchez et al. 2001a). Sera from fish recovered from experimental exposure was administred IP to naive fish, which were subsequently exposed to fresh spores. There was a delay in the establishment of the parasite in the heart when compared with non-treated fish; however, this delay did not interfere with xenoma formation in the gill (Sanchez et al. 2001a). In this case, the humoral response was either weak or unable to confer protection to fish exposed to *L. salmonae*. More studies regarding the humoral response are required to understand the role of Ab against this parasite.

The preliminary development of a potential vaccine to confer resistance in fish to *L. salmonae* has been proposed (Sanchez et al. 2001b). Partial protection in rainbow trout

occurred with an attenuated strain of *L. salmonae* (*L. salmonae* SV), which was obtained through successive passages of infective spores from chinook salmon and rainbow trout into brook trout (*Salvelinus fontinalis*) (Sanchez et al. 2001c). Complete protection to a subsequent challenge with fresh spores was not observed in this study; however, a significant diminution in the number of xenomas was observed. The use of a vaccine against *L. salmonae* opens a completely new field in the study of the resistance to this parasite. Further studies will be needed to elucidate what biochemical components of the spore are involved in the resistance to *L. salmonae*.

1.7 EVALUATION OF THE INFECTION AND RESISTANCE CAUSED BY MICROSPORIDIA

1.7.1 Exposure and re-exposure model: *Loma salmonae*

After fish survive an infection with *L. salmonae*, they are able to resist subsequent challenges with the same organism (Speare et al. 1998b). Currently, there is a well-established animal model for the study of the infection caused by this parasite, without the high mortalities encountered in naturally infected hosts (Speare et al. 1998a). The utilization of rainbow trout has facilitated studies on the use of drugs for the treatment of the disease (Speare et al. 1998d), the effect of temperature on the development of resistance to *L. salmonae* (Speare et al. 1998b, Beaman et al. 1999), and the pathogenesis of the disease (Speare et al. 1998d, Ramsay et al. 2001, Sanchez et al. 2001a,d). Several fish models have been generated to elucidate the mechanisms involved in the development of resistance to *L.* salmonae. In one study, rainbow trout exposed to fresh spores from *L. salmonae* either orally

or intraperitoneally (IP) at 15°C, developed xenomas in the gills by wk 5 PE. When these fish were re-challenged at wk 24 PE with live spores, they developed partial resistance expressed as a reduction in the number of xenomas. When fish were exposed orally to live spores and held at 10°C, they did not develop xenomas yet resisted a second challenge at 15°C with the same inoculum (Speare et al. 1998b).

The effect of the temperature on the xenoma formation has been described previously in rainbow trout (Speare et al. 1999), and it was determined that the permissive water temperature range for *L. salmonae* development was between 9° and 20°C (Beaman and Speare 1999, Beaman et al. 1999). Fish exposed beyond this temperature range never developed xenomas and when these same fish were re-exposed (at 15°C) with fresh spores, they were partially resistant, as was assessed by a non-lethal detection of cysts in the gills (Beaman et al. 1999). The experimental exposure and re-exposure models using fresh spores of *L. salmonae* have been studied and repeated in other salmonid species, like chinook salmon (Kent et al. 1999a). After the oral exposure to infective spores, the salmon exhibited xenoma formation in their gills and, after recovery, were fully resistant to subsequent re-challenges to the parasite at different times. These data suggest the possibility of a *L. salmonae* product which could be used to reduce xenoma formation and generate prolonged resistance in fish.

1.7.2 Transmission electron microscopy: Fish microsporidians

There has been considerable advancement in the understanding of fish microsporidia since they were first studied using electron microscopy (Weidner 1970, Weidner 1972). The most significant taxonomic information has been provided by transmission electron

microscopy (TEM) analysis (Hazard et al. 1981). Different genera and species within the phylum Microspora have been defined and transferred to new positions based on their ultrastructure (Kent et al. 1999b, Lom et al. 1999, Lom et al. 2000, Lom et al. 2001, Canning and Nicholas 2002). Ultra-thin sections of specimens embedded in resin generate additional knowledge, including accurate structural details of the spore and its internal structures that light microscopy or molecular biology techniques cannot provide (Hazard et al. 1981, Franzen and Muller 2001).

The morphology and development of the parasite has been described for several fish microsporidians. Detailed studies of gill microsporidia have elucidated the ultrastructural features of some parasites in gills of salmonids. Ultrastructural analysis has shown significant differences among *Loma* species, such as the size of the spore, turns of the polar tube and morphology of the xenoma (Morrison and Sprague 1981a, c, Morrison and Sprague 1983). In addition, fine details of the spore membrane system and the polar tubes have allowed a parasite, *Spraguea lophii*, from the angler fish (*Lophius americanus*), to be returned to the genus *Glugea* and be re-named *G. americanus* (Takvorian and Cali 1986). Important details regarding the life cycle of some fish microsporidia have been achieved through the ultrastructural observation of the different stages of the parasite (Bekhti and Bouix 1985a). Also, the careful ultrastructural analysis of the different developmental stages of *T. brevifilum* has allowed the description of its life cycle in the turbot (Matthews and Matthews 1980). Likewise, the developmental phases of *Microfilum lutjani* have been studied in the gills from the golden African snapper, *Lutjanus fulgens*, and a new hook-shape form of the polar tube, not seen before in microsporidia was discovered (Faye et al. 1991). The effects of *G. stephani*

on the host cell have been studied in the winter flounder using TEM, and observations revealed that the parasite development affects the host cell cystoskeleton architecture, which disappears during the hypertrophy of the infected cell (Weidner 1985). Through the examination of resin embedded specimens, it was possible to discern several structures that are surrounding or forming the xenoma wall, such as the interdigitating plasma membrane of the host cell, a dense layer of collagen fibrils outlining the cyst, and the plasma membrane of the surrounding cells (endothelial and/ or epithelial cell) (Morrison and Sprague 1981a, Bekhti and Bouix 1985a). The xenoma wall in certain microsporidia is covered by microvillus-like structures (*Tetramicra*); but in *Loma* and *Glugea* pinocytotic vesicles are observed in the same location (Canning et al. 1986).

The events and the morphological changes that occur during the germination of the spore have been extensively studied in fish. Using spores from *G. hertwigi* and *S. lophii*, it was observed that during the extrusion, the sporoplasm acquired its plasma membrane from the polaroplast membrane system. The structures involved during the SPOV wall formation were elucidated in cyprinid fishes by using TEM and it was observed that the distension of the SPOV wall occurs at the beginning of the sporogony (Pekkarinen 1996). The inflammatory tissue reaction associated with fish microsporidia has been studied at the ultrastructural level (Dyková and Lom 1980, Shaw and Kent 1999), providing identification of many of the cells that are actively participating in the elimination of spores, such as macrophages, lymphocytes, neutrophils, fibroblasts, and eosinophilic granular cells (EGCs) (Matthews and Matthews 1980, Bekhti and Bouix 1985b, Reimschuessel et al. 1987). Spores engulfed by neutrophils suffer numerous stages of degradation until their total destruction

within the phagocyte cytoplasm (Bekhti and Bouix 1985b, Canning et al. 1986). The ingested spores can follow one of two pathways: some spores are not enclosed within the classic phagocytic vacuole and apparently are not killed and can lead to autoinfection, while others remain encased within the electron-dense pansporoblastic matrix, which is gradually digested in a phagosome (Dyková and Lom 1980).

1.7.3 Immune response methods to detect fish microsporidia

Immunological approaches to detect microsporidia in fish and to evaluate the immunological status of the host are not as advanced as the study of the biology, pathogenesis, and transmission of many other parasitic diseases. Very little is known about how the resistance mechanisms of fish deal to microsporidia (Thomas and Woo 1995).

1.7.3.1 Humoral assays

A few techniques have been used for quantifying Ab levels present in fish infected with microsporidia including agglutination, single radial diffusion (SIRD), and ELISA. Direct haemagglutination was used to assess the effect of *G. stephani* infection in winter flounder. Serum from infected fish showed a lower Ab titer than those from control fish (Laudan et al. 1986b). The SIRD was used to quantify the total immunoglobulin in infected winter flounder; the total IgM levels decreased as the dosage of spores was increased, and IgM levels were lower in fish that received repeated injections of *G. stephani* (Laudan et al. 1986a, Laudan et al. 1987, Laudan et al. 1989). An indirect ELISA (Voller and Bidwell 1986, Gershwin et al. 1995) detected the Ab levels in turbot exposed with *T. brevifilum* by using particulate and

soluble Ag. Similarly, the indirect ELISA was used to measure the Ab levels in ayu infected with *G. plecoglossi* (Kim et al. 1996), in Japanese eel infected with *P. anguillarum* (Hung et al. 1996), and to quantify the Ab titers in the serum of flounder (*Platichthys flesus*) against *Glugea* (Pomport-Castillon et al. 1999).

1.7.4 Cellular assays

There is a lack of information on the mechanisms that regulate the cellular immune functions in teleosts. This is not only because of the very small number of fish species studied so far, but also due to the lack of *in vitro* culture techniques (Miller and McKinney 1994). One of the most used *in vitro* techniques for studying the cellular response to fish pathogens has been the proliferative response of lymphocytes (blastogenesis, mitogenesis) (Estepa et al. 1994, Taylor and Hoole 1994, Nie et al. 1996). Presently, there are two reports on fish microsporidia using the lymphoproliferative response technique (Wongtavatchai et al. 1995a,b). From these studies it was concluded that *E. salmonis* was able to induce proliferation of lymphocytes when fish were IP exposed with the live parasite.

1.8 RATIONALE

Aquacultural activities have increased worldwide and with the practices and conditions found in farming sites, several apparently new diseases, including microsporidiosis, have been recognized. The biological impact and the economical cost caused by *L. salmonae* is considerable. Problems associated with the disease are that a prolonged period of exposure follows the formation of xenomas with reduction of the fish

growth rate and increased mortalities. An understanding of the life cycle, mechanisms of resistance and ultrastructural localization and development in gills is imperative to obtain a complete knowledge about the pathobiology of this disease. There is significant information regarding the onset of xenoma development and the acquisition of resistance after infection and recovery. In this study, the onset of resistance in rainbow trout exposed to live and dead spore was investigated, and the progression of the immunological status of fish challenged with live and dead spores. As part of this study, an *in vitro* lymphocyte proliferation assay was performed to evaluate the cell-mediated immunity in those fish. The results of this objective will serve to determine if cellular immunity can be modified or enhanced to prevent *L. salmonae* infection through the use of a biological agent (vaccine). Finally, to understand the development of *L. salmonae* after its implantation in gills and what tissue reactions are generated during its proliferation, an ultrastructural study of infected gills from 0 to 8 wk PE was conducted. The following specific objectives were the focus of this thesis:

Objective 1: To determine, by using the exposure and re-exposure model, the onset of resistance to *L. salmonae* in rainbow trout after exposure to live and dead spores.

Objective 2: To determine, by using an *in vitro* lymphoproliferation assay, the role of the cell-mediated immunity in the establishment of resistance to *L. salmonae*.

Objective 3: To determine, by using TEM analysis, the localization and subsequent development of *L. salmonae* in the gills of infected rainbow trout.

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2 ONSET OF RESISTANCE IN RAINBOW TROUT FOLLOWING EXPOSURE TO *LOMA SALMONAE*

2.1 ABSTRACT

Resistance to re-infection of rainbow trout to the microsporidia *Loma salmonae* has been previously documented, and this paper examines how rapidly this resistance develops. Naive rainbow trout were exposed to *L. salmonae*, either as live spores given orally or with frozen-killed spores inoculated intraperitoneally (IP). Groups of the exposed fish in early, middle or late stages of the infection, were re-challenged at weekly intervals following the initial oral challenge with viable spores. Xenomas from the primary challenge were differentiated from xenomas developed during the second exposure, based on xenoma diameters. All experiments were conducted in freshwater at 15°C using juvenile rainbow trout. The establishment of partial resistance was faster in fish exposed to live spores than exposed to dead spores. However, development of complete resistance was faster in fish inoculated with dead spores. When fish received live spores orally as primary challenge, there was partial resistance by wk 2 post re-exposure (PRE) and complete resistance to re-challenge occurred within 8 wk PRE. In contrast, those fish which received non-iable spores IP as their primary challenge, began to show evidence of partial resistance by wk 3 and total resistance by wk 6 PRE. Based on the results of this experiment, the possibility of using a frozen-killed spore-based vaccine is advanced.

2.2 INTRODUCTION

Loma salmonae is an important emerging pathogen that belongs to the phylum Microspora and infects mainly salmonids of the genus *Oncorhynchus* (Shaw and Kent 1999). It is an important disease in the west coast of Canada causing severe gill damage and economic loss in chinook salmon (*O. tshawytscha*) (Hauck 1984, Constantine 1999). It can also affect coho salmon *(O. kisutch)* (Kent et al. 1986), and rainbow trout (*O. mykiss*) (Bruno et al. 1995).

Loma salmonae is an obligate intracellular organism that produces cyst-like structures, called xenomas, in the gills of the infected fish (Kent et al. 1986, Kent et al. 1995). Xenomas are complex structures formed by a hypertrophic cell which is harboring the parasite in several stages of development (Weissenberg 1968, Canning et al. 1986, Dyková 1992). Xenoma growth has detrimental effects for the host cell and for the tissue adjacent to it, causing tissue oxygen deprivation due to the high metabolic demand of the parasite during proliferation and reductions of the blood glucose levels at the end of the sporogony (Weidner et al. 1999). The role of the xenoma is to confine the parasite in one place in host tissue and to avoid the propagation of the infection, and also to protect the parasite by forming a barrier against the attack of the host defenses (Canning et al. 1986, Didier and Bessinger 1999). In addition, some microsporidia can arrest the cell cycle and prevent the host cell apoptosis (Scanlon et al. 1999, Scanlon et al. 2000), therefore enabling proliferation of the parasite.

Although the life cycle of *L. salmonae* is not fully understood, some important investigations have elucidated part of this enigma. Infection by most microsporidia in vertebrates occurs after the ingestion of infective spores (Kreier and Baker 1987, Didier et

al. 2000, Dunn and Smith 2001), and with *L. salmonae* this has been confirmed by the early appearance of spores adjacent to gastrointestinal tract epithelium during the first hours after experimental infection (Shaw et al. 1998, Sanchez et al. 2000). With other microsporidia, after spores reach the digestive tract they germinate under an appropriate stimulus (Cali and Takvorian 1999), and germinated spores inject the infective stage of the parasite, the sporoplasm, directly into the host cell cytoplasm through the polar tube (Bigliardi and Sacchi 2001). Host cell candidates could be enterocytes or intraepithelial leucocytes (Dyková 1992). The next step in the infection may involve transport of the parasite from the gut to its final site of development, which for *L. salmonae* is the gills.

Currently, it is not known whether fish host defence mechanisms are active against the parasite during the transport of the parasite to the gills. However, current research is under way to understand the cellular mechanisms that could be acting against the parasite. By using *in situ* hybridization to detect *L. salmonae*-DNA in paraffin sections of tissues from fish 0 - 7 weeks (wk) postexposure (PE), it was observed that the parasite is transported from lamina propria of the gut to the heart, where its DNA is detected at day 2 PE (Sanchez et al. 2000, Sanchez et al. 2001a). After an arrest in the heart (Sanchez et al. 2000), *L. salmonae* is detected in the gills 2 wk later (Sanchez et al. 2001a). By wk 4 PE small xenomas are observed under light microscopy in gill wet mounts and routine histological preparations.

Recently, infected cells containing only dividing meronts were detected by wk 3 PE in rainbow trout gills using high resolution high microscopy and transmission electron microscopy (Rodriguez-Tovar et al. 2002). Xenomas were accompanied by an early inflammatory reaction to mature spores at wk 4 PE; this could be considered as the first

morphological indication of host response against *L. salmonae* (Rodriguez-Tovar et al. 2002). During the final stages of *L. salmonae* development, which occurs around wk 8 PE, rupture of xenomas and subsequent release of infective spores from the gills stimulates a severe inflammatory response (Hauck 1984, Kent et al. 1986). The exact biological stimulus by which spores are released is unknown, but it has been hypothesized that certain constituents of the spore wall, chitin among them, may promote the severe inflammatory reaction seen around xenomas (Speare et al. 1998c). Chitin could be one of the antigenic components that triggers resistance to *L. salmonae*. After fish survive the disease, they are able to resist subsequent challenges with the same inoculum (Speare et al. 1998b).

Currently, there are no approved medications or vaccines in Canada or elsewhere for the treatment and prevention of the infection by *L. salmonae*. However, under experimental conditions, some drugs have demonstrated to have a valuable potential for controlling or delaying the formation of xenomas (Kent and Dawe 1994, Speare et al. 1998d). Unfortunately, there is still a considerable lack of knowledge about how to control the infection and how the recovered fish acquire resistance to *L. salmonae*. Recently, the potential for a vaccine to prevent disease was indicated in a series of experimental infections involving the route of inoculation of the parasite, the physical form of the spore, and water temperature manipulation (Beaman and Speare 1999, Beaman et al. 1999, Speare et al. 1999). In one study, xenoma formation was observed in gills after oral or IP inoculation of fish held at 15 °C, whereas xenomas were not observed in fish that were inoculated IP with inactive (dead) spores (Speare et al. 1998b). However, after being orally re-challenged with live spores, these same fish were resistant to the disease. In the same study, fish orally

exposed to live spores and held at 10°C did not develop xenomas; however they were resistant to a second challenge at 15°C when fish were exposed orally with live spores. This study indicates that fish acquired strong protection after being exposed to spores of *L. salmonae,* regardless of the viability of the spore (Speare et al. 1998b). In addition, infecting fish orally at different temperatures (above 20°C and below 9°C) did not result in development of xenomas, but it allowed those fish to be at least partially resistant to a second oral challenge at 15°C with live spores (Beaman et al. 1999). The experimental exposure and re-exposure models (ERE) using live spores of *L. salmonae* have also been studied in chinook salmon (Kent et al. 1999). Fish exhibited xenoma formation in the gills following the oral exposure with infective spores, but after their recovery, fish were fully protected to subsequent re-challenges to the parasite at different times. Similar to the rainbow trout model, chinook salmon developed strong protection to subsequent inoculations with *L. salmonae* (Kent et al. 1995). All these data point to the use of an inactivated or attenuated *L. salmonae* product to reduce the production of xenomas and generate a prolonged resistance in fish.

Recently, the preliminary development of a vaccine to confer resistance in rainbow trout to *L. salmonae* has been reported (Sanchez et al. 2001b). Partial protection in rainbow trout has been proposed to occur following inoculation with a newly discovered attenuated strain of *L. salmonae* (*L. salmonae* SV) (Sanchez et al. 2001a, Sanchez et al. 2001c, Sanchez et al. 2001b). Although complete protection was not observed in this study, a significant reduction in the number of xenomas occurred. All these findings have suggested a promising advance in the development of resistance to microsporidia in salmonids, and provided insight

into how salmonids, after recovery from this intracellular microorganism, survive further exposure to the parasite. In the present study, the ERE model was used to determine the time of onset of resistance in rainbow trout experimentally exposed to live and dead spores and subsequently re-exposed to live spores.

The aim of this investigation was to study, by using ERE, the onset of resistance in fish previously exposed to live (infective) and dead (inactive) spores and subsequently re-challenged at different times. The main objectives were to determine: 1) when resistance to *L. salmonae* begins; 2) whether there is a difference in the development of resistance in response to live or dead spores; and 3) if previous exposure to *L. salmonae* alters the xenoma development when measured under light microscopy.

2.3 MATERIALS AND METHODS

2.3.1 Source of the inoculum

2.3.1.1 Infective spores

Live (infective) spores were collected from heavily infected fish, as determined by stereomicroscope. The fish were killed with an overdose of benzocaine (150mg/L), and their opercula were removed to expose the gill arches, which were then dissected and the arch cartilage was removed. Gills were chopped into small pieces with a razor blade and the macerated gill tissue was placed into a 50 ml conical plastic tube and mixed with an equal volume of sterile distilled water. This mixture was transferred into 1 ml tuberculin syringes attached to a 5 cm long plastic tube (0.2 cm diameter), and stored at 4°C until its use (30 min).

2.3.1.2 Dead (Inactive) spores

Live spores were collected from a group of infected fish as explained above. The macerated infected gill tissue (devoid of cartilage) was placed into a tissue grinder and crushed 4 times with a mortar. The crushed tissue was placed in a 15 ml conical plastic tube and mixed with an equal volume of distilled water. This mixture was centrifuged at 800 x g for 10 min at 4°C. The pellet was suspended in sterile distilled water and passed through a 50 μm Nytex filter. The filtered gill tissue was centrifuged twice at 1,500 x g for 10 min at 4°C, resuspended in 25 ml of sterile distilled water and filtered again in 50 μm Nytex. Then, gill tissue was solubilized with 6% Triton-X 100and centrifuged at 1,500 x g for 15 min at 4°C. The pellet was washed twice at 800 x g for 5 min at 4°C. Finally, semipurified spores were resuspended in sterile distilled water, mixed with 1% (v/v) penicillin (10,000 units/ml)/ streptomycin (10 mg/ml) (Sigma) and stored at -20°C in 1 ml plastic vials for 20 days and subsequently thawed at room temperature (24°C) before their use. A small sample (20 μl) was taken for estimating the total concentration of spores per ml using an hemocytometer. To confirm lack of infectivity of the spores, a small group (n=10) of naive fish were orally inoculated with 1 x 10^6 spores ml^{-1} by using gastric intubation as previously described (Speare et al. 1998a), and were monitored for 4 months for the presence of branchial xenomas.

2.3.2 Fish and experimental design

Naive rainbow trout (*Oncorhynchus mykiss*), with an average weight of 15-20 g were acquired from a certified pathogen-free commercial hatchery in Prince Edward Island. This

hatchery had no previous history of infection by *L. salmonae*. Fish were housed in 70 L fibre-glass tanks in a continuous flow system and acclimatized at 15°C for 1 wk before starting the experiment. They received commercial food (Trout grower, Corey Feeds) every day and photoperiods of light and darkness of 12 h each. Fish showing superficial damage to the skin, opercula or eyes were eliminated from the group. The experimental procedure used during this experiment was in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care 1993). Twenty-six circular fibre glass tanks each with a capacity of 70 L and maintained at 15°C were utilized during the study, which was divided in 3 experiments.

2.3.2.1 Experiment 1. Measurement of xenoma diameters

This experiment measured the size of the xenomas at different weeks postexposure. Results obtained were used to compare new and old xenoma growth in experiments 2 and 3. A group of 80 fish was exposed orally to infective spores as describe previously by Speare et al. (1998a). Eight fish were killed every wk (0 to 9), and the gill arches of these fish were dissected and processed for conventional light microscopy analysis of paraffin embedded tissues (see section 2.3.4.1 for details). Two slides with 3 gill arches, one for each side, were chosen per fish. The diameter of all xenoma sections was measured using a light microscope with a calibrated ocular scale. For experiments 2 and 3, gill samples were collected from the left gill arch. Ten slides were randomly selected from every wk (0 to 20 wks post-exposure, PE) and for every observation (early, middle, and late). Three specimens per slide were analysed and all xenomas (large and small) observed in the gill tissue were measured. Since

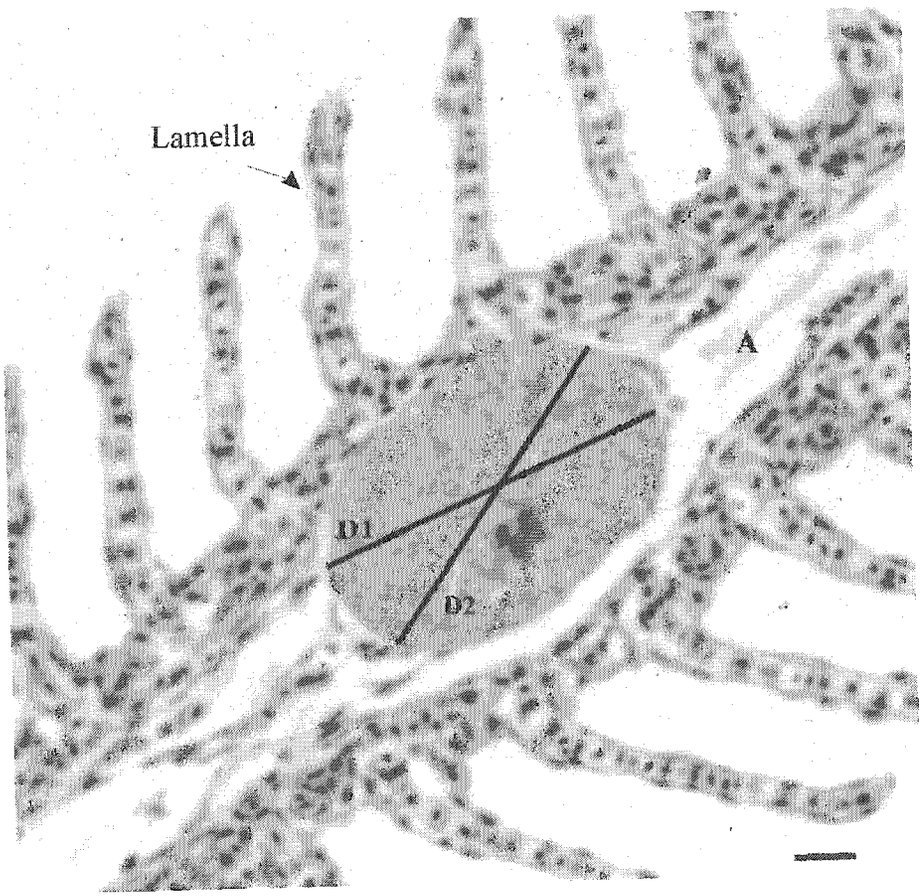
not all the xenomas were perfectly circular, for those few xenomas with an irregular appearance, the diameter was calculated using a formula for measuring sphere-like structures (Löfstedt and Ireland 2000). First, the two "best diameters" (D1 and D2) were estimated; where D1 is the first largest diameter and D2 is the second largest diameter (Figure 2.1). D1 was multiplied by D2, then the result was divided by 4, to give the final diameter.

2.3.2.2 Experiment 2. Onset of resistance in fish experimentally exposed IP to dead spores of *L. salmonae*

This experiment was designed to assess whether the previous IP exposure of fish to inactive spores of *L. salmonae* could provide protection to subsequent re-exposure to live spores, and to determine when this protection develops. This experiment was divided into 3 observation times: early, middle and late depending on the stage of infection by *L. salmonae*.

Early observations. The general experimental design is depicted in Figure 2.2 (more tanks and fish were used when necessary). Group 1 consisting of 3 tanks (n = 50 fish/tank) was designated as **"re-exposed"**. Fish in group 1 initially received an IP exposure to dead spores of *L. salmonae* at the beginning of the experiment (t = 0). Subsequently, to evaluate the development of resistance, subgroups of fish (n = 30 / tank per wk) from group 1 were orally re-exposed at wk 1, 2, 3, and 4 with live spores of *L. salmonae* and placed in a new tank at each wk. The evaluation of the onset of resistance was estimated by taking gill samples at

Figure 2.1. Measurement of an irregular xenoma in a gill filament from rainbow trout exposed orally to *Loma salmonae* live spores. This xenoma appeared to be situated in the lumen of the primary artery (A). The first 2 "best diameters" were localized in the xenoma and were assigned as D1 (the first largest diameter) and D2 (the second largest diameter). Then, D1 was multiplied by D2 and the result was divided by four. This end result was considered as the final diameter. H & E, 40X. Bar = 50 μm.
(Note: The digitalized lines only illustrate how the diameters of irregular xenomas were measured using conventional light microscopy, not by computerized programs).



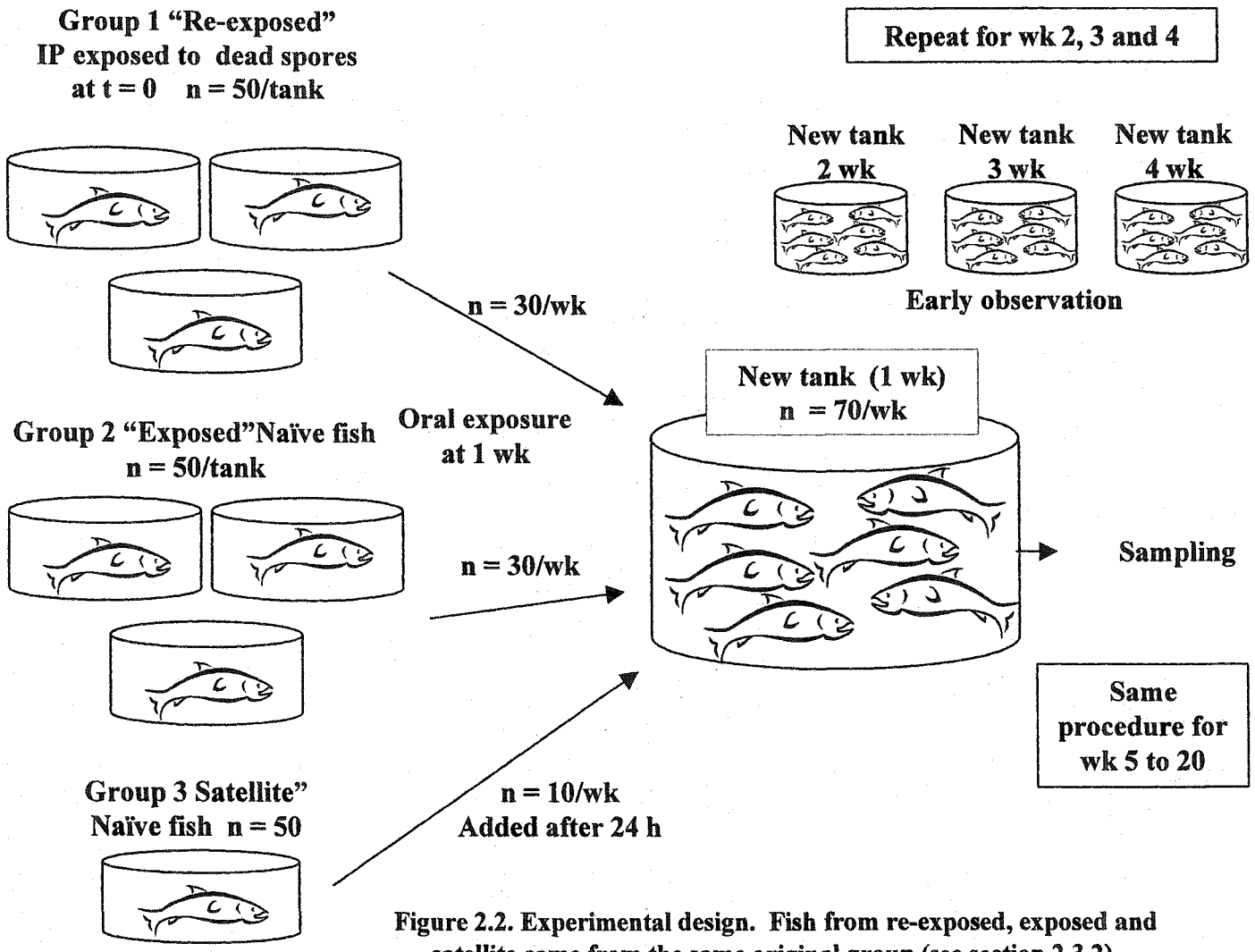


Figure 2.2. Experimental design. Fish from re-exposed, exposed and satellite came from the same original group (see section 2.3.2).

t = 0 and then every 4 wk after second challenge and measuring by light microscopy the diameters of xenomas present.

Group 2 consisting of 3 tanks (n = 50 fish/ tank) of naive, adipose fin clipped fish was designated as **"exposed"**. Fish from this group (n = 30/ tank per wk) were exposed (at the same time as the re-challenging of fish taken from group 1) with live spores of *L. salmonae*, and placed into the new tanks along with the re-exposed fish from group 1. The exposed fish (group 2) were considered as an internal positive control to assess the infectivity of the inoculum and were examined at t = 0 and every 4 wk.

Group 3, consisting of one tank of 50 naive tail-fin clipped fish were designated as "satellite". Fish from this group (n = 10) were added to the new tank along with fish from group 1 and 2, 24 h after the exposure of the fish from groups 1 and 2. The satellite fish (group 3), assessed the possible horizontal spread between tankmates that could have occurred inside each tank as a consequence of released spores from the re-exposed fish (early xenoma rupture), and were evaluated at t = 0 and then every 4 wk. The number of xenomas developed in the satellite group would be used as a correction factor to exclude those xenomas that could have developed as a result of horizontal spread.

Middle observation. Again, fish were divided into similar groups designated as re-exposed, exposed and satellite as described above. Fish from group 1 (re-exposed) received a second challenge (oral exposure with live spores) at wk 5, 6, and 7 PE and placed in a new tank at each wk. The exposed fish were inoculated orally at the same time as the re-exposed group and the satellite fish were added 24 h later.

Late observation. Fish were divided into similar groups designed as re-exposed, exposed, and satellite as described above. The re-exposed group, received a second challenge at wk 8, 12, 16 and 20 PE.

2.3.2.3 Experiment 3. Onset of resistance in fish orally exposed to live (infective) spores of *L. salmonae*

This experiment was designed to evaluate temporal the development of resistance among fish initially exposed to live spores and subsequently re-exposed to live spores. This experiment was divided in early, middle (wk 4½, 5, 5½, 6, 6½, 7, and 7½ PE), and late observations using the same number of fish, same groups and same protocol as were used in the experiment 2.

2.3.3 Method of exposure

2.3.3.1 Experiment 1 and 3. Live spores

Food was withdrawn from naive rainbow trout 24 h before inoculation with live *L. salmonae* spores. Fish were lightly anaesthetized with benzocaine (60 mg/L), manually restrained, and infected by gastric intubation. Fish were inoculated with approximately 0.1 ml of the infective gill tissue (≈1 x 10^6 spores/ml distilled water). After receiving the inoculum, fish were returned to their tanks and checked periodically for any evidence of regurgitation of the infective gill tissue. No regurgitation was noted.

2.3.3.2 Experiment 2. Inactive spores

Naive rainbow trout were lightly anaesthetized and manually restrained. Approximately 0.1 ml (1 x 10^6 spores / ml sterile distilled water) was inoculated IP into fish with a tuberculin syringe. Fish were inoculated mid-ventrally, rostral to the pelvic fins (Wall 1993), returned to their tanks for recovery and monitored periodically for mortality.

2.3.4 Sampling and evaluation of the onset of resistance

2.3.4.1 Histopathology

Histological (paraffin) sections from experiments 1, 2 and 3 were examined for the presence and size of xenomas and for any other lesions. Both left and right gill arches from each fish in experiment 1 and only the left gill arches from each fish in experiments 2 and 3, were fixed in 10% neutral buffered formaldehyde, dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin. Sagittal sections (7 μm thick) were cut and mounted on glass slides (previously coated with 2% silane-acetone solution) with three gill arches per slide. Specimens were stained according to Harris' hematoxylin and eosin procedure (Armed Forces Institute of Pathology 1992).

2.3.4.2 Wet mounts

The intensity of the infection was determined by estimating the number of xenomas per gill arch. Also, a rough determination of the initial and subsequent exposure of the disease was made with this technique. Fish from all groups (re-exposed, exposed and satellite), were killed at t = 0 and every 4 wk after the second challenge [0 to 20 wk post re-

exposure (PRE)] with an overdose of benzocaine. The first left gill arch from every fish was dissected, removed from its cartilage and placed onto a glass slide. Gills were lightly soaked in a beaker with water (from the tank) and gently flattened with a cover slide to allow a better distribution and visualization of the filaments. The wet mounts were observed under light microscope using the 40X objective. The concentration of xenomas per gill (intensity), the prevalence of the infection, and the differentiation between large and small xenomas, was assessed using these preparations. The enumeration of the xenomas was divided in 2 morphological criteria: large xenomas resulted from the initial exposure to *L. salmonae* and small xenomas represented the second exposure (second challenge for the re-exposed group, first challenge for exposed group) with the parasite (Figures 2.3A, B). This procedure was followed in experiments 2 and 3.

2.3.5 Analysis of data

Statistical analysis employed the statistical software MINITAB® (version 12). Significant differences were reported at the $\alpha = 0.05$ level of probability. For experiment 1, a regression analysis of the xenoma size vs time of the infection was used to develop a graphic mathematical model for predicting these events in rainbow trout kept at 15°C. In this model, xenoma size was the dependant variable and the time was the independent variable. Simple linear regression was used to find the best line through a graph of data points and explained by the fitted equation $\hat{Y} = b_o + b_1 x + \varepsilon$ (Motulsky 1995). In this equation b_0 (place where line crosses the axis) + b_1 (slope: change in $\hat{Y}$ for every unit change in x) were constants derived from least squares, and x was the time expressed as wk PE (for experiment

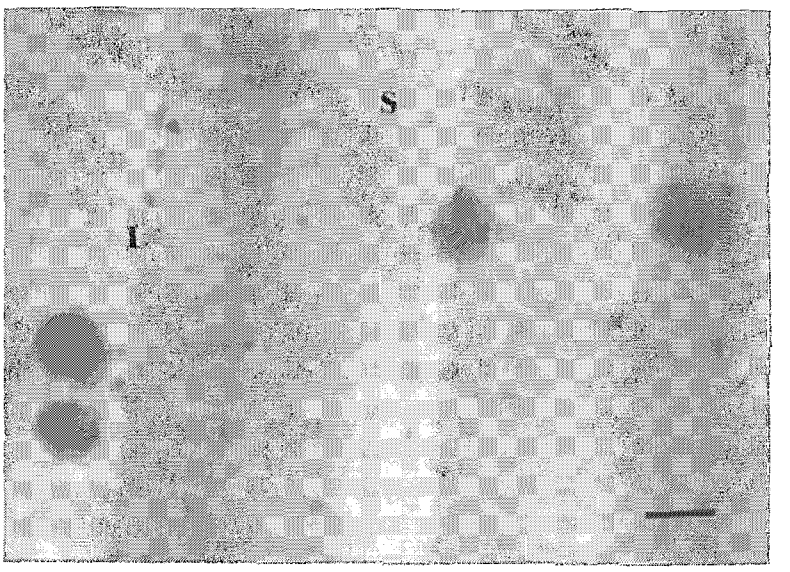


Figure 2.3A. Wet mount of rainbow trout gills exposed to live spores of *Loma salmonae*. Five weeks (wk) postexposure PE. Spherical (S) and irrgular xenomas (I). Bar = 50 μm.

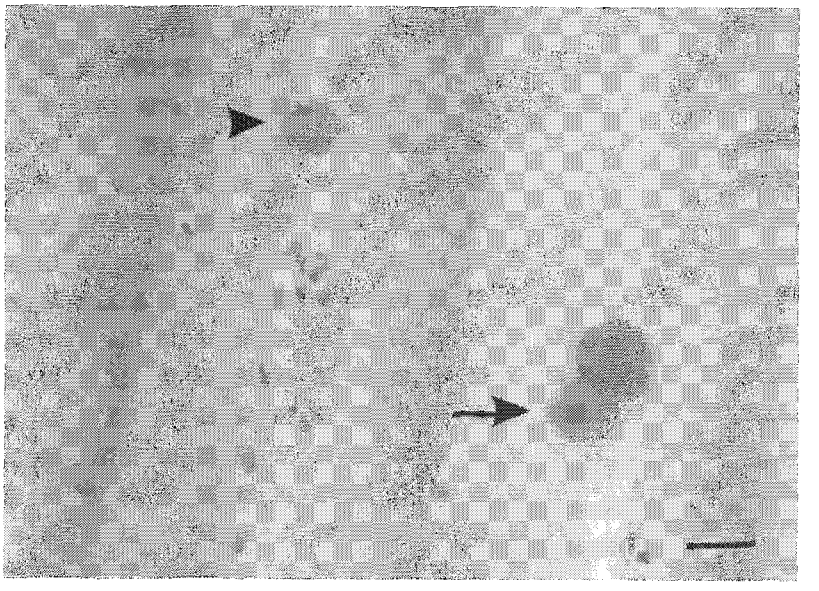


Figure 2.3B. Wet mount of rainbow trout gill re-exposed to live spores of *Loma salmonae*. Five wk PRE. Large xenomas represent the initial inoculation (arrow), small represent the second exposure (arrowhead). Bar = 50 μm.

1) and PRE (for experiments 2 and 3). The epsilon (ε) represents random variability (error). The linear regression was assessed by the coefficient of determination or R-squared (R^2) value. The R^2 was calculated from $s^2_y - s^2_e / s^2_y = SST - SSE/SST$; where SST is the total sum of the squared deviations, and SSE is the variation due to random error (Ryan et al. 1985). Results obtained from this trial were used to predict the dimension of the xenoma in the experiments 2 and 3 to distinguish the first (large xenomas) and second exposures (small xenomas), and the onset of resistance and resolution of xenomas in fish in the experiments 2 and 3. The effect of the physical state of the inoculum or treatment (live and dead spores) and the proportion of infected fish caused by *L. salmonae* in experiments 2 and 3, were determined using several comparative statistical methods dependent on the type of collected data. A one-way analysis of variance (ANOVA), followed by a Tukey's pairwise comparison, was used to compare the mean numbers of xenomas (intensity) generated per gill arch in the experiments 2 and 3. This analysis tested the null hypothesis that the intensity of the xenomas in the gills was not depending on the exposure with live or dead spores. In addition, the Chi-square analysis was used each wk PE/PRE to compare the different proportions of fish infected with xenomas in the experiments 2 and 3. This statistical analysis tested the null hypothesis that the exposure with live or dead spores had no effect on the prevalence and the number of small and large xenomas over time.

2.4 RESULTS

2.4.1 Experiment 1. Determining the xenoma diameters during the experimental infection by *L. salmonae* in rainbow trout

In this experiment, the xenoma size was modelled by a linear regression model. The regression analysis showed strong association between the size of the xenoma and the time of the infection. The linear regression fitted line is represented in the figure 2.4. On this plot, the fitted line was expressed as: $\hat{Y}(\text{Xdiam}) = b_0\,(-42.9) + b_1\,(15.3)\,wk$, where Xdiam is the xenoma diameter. The regression of the Xdiam against the time was significant, with a P value < 0.05 (Table 2.1). The regression examined was highly predictive between wk 4 and 9 PE. Observations before this time were impossible due to the smaller size of the xenomas or because they were undetected. After this time it was also difficult because most of the xenomas were completely ruptured. The coefficient of determination (R^2) was 0.86. The slope was 15.3, which means that when wk PE increases by 1 unit, the XDiam number of xenomas increases by 15.3 μm every wk. The results closely coincided for wk 4 to 5, 6 to 7, and 7 to 8 PE, but not from wk 5 to 6 and 8 to 9 PE (Table 2.2).

2.4.2 Experiment 2. Induction of resistance in fish experimentally exposed to dead (inactive) spores of *L. salmonae*

One of the most important features of this experiment was that only small xenomas were observed in the re-exposed and exposed groups. Large xenomas were never observed, and the satellite group did not develop xenomas. This indicated that dead spores used as

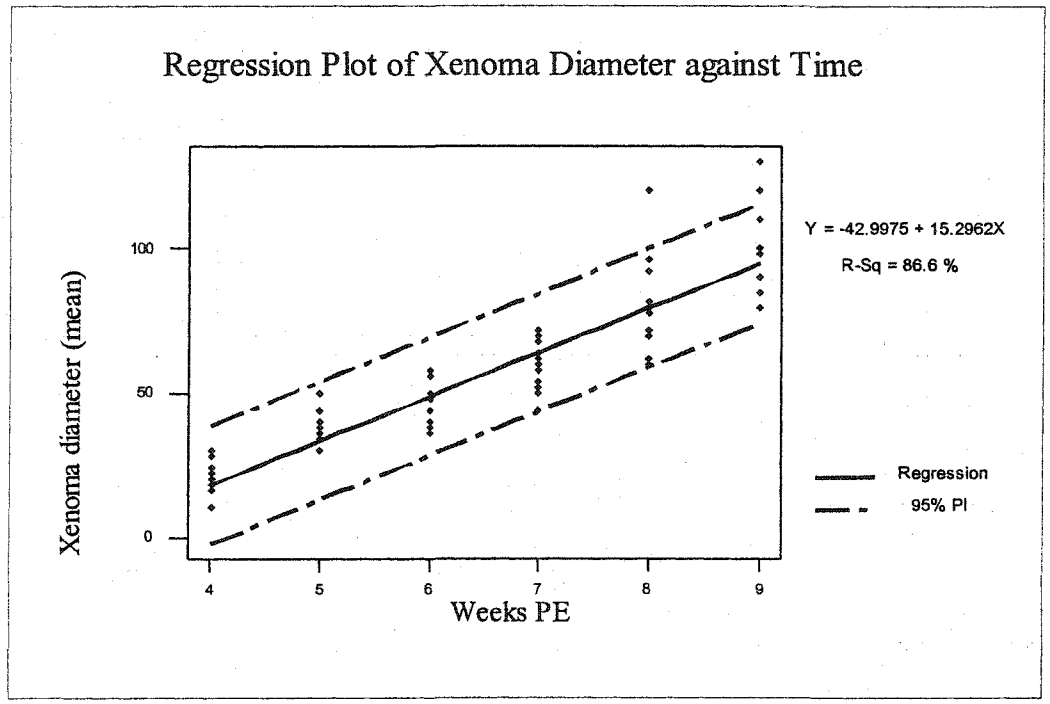


Figure 2.4. The best-fit line for the xenoma diameter (Xdiam) in experiment 1.

Table 2.1. Experiment 1. Numerical output generated from the analysis of the xenoma measurement at different intervals in rainbow trout experimentally exposed by gastric intubation with fresh spores of *Loma salmonae.*

Parameter	Expected Value	Linear Regression SE
Slope	15.3	3
Y intercept	-42.9	0.4
X intercept	2.8	
r squared	0.86	

Standard deviation of residuals from line (*Sy.x*) = 10.3
P value= 0.000 It is considered significant

Table 2.2. Experiment 1. Diameter of xenomas (Xdiam) measured under light microscopy (H&E) at 40X. Rainbow trout experimentally exposed by gastric intubation with live spores of *Loma salmonae.*

Wk PE	Xdiam (μm ± SD) Range μm (Min-Max)	* Slope 15.3 μm
3	ND	
4	21 ± 3.5 10-32	16
5	37.1 ± 5.2 30-42	6
6	43.7 ± 5.8 39-58	16
7	59.1 ± 7.1 44-72	16
8	75.4 ± 12.8 60-110	27
9	102.1±14.7 98-130	

* R-squared = 0.86

initial inoculum never developed branchial xenomas. Fish exposed to dead spores from *L. salmonae* become resistant to subsequent exposures with the same inoculum at wk 6 PE. As in the experiment 3, using the linear regression from the experiment 1 as a standard curve (Fig. 2.4) for predicting the size of the xenoma, small xenomas were inside the range of Y value corresponding for wk 4 PE, when measured from wk 1 to 6 PE. With exception of 2 fish that had a few xenomas at wk 8 PE, from wk 6 to 20 PE small xenomas were not detected. Again, the regression equation (Table 2.1) was useful for predicting the size of xenomas in re-exposed and exposed groups. Xenomas from these groups corresponded with the predicted size for small xenomas at wk 4 PE. The coefficient of determination (R^2) was 0.86. The simple linear regression proved to be useful for predicting the size of xenomas based on the time of *L. salmonae* infection.

By using Chi-square analysis, significant differences were observed between re-exposed and exposed groups at wk 1, 2, 3, 4 and 5 PRE/PE ($P<0.05$) (Fig.2.5). The maximum prevalence for re-exposed fish (80%) was observed at wk 2 PRE, whereas for the exposed group maximum prevalence (100%) was observed at wk 2, 7, 8, and 16 PE (Fig. 2.5).

The number of xenomas per gill was lower in the re- exposed group (Fig.2.6), when compared with the exposed group (Fig. 2.7). The one-way ANOVA revealed significant differences among groups at wk 1, 2, 3, 4 and 5 PRE/PE ($P < 0.05$). In addition, the mean of maximum xenoma intensity in the re-exposed group was observed at wk 1 PRE. The exposed group has a slight intensity increase at wk 12 and 16 PE. By comparing the xenoma intensity for each treatment over time, a one-way ANOVA revealed that the intensity in

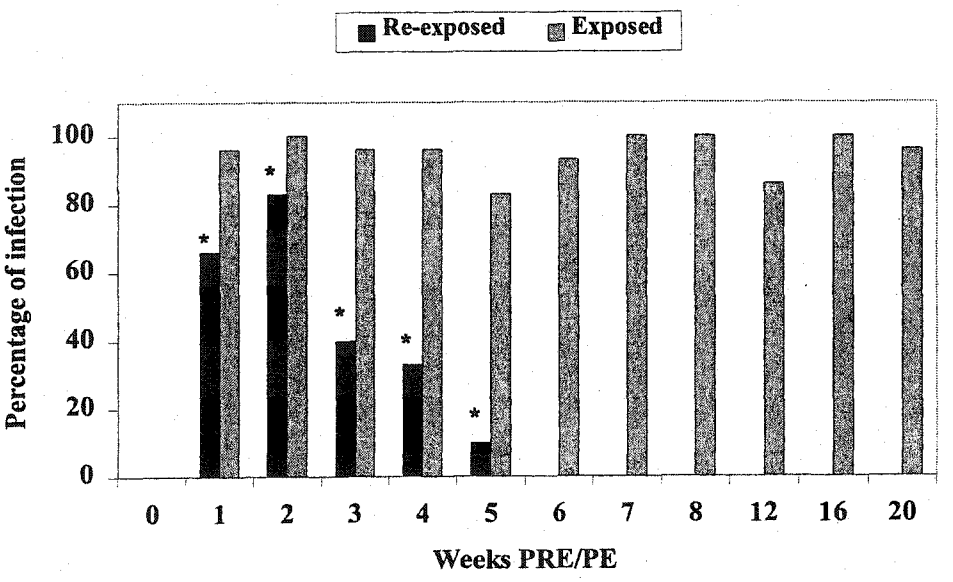


Figure 2.5. Experiment 2. Percentage of fish infected with *Loma salmonae* after challenge of naive fish (exposed) or fish inoculated with killed spores (re-exposed). The asterisk above the re-exposed group indicates significant differences between this group and the exposed group.

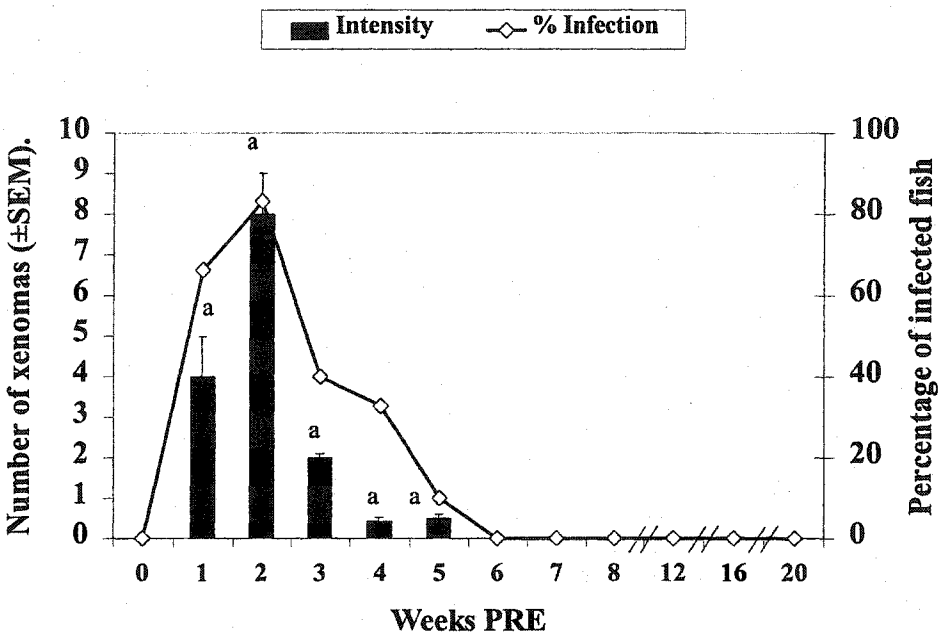


Figure 2.6. Experiment 2. Percentage of infected fish with *Loma salmonae* and intensity of infection in fish exposed to dead spores and challenged with live spores (re-exposed).
[a] Significantly different when compared with exposed group ($p < 0.05$)

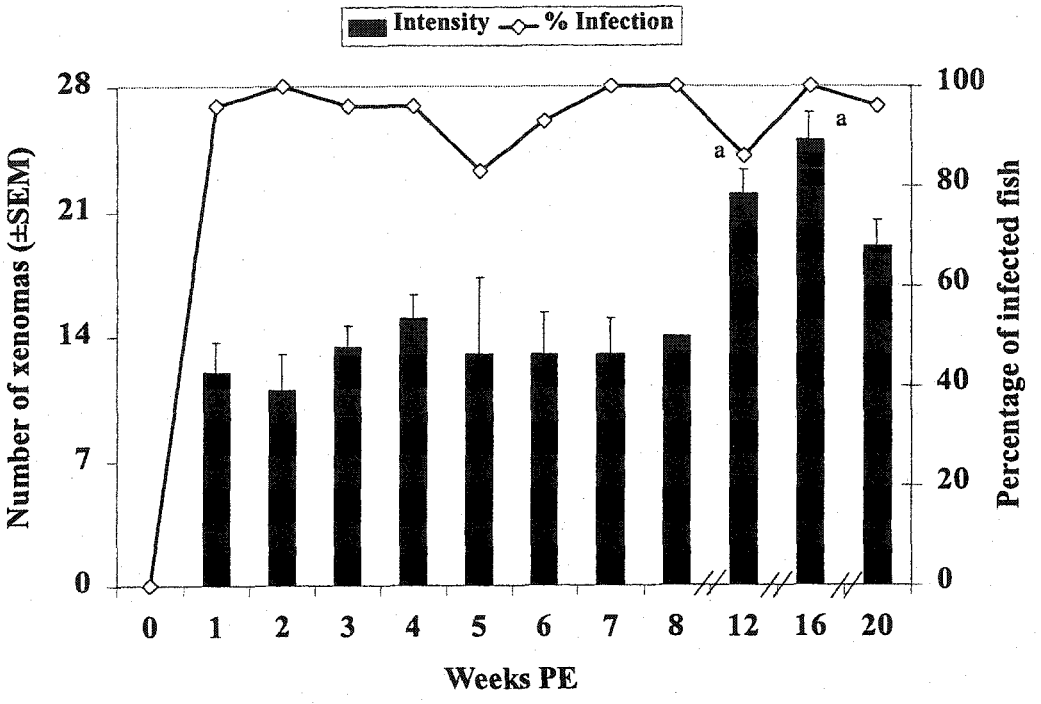


Figure 2.7. Experiment 2. Percentage of infected fish with *Loma salmonae* and intensity of infection in naive fish challenged with live spores (exposed).
[a] Significantly different within the group over the time ($p < 0.05$).

re-exposed fish is significantly different over time ($p < 0.05$), specifically in wk 1,2, 3, and 4 PRE (Fig. 2.10), whereas the intensity of the xenomas over time in exposed fish was significantly different at wk 12 and 16 PE ($p > 0.05$) (Fig. 2.5).

Gill samples taken for histopathology did not reveal any significant changes suggestive of infection in re-exposed fish during the whole study and were similar to those seen in exposed fish. Satellite fish had no any significant histopathological changes.

2.4.3 Experiment 3. Fish exposed to infective spores

Fish exposed to live spores from *L. salmonae* become resistant (no detectable small xenomas) to a subsequent exposure to the same inoculum at wk 8 PRE. Using the linear regression from experiment 1 as a standard curve for predicting the size of the xenoma, we found that the size of xenomas in this group was inside the range of Y value corresponding to wk 4 PE, when measured from wk 1 to 8 PRE. From wk 8 to 20 PRE small xenomas were not detected. The regression equation showed that the expected xenoma size was: $Y = -42.9 + 15.3$ (4 wk) = 18.5 μm; R-squared 0.86. The size of the small xenomas in the re-exposed group was within this value showing that the equation is predictive (Table 2.3). The value generated from the simple linear regression plot for wk 4 PE was 18 μm, and was predictive, with a coefficient of determination of 0.86. In addition, when the linear regression and the equation were used in exposed fish for predicting the Xdiam (Table 2.4), they proved useful since they corresponded with small xenomas at wk 4 PE. The linear regression and the regression equation for predicting the size of the large xenoma (first exposure) proved not to be useful for estimating the time of exposure in the re-exposed group. Xenomas from first

Table 2.3. Experiment 3. The diameter (μm ± SEM) of the xenomas measured by light microscopy and the expected diameter to which they would have grown.

	Small Xenomas (+ 4 wk for sampling)		**Large Xenomas (+ 4 wk for sampling)**		
Wk PRE	**Measured**	**Expected***	**Measured**		**Expected***
1	20 ± 1.1		ND		ND
2	19.7± 0.9		ND		ND
3	24 ± 1.4		30 ± 2.2		59.1 ± 2.5
			Interm.	**Large**	
4	19.4 ± 1.5	**18.5**	*35.6 ± 2*	52.2 ± 1.9	75.4 ± 1.7
5	22.4 ± 0.8		*38.4 ± 2.3*	67.7 ± 1.6	102.1 ±4
6	21.4 ± 1.1		70 ± 3		110 ± 3
7	22 ± 0.8		110.2 ±2.9		125.3 ± 2.6
8	ND		120 ± 3.3		143 ± 3.7

ND: Not Determined. Interm: Intermediate

* Based on $Y = b_0 + b_1 (x)$

Table 2.4. Experiment 3. Comparison of xenoma measurements in the exposed (Exp) and satellite (Sat) groups. Values represent the mean (μm ±SEM). Expected value Xdiam based on equation $Y = b_0 + b_1 (x) = 18.5$ μm.

	Weeks PE										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**12**	**16**	**20**
Exp	21.4 ± 0.5	22.1 ± 1.6	21 ± 1.5	19.7 ±0.9	20 ± 1	20.3 ±1.5	21.9 ±0.9	19.1 ±1.2	22.1 ±1	19 ±0.8	20.2 ±1.6
Sat	–	–	–	–	16.4 ±2.2	15.7 ± 1.9	21 ± 1.1	19.9 ±1.4	–	–	–

exposure showed differences in the Xdiam that did not correspond to the expected size between wk 4 and 8 PE. (Table 2.3). By wk 8 and 9 PRE, small and large xenomas respectively, disappeared completely and were no longer detected at wk 20 PRE. At wk 5 and 6 xenomas having the size of 5 week old xenomas as determined by the regression equation were observed and arbitrarily called "intermediate xenomas", for being an intermediate size between small and large xenomas (Table 2.3).

By using Chi-square analysis, the percentage of infected fish in the re-exposed group was significantly lower at wk 6 and 7 PRE/PE when compared to the exposed group ($P<0.05$), while no significant differences were observed at wk 1, 2, 3, and 4 PRE/PE ($P > 0.05$) (Fig. 2.8). The highest percentage (100 %) of infected fish in re-exposed fish was observed at wk 4 PRE. The exposed fish reached maximum prevalence (100%) at wk 2, 4, 8, and 12 PE, while satellite fish reached the highest percentage of infection (80%) at wk 5 PE. In addition, significant differences between small and big xenomas were seen at wk 4, 5, 6, and 7 PRE ($p < 0.05$) (Fig. 2.8). From observations in wet mounts, the maximum number of small xenomas in re-exposed fish was observed at wk 5 PRE, while the maximum number of large xenomas was observed at wk 6 and 7 PRE. In general, the number of xenomas per gill (intensity of the infection) was higher in the fish re-exposed during the early stage of the disease (Fig. 2.9), compared with the exposed (Fig.ure 2.10), and the satellite group (Fig. 2.11). The maximum intensity of infection for the re-exposed group occurred at wk 1 PRE, whereas exposed fish did not show variation in the intensity of infection. By comparing the xenoma intensity for each treatment over time, a one-way

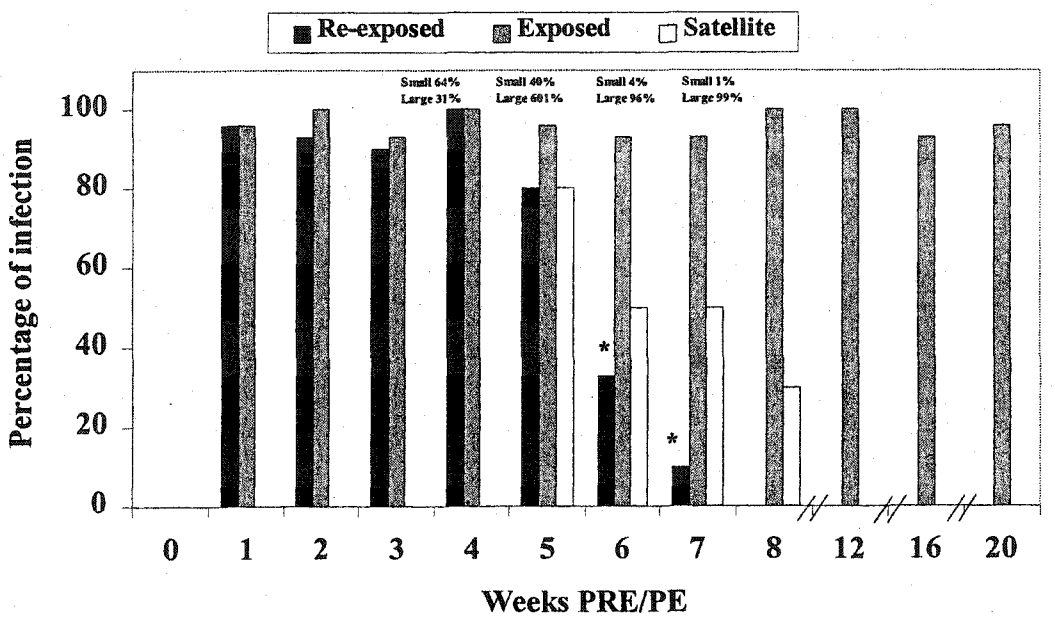


Figure 2.8. Experiment 3. Percentage of fish infected with *Loma salmonae* in the re-exposed, exposed and satellite groups. The percentage of small and large xenomas is indicated on top of the bars. The asterisk above the re-exposed group indicates significant differences between this group and the other two.

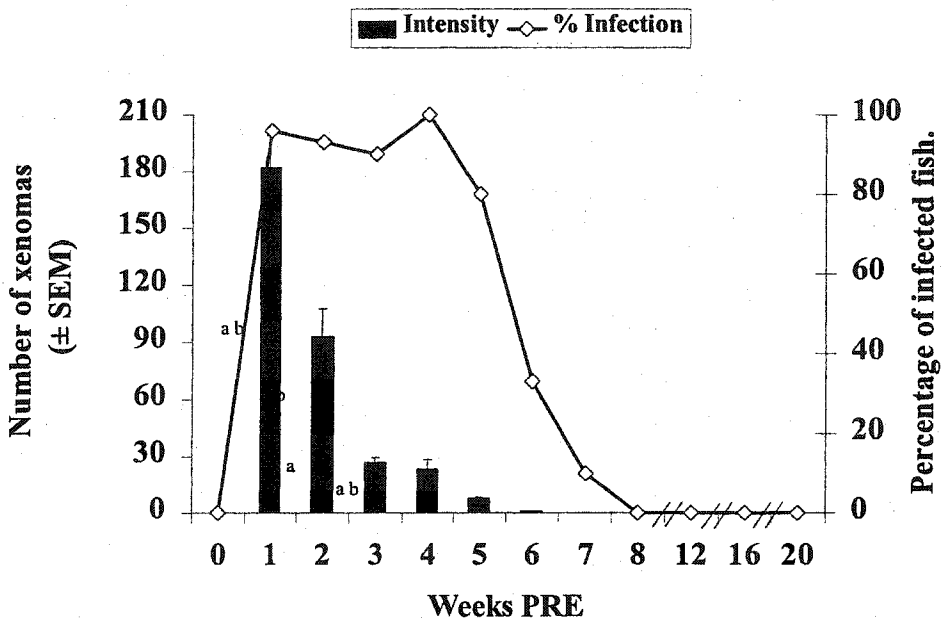


Figure 2.9. Experiment 3. Intensity of the infection and percentage of fish infected with *Loma salmonae* in the re-exposed group.
[a] Significantly different when compared with exposed group ($p < 0.05$)
[b] Significantly different within the group over the time ($p < 0.05$).

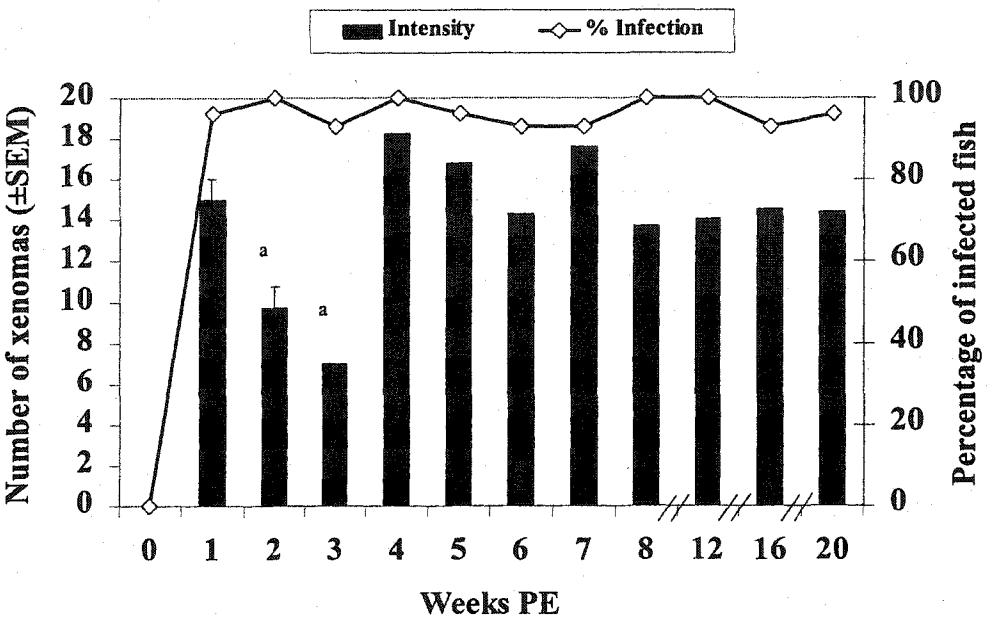


Figure 2.10. Experiment 3. Intensity of the infection and percentage of fish infected with *Loma salmonae* in the exposed group.
[a] Significantly different within the group over the time ($p < 0.05$).

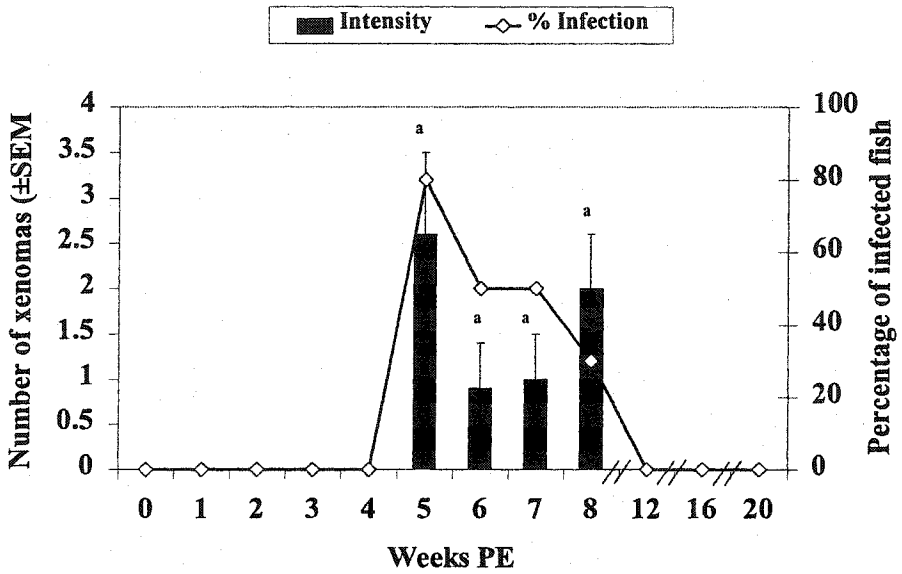


Figure 2.11. Experiment 3. Intensity of the infection and percentage of fish infected with *Loma salmonae* in the satellite group.
[a] Significantly different within the group over the time ($p < 0.05$).

ANOVA revealed that the intensity in re-exposed fish was significantly different (wk 1 to 6 PRE) (Fig. 2.12), while in exposed group the intensity of the xenoma over time was only significantly different at wk 2 and 3 PE ($p< 0.05$) (Fig. 2.10). Satellite fish differ significantly over time ($p<0.05$) from wk 5 to 7 PE (Fig. 2.11). A summary of the intensity and proportion of fish showing xenomas is presented in Fig. 2.12.

Samples collected for histopathology from the re-exposed group revealed multifocal areas of lamellar fusion, moderated filament epithelial hyperplasia and moderate granulomatous reaction (Fig. 2.13). These changes were more accentuated at wk 8 PRE. In previously exposed fish, moderate inflammatory reaction was observed around xenomas without any indication of destruction. Although satellite fish showed a few xenomas in the gills, there was no inflammatory reaction.

2.5 DISCUSSION

Resistance (or immunity) against a disease has been defined as the natural capacity of a living organism to stay unaffected by deadly pathogens that are present in its vicinity or habitat (Janeway and Travers 1997). In fish, the elements involved in resistance to pathogens are similar as those encountered in mammals (Secombes 1994, Watts et al. 2001, Pasquier 2001, Ellis 2001b). With microsporidia, most research on resistance has been done in mammals (Didier and Bessinger 1999, Desportes-Livage 2000), and in some cases a specific resistance mechanism has been defined as being protective against this pathogen (Khan et al. 1999, Braunfuchsová et al. 2001, Moretto et al. 2001). However, the onset and mechanisms of resistance in fish to microsporidia are unknown.

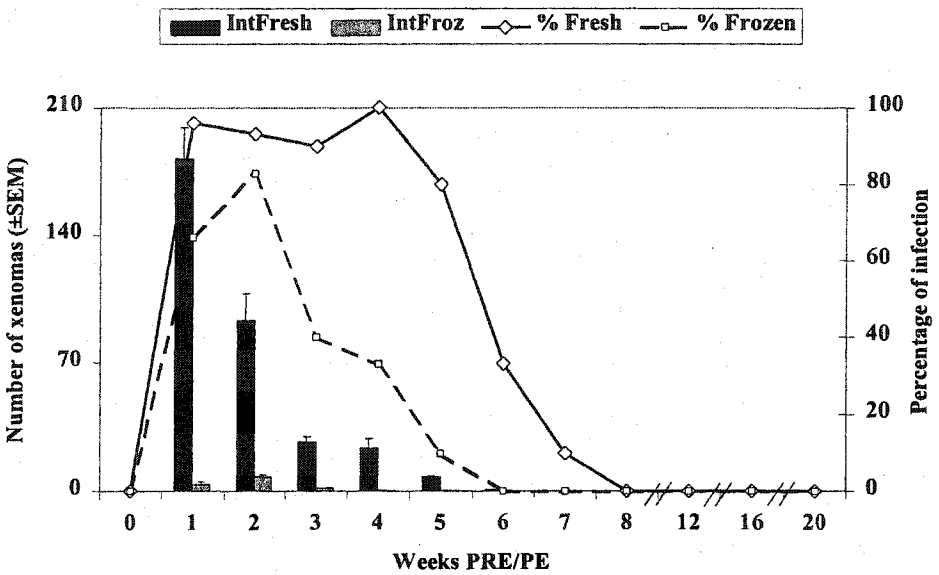
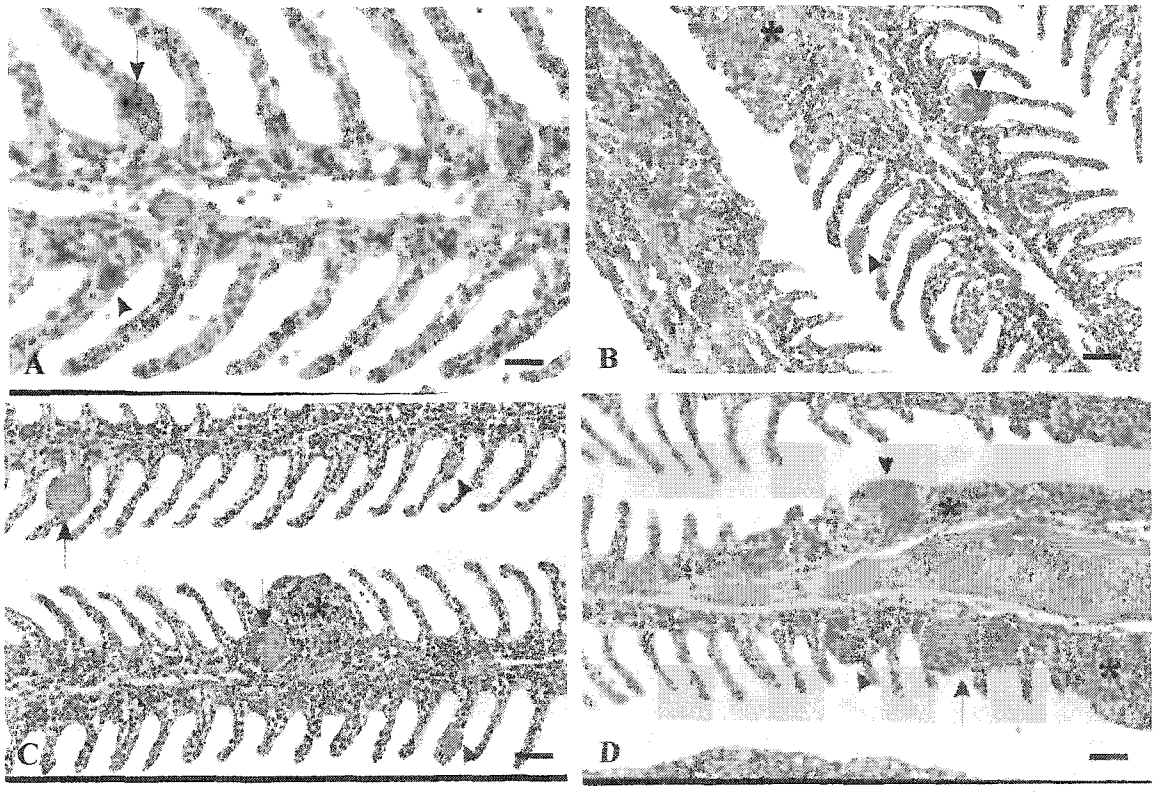


Figure 2.12. Intensity of the infection and percentage of infectedfish exposed with dead and live spores from *Loma salmonae* in the re-exposed groups at different weeks postexposure.

Fig. 2.13. Light micrographs of gills from rainbow trout re-exposed to *Loma salmonae*. A. Four wk PRE. **A.** Large (arrow) and small (arrowhead) xenomas are evident in lamellae. Bar = 40 μm. **B.** Five wk PRE. Large (arrow) and small (arrowhead) xenomas close to an area of epithelial hyperplasia and lamellar fusion (*). Bar = 100 μm. **C.** Six wk PRE. Large xenomas (arrows), one of which is associated with a granulomatous reaction (*). Small xenomas (arrowhead). Bar = 100 μm. **D.** Large (arrows) and small (arrowhead) xenomas associated with areas of lamellar fusion (*). Bar = 100 μm. H&E stain.



the onset of xenoma development and resistance (Speare et al. 1998b, Kent et al. 1999).

First experiment. A simple linear regression and equation as a mathematical model was developed to predict the size of the xenoma based on the time of the exposure. For the first time a strong association between the size of the xenoma produced by *L. salmonae* in rainbow trout and the time after exposure was demonstrated. The temperature used in this experiment was selected since it is the optimum temperature for the maximal xenoma development (Beaman and Speare 1999). However, future studies will consider the effect of variation in the temperature on the growth of the xenoma. Presently, there are no studies on parasites which generate cystic structures that relate size of the structure to the time of its development. Most analysis of cyst or mass diameters are either focused in human oncology, where the diameter of a sphere-like tumour could be used to predict the age of a tumoral mass and its severity (Maehle et al. 2001, Wellman et al. 2001), or with reproductive parameters (Löfstedt and Ireland 2000). Nevertheless, accurate measurements for monitoring the development of parasites based on time are still not available.

The simple linear regression model was highly predictive for the Xdiam within the period of 4 to 9 wk PE and most of the xenomas examined were inside the parameters of the fitted line. In this study, wk 4 PE was considered to be the earliest time for detecting xenomas under light microscopy. The analysis of Xdiam at wk 3 PE or before was difficult because the infected host cells were too small to be detected in paraffin sections. At this time *L. salmonae* are undergoing merogonic stages and the infected host cell can only be detected by analysing gill samples using high resolution light microscopy (Rodriguez-Tovar et al. 2002). Furthermore, the evaluation of the Xdiam from wk 9 PE onwards was difficult

because most xenomas were already ruptured or experiencing severe inflammatory response that interfered with the identification of the xenoma boundaries. The xenoma increase had a consistent rate of enlargement of approximately 16 μm every wk which was very close to the slope value computed and remained almost unaltered until wk 9 PE. However, temporary delay in xenoma development was observed from wk 5 to 6 PE. This delay could have resulted from an early inflammatory response that was observed in some gills when examined in paraffin sections at wk 4 PE. A few xenomas were accompanied by leucocyte (mainly macrophages) infiltration and there was some evidence of xenoma rupture and release of spores. Similar findings of early dissolution of xenomas at wk 4 PE have been described elsewhere (Speare et al. 1998c). Perhaps intracellular parasite proliferation was affected by the production of pro-inflammatory substances generated by the inflammatory reaction that temporarily retarded the intracellular *L. salmonae* multiplication. For example, in some microsporidia infections certain cytokines, such as TNF-α and IFN-γ, may contribute to the generation of resistance during the early stages of the infection (Didier and Shadduck 1994, Didier 1995). In those studies the inflammatory event caused a "parasitistatic" effect, rather than a parasiticidal activity (Didier and Bessinger 1999). If this happened in the present study, this parasitistatic effect was brief, because the rate of the xenoma enlargement returned to its original value from wk 6 onwards as predicted by simple linear regression. Research in fish cytokines is an area of active interest (Collet and Secombes 2002), and further studies will be necessary to elucidate the role of these substances as parasitistatic agents in *L. salmonae* infections.

Second experiment. Only small xenomas were detected in the gills from re-exposed fish until wk 5 PRE, and were similar in dimensions to the branchial xenomas observed in the exposed group. The simple linear regression was appropriate for determining that the size of the xenomas seen in those groups corresponded with the period when fish received the second exposure with live spores. The finding of just small xenomas in these 2 groups demonstrated that the initial IP inoculation with inactive spores did not generate branchial xenomas and that the procedure for inactivating them resulted in a complete non infectivity of the parasite (Shaw et al. 2000). This was confirmed by the total absence of xenomas in the satellite fish when examined for the presence of infection that could be generated from the re-exposed fish. In a parallel study (not described here), naive fish receiving inactive spores did not develop detectable xenomas over a period of 4 months, confirming that spores inactivated by freezing never reverted to their infectivity. It was assumed that spores did not generated branchial xenomas because they lost their viability after the freezing-thawing procedure. Similarly, Shaw et al. (2000) demonstrated that *L. salmonae* spores were completely killed under freezing-thawing conditions because fish exposed to these spores failed to develop branchial xenomas.

In the present experiment, IP inoculation was selected because it was previously proven to confer protection in rainbow trout (Speare et al. 1998b), which strongly suggests the establishment of immunological memory (= resistance). The use of IP injected *L. salmonae* inactive spores as an immunogen could have several advantages over the use of live spores (virulent and avirulent strains): 1) it could be antigenically similar to the living organism; 2) it would be processed by the immune system without the risk of producing

disease; 3) it would elicit specific immune response; and 4) it is expected to induce memory. It has been suggested that IP injection has some undesirable effects that would affect fish productivity, such as the necessity for anaesthetizing fish for the appropriate handling, the risk of fungal attack, and the formation of peritoneal adherences (Horne 1997). Although in this investigation fish were inoculated with a high concentration of inactive spores, they never showed any signs that indicated adverse reactions to the spores. In addition, during the sampling of gills, fish were inspected for the presence of peritoneal injury, without evidence of abdominal lesion.

The prevalence of infection in the re-exposed fish was significantly different when compared with the exposed group during the first 5 wk PRE/PE. The maximum prevalence for the re-exposed group occurred 2 wk earlier than that observed in experiment 3, but it was much lower (80%). The proportion of infection in the re-exposed group started to decrease at wk 3 PRE and by wk 6 PRE the parasite was unable to induce detectable xenomas following exposure with live spores. When the re-exposed group was compared with that from experiment 3, an earlier reduction in the percentage of infection and a total resistance to subsequent inoculations with live spores was observed at wk 3 and 6 PRE, respectively. The prevalence of the infection observed in the exposed group from experiment 2 was quite similar to that observed in experiment 3. This confirmed that the inoculum used for challenging fish was infective and able to generate branchial xenomas, but it was unable to induce xenomas in fish previously exposed to inactive spores at wk 6 PE. However, the presence of a few xenomas in just 2 fish 8 wk after exposure could indicate a delayed partial protection in those fish. The small size of the xenomas observed in these fish confirmed that

infection came from the second exposure and not from the initial exposure. In addition, satellite fish did not show any xenomas, corroborating that disease came from the latest inoculation.

Significant differences were observed in the intensity of the infection in the re-exposed group from experiment 2 over time. Infection was more intense at wk 2 PRE and was followed by a reduction in the number of xenomas per gill at wk 3 PRE. Nevertheless, the concentration of xenomas in this group never reached the concentration observed in the exposed fish from this same experiment and in the re-exposed and exposed groups from experiment 3. This indicated that the use of IP inactive spores induced an earlier and stronger refractory response to subsequent exposures to live spores by blocking the development of xenomas in gills.

There could be several reasons that contributed to the fast and solid resistance to live spores in the re-exposed fish from experiment 2. The inactive spores were taken up immediately by local phagocytes in the peritoneal cavity and processed for recognition by the fish immune response. Peritoneal macrophages are considered as the main resident leucocyte population in the peritoneal cavity of some salmonids, and actively participate in the ingestion of attenuated or dead microorganisms inoculated IP (Woo 1997, Afonso et al. 1998). Perhaps the IP inoculation activated a cell-mediated response contributing to the rapid elimination of the second exposure with live spores by the establishment of memory.

Recent results with rainbow trout lymphocytes have confirmed that Ag from *L. salmonae* is able to induce *in vitro* lymphoproliferation when fish were exposed to live and dead spores (Chapter 3). Similar results in lymphocyte proliferation were observed in fish

infected with *Enterocytozoon (Nucleospora) salmonis* (Wongtavatchai et al. 1995b). In experimental exposures, resistance to *L. salmonae* apparently develops after the rupture of xenomas from non-lethal infections (Speare et al. 1998b, Speare et al. 1998c, Beaman et al. 1999, Kent et al. 1999).

The development of resistance after infection occurs with other parasites in fish, such as *Ichthyophthirius multifiliis* (McCallum 1986), *Gyrodactylus derjavini* (Lindenstrom and Buchmann 2000), and *Trypanosoma danilewskyi* (Islam and Woo 1991). However, results from the present experiment suggest that resistance to *L. salmonae* does not require completion of the parasite life cycle, as recently suggested (Speare et al. 1998b), or the requirement of a living organism to generate protection. The early diminution of the number of xenomas in the re-exposed group from this experiment indicated that the development of some early partial resistance began before live spores could form branchial xenomas. In addition, the failure to produce xenomas at wk 6 PE in fish IP inoculated with inactive spores indicated that antigenic similarities from the live parasite are shared in the inert organism, and could be considered in the future as a potential biological agent for preventing the disease, as was proposed earlier (Kent et al. 1999). The induction of resistance to parasites in fish via IP have been reported in channel catfish inoculated with killed trophonts of *I. multifiliis* (Burkart et al. 1990), and in rainbow trout inoculated with different antigenic forms of *Diplostomum spathaceum* (Stables and Chappell 1986, Whyte et al. 1990).

Previous studies have indicated that the presence of a resistance state in fish recovered from *L. salmonae* infection, affected the parasite development by delaying or avoiding its implantation in the gills (Sanchez et al. 2001a). If this was the case in the present

experiment, then the absence of xenomas in re-exposed fish, at wk 6 PE and wk 8 PE, in experiments 2 and 3 respectively, indicated the establishment of complete resistance, which was interpreted as the inability of the parasite to produce xenomas after subsequent exposures with live spores.

Currently, there is no information about any biological product developed to confer protection against fish microsporidia, and only a few experimental assays have been conducted to explore the effect of an attenuated *L. salmonae* for the generation of immunity against this parasite (Sanchez et al. 2001b, c). However, the exact mechanisms of resistance participating in the rejection of microsporidia both in fish and mammals still are unknown (Didier 2000)

The ERE model in the present study allowed us to determine when rainbow trout developed partial and complete resistance when exposed to live and dead spores. As described in experiment 2, although partial resistance was reached during the first month PRE, one important consideration for use of the inactive form of the parasite is that it did not revert to its pathogenic state. In our study, contrary to the partial protection observed in a preceding investigation (Speare et al. 1998b), complete resistance to re-exposure with live spores was observed at wk 6 PRE, which persisted until wk 20 PRE. Probably in the earlier study, by the time the fish were examined for the presence of xenomas (wk 24 PE), resistance was in decay and it could have generated xenoma formation following oral exposure. Further research will be needed to determine the dosage, timing and method of administration of *L. salmonae* dead spores to obtain maximum resistance.

Third experiment. In this experiment the use of the regression line as a standard curve and the equation modelled from experiment 1 proved useful in predicting the size of the small xenomas and to estimate the onset of resistance in rainbow trout, which occurred at wk 8 PE. This period was characterized by the presence of a severe inflammatory reaction to xenomas. From the first experiment, the earliest period for detecting small xenomas was 4 wk after the last exposure, and when gills from the re-exposed group were examined to detect 4 wk old xenomas, the presence of these xenomas was consistent until wk 7 PRE, but could not be observed from wk 8 until 20 PRE. The dimension of those small xenomas observed in the re-exposed fish suggested that the origin was from the re-exposure, which had occurred 4 wk previously. The presence of "intermediate"xenomas was an interesting finding in some re-exposed fish at wk 5 and 6 PRE, roughly coinciding with the time when reduction in the xenoma growth was observed in experiment 1. The presence of these intermediate xenomas could be explained as by of four possibilities: 1) they originated as a consequence of sectioning the xenomas in different planes during the histological process. Nevertheless, results obtained from the experiment 1 did not show such variation in the dimension of the xenomas; 2) they represented overgrown xenomas originating from the second exposure with live spores, perhaps as a result of immunosuppression generated by the first inoculation. However, this possibility was considered unlikely because a previous observation from other experiments (Chapter 3) clearly demonstrated that immunosuppression, at wk 5 and 6 PE did not occur; 3) they originated from a subsequent autoinfection from the same re-exposed fish. Some satellite fish were also infected by cohabiting with the re-infected group, and although they also showed the highest

concentration of xenomas per gill at wk 5 PE, they did not present any xenomas with the dimensions of the intermediate ones. In addition, these xenomas were not found in the exposed group. Nevertheless, the period for sampling the gills in those fish did not allow the detection of such intermediate xenomas, therefore the possibility of autoinfection cannot be disregarded; or 4) they were a product of the first inoculation, but their development was retarded as a result of partial immunity during the middle observation which was subsequently enhanced after the second exposure with live spores. This possibility is supported by the smaller size of the "large" xenomas in the re-exposed fish, when compared with the standard curve generated in the experiment 1. In addition, the period when these xenomas were seen coincided with the presence of an inflammatory reaction. Unfortunately, the time frame for observing substantial differences in the intermediate xenomas was particularly brief, and consequently additional studies including morphometry techniques are necessary to clarify the origin of this type of xenoma. Based on these results obtained from measuring xenomas, a complete resistance to subsequent exposures with live spores occurred at wk 8 PE, and it was preceded by a delayed xenoma growth at wk 5 PE, which coincided with an early release of spores. With those large xenomas that exhibited reduction, some showed no evidence of an inflammatory reaction, suggesting that an unidentified substance produced in the re-exposed fish could have interfered with the normal xenoma development (parasitistatic effect).

In mammals IFN-γ is essential for controlling and preventing the intracellular replication of *Toxoplasma gondii* (Nare et al. 2002, Suzuki 2002) and *T. cruzi* (Teixeira et al. 2002). In fish, the presence of an IFN-like substance has been demonstrated (Secombes

1994, Manning and Nakanishi 1996, Press 1998, Ellis 2001a), and it is possible that an IFN-like substance could have been participating with macrophages to interfere with the intracellular development of *L.* salmonae in this study. Future research is necessary to determine what biochemical substances are produced in response to *L. salmonae* and elucidate their role in xenoma resolution.

Significant differences in the prevalence of the infection among the different groups in experiment 3 were observed during the middle observation. In general, the proportion of infected fish in the re-exposed group started to decrease at wk 5 PRE, coinciding with the period when delayed xenoma growth was observed in experiment 1, and with the presence of intermediate xenomas in experiment 3. This was followed by a continuous reduction both in the proportion of infected fish and in the number of detectable small xenomas per gill when observed in wet mounts, until a complete absence of infection by wk 8 PRE. Histopathological examination of the gills from re-exposed fish at wk 4 PRE confirmed the presence of an inflammatory response around xenomas; most showed evidence of rupture and release of spores. This suggests that partial resistance to subsequent exposures with the parasite started soon after the early release of spores into the aquatic environment.

The period when the prevalence was being reduced in the re-exposed fish coincided with the highest prevalence of infection in the satellite group. The size of the small xenomas in the satellite group was similar to that observed both in re-exposed fish and in the exposed fish, suggesting that when satellite fish were introduced into the tanks infective spores were already free in the tanks. The period when spores were released from the re-exposed group was similar to that observed in other studies regarding horizontal transmission of *L.*

salmonae in rainbow trout (Speare et al. 1998b, Speare et al. 1998c). The maximum prevalence for the satellite group was lower (80%) than the exposed group (100%), which remained almost invariable during the experiment, demonstrating the infectivity of the inoculum used for reinfection. There were several factors that probably led to the lower prevalence of infection in the satellite fish, such as the concentration of spores that were released into the tanks (confirmed by the low concentration of spores seen in their gills), the susceptibility of the host used in the experiment and the rate of water turnover (Ramsay 2000).

Significant differences in the intensity of the infection were found in the re-exposed group over time. Our results indicated that the maximum intensity occurred at 1 wk PRE, in contrast with the number of xenomas observed in the exposed group. By comparing both groups, the higher number of xenomas seen in the re-exposed group seem to have been caused by the establishment of a superinfection state. This could imply that a temporary immunosuppressive effect was generated during the first wk PRE, leaving the fish susceptible for the development of more xenomas originating from the second exposure. Based on the time of sampling, the high concentration of xenomas observed in that group could not have originated from autoinfection. This was corroborated by measuring the xenomas with light microscopy and analysing wet mounts from the satellite group. Satellite fish remained uninfected during the first 4 wk PE of this experiment, suggesting that there was no xenoma rupture from the re-exposed fish during that time. Although at wk 1 PRE it was impossible in re-exposed fish to distinguish the small xenomas coming from the first and

second exposures, due to the similarity in their size, it was probable that a high proportion of those xenomas were coming from the second exposure.

Immunosuppression caused by microsporidians has been reported in fish infected with *E. (Nucleospora) salmonis* (Wongtavatchai et al. 1995a), and in mammals, where a superinfection following immunosuppression was reported in mice with toxoplasmosis (Vollmer et al. 1987). In the present investigation, a slight suppression of the lymphocyte proliferation to ConA was observed at 1 wk PRE in fish IP inoculated with live spores (Chapter 3), thus the superinfection observed in this experiment could be caused by the second exposure with live spores 1 wk PRE. Although the number of total xenomas per gill in re-exposed fish was significantly higher than that observed in the exposed and satellite groups until wk 4 PRE, a substantial reduction in the number of xenomas was apparent at wk 2 PRE, where the concentration of xenomas was drastically reduced by half. This could indicate an early resistance and could be related with the natural high capacity that rainbow trout has for resisting the infection with *L. salmonae* (Speare et al. 1998a), as was demonstrated by the absence of clinical signs of disease in the present study.

Evidence validating early resistance to *L. salmonae* comes from results in Chapter 3 with head kidney lymphocytes from rainbow trout exposed to live spores of *L. salmonae*. Lymphocytes proliferated as early as 2 wk PE when they were stimulated *in vitro* with dead spores and crude soluble extract from *L. salmonae*. Thus, after an apparent immunosuppression, an early weak resistance could be initiated as early as wk 2 PRE. By wk 5 PRE, the intensity of the infection in the re-exposed group was significantly lower than the other two groups. This was confirmed when the proportion of small and large xenomas

was analysed in wet mounts and then compared over time. Clearly, the proportion of small xenomas was reduced from nearly 70% to 1% over a period of 4 wks. No small xenomas were observed at wk 8 PRE, although fish received a considerable concentration of infective spores, indicating that by this time, the parasite was unable to complete its life cycle and produce xenomas. However, resistance does not mean complete elimination of the parasite, since it was previously noted that *L. salmonae* could persist for several wk PE in the heart of recovered fish. Thus, although the parasite development in the gills was blocked, it survived inside resistant hosts for an extended period (Sanchez et al. 2001a). This carrier state could have serious repercussions in aquaculture since recovered fish would act as reservoirs of the disease. In a recent study, naive rainbow trout were infected with viscera from recovered fish that were free of branchial xenomas for as long as 20 wk PE (Ramsay et al. 2001), suggesting that the infection could be maintained and perpetuated in fish farms where smolts are raised in the same site with older recovered fish (Shaw and Kent 1999). Likewise, in recovered chinook salmon *L. salmonae* spores were observed under light microscopy in the kidneys as late as wk 29 PE. Although this carrier state could enhance the resistance status in fish, recently it was indicated that urine of infected fish could be considered as another source of transmission for *Loma* sp. (Hauck 1984). In the present study, the infection in other viscera was not explored and the presence of parasites in sites other than gills is unknown. Further studies will be necessary to establish if the parasite is complete eliminated from recovered fish.

The study of vaccines against fish parasites is a relatively new field in comparison with bacteria and viral vaccines research (Gudding et al. 1999). Nevertheless, there is

increasing interest for developing strategies to control parasitic fish diseases, although it has been under experimental conditions (Woo 1997). For *L. salmonae*, further work has already commenced to elucidate the most important antigenic components involved in the development of resistance to this parasite.

In conclusion, the ERE model used in this experiment was appropriate to determine the onset of resistance in rainbow trout infected with *L. salmonae* at 15°C. The use of the simple linear regression for the development of a linear regression model was useful for predicting the xenoma dimension based on the time of exposure. The simple linear regression was also useful for distinguishing early and late exposures of *L. salmonae*, and for determining of the onset of resistance in rainbow trout exposed to dead and live spores. The generation of a partial and complete protection occurred at different times when dead and live spores were used as initial inoculum in experiments 2 and 3. The establishment of a partial resistance was faster in fish exposed to live spores than exposed to dead spores. Partial and complete resistance in experiment 2 occurred at wk 3 and 6 PRE, respectively. In the case of fish exposed to live spores, this period was observed at wk 2 and 8 PE respectively. The use of inactive spores seems to be an excellent option for preventing or reducing the infection in gills.

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3 CELL-MEDIATED IMMUNITY OF RAINBOW TROUT EXPOSED TO DEAD AND LIVE SPORES OF *LOMA SALMONAE*

3.1 ABSTRACT

Loma salmonae is a strict intracellular parasite that infects salmonids of the genus *Oncorhynchus* and is characterized by the presence of spore-filled xenomas in the gills. Fish that survive infection after oral exposure to live spores or after intraperitoneal inoculation with live or dead (inactive) spores, remain resistant to subsequent xenoma formation upon re-exposure to the same parasite. The nature of this resistant state is unknown although cellular mechanisms have been postulated to be involved. Research in cellular immunity to microsporidia is still in its infancy and the role of this immune response in fish has not been investigated. The main objective of this study was to determine if leucocytes from recovered, exposed, and unexposed fish could respond to antigens from *L. salmonae* when measured using *in* a vitro lymphoproliferation assay. When fish were exposed orally to live spores from *L. salmonae,* they reached a maximum proliferation at wk 6 and 8 PE, when cultured *in vitro* with the crude soluble extract and intact dead spores respectively. In the second experiment, when fish that had recovered from an initial infection were re-challenged at wk 14 with live spores, an increase in the proliferative response to the dead spores was evident at wk 15 PE. Lymphoproliferation to crude soluble extract was similar to that seen in experiment 1. Fish from experiment 2 never developed xenomas after reinfection. In experiment 3, lymphocytes from fish exposed to dead spores proliferated as early as wk 2 PE, and this proliferation was higher when cells were stimulated *in vitro* with dead spores.

Finally, in the fourth experiment, lymphocytes from fish exposed to dead spores and then re-exposed to fresh spores at wk 22 PE, had the highest proliferative response when stimulated *in vitro* with both Ag. However, a few fish developed sporadic branchial xenomas. It appeared that IP inoculation with dead spores generated a stronger *in vitro* proliferative response to the particulate Ag than when inoculated with infective spores via gastric intubation. Importantly, the inoculation of dead spores reduced the number of branchial xenomas in fish re-exposed to fresh spores. This suggested that an inactive form of the parasite could be used in the future as a candidate vaccine for controlling or preventing the disease.

3.2 INTRODUCTION

Loma salmonae is an obligate intracellular parasite that infects the gills of salmonids of the genus *Oncorhynchus*. Chinook salmon (*O. tshawytscha*) (Hauck 1984, Constantine 1999), coho salmon (*O. kisutch)*(Kent et al. 1986), and rainbow trout (*O. mykiss*) (Bruno et al. 1995) are the most commonly affected species. *Loma salmonae* belongs to the phylum Microspora and is characterized by the production of infective spores that contain a unique and sophisticated extrusion apparatus for the transmission of the disease agent (Cali and Takvorian 1999). The life cycle of *L. salmonae* is poorly understood, but the general model of infection for microsporidia suggests that the mode of entry is by direct ingestion of spores (Bigliardi and Sacchi 2001, Sanchez et al. 2001b). In experimentally infected rainbow trout, *L. salmonae* spores were found in close association with the gastrointestinal epithelium (Shaw et al. 1998, Sanchez et al. 2001b) soon after ingestion. For most microsporidia, *L.*

salmonae included, infective sporoplasms are released through the polar tube into the cytoplasm of the host cell, which could be an enterocyte or an intraepithelial leukocyte (Canning et al. 1986, Dyková 1992). Recently, by using the polymerase chain reaction (PCR), it was observed that the parasite moves from the gut to the gills in approximately 2 wk through a mechanism that could involve intraepithelial leucocytes acting as transport vehicles (Sanchez et al. 2001b).

High mortalities in fish are associated with the presence of xenomas, containing parasites in multiple stages of development, in their gills. The rupture of xenomas and the release of the spores occurs by wk 7 or 8 postexposure (PE), generating severe branchitis, granuloma formation and finally death due to asphyxia (Speare et al. 1998a, Speare et al. 1998c). Fish that survive the infection, either after oral exposure to live spores or after intraperitoneal (IP) inoculation with live or dead spores, remain resistant to subsequent experimental exposures with the same parasitic agent (Speare et al. 1998b). However, little is known about the role of humoral and cellular immunity in this resistance to *L. salmonae* (Sanchez et al. 1999). The protective role of the humoral immunity in microsporidia infection is controversial. In some cases a specific humoral response apparently occurs with certain microsporidia (Leiro et al. 1993, Hung et al. 1996, Didier 2000); and in other cases the infection causes immunodepression (Laudan et al. 1986b, Laudan et al. 1986a, Laudan et al. 1987). More recently, attempts to induce humoral response in fish or to transfer immunity through hyperimmune serum have indicated that antibodies do not have an important role in the defence to *L. salmonae* (Sanchez et al. 2001a). Under experimental

conditions certain factors contained in fish plasma can enhance macrophage uptake of this parasite (Shaw et al. 2001).

In fish, the use of adoptive immunity by transferring T cells from sensitized fish into unsensitized fish to demonstrate the role of cellular immunity is problematic because there are no histocompatible lines of fish (Komen et al. 1990, Manning and Nakanishi 1996). In addition, to demonstrate the *in vivo* role of these sensitized cells without interference of fish receptor T cells, requires thymic ablation by surgery or radiation treatment. These methods take time and the use of sophisticated and expensive equipment (Nakanishi 1990).

Culture systems, like the *in vitro* proliferation of lymphocytes (blastogenesis or mitogenesis), represent an extremely useful immunological tool for studying fish cellular immunity (Miller and McKinney 1994). In general, lymphocyte proliferation is a procedure that involves the *in vitro* culture of lymphocytes either from peripheral blood (Wang et al. 1997) or from lymphoid tissue, like the head kidney (Nie et al. 1996, Galeotti et al. 1999). Lymphocytes are cultured in the presence (or absence) of an antigenic or mitogenic stimulus (Maluish and Strong 1986) and the proliferative response is detected by the incorporation of ^{3}H-thymidine into the nuclei of the host cells. Although lymphocyte proliferation assay only measures one component of cellular immunity, a correlation between this *in vitro* event and the cellular response in an exposed living organism has been identified (Gershwin et al. 1995, Pund et al. 1998).

Lymphocyte proliferation has been studied in rainbow trout (Warr and Simon 1983, DeKoning and Kaattari 1991, Estepa and Coll 1992), carp (Rosenberg-Wiser and Avtalion 1982, Caspi et al. 1984, Richards et al. 1996), and channel catfish (Sizemore et al. 1984,

Miller et al. 1986, Luft et al. 1991). There are considerable gaps in the identification of different subpopulations of fish T cells (CD4+, CD8+) (Manning and Nakanishi 1996). However, two lymphocyte populations, T-like cells (sIg^- cells) and B-like cells (sIg^+ cells), have been demonstrated in fish, based on the ability of these cells to proliferate in response to mitogens, such as concanavalin A (ConA), phytohemagglutinin (PHA), or lipopolysaccharide (LPS) (Etlinger et al. 1976, Warr and Simon 1983, Sizemore et al. 1984, Miller and McKinney 1994, Koumans-van Diepen et al. 1994). Lymphocyte proliferation has been an extremely useful tool to elucidate the cellular response of fish to different parasitic antigens (Taylor and Hoole 1994, Nie et al. 1996, Richards et al. 1996), different mitogens (Tillitt et al. 1988, Koumans-van Diepen et al. 1994), and bacterial agents (Pourreau et al. 1987, Yui and Kaattari 1987, Tatner 1990, Marsden et al. 1996), and for the evaluation of fish vaccines (Estepa et al. 1994, Pund et al. 1998). However, very little is known about the specific cellular immune mechanisms involved in the resistance of fish to microsporidia compared with that obtained from mammalian models (El Naas et al. 1999, Khan and Moretto 1999, Khan et al. 1999, Khan et al. 2001). Recently, the *in vitro* stimulation of lymphocytes has been used to investigate the immunological status of chinook salmon during infection with the intranuclear microsporidian *Enterocytozoon (Nucleospora) salmonis* (Wongtavatchai et al. 1995a).

The objective of this study was to use the *in vitro* lymphocyte proliferation assay to determine whether intact spores and crude soluble extract from *L. salmonae* induce proliferation of the head kidney lymphocytes from fish exposed to live and dead spores. Also, the temporal development of the cellular immune response to *L. salmonae* and its

relationship to resistance to reinfection was investigated. The response to specific Ags was compared with that to the mitogen ConA. This is the first time the cellular response of trout exposed to live and dead *L. salmonae* spores have been studied by using dead spores and crude soluble extract of this parasite as *in vitro* challenging agents.

3.3 MATERIALS AND METHODS

3.3.1 Fish population

Naive rainbow trout (*O. mykiss*) of both sexes with an average weight of 50 g were obtained from a commercial hatchery on Prince Edward Island with no previous history of *L. salmonae* infection. They were kept in 70 L fibreglass tanks at 15°C, with a continuous flow of water and photoperiods of light and darkness of 12 h each. Fish exhibiting superficial damage to the fins, gills or skin were eliminated. All procedures followed the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

3.3.2 Experimental design

Experiment 1. *Lymphoproliferative response of rainbow trout exposed to live spores of L. salmonae.* Fish were divided in 2 groups and maintained in separated tanks. The first group (n=64) was exposed orally (primary exposure) to infective spores of *L. salmonae* (~1x 10^6 spores/0.1ml). The second group (n=40) did not receive any inoculum and acted as negative controls. Fish from both the infected and naive groups were killed with an overdose of benzocaine (150 mg/L) at 0, 2, 4, 6, 8, 12, 16, and 22 wk PE. Head kidney lymphocytes were

removed, cultured and stimulated *in vitro* with dead spores and crude soluble extract from *L. salmonae* spores.

Experiment 2. *Lymphoproliferative response of rainbow trout re-exposed to live spores of L. salmonae.* Fish were divided in 3 groups and maintained in separated tanks. The first group (re-exposed) (n= 40) had recovered from a previous infection (primary response) after oral challenge with infective spores of *L. salmonae.* Fish were orally re-exposed (secondary exposure) with live spores (~1 x 10^6/spores/0.1ml) at wk 14 PE. The second group (exposed) (n=40) received one oral inoculation, same dose, when fish from group one were rechallenged. Group 2 was used to assess the infectivity of the inoculum and were considered as positive controls. The third group (n = 10) did not receive any inoculum and served as negative controls. Fish from the 3 groups were killed at 0, 1, 2, 4, and 8 wk PE. Lymphocytes were stimulated as above.

Experiment 3. *Lymphoproliferative response of rainbow trout IP inoculated with dead spores of L. salmonae.* Fish were divided in 2 groups and maintained in separated tanks. The first group (n= 64) received an IP inoculation of dead spores (~1 x 10^6/spore/ml) of *L. salmonae.* An additional group of 8 fish receiving dead spores was reserved for experiment 4. The second group (n=40) did not receive any inoculation and was considered the negative control. Fish from both groups were killed at 0, 2, 4, 6, 8, 16 and 22 wk PE. Lymphocytes were recovered and stimulated as described above.

Experiment 4. *Study of the lymphoproliferative response of rainbow trout IP inoculated with dead spores of L. salmonae and then re-exposed with live spores.* Fish were divided in 3 groups and maintained in separated tanks. The first group (n= 8) had received a previous IP inoculation (experiment 3) with dead spores of *L. salmonae* (primary exposure). These fish were orally re-exposed (secondary exposure) with live spores (~ 1 x 10^6 spores/0.1 ml) at wk 22 PE. The second group (n= 8) received one oral inoculation with live spores (primary exposure) when group one was rechallenged. Group 2 was used to assess the infectivity of the inoculum and were considered as positive controls. The third group (n = 8) did not receive any inoculum and served as negative controls. Fish from the 3 groups were killed 4 wk later and their lymphocytes were stimulated as described above.

3.3.3 Exposure and sampling

Infective spores were obtained from heavily infected rainbow trout. Under light anaesthesia, these fish were screened for xenomas with a stereomicroscope and then were killed by using an overdose of benzocaine (150 mg/L) as reported previously (Speare et al. 1998b). The operculum on both sides of the fish was removed and all gill arches were dissected. The cartilage was completely removed with a razor blade. The gill filaments were chopped finely until a soft mass remained. This mass was transferred into a 50 ml conical polystyrene tube (Falcon) and distilled water was added (v/v). For estimating the number of spores, aliquots of 100 µl were collected and stored at 4°C for 1 h (see section 3.3.7). The mixture was collected into tuberculin syringes attached to small plastic tubes of 5 cm long and 5 mm in diameter.

For the infection procedure, rainbow trout were lightly anaesthetized with benzocaine and approximately 0.1 ml of the inoculum (~1 x 10^6 spores) was given orally to each fish by gastric intubation (Speare et al. 1998a). Trout were returned to their tank to recover and were periodically observed for any evidence of regurgitation, which was not apparent. At scheduled intervals after exposures fish were killed with an overdose of benzocaine. The fish were dried with paper towel and laid on their right side on a paper towel-covered polystyrofoam tray and the skin was disinfected with 70% ethanol.

Blood was obtained by severing the caudal peduncle, and collected in heparinized capillary tubes. Blood smears were prepared and stained with a commercial kit (LeukoStat™. Phy-Lab. Trend Scientific Inc.). One hundred white cells were counted and the proportion of granulocytes and mononuclear cells was determined, as previously described by Peutz et al. (1996). For the lymphoproliferative experiments, the complete head kidney was removed under aseptic conditions after making a mid-ventral incision (with scissors) from the pectoral to the pelvic fins. The abdominal viscera were retracted and the head kidney was exposed and withdrawn with soft scraping movements of the scalpel. Head kidney samples were stored in tubes containing 3 ml of L-15 culture medium (see section 3.3.6) with antibiotics [penicillin (100 units/10µl)/streptomycin (100 µg/10µl) (Sigma)] at 4°C for 20 min. Finally, to assess the presence of xenomas, the left gill arch from every fish was removed and wet mounts were observed under light microscopy.

3.3.4 Isolation of rainbow trout lymphocytes

Cell suspensions free of large tissue fragments were obtained by mincing and sieving the head kidney through a fine stainless steel mesh (80 msh, 190 μm) (Cellector®, Tissue Sieve. Bellco Glass Inc.) over a plastic Petri dish. The cell suspension was aspirated and transferred into 10 ml polystyrene assay tubes (Simport) with L-15 culture medium plus antibiotic at 4°C until use (30 min). To separate the mononuclear cells from the rest of the head kidney tissue and remaining blood cells, 3 ml of the cell suspension was layered on top of a discontinuous gradient [51% (3.5 ml)/34% (3.5 ml)] of sterile Percoll (Sigma) (Rowley 1990) in a 15 ml conical tube. This gradient was prepared by using L-15 culture medium as a diluent for the Percoll. Cell suspensions were centrifuged (Refrigerated Centrifuge TJ-6. Beckman) at 700xg for 30 min at 4° C.

3.3.5 Viability and enumeration of mononuclear cells

After centrifugation, the layer of cells at the interface between the 34%/51% Percoll, was carefully aspirated and washed twice with 10 ml of L-15 culture medium containing antibiotics at 500 x g for 5 min at 4°C. Cells were resuspended in 1 ml of L-15 culture medium with antibiotics plus 1.5% rainbow trout serum, and stored at 4°C until their use (30 min). Small samples of this cell suspension (10 μl) were diluted 1:40 with 0.85% saline solution and 4% Trypan blue. Cells were counted and assessed for their viability by stain exclusion in a hemocytometer and adjusted to a final concentration of 5×10^5 lymphocytes/ ml with L-15 culture medium.

3.3.6 *In vitro* conditions: medium, supplements

Leibovitz's L-15 powder (GibcoBRL, Life Technologies) was dissolved into 1 L of distilled water, adjusted to pH 7.2 and sterilized through a 0.22 μm pore diameter filter (Millex. Millipore). Serum used as a medium supplement source was drawn from healthy rainbow trout (average weight 400 g) by puncturing the caudal vein with a 10 ml syringe. Blood was collected into a 50 ml conical vial, pooled and stored at room temperature (24°C/1 h). Sera were separated from the clot by centrifugation at 200 x g for 10 min at 4°C. Sera were sterilized through a 0.22 μm filter, collected into 5 ml polystyrene assay tubes, heat inactivated at 45°C for 25 min and stored at -20°C until use. The serum was previously tested in pilot studies for its efficiency in proliferative response. For the blastogenesis experiment 1.5% of serum (v/v) and antibiotics were added to the culture medium.

3.3.7 Mitogens and antigens

Concanavalin A. A stock solution of concanavalin A (ConA, Sigma) was dissolved in L-15 culture medium to a final concentration of 1,000 μg/ml, sterilized through a 0.22 μm filter and stored at -20°C in 100 μl aliquots until used.

Dead spores (Inactive parasites). After killing heavily infected fish, gills were dissected, freed of cartilage and ground in a tissue grinder (Wheaton). The crushed gill tissue was resuspended in an equal volume of sterile distilled water and centrifuged at 800 x g for 10 min at 4°C. The pellet was resuspended in sterile distilled water and filtered through a wire mesh of 50 μm (Nytex) to eliminate large debris. This step was repeated twice. The remaining filtered gill sample was mixed with 6% (v/v) Triton-X 100 in a vortex (Fisher

Scientific) and centrifuged twice with sterile distilled water at 1,500 x g for 15 min at 4°C. *Loma salmonae* spores were counted and adjusted in a hemocytometer to a final concentration of 1 x 10^8 spores/ml of sterile distilled water with antibiotics. Subsequently spores were stored at -80°C for 1 to 6 months to ensure their inactivation (Shaw et al. 2000).

Crude soluble extract. The crude soluble extract was obtained from xenoma-bearing gills as follows: spores were purified as described above. Five hundred microlitre aliquots of the spores were deposited into screw cap vials and mixed with an equal volume of 50 μm diameter glass beads (Sigma). Vials were placed in a glass bead beater (Mini-Beadbeater 2110BX, BioSpec Products, Inc) and spores were broken in 3 series of 30 sec at 2,000 rpm each. Subsequently, this mixture was left to settle for 3 min and the supernatant collected in a 15 ml conical polystyrene tube. This process was repeated 3 times with sterile distilled water. After centrifugation at 800 x g for 15 min at 4°C, the resultant supernatant was sterilized through a 0.22 μm pore diameter filter and then analysed to determine the total concentration of protein with a commercial kit (Proteo Kit Assay, Pierce). Finally, crude soluble extract was stored at -20°C in 100 μl aliquots until use.

3.3.8 Proliferative response of lymphocytes and statistical analysis

All culture conditions were determined from previous studies with rainbow trout head kidney lymphocytes in our laboratory (not shown in this thesis). For the blastogenesis experiment, 100 μl of the fish cell suspensions (adjusted to 5 x 10^5 cells/ml) were cultured in individual wells of a 96-well, flat bottom tissue culture plate (Corning Incorporated, Costar) (Figure 3.1). A hundred microlitres of mitogen or antigens were added to each well

to the final concentrations of 5 μg ml^{-1} for ConA, 100,000 spores ml^{-1} for frozen and thawed *L. salmonae* spores and 1.5 μg ml^{-1} for the *L. salmonae* soluble antigen. Unstimulated cultures received only 100 μl of L-15 culture medium plus serum.

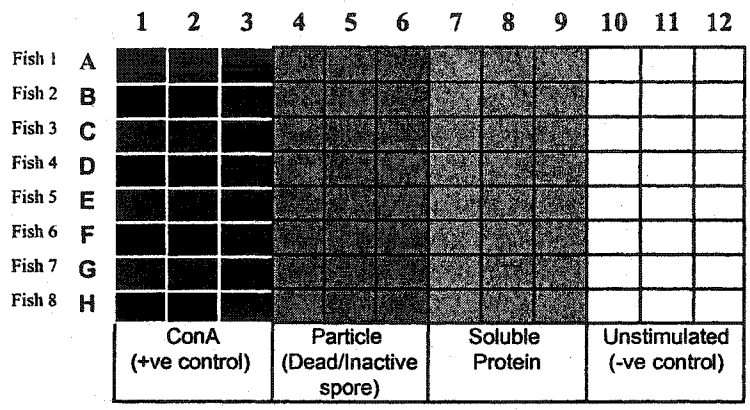

Figure 3.1. General design of the culture of lymphocytes with either mitogen or antigen. Each dosage was effectuated by triplicate.

The rainbow trout lymphocytes were cultured at 18°C for 5 days and were periodically observed for any evidence of contamination. Eighteen hours before harvesting, cells were pulsed with 0.05 μCi of tritiated thymidine (^{3}H-thymidine, Amersham) per well, by using a 50 μl Hamilton syringe. Cells were collected in an automated harvester (Skatron Inc.), lysed with distilled water, and cell debris and DNA were transferred onto glass filter paper disks (Skatron Inc.). After drying in an incubator at 37° C overnight, filter disks were placed in 12-well plastic plates (Wallac) and 0.5 ml of scintillation cocktail fluid (Betaplate Scint, Wallac) was added to each well. The uptake and incorporation of ^{3}H-thymidine in the DNA rainbow trout lymphocytes were measured in a microbeta liquid scintillation and luminescence counter (Wallac, Trilux). The results were expressed as either the number of

counts per minute (CPM) or the stimulation index (SI) and calculated as the mean (± SE) of the triplicate cultures. The SI was obtained by dividing the CPM of the stimulated cultures by the CPM of the unstimulated cultures: SI = CPM stimulated cells/CPM control cells. Results were expressed as mean ± SE of cpm and SI. The effect of the particulate and crude soluble extract was determined by several comparative statistical methods depending on the type of collected data. A Student's t test was used to compare the mean cpm and SI obtained between re-exposed, exposed and control groups. This analysis tested the null hypothesis that the cpm and SI values did not depend on the exposure with live or dead spores. A one-way analysis of variance was used to compare the mean cpm and SI over time within groups. This statistical analysis tested the null hypothesis that the exposure with live or dead spores had no effect on the cpm and SI over time. Data were considered as statistically significant at the 0.05 level and were analysed using the software Minitab® version 12.

3.4 RESULTS

3.4.1 Experimental infection in general

The oral inoculation of fish with *L. salmonae* spores caused infection in all fish (n=64). Small xenomas were detected in the gills by stereomicroscopy at 4 wk PE and by 8 wk PE an inflammatory reaction adjacent to xenomas was observed as pale and swollen areas on the gill surface. No clinical signs were observed in infected fish or control fish. By wk 12 PE and until the termination of the study, no evidence of xenomas was observed in infected fish.

3.4.2 Lymphocyte isolation and viability

Based on their morphological appearance, cells collected from Percoll gradients were mainly composed of lymphocytes with the rest being monocytes and granular leucocytes. Red blood cells were a minimal constituent of this cell preparation. Viability of the lymphocytes, determined by trypan blue dye exclusion, was greater than 90%.

3.4.3 The effect of *L. salmonae* on the proliferative response of lymphocytes

Lymphocytes were isolated from exposed and control rainbow trout and stimulated *in vitro* at different weeks depending on the experiment. The proliferative response to mitogen (ConA) or to Ag (dead spores or crude soluble extract) stimulation was determined as the arithmetical mean of either SI or cpm ± SE.

3.4.3.1 Response of lymphocytes to *Loma salmonae* dead spores (inactive parasites)

Experiment 1. A single oral exposure of fish to live spores produced a consistent proliferative response as tested by *in vitro* stimulation of lymphocytes. The addition of dead spores to sensitized lymphocytes resulted in an increase in the proliferative response at wk 4 and 8 PE (Figure 3.2; Table 3.1), respectively. A Student's t test showed significantly higher differences ($p < 0.05$) when the re-exposed group was compared to controls from wk 2 to 22 PE. A one-way ANOVA of exposed groups showed significant differences over time ($p<0.05$), mainly at wk 8 PE. Lymphocytes from the control group had no significant response when stimulated with the same Ag (Figure 3.3; Table 3.2).

Experiment 2. Proliferative response from the re-exposed group was maximal at wk 15 PE = wk 1 post re-exposure (PRE) (Figure 3.4; Table 3.3). Results during the following weeks, were similar to those observed both in the group exposed once from this same experiment (Figure 3.5; Table 3.4) and in the exposed group from experiment 1. Significant differences between re-exposed and once exposed groups ($p < 0.05$) were observed only at wk 1 PRE; however both were significantly higher than the proliferative response of the control cultures throughout the experiment. The only significant differences over time were observed at wk 15 PE both in cultures from re-exposed and exposed fish ($p< 0.05$). Control proliferation (Figure 3.6; Table 3.5) was similar to that observed in control culture from experiment 1.

Experiment 3. Cultures from fish exposed to dead spores had a significantly higher proliferation ($p < 0.05$) when were compared to control cultures during the experiment. Proliferation of cells from the exposed group remained without significant changes over time ($p < 0.05$). Proliferative response was significantly ($p<0.05$) higher (Figure 3.7; Table 3.6) in the cultures from fish exposed to dead spores than that observed in the cultures from the orally exposed fish in experiments 1 and 2. Lymphocyte proliferation from the control culture (Figure 3.8; Table 3.7) was comparable to that observed in the control cultures in experiments 1 and 2.

Experiment 4. The proliferative response from IP injected fish had the highest SI when re-challenged with live spores at wk 22 PE (Figure 3.9; Table 3.8). This response was much higher when compared to the group given once live spores orally (Figure 3.7; Table 3.6) in

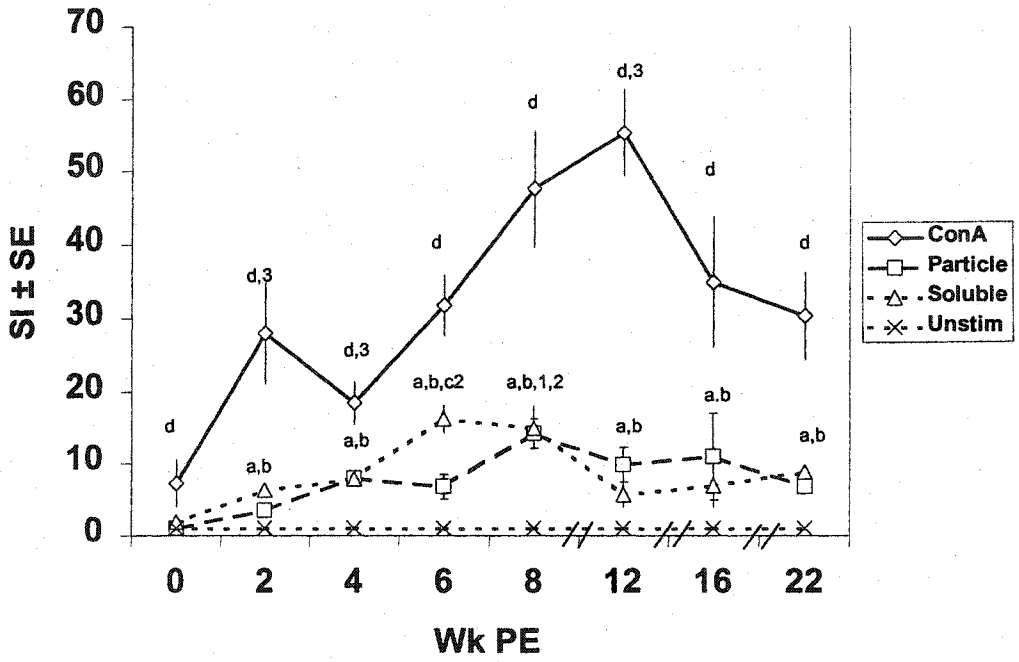

Figure 3.2. Experiment 1. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from rainbow trout **exposed to live spores via gastric intubation.** Data are presented as the mean SI ± SE of triplicate cultures.

a. Exposed group challenged with the particulate Ag are significantly different from unstimulated cells ($p < 0.05$). **b.** Exposed group challenged with the crude soluble extract different from unstimulated cells ($p < 0.05$). **c.** *In vitro* challenge with particulate Ag significantly different from the challenge with the crude soluble extract. **d.** Exposed group challenged with ConA different from unstimulated cells ($p < 0.05$) with the particulate Ag different from unstimulated cells ($p < 0.05$). **1.** Challenge with the particulate Ag different over the time ($p < 0.05$; **2.** Challenge with crude soluble extract different over the time ($p < 0.05$); **3.** Challenge with ConA different over the time ($p < 0.05$).

Table 3.1. Experiment 1. Proliferation of head kidney lymphocytes from rainbow trout orally exposed with infective spores of *Loma salmonae*. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

	Weeks postexposure (PE)							
***In vitro* treatment**	**0**	**2[abc]**	**4[abc]**	**6[abc]**	**8[abc]**	**12[abc]**	**16[abc]**	**22[abc]**
ConA	2738 ±464	6045 ±450	11380 ±354	12977 ±127	21721 ±357	18910 ±202	14860 ±762	9056 ±114
Particle	162.6 ±15	1555 ±466	5050 ±192	3054 ±841	7298 ±165	3442 ±1765	2269 ±242	1946 ±323
Soluble	912 ±120	1692 ±666	4692 ±1285	6973 ±988	8261 ±225	3187 ±923	1502 ±78	2527 ±27
Unstim	527.5 ±89.4	207.9 ±56	507 ±207	438 ±55	637 ±162	563 ±83	410 ±84	282 ±23

Table 3.2. Proliferation of head kidney lymphocytes from naïve rainbow trout. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 5 fish per observation)

	0*	**2**	**4**	**6**	**8**	**12**	**16**	**22**
ConA	2738 ±464	1480 ±131	2335 ±765	3185 ±74	7300 ±721	3357 ±799	2815 ±550	1486 ±333
Particle	162.6 ±15	361 ±189	490 ±29	273 ±6	485 ±100	370 ±96	370 ±30	254 ±39
Soluble	912 ±120	342 ±151	550 ±88	383 ±9	491 ±143	288 ±66	287 ±45	232 ±68
Unstim	527.5 ±89.4	188.5 ± 50	370 ±23	306 ±6	579 ±160	342 ±104	340 ±99	128 ±88

* Only time 0 involved same observations both in infected and naive fish

a. ConA different from naive fish ($p<0.05$)

b. Particulate Ag different from naive fish ($p<0.05$)

c. Crude soluble extract different from naive fish ($p<0.05$)

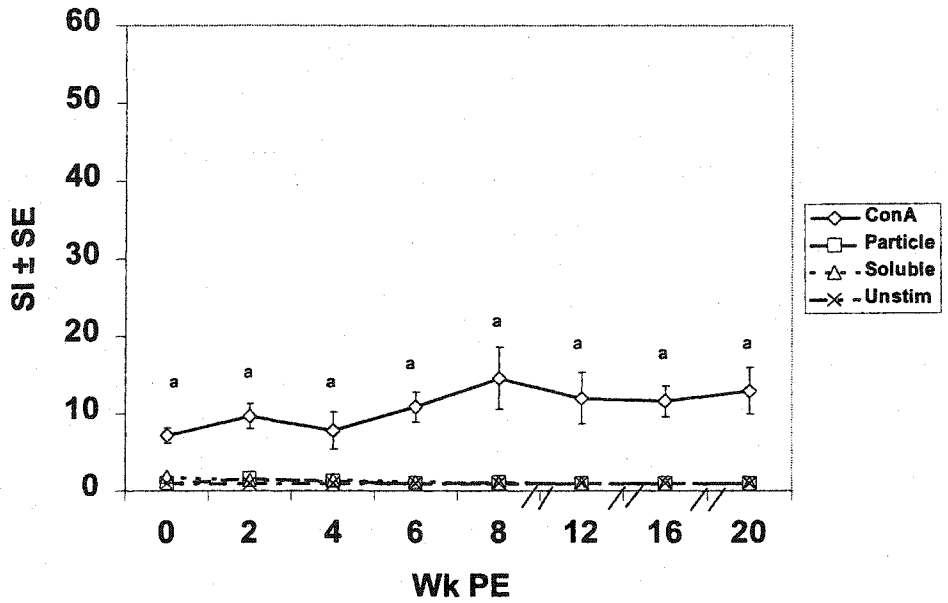

Figure 3.3. Experiment 1. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from naive rainbow trout (control group). Data are presented as the mean SI ± SE of triplicate cultures.
a. Naive fish lymphocytes challenged with ConA are significantly different from unstimulated cells ($p < 0.05$).

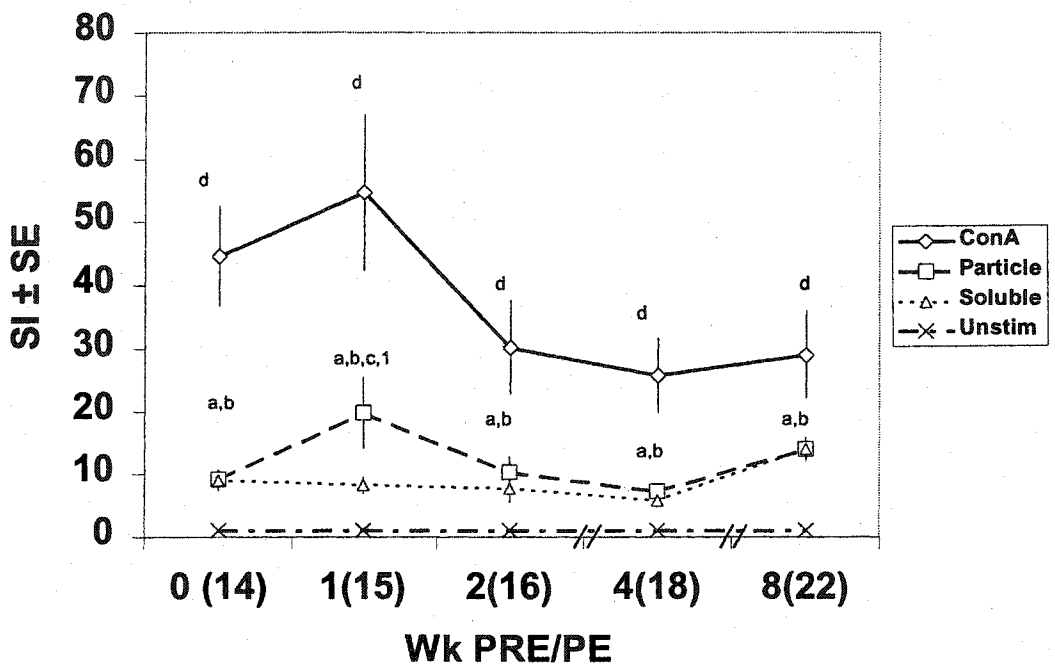

Figure 3.4. Experiment 2. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from recovered rainbow trout **re-exposed to live spores via gastric intubation.** Data are presented as the mean SI ± SE of triplicate cultures.

a. Re-exposed group lymphocytes challenged with the particulate Ag are significantly different from unstimulated cells ($p < 0.05$). **b.** Re-exposed group challenged with the crude soluble extract different from unstimulated cells ($p < 0.05$). **c.** *In vitro* challenge with particulate Ag different from the challenge with the crude soluble extract. **d.** Re-exposed group challenged with ConA different from unstimulated cells ($p < 0.05$) with the particulate Ag different from control group ($p < 0.05$).
1. Challenge with the particulate Ag different over the time ($p < 0.050$;
2. Challenge with ConA different over the time ($p < 0.05$).

Table 3.3. Experiment 2. Anamnestic response. Proliferation of head kidney lymphocytes from recovered rainbow trout orally re-exposed with infective spores of *Loma salmonae*. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

In vitro treatment	Weeks postexposure (PE)				
	14[abcdef]	15[abcdef]	16[abe]	18[abcdef]	22[bcdef]
ConA	9490 ±1912	12160 ±1424	12112 ±3827	6052 ±1236	7411 ±83
Particle	1999 ±408	5033 ±1414	4185 ±1566	1702 ±191	3619 ±42
Soluble	1712 ±307	3061 ±1638	2953 ±1284	1489 ±103	3830 ±67
Unstim	210 ±102	212 ±296	410 ±95	213 ±43	236 ±9

Table 3.4. Positive control. Proliferation of head kidney lymphocytes from rainbow trout orally exposed with infective spores of *Loma salmonae*. Data are expressed as mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

	14*	15	16	18	22
ConA	4215 ±108	1858 ±128	17595 ±1280	8760 ±145	8176 ±54
Particle	482 ±116	1443 ±99	4544 ±1245	2614 ±38	2619 ±100
Soluble	260.7 ±29	981 ±123	5954 ±1096	2611 ±580	2912 ±67
Unstim	287 ±55	217.8 ±87	489 ±225	209 ±10	187 ±13

Table 3.5. Negative control. Proliferation of head kidney lymphocytes from rainbow trout. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 5 fish per observation).

	14*	15	18	24
ConA	4215 ±482	3679 ± 435	1681 ± 109	1625 ± 301
Particle	482 ±116	562 ± 24	515 ± 23	251 ±23
Soluble	260 ±29	517 ± 67	365 ± 39	271 ±67
Unstim	287 ±55	422 ±34	391 ± 67	234 ± 56

*** Only time 14 involved same observations both in infected and naive fish**

a. ConA different from exposed fish; b. ConA different from naive fish ($p<0.05$)

c. Particulate Ag different from exposed fish; d. Particulate Ag different from naive fish ($p<0.05$)

e. Crude soluble extract different from exposed fish; f. Crude soluble extract different from naive fish($p<0.05$)

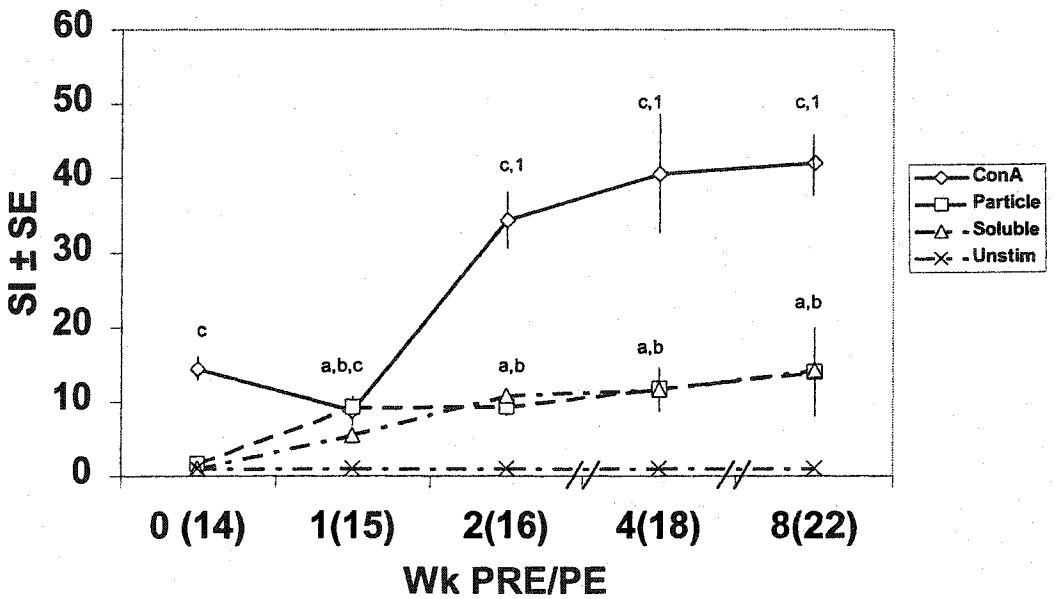

Figure 3.5. Experiment 2. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from rainbow trout **exposed to live spores via gastric intubation.** Data are presented as the mean SI ± SE of triplicate.
a. Exposed group challenged with the particulate Ag different from unstimulated cells ($p < 0.05$). **b.** Exposed group challenged with the crude soluble extract different from unstimulated cells ($p < 0.05$). **c.** Exposed group challenged with ConA different from unstimulated cells ($p < 0.05$) with the particulate Ag different from unstimulated cells ($p < 0.05$). **1.** Challenge with ConA different over the time ($p < 0.05$).

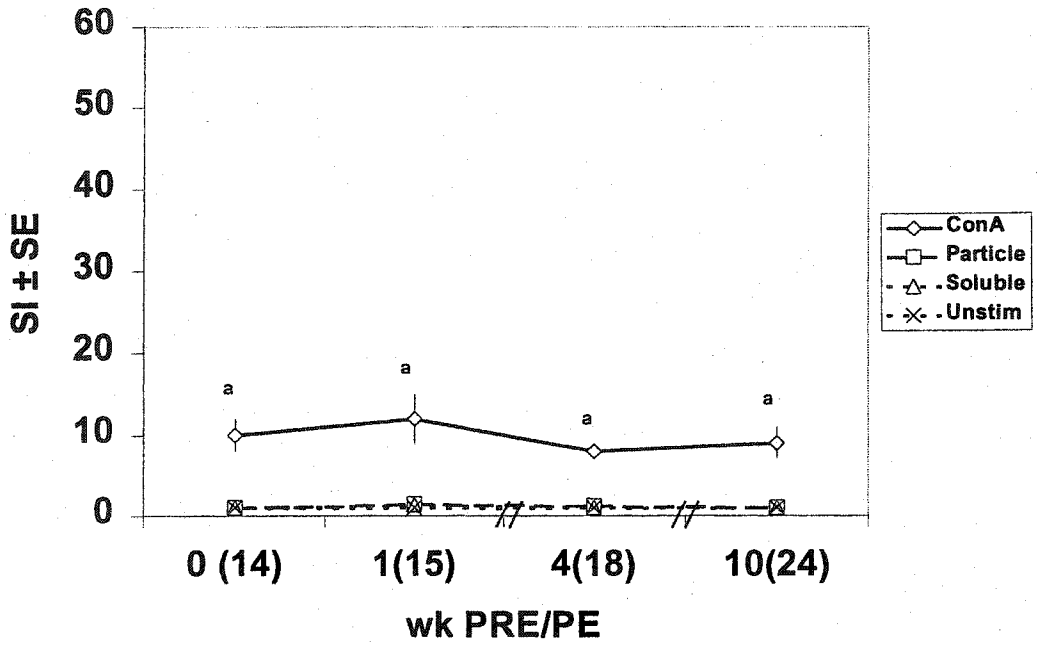

Figure 3.6. Experiment 2. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from **naive** rainbow trout (control group). Data are presented as the mean SI ± SE of triplicate cultures.
a. Naive fish challenged with ConA different from unstimulated cells ($p < 0.05$).

this same experiment and from the fish exposed orally once or twice in previous experiments. Significant differences between cultures from re-exposed and exposed groups were observed at wk 26 PE ($p<0.05$), and between re-exposed and control cultures from wk 2 to 22 PE ($p<0.05$). Significant differences over time ($p<0.05$) for the re-exposed group occurred at wk 26 PE (Figure 3.9 and Table 3.8), but not for the control group (Figure 3.10; Table 3.9), which remained similar to that observed in experiments 1 to 3.

3.4.3.2 Response of lymphocytes to *Loma salmonae* crude soluble extract

Experiment 1. The addition of the crude soluble extract to lymphocytes from exposed fish induced a good proliferative response (Figure 3.2; Table 3.1), and in general, the pattern was very similar to that observed with the cells stimulated with dead spores. Proliferation was maximal at wk 6 and 8 PE, and significantly higher at wk 6 PE when compared to cells stimulated with dead spores. Blastogenesis remained constant until wk 22 PE. Proliferative response was significantly higher in the exposed group (Fig. 3.2; Table 3.1) when compare to the control cultures (Fig. 3.3 , Table 3.2) along the experiment. A one-way ANOVA of the SI and cpm from the cells stimulated with the crude soluble extract showed no significant difference over time, except at wk 6 and 8 PE ($P< 0.05$).

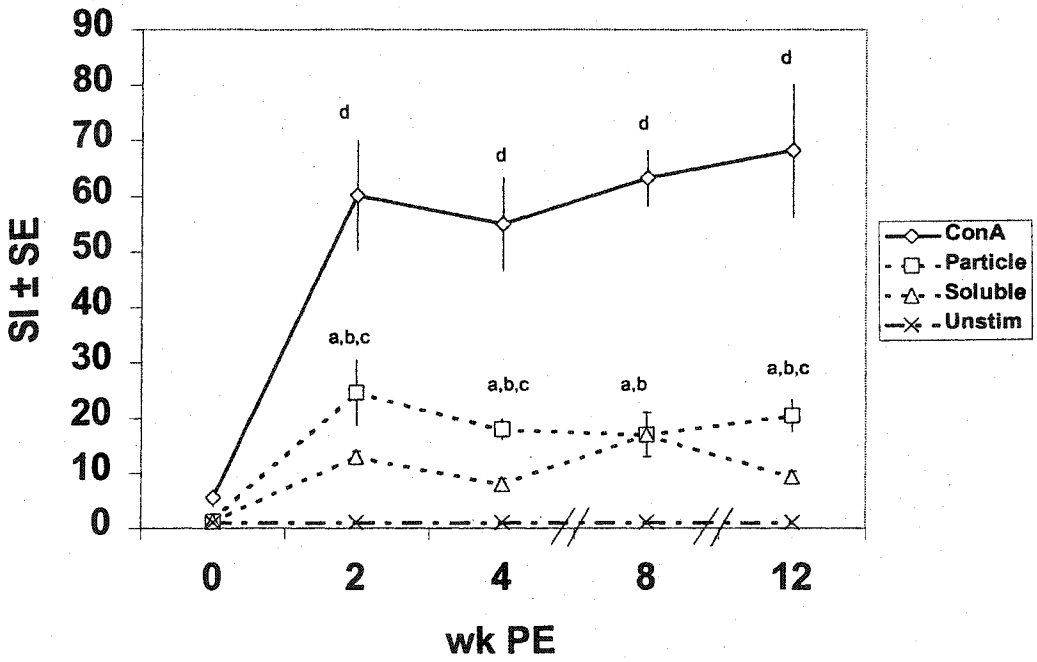

Figure 3.7. Experiment 3. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from rainbow trout **IP exposed to dead spores.** Data are presented as the mean SI ± SE of triplicate cultures.
a. Exposed group challenged with the particulate Ag are significantly different from unstimulated cells ($p < 0.05$). **b.** Exposed group challenged with the crude soluble extract different from unstimulated cells ($p < 0.05$). **c.** *In vitro* challenge with particulate Ag different from the challenge with the crude soluble extract. **d.** Exposed group challenged with ConA different from unstimulated cells ($p < 0.05$) with the particulate Ag different from unstimulated cells ($p < 0.05$).

Table 3.6. Experiment 3. Proliferation of head kidney lymphocytes from rainbow trout IP inoculated with dead spores of *Loma salmonae*. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

In vitro treatment	Weeks postexposure (PE)				
	0*	2[abc12]	4[abc12]	8[abc12]	12[abc1]
ConA	2291 ±356	32744 ±7681	19189 ±1619	11940 ±764	12221 ±486
Particle	542 ±87	14222 ±5199	6957 ± 1165	2885 ±509	3680 ±176
Soluble	576 ±82	10915 ±4585	3739 ±1191	3160 ±891	1712 ±100
Unstim	518 ±73	869 ±377	358 ±141	196 ± 20	193.8 ±28

Table 3.7. Proliferation of head kidney lymphocytes from naive rainbow trout. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 5 fish per observation).

	0*	2	4	8	12
ConA	2291 ±356	4188 ±705	1750 ±246	1377.3 ±84	1949 ±50
Particle	542 ±87	435 ±74	350 ±91	394.7 ±63	213 ±20
Soluble	576 ±82	341 ±131	471 ±97	417 ±112	397 ±67
Unstim	518 ±73	331 ±86	371 ±95	393.7 ±60	275 ±37

* Only time 0 involved same observations in exposed and naive fish
a. ConA different from naive fish ($p<0.05$)
b. Particulate Ag different from naive fish ($p<0.05$)
c. Crude soluble extract different from naive fish ($p<0.05$).
1. Significantly higher than the exposed fish in experiment 1 ($p<0.05$)
2. Significantly higher than exposed fish in experiment 2 ($p<0.05$)

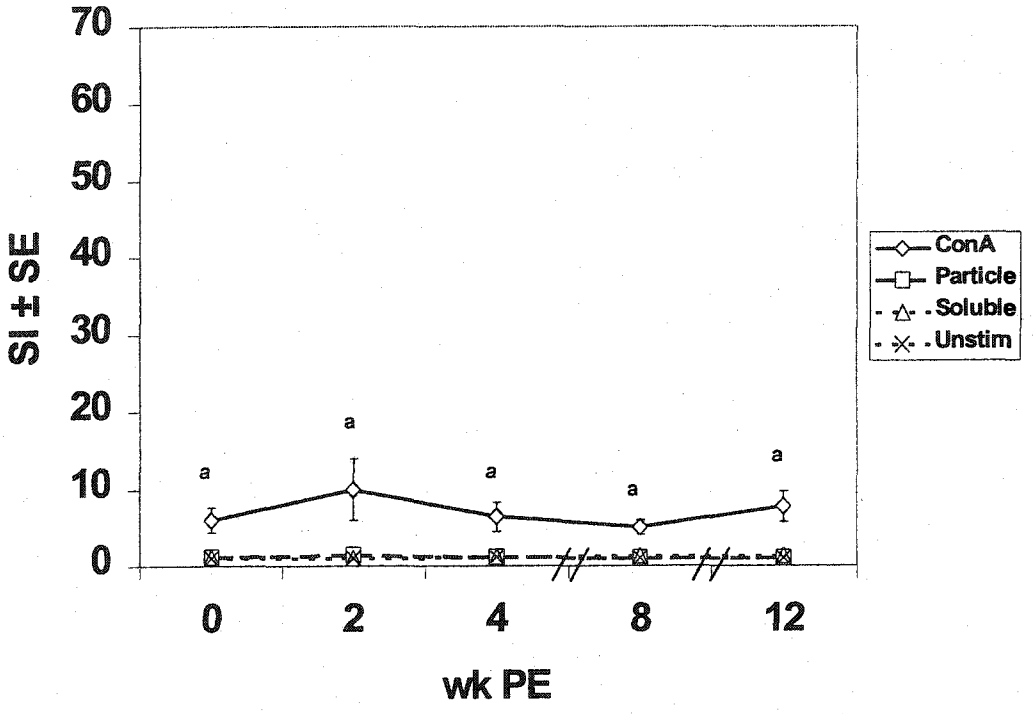

Figure 3.8. Experiment 3. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from **naive** rainbow trout. Data are presented as the mean SI ± SE of triplicate cultures.
a. Naive fish challenged with ConA are significantly different from unstimulated cells ($p < 0.05$).

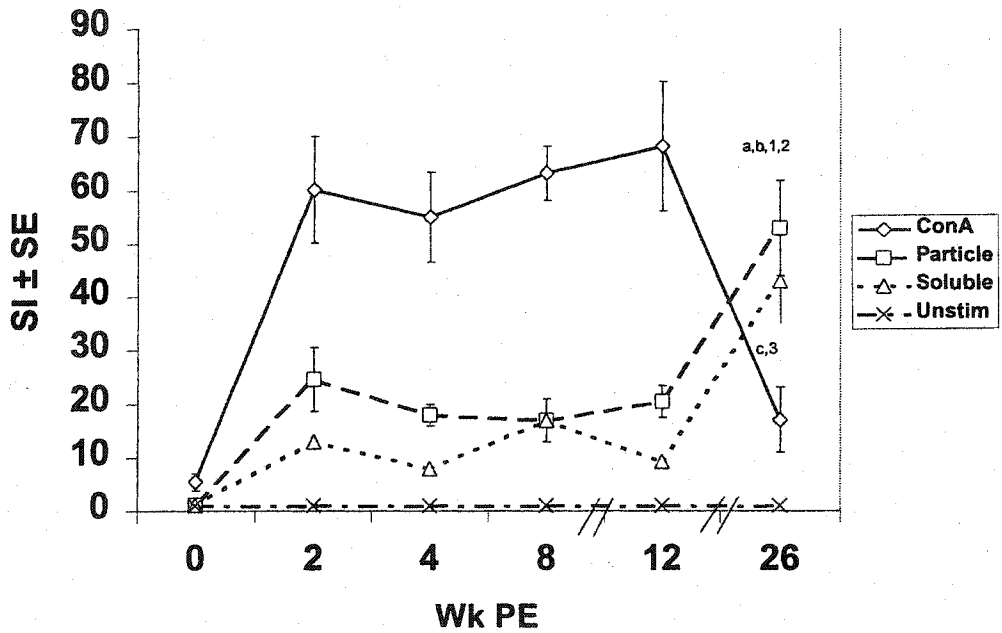

Figure 3.9. Experiment 4. *(This table is similar to Fig. 3.7. Only observation at wk 26 was added).* Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from rainbow trout **IP exposed to dead spores and re-exposed at wk 22 PE to live spores via gastric intubation**. Data are presented as the mean SI ± SE of triplicate cultures.

a. Exposed group challenged with the particulate Ag is significantly different from unstimulated cells ($p < 0.05$). **b.** Exposed group challenged with the crude soluble extract different from unstimulated cells ($p < 0.05$). **c.** Exposed group challenged with ConA different from unstimulated cells ($p < 0.05$) with the particulate Ag different from control group ($p < 0.05$). **1.** Challenge with the particulate Ag different over the time ($p < 0.05$; **2.** Challenge with the crude soluble extract different over the time ($p < 0.05$); **3.** Challenge with ConA different over the time ($p < 0.05$).

Table 3.8. Experiment 4. Proliferation of head kidney lymphocytes from rainbow trout IP exposed to *Loma salmonae* dead spores and then orally exposed at wk 22 PE with infective spores. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

In vitro treatment	wk PE	
	0*	26[abc]
ConA	2738 ±464	2673 ±107
Particle	162.6 ±15	8938 ±281
Soluble	912 ±120	7439 ±249
Unstim	527.5 ±89.4	177 ±16

Table 3.9. Experiment 4. Proliferation of head kidney lymphocytes from naive rainbow trout. Data are expressed as the mean counts per minute (cpm ± SE) of tritiated thymidine (n = 8 fish per observation).

In vitro treatment	wk PE	
	0*	26[abc]
ConA	2738 ±464	1496 ±177
Particle	162.6 ±15	316.7 ±29.2
Soluble	912 ±120	380 ±73.4
Unstim	527.5 ±89.4	305 ±23

* Only time 0 involved same observations in exposed and naive fish
a. ConA different from naive fish ($p<0.05$)
b. Particulate Ag different from naive fish ($p<0.05$)
c. Crude soluble extract different from naive fish ($p<0.05$)

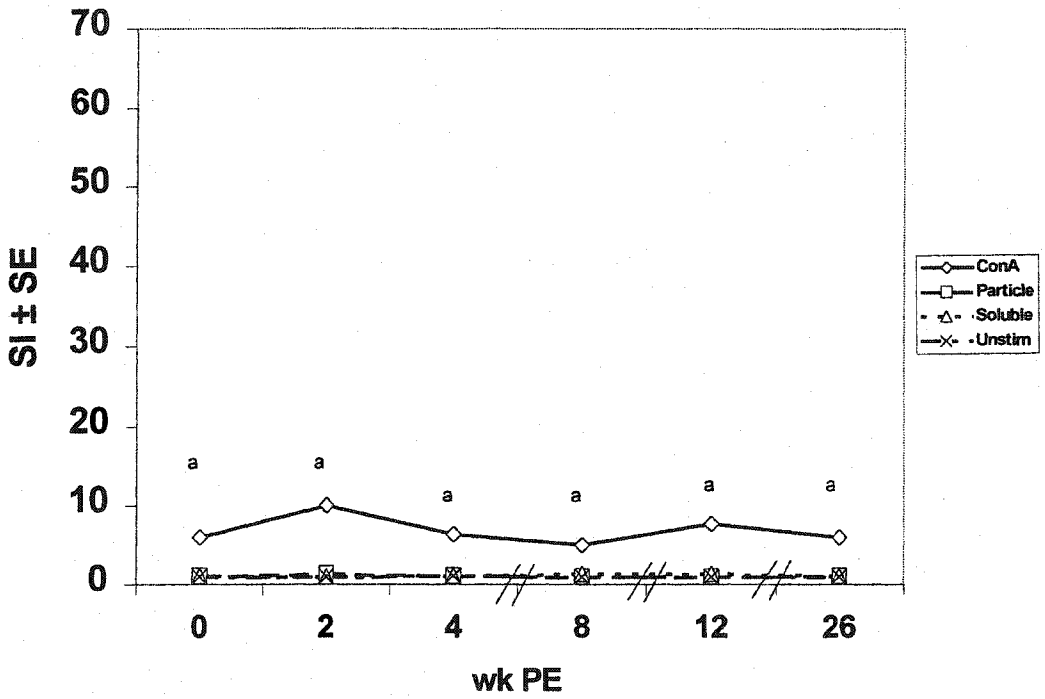

Figure 3.10. Experiment 4. Proliferative response to dead spores and crude soluble extract from *Loma salmonae*, and ConA by head kidney lymphocytes from naive rainbow trout. Data are presented as the mean SI ± SE of triplicate cultures. **a.** Naive fish challenged with ConA is significantly different from unstimulated cells ($p < 0.05$).

Experiment 2. The proliferative response in the re-exposed fish (Figure 3.4; Table 3.3) was not as different as that observed from group exposed once (Figure 3.5; Table 3.4) and that from the same group in the experiment 1. There was no significant difference between the SI from re-exposed and exposed groups ($p < 0.05$), whereas significant differences were observed when compared with the control culture ($p < 0.05$). No statistical differences over time ($p < 0.05$) were observed in the cultures from re-exposed and exposed groups. Proliferative response in the control group was similar to that observed in experiment 1 (Figure 3.6; Table 3.5).

Experiment 3. The proliferative response to crude soluble extract from wk 2 to 12 PE remained slightly lower than cells stimulated with dead spores (Figure 3.7; Table 3.6), and comparable to the SI and cpm observed in the exposed group from experiment 1, and the re-exposed and exposed groups from experiment 2. The proliferative response was significantly higher in the exposed group when compare to the control group from wk 2 to 12 PE (Figure 3.8; Table 3.7) ($p < 0.05$), whereas no significant differences over time were observed in the exposed group ($p < 0.05$). Proliferative response in the control group remained similar to that from experiments 1 and 2.

Experiment 4. The proliferative response of IP injected fish was highest when re-challenged with live spores at wk 22 PE (Figure 3.9; Table 3.8). In general, this response was similar to that observed with lymphocytes stimulated with the particulate Ag.

3.4.3.3 Response of cells to Concanavalin A

Experiment 1. Although there was variability among fish per wk, head kidney lymphocytes from exposed animals showed a proliferative response to ConA (Figure 3.2; Table 3.2). An increased proliferative response was observed at wk 2 PE when compared to pre-exposure values. This proliferation decreased by wk 4 PE and reached a second maximal stimulation by wk 12 PE. The proliferative response of the exposed group was significantly higher than the unstimulated lymphocytes ($p < 0.05$)(Figure 3.3; Table 3.2) from wk 2 to 20 PE. The ANOVA test of the SI of re-exposed fish showed significant differences over time (wk 2 and 12 PE) ($p<0.05$) (Figure 3.2; Table 3.1). Concanavalin A was also mitogenic for the head kidney lymphocytes of the naive fish; however the SI and cpm were considerably lower than those observed in the exposed group from wk 2 to wk 22 PE.

Experiment 2. Mitogenic response to ConA from recovered fish lymphocytes showed strong proliferation (Figure 3.4; Table 3.3) when compared to the proliferation in lymphocytes from the exposed once group (Figure 3.5; Table 3.4). Lymphocytes from the group exposed once had a reduction of the proliferative response at wk 15 PE (Figure 3.5; Table 3.4), but this was followed by an increase in the mitogenesis by wk 16 PE and remained without significant changes until wk 22 PE. Significant differences between re-exposed and exposed groups ($p < 0.05$) occurred at wk 14 and 15 PE, and between re-exposed and control groups differences were observed from wk 2 to 22 PE ($p<0.05$). Significant differences ($p<0.05$) over time for the re-exposed and exposed groups were observed at wk 14 and 15 PE, but not for controls

($p < 0.05$). The SI and cpm for controls (Figure 3.6; Table 3.5) were similar to that observed in experiment 1.

Experiment 3. Head kidney lymphocytes (from the exposed group) stimulated with ConA had higher SI to ConA when compared to those observed in experiments 1 and 2, and this persisted until wk 12 PE (Figure 3.7; Table 3.6). Significant differences ($p < 0.05$) between the re-exposed and control cultures were observed during the experiment. Excepting by a minimal increase in the proliferative response at wk 8 PE, no significant differences over time ($p > 0.05$) were observed in the cultures from exposed group (Figure 3.7; Table 3.6). Concanavalin A was also mitogenic for the control cultures (Figure 3.8; Table 3.7), but similar to that observed in experiments 1 and 2.

Experiment 4. Proliferation of lymphocytes from the re-exposed group to ConA showed a significant decrease at wk 26 PE (Figure 3.9; Table 3.8), when compared to the response in similar groups in experiments 1, 2 and 3 ($p < 0.05$); however this proliferative response was significantly higher than the control cultures.

3.4.4 The effect of *L. salmonae* on the leucocyte population

Leucograms from fish exposed either to live or dead spores of *L. salmonae* showed changes in the concentration of granulocytes and mononuclear cells (MN) during the progress of the infection.

Experiment 1. Mononuclear cell counts were not different from those observed in the control group (Table 3.10). However, granulocyte counts from exposed fish were significantly different from the control fish from wk 2 to 8 PE (Table 3.11).

Experiment 2. Mononuclear cell counts from re-exposed fish were significantly lower ($p<0.05$) from those observed in the exposed and control fish especially at wk 16 PE (Tables 3.12 to 3.14). Granulocyte cell counts from recovered fish were significant different ($p<0.05$) from those observed in the control group particularly at wk 16 and 20 PE. No differences were encountered when compared with the exposed fish. Mononuclear cell from exposed fish did not show differences when compared to that from naive fish. Granulocyte cell counts from exposed group were significantly different ($p<0.05$) when compare to that observed in the control group at wk 18, 20 and 22 PE.

Experiment 3. Mononuclear cell counts from fish IP exposed to dead spores were not significantly different along the experiment ($p<0.05$) from those observed in the exposed and control fish particularly at wk 2 PE (Table s 3.15 to 3.17). Granulocyte cell counts from fish IP exposed were significant higher ($p<0.05$) at wk 2 to 8 PE when compared to those observed in the exposed and naive fish.

Experiment 4. Mononuclear and granulocyte cell counts from recovered fish were significantly lower ($p<0.05$) from those observed in the exposed and control fish particularly at wk 26 PE (Tables 3.15 to 3.17).

Table 3.10. Experiment 1. Leucogram of fish orally exposed with live spores of *Loma salmonae*. Data are expressed as the mean percentage (% ± SE) of 100 counted white cells (n = 8 fish per observation).

	Weeks postexposure (PE)							
	0	2[a]	4[a]	6[a]	8[a]	12	16	22
Mononuclear cells	98.75 ±0.2	98.87 ±0.3	98.38 ±0.5	98.5 ±0.6	98.87 ±0.4	98.13 ±0.5	98.38 ±0.8	96.5 ±0.7
Granulocytes	0.4 ±0.2	1.12 ±0.3	1.6 ±0.5	1.5 ±0.6	1.12 ±0.4	1.8 ±0.5	0.87 ±0.2	0.87 ±0.2

Table 3.11. Experiment 1. Leucogram of naive fish. Data are expressed as the mean percentage (% ± SE) of 100 counted white cells (n = 4 fish per observation).

	0	2	4	6	8	12	16	22
Mononuclear cells	98.7 ±0.2	100 ±0	99.7 ±0.2	96.5 ±2	99.5 ±0.2	98.7 ±0.6	99 ±0.7	100 ±0
Granulocytes	0.7 ±0.2	0 ±0	0.2 ±0.2	0.5 ±2	0.5 ±0.2	1.2 ±0.6	1 ±0.7	1 ±0.2

a. Granulocyte concentration is different from naive fish ($p<0.05$).

Table 3.12. Experiment 2. Leucogram from recovered fish and re-exposed to fresh spores of *Loma salmonae* by gastric intubation. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 8 fish per observation)

	Weeks postexposure (PE)				
	15	**16[abcd]**	**18[c]**	**20[d]**	**22**
Mononuclear cells	99.6 ±0.2	82.5 ±1.1	99.8 ±0.2	97.8 ±0.5	98.6 ±0.3
Granulocytes	0.3 ±0.2	2.1 ±0.4	0.2 ±0.2	2.1 ±0.5	1.3 ±0.3

Table 3.13. Experiment 2. Leucogram from fish exposed to fresh spores of *Loma salmonae* by gastric intubation. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 8 fish per observation).

	15	**16**	**18**	**20**	**22**
Mononuclear cells	99.6 ±0.2	99.6 ±0.2	97.8 ±0.5	97 ±1.5	98.3 ±0.9
Granulocytes	0.3 ±0.2	0.5 ±0.3	2.2 ±0.5	3 ±1.5	1.6 ±0.9

Table 3.14. Experiment 2. Leucogram from naive fish. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 4 fish per observation).

	15	**16**	**18**	**20**	**22**
		Weeks postexposure (PE)			
Mononuclear cells	99.6 ±0.2	99.5 ±0.3	99.4 ±0.4	99.5 ±0.3	99.5 ±0.3
Granulocytes	0.3 ±0.2	0.5 ±0.3	0.6 ±0.4	0.5 ±0.3	0.5 ±0.3

a. Mononuclear different from infected fish ($p<0.05$)
b. Mononuclear different from naive fish ($p<0.05$)
c. Granulocytes different from infected fish ($p<0.05$)
d. Granulocytes different from naive fish ($p<0.05$)

Table 3.15. Experiment 3. Leucogram from fish IP exposed with dead spores of *Loma salmonae*. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 8 fish per observation)

	Weeks postexposure (PE)				
	0	**2[abcd]**	**4[c]**	**8[cd]**	**26[abcd]**
Mononuclear cells	99.6 ±0.2	99.3 ±0.4	99.2 ±0.3	99.5 ±0.2	80.1 ±0.1
Granulocytes	0.4 ±0.2	0.6 ±0.4	0.7 ±0.3	0.3 ±0.2	1 ±0.3

Table 3.16. Experiment 3. Leucogram from exposed fish with live spores of *Loma salmonae* by IP administration. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 8 fish per observation)

	0	**2**	**4**	**8**	**26**
Mononuclear cells	99.6 0.2	94.3 ±1.8	99.5 ±0.2	92.1 ±1.8	90 ±1.2
Granulocytes	0.3 ±0.2	5.6 ±1.8	0.5 ±0.2	7.8 ±1.8	6.7 ±1.3

Table 3.17. Experiment 3. Negative control. Leucogram from naive fish. Data are expressed as the mean percentage (%±SE) of 100 white cells (n= 4 fish per observation)

	0	**2**	**4**	**8**	**26**
Mononuclear cells	99.6 ±0.2	97.8 ±1.7	98.1 ±0.4	98.1 ±0.4	99 ±1.2
Granulocytes	0.3 ±0	2.2 ±0.2	2 ±0.3	2 ±0.2	1.8 ±0.6

a. Monocytes different from infected fish ($p<0.05$)
b. Monocytes different from naive fish ($p<0.05$)
c. Granulocytes different from infected fish ($p<0.05$)
d. Granulocyte different from naive fish ($p<0.05$)

Table 3.18. Presence of branchial xenomas in experiments 1 to 4. Results are expressed as the number of affected fish/total fish and mean (±SE) number of xenomas per gill.

Wk PE		0	1	2	4	6	8	12	16	22	26
Experiment 1	Exposed	0	ND	0	8/8 17±3	8/8 25±2	4/8 12±2.1	0	0	0	ND
	Naïve	0	0	0	0	0	0	0	0	0	ND
Experiment 2	Re-exposed	0	0	0	0	ND	0	ND	ND	ND	ND
	Exposed	0	0	0	8/8 31±4.3	ND	40 20±4	ND	ND	ND	ND
	Naïve	0	0	0	0	ND	0	0	0	0	ND
Experiment 3	Exposed	0	ND	0	0	ND	0	0	ND	ND	ND
	Naïve	0	ND	0	0	ND	0	0	ND	ND	ND
Experiment 4	Re-exposed	0	ND	0	0	ND	0	0	ND	ND	2/8 2.5± 0.2
	Naïve	0	ND	0	0	ND	0	0	ND	ND	0

ND: Not determined

3.4.5 Presence of *Loma salmonae* in rainbow trout at different times

Fish gills were sampled prior to infection to *L. salmonae* and were negative for branchial xenomas. Xenomas were observed in exposed fish gills from experiment 1 at wk 4 PE and disappeared at wk 8 PE (Table 3.18). Fish initially exposed orally to live spores never developed xenomas after re-exposure with the same organism. A few fish initially exposed to dead spores (experiment 4) presented a few branchial xenomas 4 wk after being exposed to live spores of *L. salmonae*.

3.5 DISCUSSION

This is the first report of the proliferative response of rainbow trout head kidney lymphocytes following experimental exposure to *L. salmonae*. This study showed that head kidney lymphocytes from the exposed rainbow trout proliferated in response to both particulate and crude soluble extract from *L. salmonae* and had enhanced response to the T cell mitogen ConA.

The *in vitro* lymphocyte proliferation assay has been used extensively in mammalian models to study the cellular immune response to microsporidia (Liu et al. 1989, Didier and Bertucci 1996). However, the study of the immune response to microsporidia in fish is limited (Leiro et al. 1993), and focuses either on the study of the humoral response (Estevez et al. 1995, Hung et al. 1996, Kim et al. 1996, Leiro et al. 1996a, Pomport-Castillon et al. 1999) or on studies of the innate immune response to this parasite (Leiro et al. 1996b, Kim et al. 1998, Leiro et al. 2000, Leiro et al. 2001). Currently, the study of cell-mediated immune response to various fish pathogens, and in particularly to microsporidia, remains in

its infancy. Information regarding mechanisms of resistance to infections caused by *L. salmonae* is not available. However, some arguments that suggest the participation of cell-mediated immunity (CMI) in the resistance to this parasite on fish are: 1) *L. salmonae* is an intracellular parasite (Shaw and Kent 1999); 2) the humoral response does not appear to be effective to protect fish (Sanchez et al. 2001a); 3) the cells observed around the xenoma after its rupture are mainly mononuclear cells (Speare et al. 1989, Kent et al. 1995); 4) *Loma salmonae* causes granuloma formation (Speare et al. 1998a), 5) it is a chronic disease; and 6) infection induces a strong protection in recovered fish (Speare et al. 1998b, Kent et al. 1999), which is not attributable to a humoral response.

In this study, gastric intubation and IP inoculation were used to administer live and dead spores respectively. As mentioned in Chapter 2, Shaw et al. (2000), demonstrated that *L. salmonae* spores were completely killed under freezing-thawing conditions because naive fish exposed to these spores never develop branchial xenomas. However, the possibility that the viability of these spores was reduced cannot be disregarded and in this thesis the terms dead or inactive spores were use indistinctively.

The *in vitro* lymphocyte proliferation assay was used to evaluate the response to 2 different antigenic preparations of *L. salmonae*, to determine the specificity of those components on the fish cellular response and to associate this *in vitro* assay with resistance. Lymphocytes from the head kidney were used because this organ contains a large lymphocyte population and much of the fish immune response is induced and elaborated in this organ (Etlinger et al. 1976, Press 1998, Press and Evensen 1999). Lymphocytes from the spleen were not used in this experiment, but future research could simultaneously compare

the fish immune response of cells from the head kidney, spleen and blood. The isolation of leucocytes with a Percoll gradient produced a good recovery of lymphocytes with viability greater than 90%. Other cells observed were monocytes and a few neutrophils. Red blood cell numbers were insignificant. The high proportion of lymphocytes observed in the present research contrast with some studies where the lymphocyte proliferation assays used either debris-free cell suspensions derived by teasing apart whole lymphoid organs (Etlinger et al. 1976, Estepa and Coll 1992), whole blood (Wang et al. 1997), or pools of lymphocytes of 2 or 5 fish (Warr and Simon 1983, Yui and Kaattari 1987). In such systems, an elevated proportion of other cell types could be expected, and although good proliferative responses have been obtained from those studies, it is uncertain if the presence of additional cells could have influenced those experiments. The presence of antigen presenting cells (APC), like monocytes, in fish blastogenesis assays has been recommended for an appropriate proliferative response (Sizemore et al. 1984, Clem et al. 1985). Lymphocytes, particularly T cells, *per se* cannot recognize specific Ag, and APC are required in a lymphocyte culture system to present the Ag. Therefore, monocytes observed in our cell suspension could be considered as the APC required for proper stimulation of lymphocytes.

First experiment: The *in vivo* exposure of fish to live spores from *L. salmonae* sensitized rainbow trout head kidney lymphocytes to proliferate when stimulated *in vitro* with dead spores or crude soluble extract. The vehicle (distilled water) used for administrating the inoculum did not have any effect on the lymphocyte proliferation when administered alone to fish. Lymphocytes from exposed fish also had an enhanced proliferative response to ConA compared with that observed in naive fish. This is in contrast with other studies on

microsporidiosis where a suppression of the proliferative response to ConA, PHA, and LPS was observed in fish (Wongtavatchai et al. 1995b) and in mammals (Niederkorn et al. 1981, Didier and Bessinger 1999). Suppression of the proliferative response was not observed when lymphocytes from exposed rainbow trout were stimulated both with the dead spores and with the crude soluble extract from *L. salmonae* at wk 2 PE.

Lymphocytes stimulated with the particulate Ag showed increased proliferation at wk 2 PE reaching maximum proliferation at wk 8 PE, and maintained this level without significant changes until wk 22 PE. Although lymphocytes from control fish occasionally showed limited proliferation when stimulated with the same Ag, this response was minimal. Similar results were obtained in mice exposed to *E. cuniculi*, where the lymphoproliferative response was higher than that observed in naive mice (Didier and Shadduck 1988, Liu et al. 1989, Didier 2000).

Although there is no information on the lymphocyte proliferation at wk 1 PE in the first experiment, the presence of reactive cells at wk 2 PE coincided with the time when *L. salmonae* was detected in rainbow trout gills (Sanchez et al. 2000, Sanchez et al. 2001a, Sanchez et al. 2001b). The observation of lymphoproliferation at this time suggests that this parasite is recognized early in infection, before the parasite moved to and infected the gills.

It has been indicated that the transit of *L. salmonae* through the gut occurs relatively quickly (Shaw et al. 1998, Sanchez et al. 2001b); however it is uncertain when and how fish first recognize the parasite for first time. In the present research it is unlikely that the apparent early response observed to infection represented a prior exposure to *L. salmonae* when fish were raised in the hatchery. The hatchery facilities were free of this parasite and

infections by *Loma* spp. have not been reported. Furthermore, cells from the naive fish (from the same population) did not proliferate as the exposed fish lymphocytes did when they were *in vitro* stimulated with the same particulate or crude soluble extract. Similarly, fish could have been exposed to Ags analogous to some from *L. salmonae* developing a cross-reaction, but this event was also dismissed for the same reason explained above. Thus, the early response appears to be directly against the infective inoculum.

In fish, the second segment of the hindgut is able to take up and process Ag, like bacteria, that have been ingested (Quentel and Vigneulle 1997). These Ag can be taken up by intraepithelial leucocytes and be transported to mucosal lymphoid aggregates, and subsequently initiate an immune response (Kaattari and Piganelli 1996). In this study, there were 2 periods in the life cycle of *L. salmonae* where the viable organism could be recognised, taken up and processed by leucocytes. One occurred shortly after oral administration (Time 0). Mature spores of *L. salmonae* have been detected in close association with the epithelium of salmonid gut (Shaw et al. 1998, Sanchez et al. 2001b), and here the parasite could have established a first contact with the resident leucocytes from the gastrointestinal tract, and initiated parasite recognition.

The other period occurred during the xenoma rupture at wk 4 and wk 8 PE (Speare et al. 1998c). Released mature and immature parasites were subject to an inflammatory reaction characterized by the presence of mononuclear cells. As suggested by *in vitro* proliferation at wk 8 PE, the rupture of the xenomas could attract phagocytic cells and specific lymphocytes (already primed) to this site and with an immune response to them, as was evidenced by the significant changes that were observed in the proportion of

granulocytes in the leucograms of those fish at wk 8 PE when compared with those from the control group.

The proliferative response of rainbow trout (exposed to live spores) lymphocytes *in vitro* stimulated with the crude soluble extract had a moderate although not significant, increase around wk 2 PE when compared to the cells stimulated with dead spores. This response reached its maximum proliferation at wk 6, persisting until 8 PE, and moderately declined until wk 22 PE.

The maximal stimulation of lymphocytes stimulated with the crude soluble extract occurred 2 wk before the lymphocytes stimulated with the particulate Ag. This suggests an initial recognition to some soluble component that could be "leaking"from the cyst wall at wk 6 PE, previous to the rupture of the xenoma. To validate this, there is ultrastructural evidence (Chapter 4) that suggests the presence of a discontinuity in the xenoma plasmalemma, and many inflammatory cells were evident in these areas. Through this discontinuity, some *L. salmonae*-Ag could have escaped into the interstitial tissue and been recognized by leucocytes. The presence of soluble Ag from parasitic cysts has been reported in *Toxoplasma gondii* (Reiter-Owona et al. 1996).

A subsequent antigenic recognition accompanied with inflammatory reaction could occur when spores were released into the gill interstitial tissue. Based on the observation of the proliferative response around wk 8 PE, the discharge of Ag from the xenoma possibly persisted until the establishment of an inflammatory reaction in gills. Subsequently, the moderate but not significant decay of the proliferative response that was observed until wk

22 PE could be interpreted as the catabolism and subsequent clearance of *L. salmonae*-Ag from the fish, with persistence of memory cells against the parasite.

Rainbow trout lymphocytes from infected fish were strongly stimulated with ConA at wk 2 PE. However, a decrease in the lymphocyte stimulation was observed at wk 4 PE in some of the exposed fish. Probably this decrease was due to the generation of an early inflammatory reaction to the xenomas in the gills, as was observed under stereomicroscope. This caused mobilization of specific lymphocytes from the head kidney, as occurred in certain fish trematode infections (Richards et al. 1994). However, certain microsporidia can cause the generation of prostaglandins by macrophages during the inflammatory process resulting in the suppression of the lymphoproliferation to mitogens (Liu et al. 1989, Didier and Bessinger 1999). Also, the possibility that *L. salmonae* could be generating some immunosuppresive substance cannot be disregarded. In our investigation, possibly one or all of these events were implicated in the temporary reduction in the proliferation of lymphocytes at wk 4 PE when stimulated with ConA. In general, lymphocytes from fish exposed to *L. salmonae* and *in vitro* stimulated with ConA had significantly greater proliferative response when compared to cultures from uninfected controls.

Although the *in vitro* lymphocyte proliferation correlates with the existence of CMI *in vivo* (Maluish and Strong 1986), this technique has its limitations. This system only shows the capacity of these cells to respond to an activation signal (Reitan and Thuvander 1991), but can neither define what proportion of those cells will have an effector function nor what phenotype of the lymphocyte (T or B cell) is involved.

Means for detecting different fish lymphocyte subpopulations ($CD4^+$, $CD8^+$, etc) are not presently available. Nevertheless, by comparing both the infected and uninfected fish lymphocytes stimulated *in vitro* with the mitogen, the higher SI observed in lymphocytes from exposed fish could suggest presence of responding T cells that were generated during the infection by *L. salmonae*. Furthermore, the *in vitro* lymphoproliferative response of cells from naive fish to both the particulate and the crude soluble extract remained very similar to the results obtained from the unstimulated cells. This could imply that the mitogenic effect that has been observed in cultures stimulated with supernatants from microsporidia-infected cells was not occurring in the present experiment (Wongtavatchai et al. 1995a, Wongtavatchai et al. 1995b). Thus, lymphocytes from fish exposed to live spores most likely responded in an antigen-specific manner when they were cultured in the presence of the particulate and crude soluble extract *L. salmonae* Ag.

Second experiment. Studies of mammalian and fish lymphocyte cultures have demonstrated that a secondary exposure usually produces a greater response than the initial exposure (Estepa and Coll 1992, Marsden et al. 1996, Amaral et al. 2001). However, in the present study lymphocytes from fish re-exposed at wk 14 PE did not show an increase in response when compared to the group exposed once. There was a significant increase in the lymphocyte proliferation in cells stimulated with dead spores, but not with crude soluble extract, at wk 15 PE, suggesting that an anamnestic response was present and more selective to the complete spore than to the crude soluble extract one wk after re-exposure. However, during the following weeks there was no significant difference in the cultures stimulated with the particulate and crude soluble extract, and the proliferative response was quite similar to

that observed in the proliferative response in the exposed group on this experiment and from experiment one.

The reason why there was not a greater proliferative response (when compared to exposed fish from experiment 1) upon re-exposure of fish to *L. salmonae* is unknown. A similar lack of increase in proliferation after re-exposure has been described before in mammals (Lambot et al. 2001). In that study, insufficient production of IL-2, which induces the proliferation of T cells (Manning and Nakanishi 1996), or the rapid consumption of this interleukin, was thought to contribute to the low lymphoproliferation. If these events occurred in the present research, then this could have been reflected in the similar proliferative response observed in exposed and re-exposed fish from experiments 1 and 2, respectively. Perhaps recovered rainbow trout respond to *L. salmonae* in this manner after re-exposure. In addition, the expression of the receptor for IL-2 in carps is transient (Manning and Nakanishi 1996). Currently, there is very little information about IL-2 in fish (Press 1998, Secombes et al. 2002) and further information regarding this cytokine in the generation of lymphocyte proliferation during *L. salmonae* infection is required. If similar events occurred in the present experiments, then this could explain the similar proliferative response observed in exposed and re-exposed fish from experiments 1 and 2, respectively. Perhaps recovered rainbow trout respond to L. salmonae in this manner after re-exposure.

In mammals, particularly foxes, it has been suggested that in order to be considered significant, proliferative responses measured as SI, have to be equal or higher than 2 (Lambot et al. 2001). In the present research, the SI observed in the lymphocytes from re-exposed and exposed groups (experiment 2) stimulated with either mitogen or Ag was higher than this

value. Thus, although a proliferative response higher than that of the cells from experiment 1 was not observed, the SI value was consistent and maintained within certain limits. It seems that at least in rainbow trout exposed to *L. salmonae*, head kidney lymphocytes stimulated with dead spores and crude soluble extract, had a tendency to return to a consistent SI value, which ranged from 10 to 15 and persisted until wk 22 PE. However, this value was always higher and significantly different than that observed in the control group, suggesting the presence of specificity to the Ag used for the *in vitro* challenge. Further research is necessary to elucidate if this same pattern could occur in the other two common hosts of *L. salmonae*, chinook and coho salmon..

Lymphocyte proliferation to ConA in re-exposed fish peaked at wk 1 PE which contrasts with the lymphoproliferation observed in exposed group cultures. In addition, the peak observed in the re-exposed fish coincided with maximum stimulation observed for cultures stimulated with the particulate Ag, which suggests the establishment of an anamnestic response in this group.

The group exposed once to live spores had a decrease in the SI 1 wk after the inoculation when stimulated *in vitro* with the mitogen. A similar decrease in the stimulation to ConA was observed in lymphocytes from mice exposed 1 wk PE to *E. cuniculi* (Didier and Shadduck 1988). It was suggested that this reduction was due to an increase in the number of lymphoblasts, which were not able to respond to an additional stimulus from the mitogen, causing their exhaustion. The exposure to *L. salmonae* live spores could have stimulated the lymphocyte cell division in the head kidney, which is known to trapp and present Ag. It is possible that one week after exposure, a considerable proportion of

lymphoblasts could have been present in this organ and when they were *in vitro* stimulated with the mitogen, they did not proliferate (Rowley et al. 1988, Vallejo et al. 1992). However, the production by the parasite of immunosuppressive substances cannot be disregarded.

Complete exhaustion of lymphocytes probably did not occur in our study because cells stimulated with the particulate and crude soluble extract did not show diminution in the proliferative response. As was indicated before, probably a large number of lymphocytes were mobilized to other organs (cell traffic), reducing the lymphocyte population in this organ. This coincided with a significant reduction in the proportion of mononuclear cells from blood.

Third experiment. The proliferative response of lymphocytes of fish inoculated with inactivated spores via IP had a maximal proliferation when stimulated *in vitro* with either Ag or mitogen, when compared with infected fish from experiments 1 and 2. A high SI was observed at wk 2 PE and remained consistent until wk 12 PE. It seemed that the IP inoculation with dead spores rendered a greater *in vitro* proliferative response to the particulate Ag than did oral exposure with live spores (experiments 1 and 2). In addition, the stimulating effect of the particulate Ag in cultures was also reflected in the proportion of the circulating mononuclear cells from fish IP inoculated with dead spores. This elevated proportion of mononuclear cells was maintained until wk 8 PE, and was significantly different when compared with the proportion of mononuclear cells from exposed and control groups. This suggests that the use of an inactive form of *L. salmonae* could be considered in the future for inducing resistance to this parasite.

The results reported here are preliminary and other factors need consideration including the age or size of the immunized fish. Further knowledge about dosage, routes, frequency of inoculations and relationship with resistance are also necessary to determine the real protective effect of the inactive spores.

The proliferative response to the crude soluble extract was quite similar to that observed in experiments 1 and 2. It seemed that the *in vivo* IP inoculation with dead spores only generated a higher but non significant *in vitro* lymphoproliferation at wk 8 to some related compound also present in the crude soluble extract. The nature of this compound is currently unidentified but important advances in mammalian systems have identified some microsporidial protein fractions involved in the regulation of the lymphocyte proliferation (Didier and Bertucci 1996). However, in the case of *L. salmonae*, a particular protein responsible for lymphoproliferation has not yet been recognized. In addition to proteinaceous compounds, complex carbohydrates, like chitin, could be participating in the inflammatory reaction to this parasite (Kent et al. 1995, Speare et al. 1998b, Speare et al. 1998c). It is unknown if this polysaccharide could be involved in resistance as well, but in microsporidia this substance represents a high percentage of the spore wall (Méténier and Vivarès 2001) and currently its role as a fish immune modulator is gaining importance (Sakai et al. 1992, Sakai 1999, Esteban et al. 2000, Seferian and Martinez 2000, Esteban et al. 2001). Current work is underway to determine the probable participation of this substance in the generation of resistance in fish.

The lymphoproliferation observed in response to ConA in lymphocytes from rainbow trout exposed to dead spores, was higher and more sustained when compared to the

lymphocyte proliferation of cells from treated fish in experiments 1 and 2. In contrast, although control cultures were also stimulated with this mitogen, they continued to have lower lymphocyte proliferation than exposed fish.

Fourth experiment. When a group of fish derived from experiment 3 was orally re-exposed to live spores at wk 22 PE, they developed the highest response when stimulated *in vitro* at wk 26 PE with the particulate and crude soluble extract, but not with ConA. At wk 26 PE, there was no significant difference between the SI and cpm of the cells stimulated with either Ag and both measures were significantly higher when compared to those from experiments 1 to 3.

The elevated proliferation of lymphocytes stimulated *in vitro* with the particulate and the crude soluble extract at wk 26 PE may suggest the presence of an anamnestic response to the parasite. Some of the re-exposed fish developed a few branchial xenomas, but not as many as those seen in the group exposed once, and an inflammatory response to these xenomas was absent.

The significant proliferative response, the low concentration of xenomas, and absence of inflammatory reaction observed in the re-exposed group suggests the establishment of partial protection. The significant low lymphocyte proliferation to ConA could suggest an active recognition of the parasite and an intense mobilization of lymphocytes from the head kidney in response to the parasite. Previous studies with other inoculated fish parasites have obtained comparable results as measured by reduction in the number of parasites (Woo and Li 1990, Woo 1997). Additionally, a nearly complete immunity involving a significant reduction of parasites has been considered as an acceptable criterion for potential vaccines

(Burkart et al. 1990, Woo and Li 1990, Woo 1997). In this regard, the *in vitro* lymphocyte proliferation assay has been used in fish to evaluate the effects of antigenic preparations from microorganisms as potential biological agents for protection (Tatner 1990, Reitan and Thuvander 1991, Estepa et al. 1994, Marsden et al. 1996, Nie et al. 1996, Richards et al. 1996).

Results from the present experiment indicated that the IP inoculation with dead spores induced an immune response stronger than the oral inoculation with live spores and was effective in reducing considerably the number of branchial xenomas. However, the difference in protection observed with the administration of live and dead spores could suggest that the immunogen is a substance that is transiently produced in the live parasite, such as membrane, secretory or excretory proteins (Burkart et al. 1990), and could have contributed to the partial protection observed with the inoculation with dead spores.

Presently, the effector mechanisms responsible for immunity to *L. salmonae* are unknown and as it was observed in this experiment, high proliferative responses do not necessarily mean complete protection, but they indicate an active immune response against the parasite. Results from experiment 4 contrast to what was observed in the second experiment, and another probable explanation for this is that the freezing procedure for killing spores denatured an important Ag involved in complete protection. Perhaps an effective vaccine to *L. salmonae* should use both the particulate and the crude soluble extract simultaneously. Nevertheless, more elements of the effector cells, such as the spectrum of cytokines released during the infection and the phenotype identification of the cells

participating in the disease need to be explored in the future to clarify the responses of live versus dead Ag and correlation with real protection.

In summary, this is the first research reporting the use of lymphocyte proliferation assay and leucograms to study the CMI of rainbow trout to *L. salmonae*. Although we found individual variability in the SI and cpm, as has been reported previously (Rosenberg-Wiser and Avtalion 1982, Tillitt et al. 1988, DeKoning and Kaattari 1991), all the experiments showed increased lymphocyte proliferation to the particulate and the crude soluble extract in the exposed and re-exposed groups compared to the control groups. This suggested that there is a specific antigenic component in the response.

The anamnestic response from recovered fish was not as high as expected and was lower than the observed in fish exposed to dead spores. Recovered fish that were re-exposed to live spores never developed xenomas. Although fish that were exposed to dead spores and then re-challenged with infective ones developed a higher proliferative response, parasitosis was significantly reduced after re-exposure with live spores. This suggested that an inactive form of the parasite could be used as a candidate vaccine in the future for controlling or preventing the disease.

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4 ULTRASTRUCTURAL STUDY OF THE LOCALIZATION AND DEVELOPMENT OF *LOMA SALMONAE* IN THE GILLS OF EXPERIMENTALLY INFECTED RAINBOW TROUT

4.1 ABSTRACT

There are no comprehensive studies of the events that occur during the localization and subsequent development of *Loma salmonae* in the gill and the cell or cells that harbor the parasite. The main objective of the present research was to examine the ultrastructural features of gills from rainbow trout experimentally infected with *L. salmonae* to identify possible mechanisms of infection in gill lamellae. No parasitic stages were identified during the first two weeks (wk) of the infection. By 3 wk postexposure (PE) uninucleate and binucleate meronts were recognized within host cells associated with the capillary channels of gill lamellae and lamellar arteries before the appearance of xenomas. An inflammatory reaction was absent. In gill lamellae, infected cells were isolated from the capillary lumen and some of the infected cells were recognized as pillar cells. In lamellar arteries, infected cells were localized beneath endothelium and not in the lumen. At wk 4 PE xenomas were observed isolated from gill lamella capillaries, beneath lamellar artery endothelium and within the interstitial tissue of the filament. Inflammatory reaction and destruction of parasites inside blood cells in the lumen of lamellae capillaries were also observed by 4 wk PE. Peripheral distribution of the mature parasites inside xenomas was observed at wk 5 and 6 PE, but eventually the parasite occupied the entire xenoma. Degenerative changes were observed only in immature parasites at wk 7 PE and eventually an inflammatory reaction

with a cellular infiltration was directed against mature spores. Round, flattened and irregular shaped xenomas were observed at wk 8 PE. The round xenomas showed a severe inflammatory response with disintegration of the xenoma membrane. This event was accompanied by the eversion of polar tubes within the affected xenoma, and by the simultaneous presence of two parasite appendages, the type I and II tubules. Flattened xenomas were observed underneath the endothelium of gill lamella arteries. The irregular xenoma was located in the connective tissue of the gill filament and showed multiple projections occupied by spores. Both xenomas showed no evidence of inflammatory reaction. Three hypotheses: Isolation, Internalization and Evasion, are proposed to explain the localization of *L. salmonae* in the gills.

4.2 INTRODUCTION

Loma salmonae is a member of the phylum Microspora, which are obligate intracellular microorganisms characterized by the production of spores (Canning et al. 1986). Recent molecular studies have indicated that microsporidia are more closely associated to fungi than to protozoa (Keeling and McFadden 1998, Keeling et al. 2000, Van de et al. 2000). Although microsporidia infect all five classes of vertebrates, fish are the most common hosts.

Infections caused by *L. salmonae* are gaining importance in salmon aquaculture because of its high prevalence, severe mortality, and economical loss (Magor 1987, Markey et al. 1994, Bruno et al. 1995, Bader et al. 1998, Constantine 1999). Although all species of *Oncorhynchus* are susceptible to infection with this parasite, chinook salmon (*O.*

tshawytscha) is the most commonly affected (Kent et al. 1995). It has been reported that gills and endothelial cells are the most important sites of the infection (Shaw et al. 1998), although other organs, including heart, spleen and kidney are also infected (Hauck 1984, Kent et al. 1986, Sanchez et al. 2000).

In gills of salmonids, *L. salmonae* produces white round cysts, called xenomas, that vary in size depending on the stage of the infection. Light microscopy analysis of paraffin sections has revealed that xenomas apparently infect endothelial and pillar cells of the gills (Hauck 1984, Kent et al. 1986), but this has yet to be confirmed. Experimental infections in rainbow trout (*O. mykiss*) held at 14.5° C, demonstrated that branchial xenomas are detected by week (wk) 5 and disappear by 10 wk postexposure (PE) (Speare et al. 1998a). However, in a study using polymerase chain reaction (PCR), *L. salmonae* DNA was detected in the heart and the gills of experimentally infected rainbow trout as early as 3 days and 2 wk PE respectively (Sanchez et al. 2000). Spores are released by wk 7 PE and the tissue reaction generated during this event includes severe branchitis, with distortion, fusion and hyperplasia of gill lamellae. As a consequence, fish may die because of loss of an active respiratory surface (Speare et al. 1998b).

Details regarding the life cycle of *L. salmonae* are still somewhat vague (Sanchez et al. 2000). In general, during microsporidial infection mature spores reach the fish gastrointestinal tract via ingestion, where they germinate due to the acidic gut environment (Undeen 1990, Cali and Takvorian 1999, Bigliardi and Sacchi 2001). Spores contain a sophisticated hatching mechanism that allows them to transmit the disease. The main structures involved in the cell invasion are the polar tube, the polaroplast, and the posterior

vacuole (Desportes-Livage 2000, Bigliardi and Sacchi 2001). Sudden hydrostatic pressure generated within the spore causes the polaroplast and the posterior vacuole to swell resulting in the eversion of the polar tubes (Vávra and Ronny-Larson 1999). The sporoplasm is rapidly expelled through the polar tube, which has punctured a host cell membrane, and is injected directly into target host cells, which might be epithelial cells and/or intraepithelial leucocytes (Weidner 1972, Canning et al. 1986, Dyková 1992, Cali and Takvorian 1999). What happens next with *L. salmonae* until it reaches the gill is still unknown. Several mechanisms of transportation of microsporidia within the host, including leucocytes (macrophages), undifferentiated mesenchyme cells and body fluids (blood), have been suggested (Canning et al. 1986, Dunn and Smith 2001). In addition, it has been proposed that the pillar cells can act as phagocytic cells within the gill lamellae and that endothelial macrophages eventually transform into xenomas after the ingestion of spores of *Loma* sp. (Hauck 1984).

Currently, most reports of fish microsporidiosis are limited to the histopathological or the ultrastructural descriptions of the parasite and the tissue reactions generated during the last stages of the disease, when the xenomas are filled with parasites in all stages of development (Dyková and Lom 1980, Morrison and Sprague 1981a). These details are essential for a correct taxonomic identification of the parasite (Hazard et al. 1981, Morrison and Sprague 1981b, Morrison and Sprague 1981c, Morrison and Sprague 1983, Bekhti and Bouix 1985a) as well as for a detailed study of the cells that are involved in the destruction of spores (Kent et al. 1986). However, comprehensive studies of the events that occur during the localization and subsequent development of the parasite in the gill and the cell or cells that harbor the parasite have not been conducted. The main objective of the present research

was to examine the ultrastructural features of gills from rainbow trout experimentally infected with *L. salmonae* to determine the localization and development of the parasite, and to suggest the possible mechanisms of infection in gill lamellae.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Naive rainbow trout (*O. mykiss*) of approximately 20 g and 15 cm long were used for this experiment. All fish came from a hatchery on Prince Edward Island with no history of previous infection by *L. salmonae*. Fish with any sign of injury, such as abrasions or damaged fins and tails were not used. The selected fish were allocated randomly in 70 L fiberglass tanks containing water at 15 ± 0.5C, and with photoperiods of light and darkness of 12 h each. They were allowed to acclimate for one week before beginning the study. All procedures carried out on the fish conformed to the guidelines of the Canadian Council on Animal Care (1993).

4.3.2 Source of spores

Gill tissue with *L. salmonae* was collected from infected *O. mykiss* which had been experimentally infected (in facilities at the Atlantic Veterinary College) 6 to 8 wk prior to use. These fish were lightly anesthetized with benzocaine in a dose of 100mg/L of water as previously described (Speare et al. 1998a), and were examined with a stereomicroscopy for the presence of xenomas in the gills. Fish exhibiting heavy infection with xenomas were killed with an overdose of benzocaine. After removal of the opercula, the gills from both

sides were collected and the cartilage was removed with scissors. The remaining gill tissue was finely minced using a razor blade. Approximately 5 g of minced tissue was then placed in a 50 ml conical tube, distilled water was added to a final volume of 15 ml, and the mixture refrigerated at 4° C until its use (approximately one hr). Aliquots of 0.5 ml were collected and diluted 1:100 in distilled water and the concentration of spores was estimated using a hemocytometer. The final concentration of the inoculum was adjusted to 1 x 10^6 spores/ml.

4.3.3 Experimental design and infection

Forty-five naive rainbow trout (~ 20 g) were divided into 2 groups: Fish in group 1 (n = 25) were used for analysis of the early stages of the parasite development and were sampled at wk 0, 1, 2, 3 and 4 PE. Fish from the group 2 (n = 20) were used for analysis of the late stages of the parasite development and were sampled at wk 5, 6, 7 and 8 PE. The two groups of fish were kept in separated tanks at 15°C and food waswithheld for 24 hr before inoculation. The fish were lightly anesthetized with benzocaine, restrained manually and given a dose of 0.1 ml of infected material (~1x10^5 spores) by oral intubation with tuberculin syringes attached to silicone tubes 5 cm long and 3 mm outer diameter. The fish were returned to their tanks and allowed to recover. They were observed periodically for any signs of regurgitation. Forty-five naive fish did not receive the challenge and were held in another tank as the negative control of the infection.

4.3.4 Preparation of the samples for high resolution light microscopy (HRLM) and transmission electron microscopy (TEM)

4.3.4.1 Sampling and tissue processing

Samples from five infected and five control fish were collected at 0, 1, 2, 3, and 4 wk PE for the early stages, and 5, 6, 7 and 8 wk PE for the late stages. All fish were euthanized with benzocaine prior to the tissue collection. The presence or absence of xenomas was determined by stereomicroscopy. The first gill arch from the left side of every fish was removed immediately after death and fixed by immersion in vials containing 2% glutaraldehyde in a 0.1 M phosphate buffer pH 7.2 for 2 hr at room temperature (24° C). Subsequently, each gill was cut into many small pieces of ≈ 1 mm^3 and transferred to fresh buffered 2% glutaraldehyde for 24 hr at 4° C.

Samples were washed in phosphate buffer (2 x 15 min) on a rotator, and then postfixed in phosphate buffered 1% osmium tetroxide (Marivac Ltd, Halifax, NS), for 1 hr at room temperature. Subsequently, samples were dehydrated through a graded series of alcohol solutions on a rotator (50% ethanol, 2 x 15 min; 70% ethanol, 2 x 15 min; 95% ethanol, 2 x15 min; and 100% ethanol, 2 x 30 min) to propylene oxide (PO) (2 x 10 min). Samples were infiltrated using increasing proportions of Spurr's resin (Marivac, Ltd) (50%/50% Spurr/PO, overnight; 75%/25% Spurr/PO overnight, and 100% Spurr in vacuum desiccator overnight). Five gill pieces were randomly selected from each fish and eventually embedded separately in 100% Spurr's resin in labeled conical truncated BEEM capsules

(Marivac, Ltd). Resin containing the tissue was polymerized in a vacuum oven (Model 1410, Johns Scientific Inc., ON) overnight at 70°C.

4.3.4.2 Semi-thin sectioning and HRLM

Semi-thin sections (0.5 μm) were cut from the five gill pieces of each fish using glass knives in an ultramicrotome (Ultracut E Ultra Microtome, Reichert Jung, Austria). The sections were mounted onto pre-cleaned glass slides and then stained with 1% toluidine blue in 1% sodium tetraborate solution for HRLM analysis using a Nikon Labophot (Nikon Canada Inc., ON) light microscope. Samples containing the parasite or the xenoma in the semi-thin sections were photographed with a camera mounted onto a light microscope using 35 mm Kodak film (Ektachrome 64T Tungsten EPY 135-36).

4.3.4.3 Ultrathin sectioning, TEM and image processing

Those specimens chosen from HRLM observations were recut for TEM analysis. Ultrathin sections were cut at 70-90 nm thickness with a diamond knife (Diatome, 45° angle, Marivac, Ltd) in the same ultramicrotome. Sections were retrieved onto uncoated 200-mesh copper super grids and contrasted with 5% uranyl acetate in 50% ethanol for 30 min. After rinsing with distilled water the samples were subsequently stained with lead for 2 min (Sato 1968), rinsed again with distilled water, and air dried. The sections were examined and photographed using a Hitachi H7000 scanning transmission electron microscope, Hitachi H600 or H7500 transmission electron microscope (Nissei Sangyo. Canada, Inc) operated at 75 kV, 75 kV and 80 kV respectively. When necessary, serial sections for further analysis

were made to confirm the individuality of the parasite (uni or binucleate) and the localization of the infected cells.

The images were recorded on 8.3 cm x 10.2 Kodak films (Estar Thick Base 4489 Electron Microscope film). The negatives were processed at room temperature following the protocol established by the EM laboratory at AVC. Negatives were immersed for 4 min in a developer solution (Developer D-19), then fixed for 2 ½ min (Kodak Rapid Fixer), followed by an immersion for 10 sec in a wetting agent (Kodak Photo-flo 200). Finally the negatives were rinsed in distilled water and air dried for 30 min. The working and final prints were generated with an enlarger (Durst Laborator S-45 Special Enlarger. Bolzano, Italy) and photographic paper (Kodak Polycontrast III RC Paper) and developed in a Kodak Ektamatic Processor (Rochester, NY).

4.4 RESULTS

A summary of the main features of *L. salmonae* development is presented in table 4.1.

4.4.1 Early stages of the xenoma development

4.4.1.1 HRLM

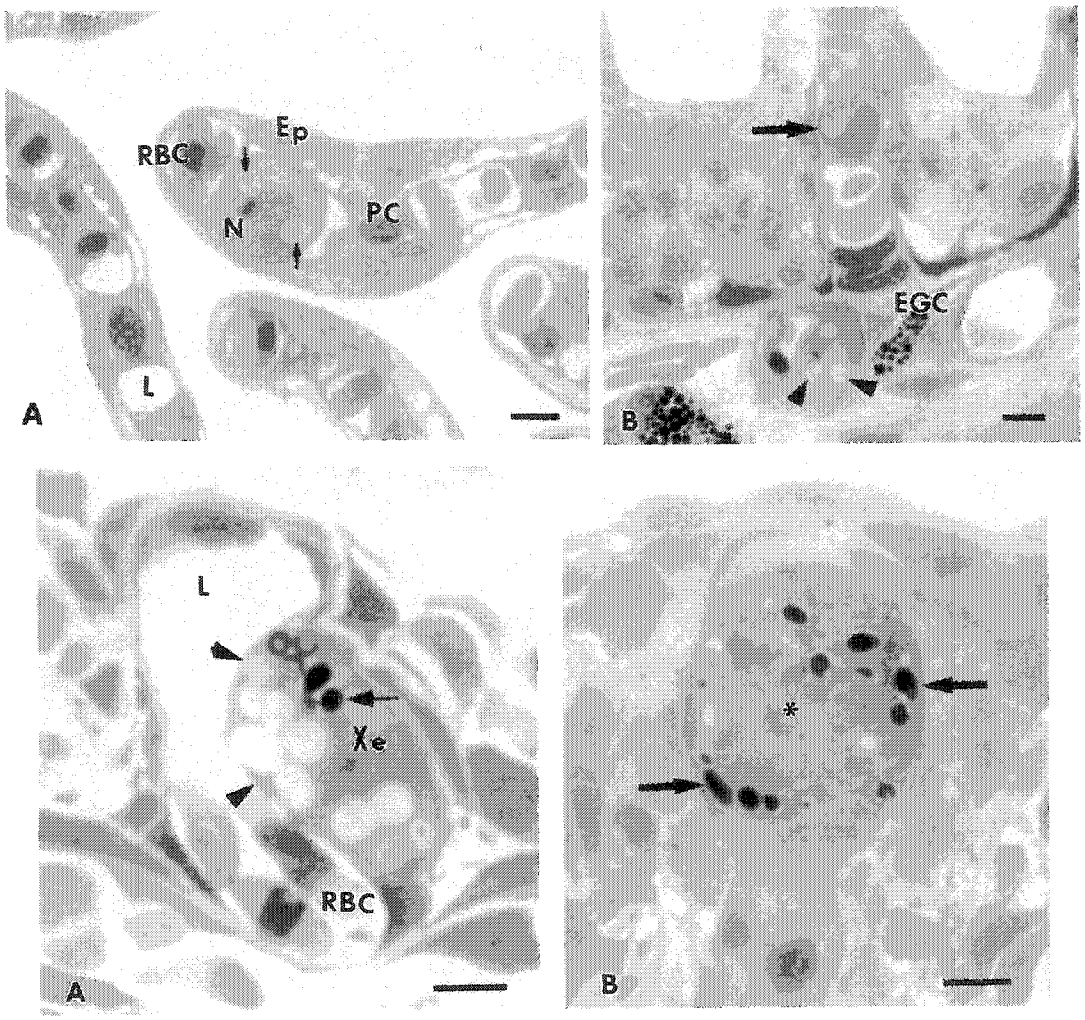
No parasites were identified in toluidine blue stained sections from wk 1 and 2 PE. Gill tissue from infected fish was comparable to that observed in the control fish. Parasitic structures were recognized as translucent areas within the cytoplasm of cells (Fig. 4.1A) in samples collected at week 3 PE. Infected cells were identified in association with the capillaries of lamellae and lamellar arteries (Fig. 4.1B). In gill lamellae, infected cells

Table 4.1. Summary of the main morphological features and location of *Loma salmonae* in gills from experimentally infected rainbow trout.

Wk PE	Important features
1 & 2	No morphological evidence of the parasite.
3	Infected cells contain only meronts. Localization: 1. Subendothelial in lamellae, outside of blood spaces, confined between flanges of non-infected pillar cells and basement membrane (BM) of pavement cells. 2. Base of lamellae beneath endothelium of arteries. No inflammatory reaction against infected cells.
4	Round xenomas present, no recognizable pattern of spore arrangement. Localization: 1. Subendothelial in lamellae outside of blood space, same as wk 3 PE. 2. Subendothelial at base of lamellae, same as wk 3 PE. 3. Within connective tissue (CT) of the filament, close to blood vessels Inflammatory reaction against parasite within capillary channels.
5	Peripheral distribution of spores within round xenomas. Localization: Similar to that seen at wk 4 PE. Abundance of inflammatory cells next to most xenomas in CT
6	Xenomas similar in appearance to those seen at wk 5 PE. Localization: Similar to that seen at wk 4 PE. Areas of the xenoma plasmalemma exposed to CT.
7	Some xenomas now have: 1) degenerative changes to meront, 2) neutrophils with internalized mature spores. Inflammatory cells associated with most xenomas Macrophages in CT contain degraded spores from destroyed xenomas.
8	Three types of xenomas are present: **Round**- some in CT with inflammatory reaction. Type I and II tubules. Everted polar tubes in host cell cytoplasm and inside phagocytes within the xenomas. **Flattened**- beneath endothelium of lamellar arteries. No associated inflammatory reaction. **Irregular shaped**- in CT, have large projections. No associated inflammatory reaction.

Fig. 4.1. High resolution light micrographs (HRLMs) of *Loma salmonae* in rainbow trout gills, wk 3 PE. **A.** Meronts (arrows) within a host cell that is apparently free in the lumen (L) of a lamella capillary; N= host cell nucleus; PC= pillar cell; RBC= red blood cell; Ep= epithelial cell. Bar = 4 μm. **B.** Intracellular meronts associated with lamella (arrow) and lamellar artery (arrowheads); EGC = eosinophilic granular cells. Bar = 5 μm.

Fig. 4.2. HRLMs of *Loma salmonae* in gills, wk 4 PE. **A.** Xenoma (Xe) with several parasitic stages including spores (arrow) associated with the endothelium of a lamellar artery (arrowheads); RBC = red blood cells; L = lumen. Bar = 6.5 μm. **B.** Spores (arrows) embedded in a dense material (*) in a lamella. Bar = 6.1 μm.



were rounded or ovoid in shape and appeared in close proximity to pillar cells. In some, parasites were peripherally distributed within the host cell cytoplasm. Infected cells in lamellar arteries appeared to be in the lumen as free elements filled with parasites (Fig. 4.1B). Inflammatory cells were absent.

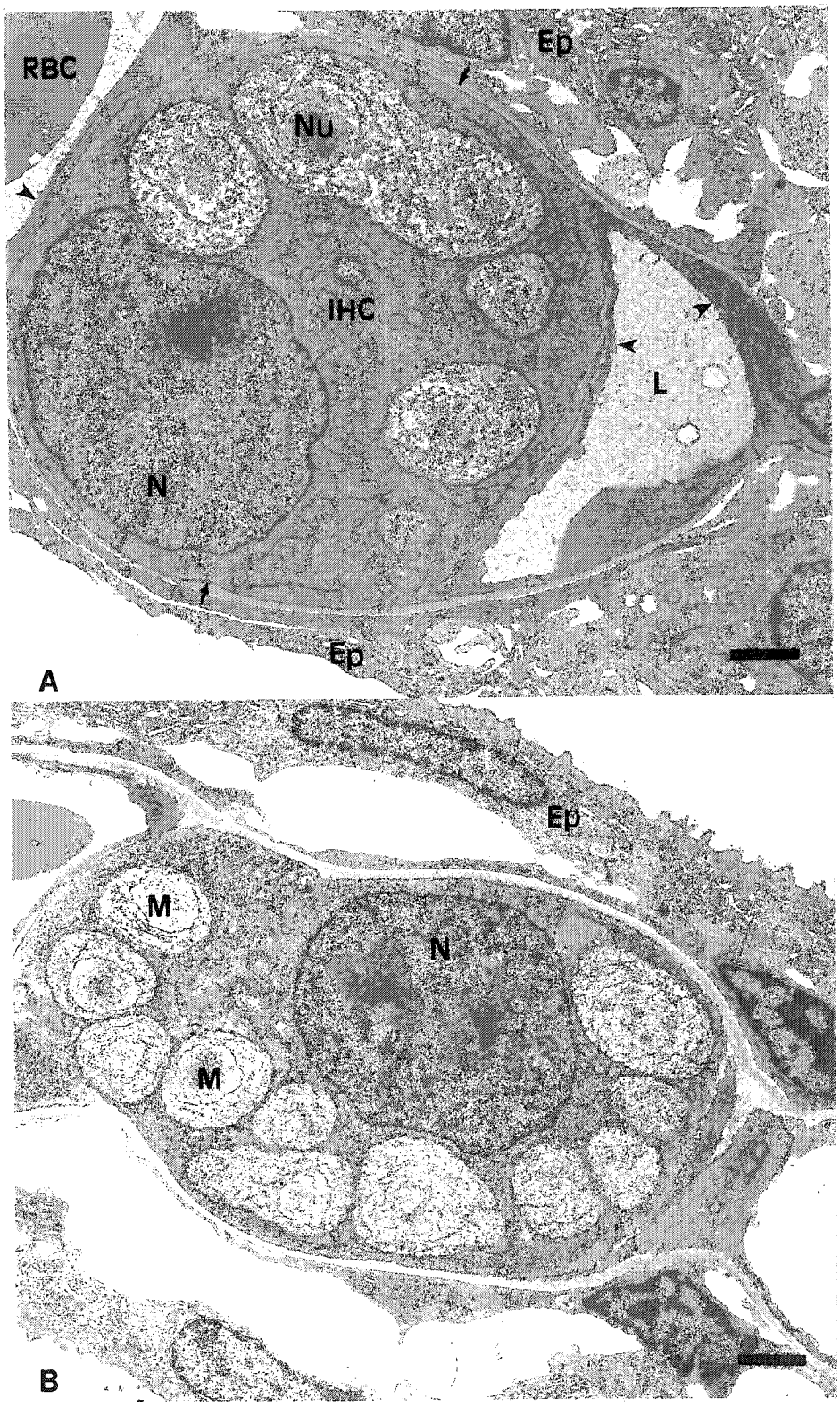
Semi-thin sections from week 4 PE contained several xenomas filled with mature spores and immature parasites (meronts) associated with the lamellar artery (Fig. 4.2A). Some xenomas were accompanied by a discrete inflammatory response characterized by the presence of leucocytes. In some capillary channels, groups of intact spores appeared to be in the lumen associated with a dense material resembling a clot (Fig. 4.2B). Numerous eosinophilic granular cells (ECG), mainly localized in the adventitia of the central venous sinus (CVS), were commonly observed at 3 (Fig. 4.1B) and 4 wk PE but not in controls.

4.4.1.2 TEM

Since there was no indication of parasites at wk 1 and 2 PE by HRLM, these samples were not analysed using TEM.

Morphology of the parasite at 3 wk PE: The only parasitic stage recognizable at this time was the meront. All meronts were observed as uninucleate or binucleate intracellular structures up to 3 μm in diameter (Figs. 4.3A, B). The parasite cell membrane had a wavy appearance and was in close contact with the surrounding host cell membrane. Meronts were sometimes localized in the peripheral region of the host cytoplasm (Fig. 4.3B).

Fig. 4.3. TEMs of *Loma salmonae* in gills, 3 wk PE. **A.** Detail of a portion of Fig. 4.1A. Infected host cell (IHC) in close association with the basement membrane (arrows) and with the flanges of adjacent pillar cells (arrowheads). Ep = epithelial cell; L= capillary lumen; N = host cell nucleus; Nu = parasite nucleolus; RBC = red blood cell. Bar = 1.25 μm. **B.** Intracellular meronts (M) distributed in the periphery of a host cell cytoplasm; Ep= epithelial cell; N = hypertrophic host cell nucleus. Bar = 1.82 μm.

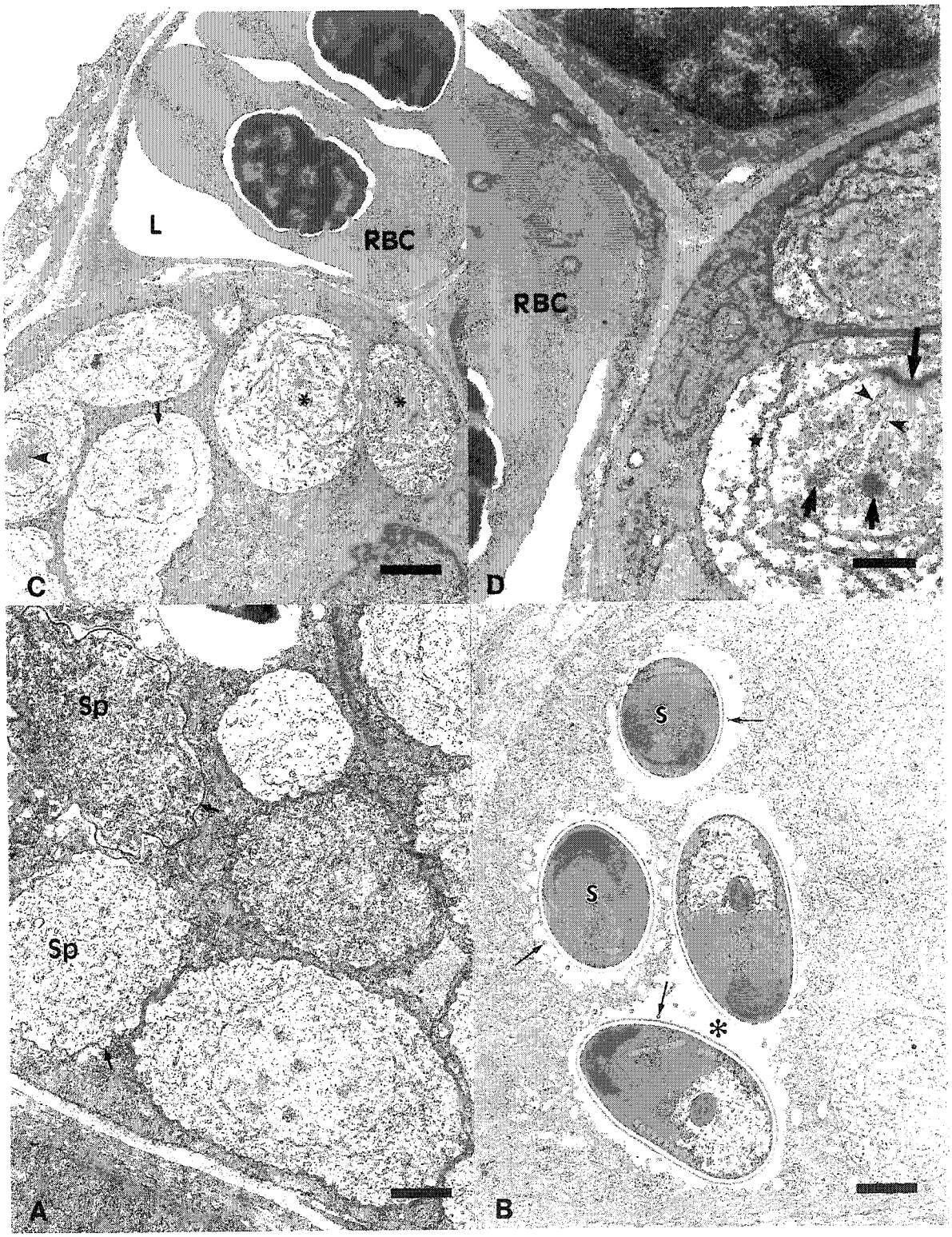


The nuclear envelope of the parasite had a double membrane arrangement with irregular boundaries. A large nucleolus was prominent in some meronts (Figs. 4.3A, C). A few meronts contained a dense spindle plaque localized in a small concavity of the nuclear envelope (Figs. 4.3C, D). Microtubules radiated from this structure towards condensed chromatin, suggesting that parasite division was occurring inside the host cell (Fig. 4.3D). Ribosomes and cisternae of rough endoplasmic reticulum (RER) were scattered throughout the cytoplasm of the meront (Fig. 4.3D). The Golgi apparatus consisted of a series of flattened sacs or vesicles.

Morphology of the parasite at 4 wk PE: Because infected cells exhibited parasites in all stages of development, including spores, these structures could now be called xenomas. Typical meronts were identified within the xenomas. Sporogony was recognized by the presence of sporonts. Sporonts showed a separation of their irregular membranes from the cell membrane of the host, an electron-dense coat was formed on the parasite surface and abundant membranous organelles were observed in their cytoplasm (Fig. 4.4A). Transition to sporoblast was not recognized in any of the samples. Mature spores within parasitophorous vacuoles (PV) showed coiled polar tubes (Fig. 4.4B). The polaroplast was localized immediately posterior to the anterior dense polar sac and was formed by flattened sacs and vesicles. A single nucleus was situated in the middle portion of the spore. Typical small tubular appendages (Type II), that morphologically resembled the cable-like description in previous studies of microsporidians (Takvorian and Cali 1983, Moore and Brooks 1992, Cali and Takvorian 1999) were observed within the sporophorous vesicles (SPOVs) and surrounding the spores (Fig. 4.4B).

Fig. 4.3. TEMs of *Loma salmonae* in gills, wk 3 PE. **C.** Intracellular meronts showing evidence of cell division (*). Meronts have irregular nuclear envelopes (arrow) and large nucleolus (arrowhead). L = capillary lumen; RBC = red blood cell. Bar = 1.3 μm. **D.** Higher magnification of a portion of Figure 4.3C showing a meront nucleus with a spindle plaque (long arrow), microtubules (arrowheads) and chromosomes (short arrows); (★) = cisternae of rough endoplasmic reticulum; RBC = red blood cell. Bar = 0.5 μm

Fig. 4.4. TEMs of *Loma salmonae* in gills, wk 4 PE. **A.** Sporonts (Sp) characterized by an electron-dense coat (arrows). Bar = 0.7 μm. **B.** Mature spores (S) within a sporophorous vesicle (*); arrows = tubules. Bar = 0.9 μm.



Morphology and localization of the infected cell at 3 wk PE: In capillary channels, infected cells appeared to be completely isolated from the lumen. The large nucleus exhibited a prominent nucleolus and dispersed chromatin (Figs. 4.3A, B). The cytoplasm was filled with mitochondria (Fig. 4.3A), ribosomes and RER, some of which showed a dilated lumen. The cuboidal-shaped infected cells were bounded on two opposing sides by the basement membrane associated with the pavement epithelium (Fig. 4.3A). The remaining two sides were separated from the capillary lumen by flanges of pillar cells (Figs. 4.3A, 4.5A). The lumina of the adjacent capillaries were filled with a dense flocculent plasma containing membrane-bound structures (Figs. 4.3A, 4.5A). Pillar cells identified by their cytoplasm rich in microfilaments and columns of collagen fibrils surrounding the main body of the cell, were infected with meronts (Fig. 4.5B). There was no evidence of degeneration of the meronts within the pillar cell cytoplasm and inflammatory cells were not observed. In addition, a putative meront was observed within the flange of a pillar cell (Fig. 4. 5C), and the surface of some infected cells was partially exposed to the capillary lumen (Fig. 4.5D).

An infected cell, which in HRLM was associated with a lamellar artery (Fig. 4.1B), was clearly resolved as being situated beneath the endothelium and not in the lumen (Fig. 4.5E). The cytoplasm of the infected cell was rich in ribosomes, mitochondria and contained some lysosomes. The large nucleus was irregularly-shaped. The infected cell was covered by endothelium which was slightly bulging into the lumen of the blood vessel. Morphologically, the parasite was recognized as an intact meront with no evidence of degradation. Eosinophilic granular cells (EGC) were commonly observed both in the adventitia of the CVS and lamellar arteries of the infected gills (Fig. 4.5E).

Fig. 4.5. TEMs of *Loma salmonae* in gills, wk 3 PE. **A.** Higher magnification of a region from Fig. 4.3A showing dense material in the lumen (L) of a lamella capillary and membranous bound structures (*). Flanges of a pillar cell (arrows) cover the infected host cell (IHC); PC = pillar cell. Bar = 0.8 μm. **B.** Infected pillar cell (IPC) containing a large meront (M). Columns of collagen (arrows) are distinguished on both sides of the main cell body; N= nucleus of the IPC. Bar = 0.8 μm.

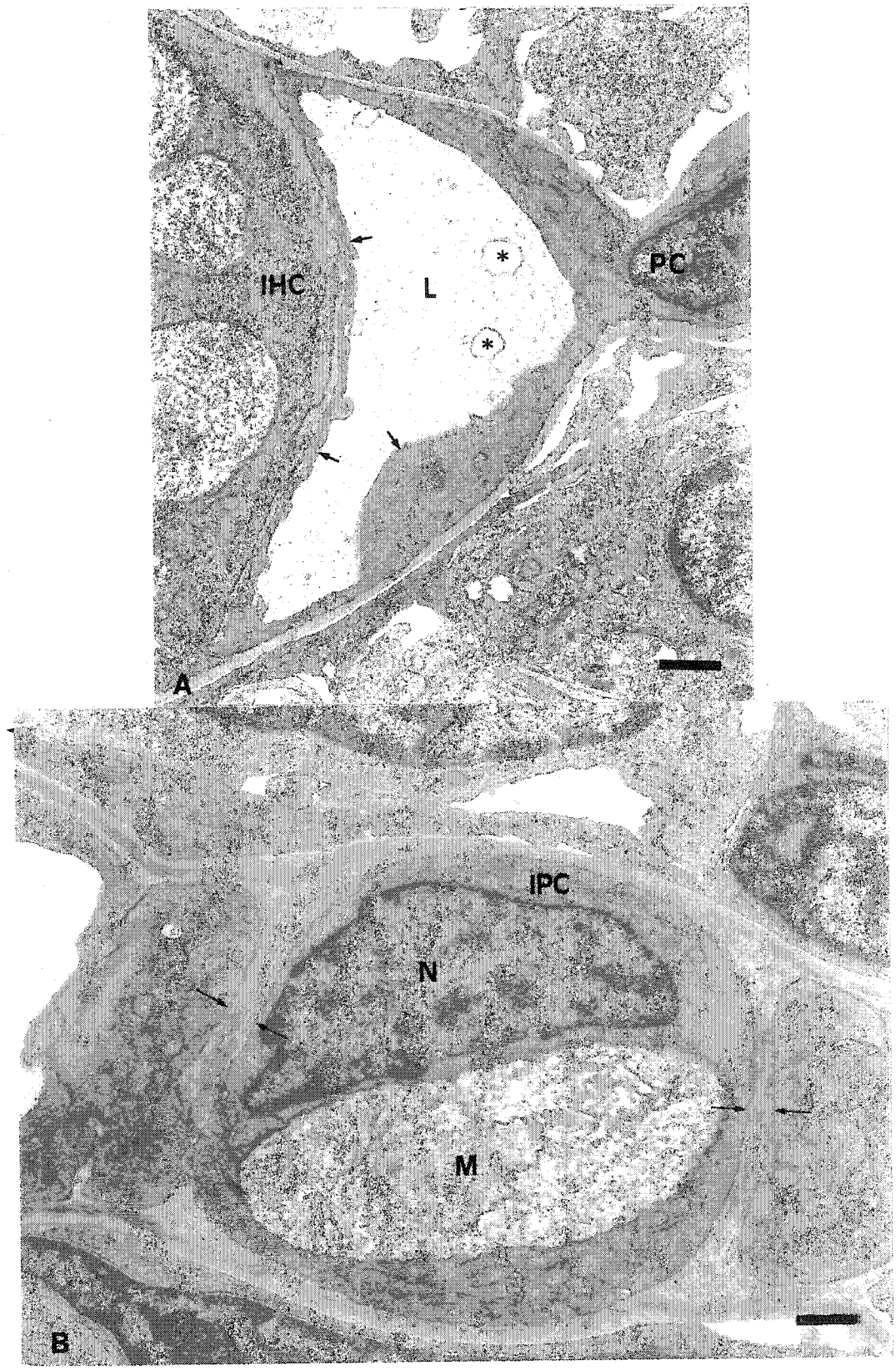
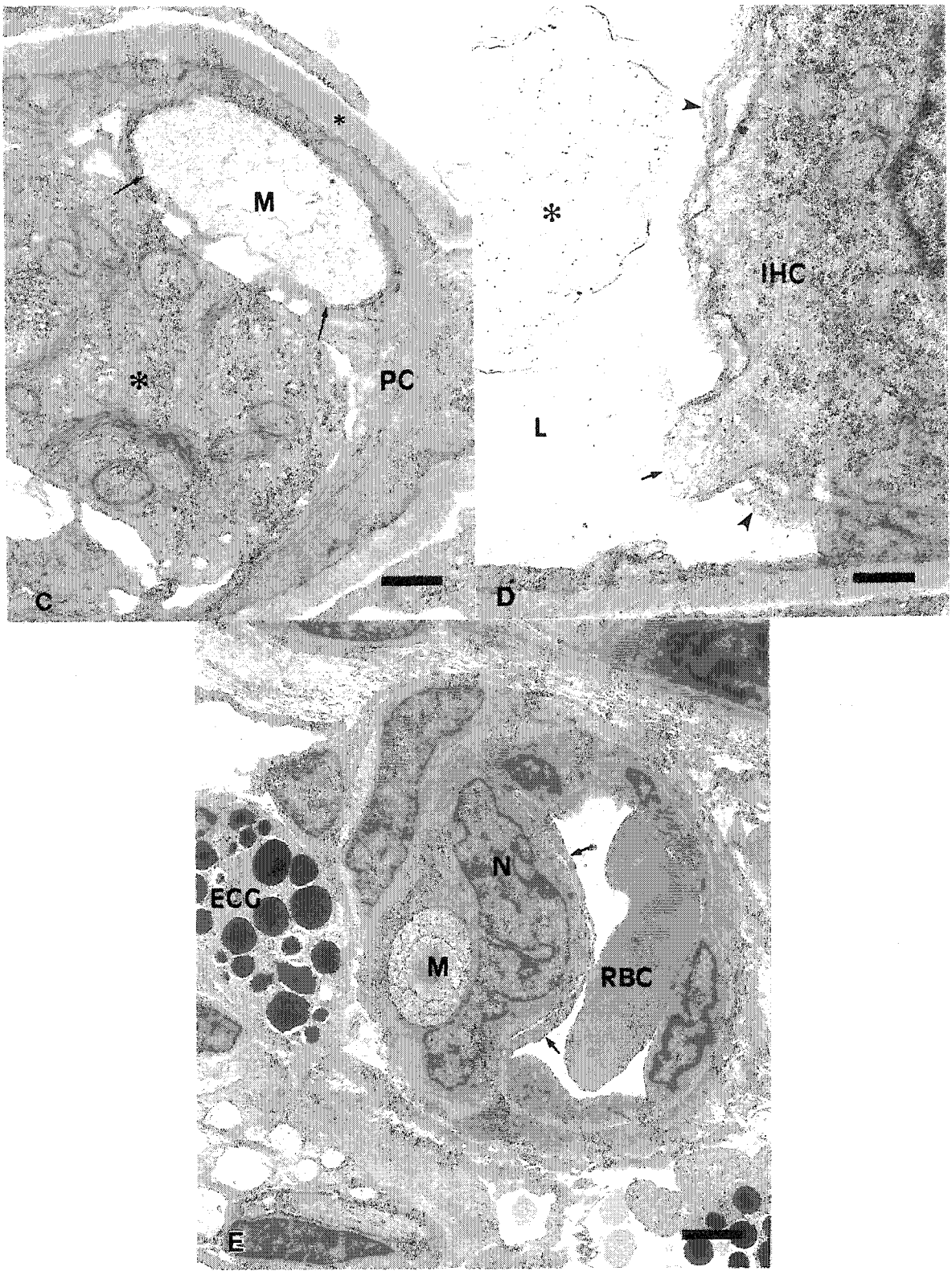


Fig. 4.5 TEMs of *Loma salmonae*, wk 3 PE. **C.** Meront (M) associated with a pillar cell (PC) flange (arrows). (*) = Basement membrane; (*) Unknown blood cell. Bar = 0.3 μm. **D.** Margin of an infected host cell (IHC) partially exposed (arrow) to the capillary lumen (L). Flanges of a pillar cell (arrowheads). A double membrane structure (*) is located in the lumen. Bar = 0.4 μm. **E.** An infected cell with intracellular meront (M) localized beneath the endothelium (arrows) of a lamellar artery. Note the eosinophilic granular cell (EGC) in close association with the blood vessel; N = host cell nucleus; RBC = red blood cell. Bar = 1.8 μm.



Morphology and localization of the xenomas at 4 wk PE: Similar to the localization of infected cells seen at wk 3 PE, xenomas were located in lamellae outside of the capillary channels and beneath the endothelium of lamellar arteries (Fig. 4.6 A). Round xenomas were also found within connective tissue of the filaments in close proximity to blood vessels. Inside the xenoma, numerous host cell mitochondria and dilated RER were common features.

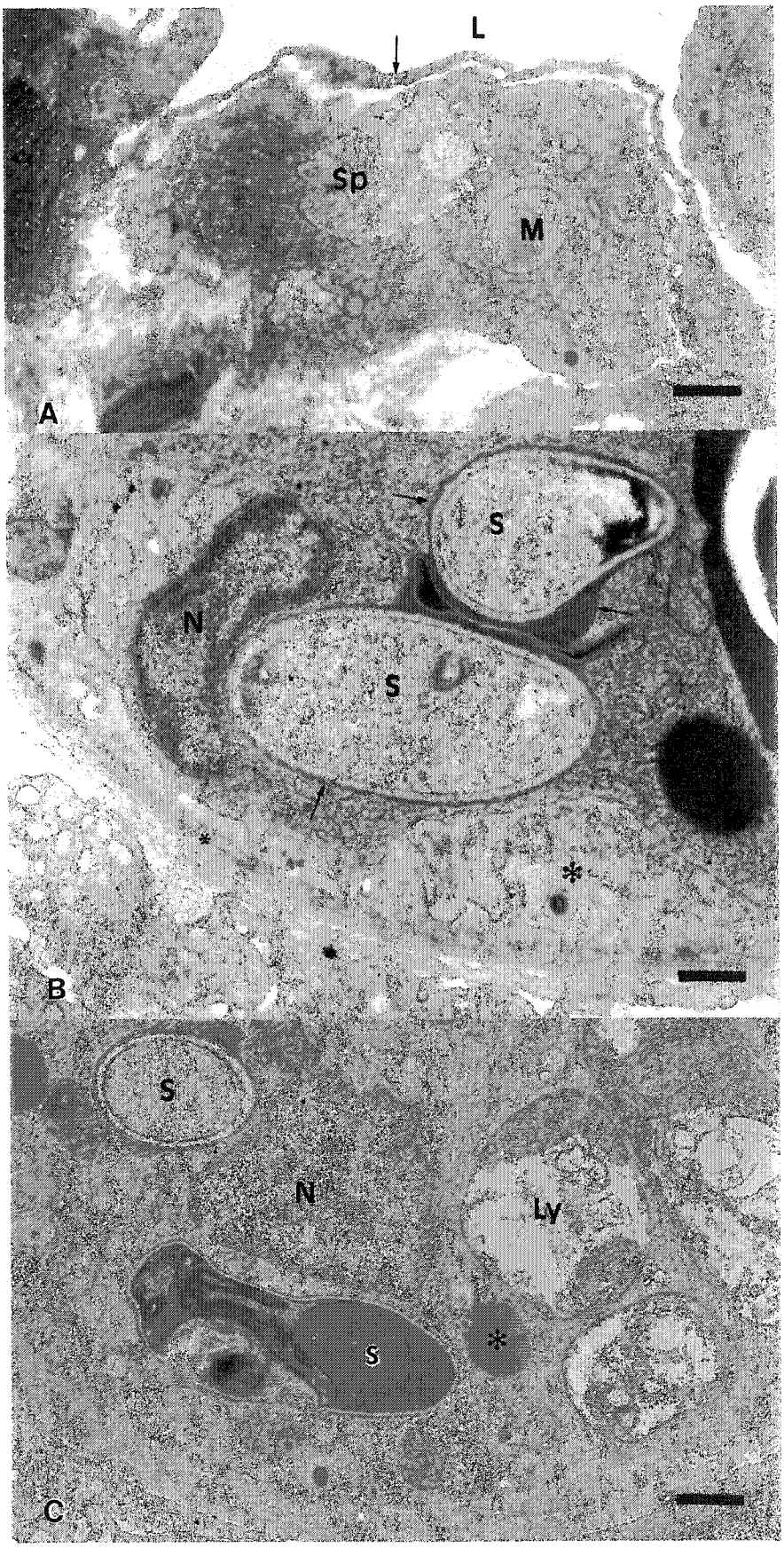
Evidence of an inflammatory reaction due to the parasite appeared to be present inside capillary channels of lamellae. Groups of spores in different levels of destruction were observed within the cytoplasm of blood cells. Spores appeared empty, collapsed and surrounded by an electron-dense membrane called the pansporoblastic matrix (Dyková and Lom, 1980) (Figs. 4.6B, C). The nuclei of some of the blood cells appeared segmented. Cytoplasm of the cell was characterized by abundant ribosomes, round vesicles with single membrane, smooth and rough endoplasmic reticulum, prominent Golgi apparatus, mitochondria and lysosomes. Secondary lysosomes were also seen (Fig. 4.6C). The blood cells appeared to be embedded in a dense and granular material (Fig. 4.6B), which in certain areas contained electron-dense irregular structures. Capillary endothelium was absent in these areas although the basement membrane remained (Fig. 4.6B)

4.4.2 Late stages of the xenoma development

4.4.2.1 HRLM

Semi-thin sections from gills at wk 5 and 6 PE showed xenomas loaded with mature and immature parasites. There were uni- and binucleate meronts, and evidence of plasmodia. The localization of the xenomas was similar to that observed at wk 3 and 4 PE,

Fig. 4.6. TEMs of *Loma salmonae* in gills, wk 4 PE. **A.** Xenoma beneath the endothelium of a lamella artery (arrow) containing a meront (M) and a sporont (Sp). L = lumen. Bar = 1.8 μm. **B.** Cytoplasm of a blood cell containing digested spores (S) which are covered by an electron-dense substance, the pansporoblastic matrix (arrows). Dense material (*) is surrounding the phagocytic blood cells; (*) = basement membrane of lamella capillary without the presence of endothelium; N = nucleus of phagocytic blood cell. Bar = 0.6 μm. **C.** Spores in different stages of degradation (S) within a phagocytic blood cell; (*) = lipid droplets; Ly = secondary lysosomes; N = nucleus of phagocytic blood cell. Bar = 0.8 μm.



associated with the lamella capillary channel (Fig. 4.7A). Mature spores appeared congregated toward the peripheral region in some xenomas at wk 5 and 6 PE (Fig. 4.7B), but by wk 7 and 8 PE spores occupied the entire xenoma. Inflammatory response was not detected in the specimens observed at wk 5 PE, but at wk 6 PE a mild infiltration by leucocytes was observed around some xenomas.

By wk 7 PE the size of the xenomas was larger due to a higher concentration of parasites (Chapter 2). Small foci of inflammatory reaction were observed in the filaments and the basal part of the lamellae (Fig. 4.8A). Numerous neutrophils, mononuclear cells, fibroblasts, and EGCs contributed to the inflammatory reaction around the xenomas (Fig. 4.8B). A few spores, apparently engulfed by phagocytes, were observed in the centre of the inflammatory reaction (Fig. 4.8C). However, many xenomas remained unaffected by the tissue reaction. Round and flattened type xenomas were observed at wk 8 PE. The severity of the inflammatory reaction was higher in some of the round xenomas observed by wk 8 PE, which were surrounded (Fig. 4.9A) or invaded by leucocytes (Fig. 4.9 B). Eosinophilic granular cells, neutrophils and mononuclear cells were always observed near xenomas and adventitia of CVS from wk 5 PE onward and were most numerous by wk 8 PE (Fig. 4.9A,B). The flattened type xenoma (Fig. 4.10), was located underneath the endothelium at the base of the gill lamellae. Mature spores, the main parasitic stage observed within this structure, appeared to be aligned along the xenoma giving an appearance of peas in a pod. No evidence of inflammatory response was observed against this structure.

Fig. 4.7. HRLMs of *Loma salmonae,* wk 5 and 6 PE. **A.** Xenoma associated with a lamella capillary channel. Arrows = spores; IP = immature parasites (probably meronts); PC = pillar cell; L = lumen. Bar = 6.3 μm μm. **B.** Spores (arrows) peripherally oriented within a round xenoma in filament connective tissue. Inflammatory cells are close to the xenoma (arrowhead). Bar = 10 μm.

Fig. 4.8. HRLMs of *Loma salmonae,* wk 7 PE. **A.** Inflammatory reaction adjacent to a xenoma in connective tissue. Neutrophils (arrows) and mononuclear cells (arrowheads) formed the main leucocyte population. A spore-like structure inside a leucocyte (open arrowhead). Bar = 10 μm. **B.** Eosinophilic granular cells (arrows), neutrophils (arrowheads), and mononuclear cells (open arrowhead) adjacent to another round xenoma in connective tissue. Bar = 10 μm. **C.** A spore engulfed by a leucocyte (arrow) in an area of inflammatory reaction. Bar = 6.1 μm.

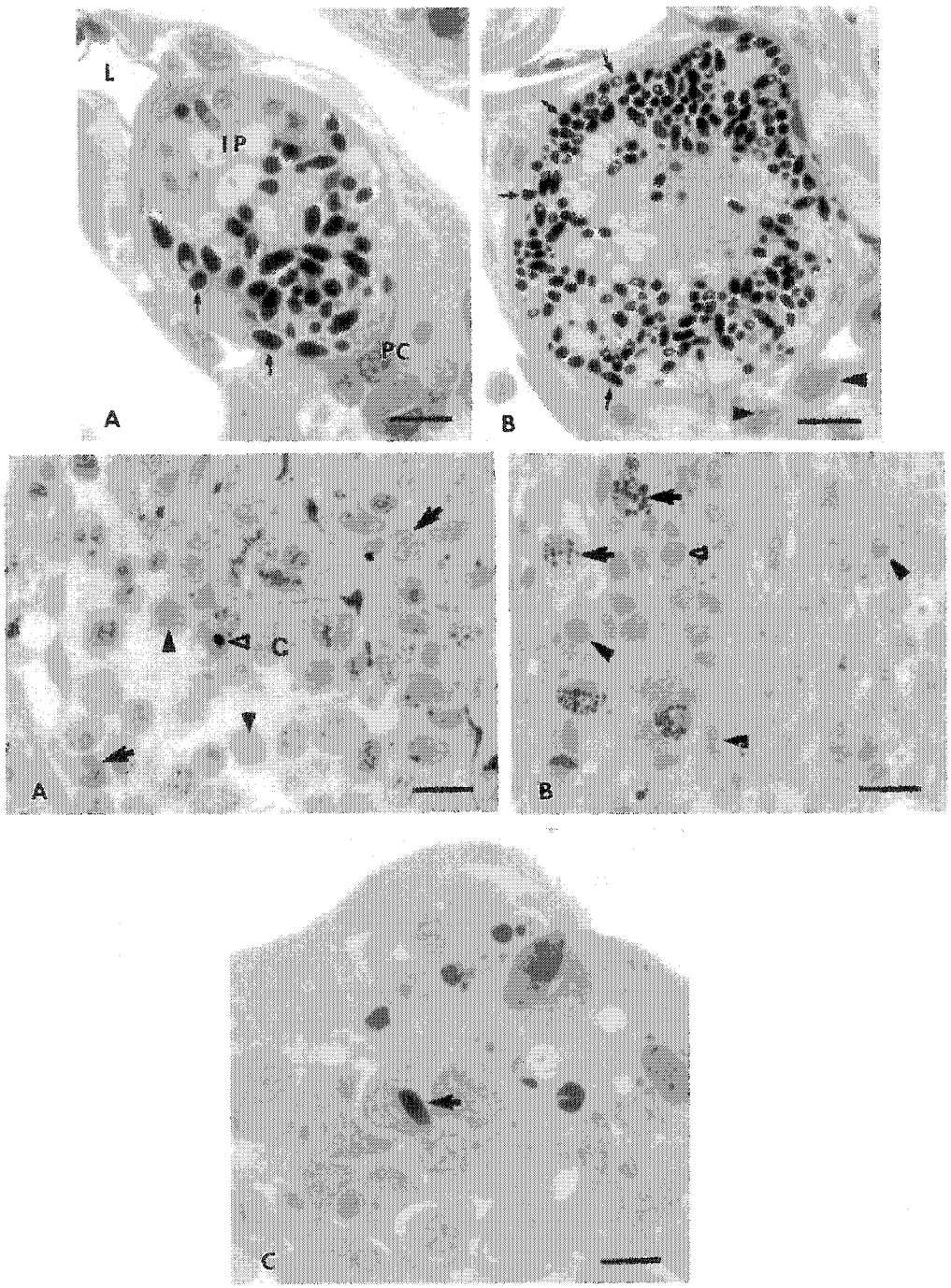
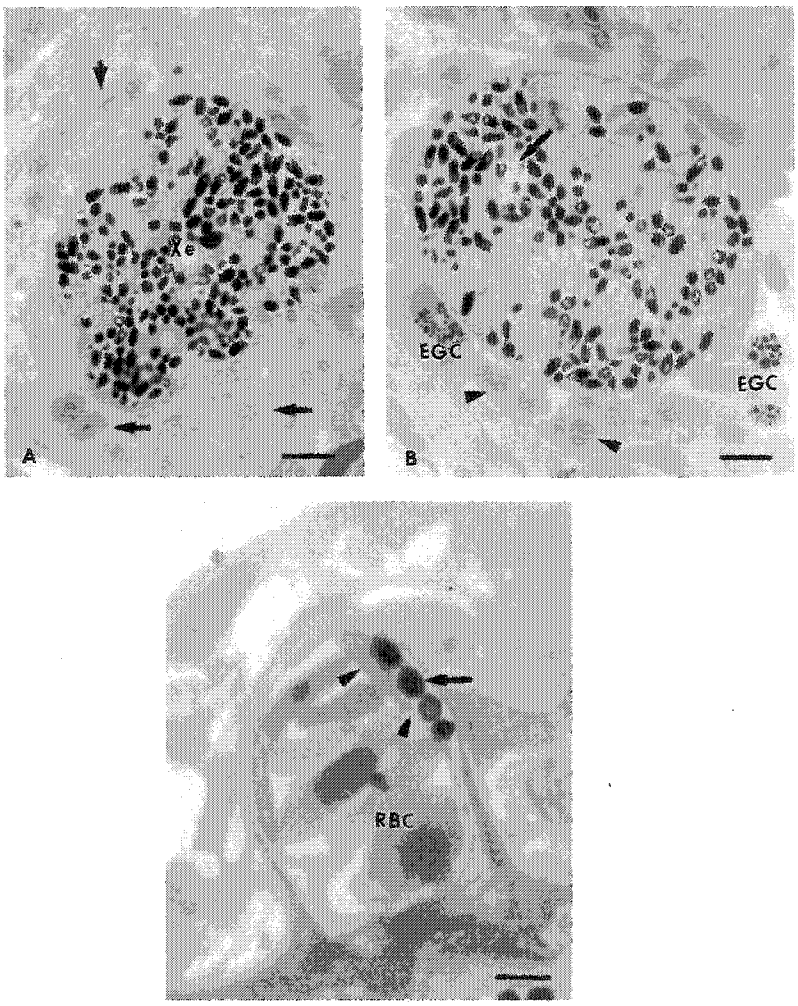


Fig. 4.9. HRLMs of *Loma salmonae,* wk 8 PE. **A.** Round xenoma (Xe) in connective tissue surrounded by numerous inflammatory cells (arrows). Bar = 10 μm. **B.** A leucocyte (arrow) within a round xenoma phagocytosing spores. The xenoma wall appears intact. Eosinophilic granular cells (EGC), and neutrophils (arrowheads) surround the xenoma. Bar = 10 μm.

Fig. 4.10. HRLM of *Loma salmonae,* wk 8 PE showing aligned spores (arrow) within a flattened xenoma associated with the endothelium (arrowheads) at the base of a lamella. RBC = red blood cell. Bar = 6 μm.



4.4.2.2 TEM

Due to the absence of significant morphological changes observed by HRLM from those observed at wk 4 PE, gill samples from wk 5 PE were not analysed using TEM.

Morphology and localization of xenomas at wk 6 and 7 PE. Xenomas originating from infected pillar cells of the lamellae contained meronts and mature spores that were separated by columns of collagen, giving the impression of being separated into 2 or 3 parts (Figs. 4.11A). Leucocytes were closely associated with these xenomas and with xenomas in filament connective tissue, but evidence of cell damage was not observed (Figs. 4.11A, B). Xenomas in the filament were surrounded by a wall of extracellular matrix and covered by attenuated fibroblast-like cells (Fig. 4.11B). In some xenomas, gaps in the layer of fibroblast-like cells allowed small areas of the plasmalemma to be exposed to leucocytes in the filament connective tissue (Fig. 4.11C). In connective tissue some round xenomas were surrounded by layers of collagenous material (Fig. 4.11B).

At wk 7 PE, round xenomas in the connective tissue of filaments showed what appeared to be degenerative changes to some immature parasites, meronts and sporonts (Fig. 4.11D). Parasite cell membranes looked distorted and the cytoplasm showed empty spaces. The nuclear membrane seemed undulated and separated in certain points, and the nuclear material was dissolved. Their RER was disorganized and scattered through the cytoplasm. In general, mature spores showed no indication of damage or degenerative changes, but some SPOV containing spores appeared dilated and filled with what seemed to be the remnants of the tubules (Fig. 4.11E). Some PMN neutrophils, plasma cells and numerous EGCs were observed adjacent to the xenoma wall (Fig. 4.11F, G). Neutrophils were identified by their

Fig. 4.11. TEMs of *Loma salmonae*, wk 6 and 7 PE. **A.** Xenoma originated from a pillar cell containing spores (S) and meronts (M). Columns of collagen (*) split the xenoma into two parts. PMN = neutrophil; arrowhead = basement membrane. Bar = 1.8 μm. **B.** Xenoma (Xe) in filament connective tissue showing a neutrophil (PMN) close to a layer of fibroblast-like cell (F) adjacent to the xenoma wall (arrows). Bar = 1.2 μm. **C.** An area of xenoma plasmalemma (arrow) and wall not covered by neighbouring fibroblast-like (F) cells. Leucocyte adjacent to the exposed area (Lu). S = spore. Bar = 0.6 μm.

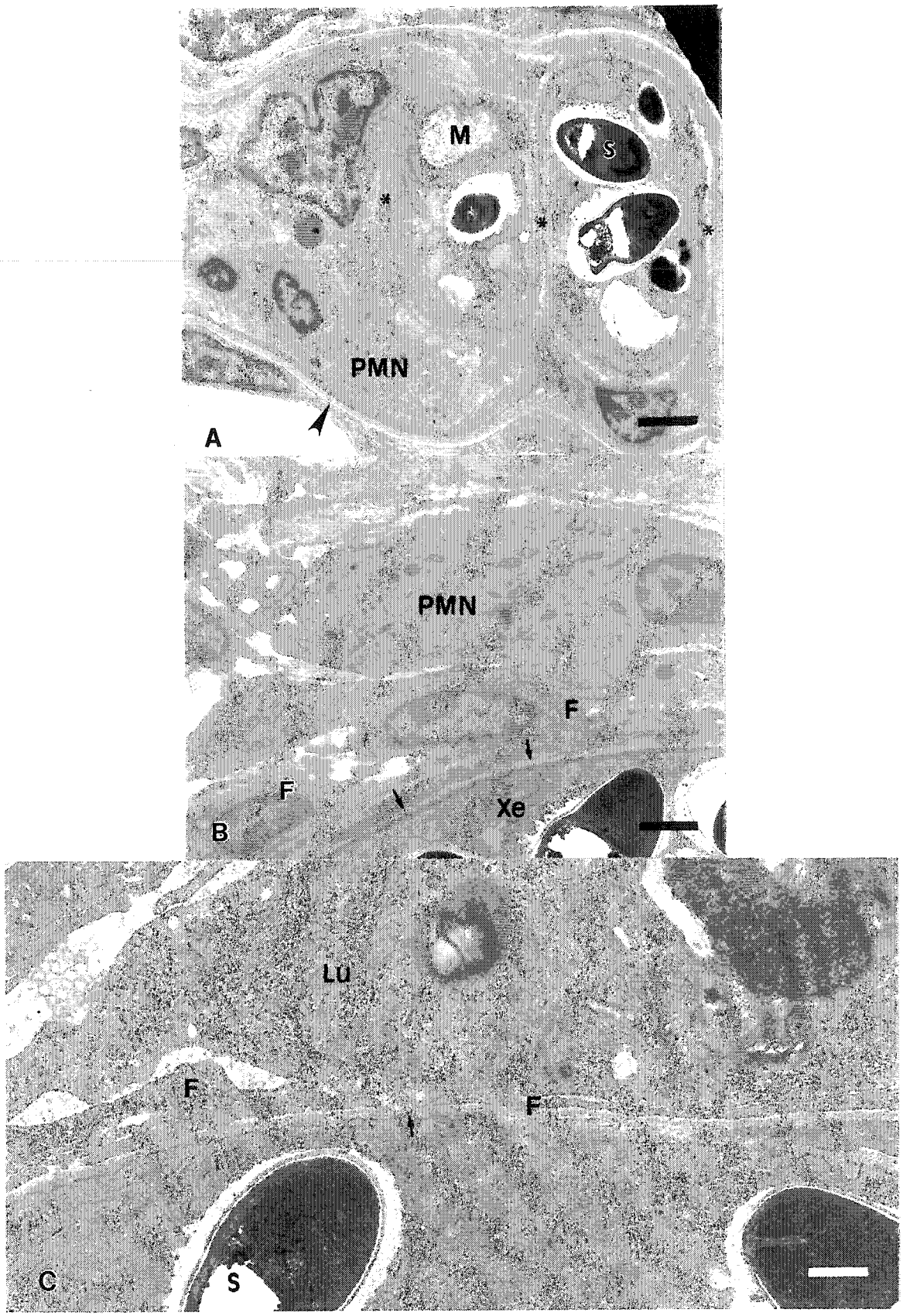
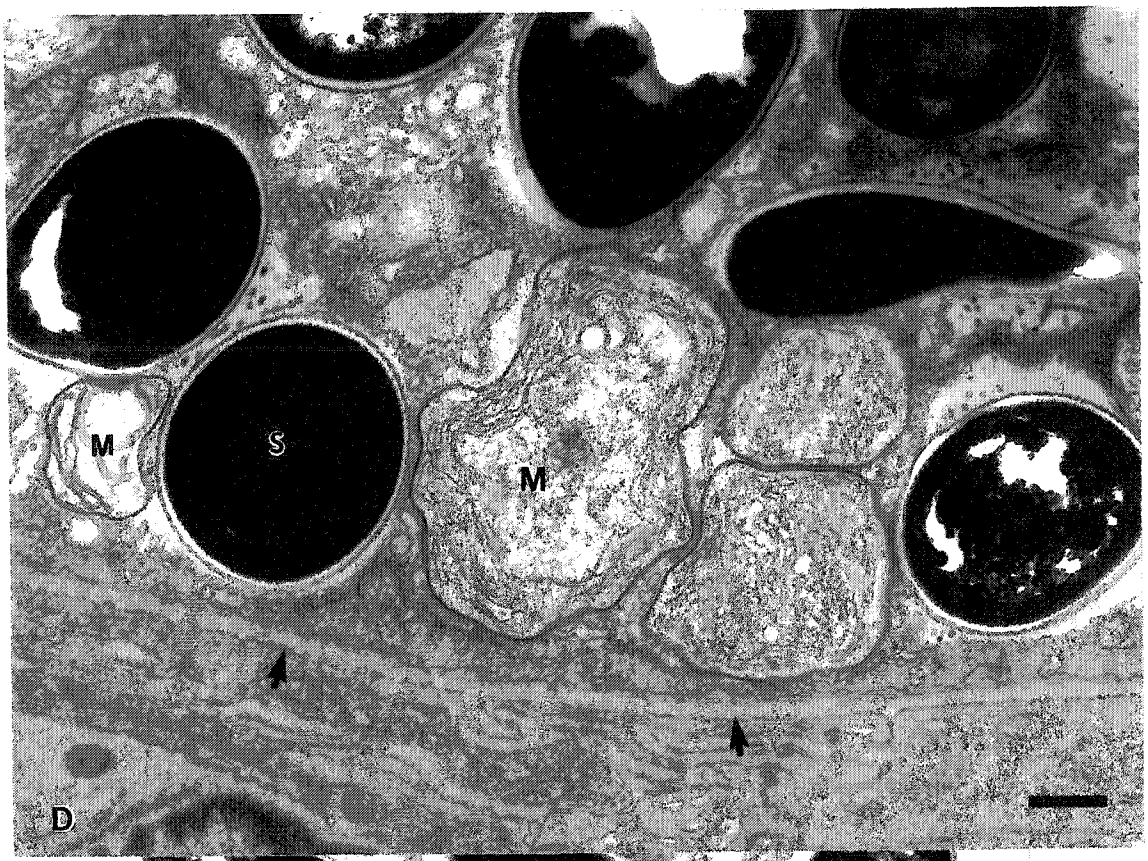


Fig. 4.11. TEMs of *Loma salmonae*, wk 7 PE. **D.** Round xenoma in connective tissue with degenerated meronts (M). Spores (S) look intact. Arrows = xenoma wall. Bar = 0.4 μm. **E.** Intact spores (S) contained in a dilated sporophorous vesicle (SPOV) showing degenerated tubules (arrows). Bar = 0.8 μm.



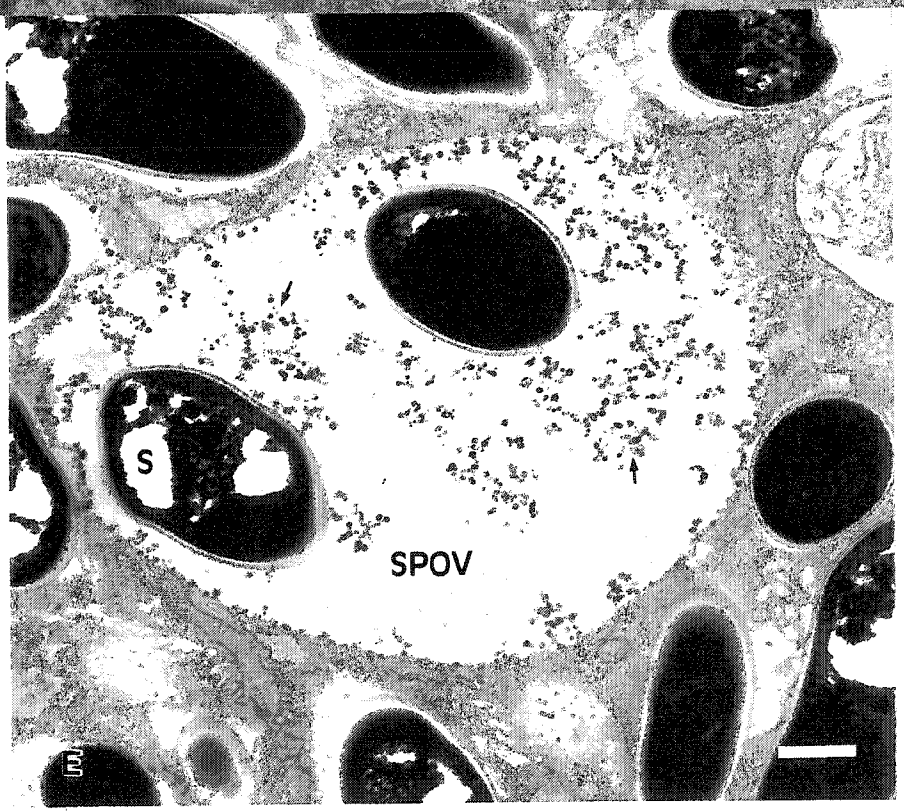
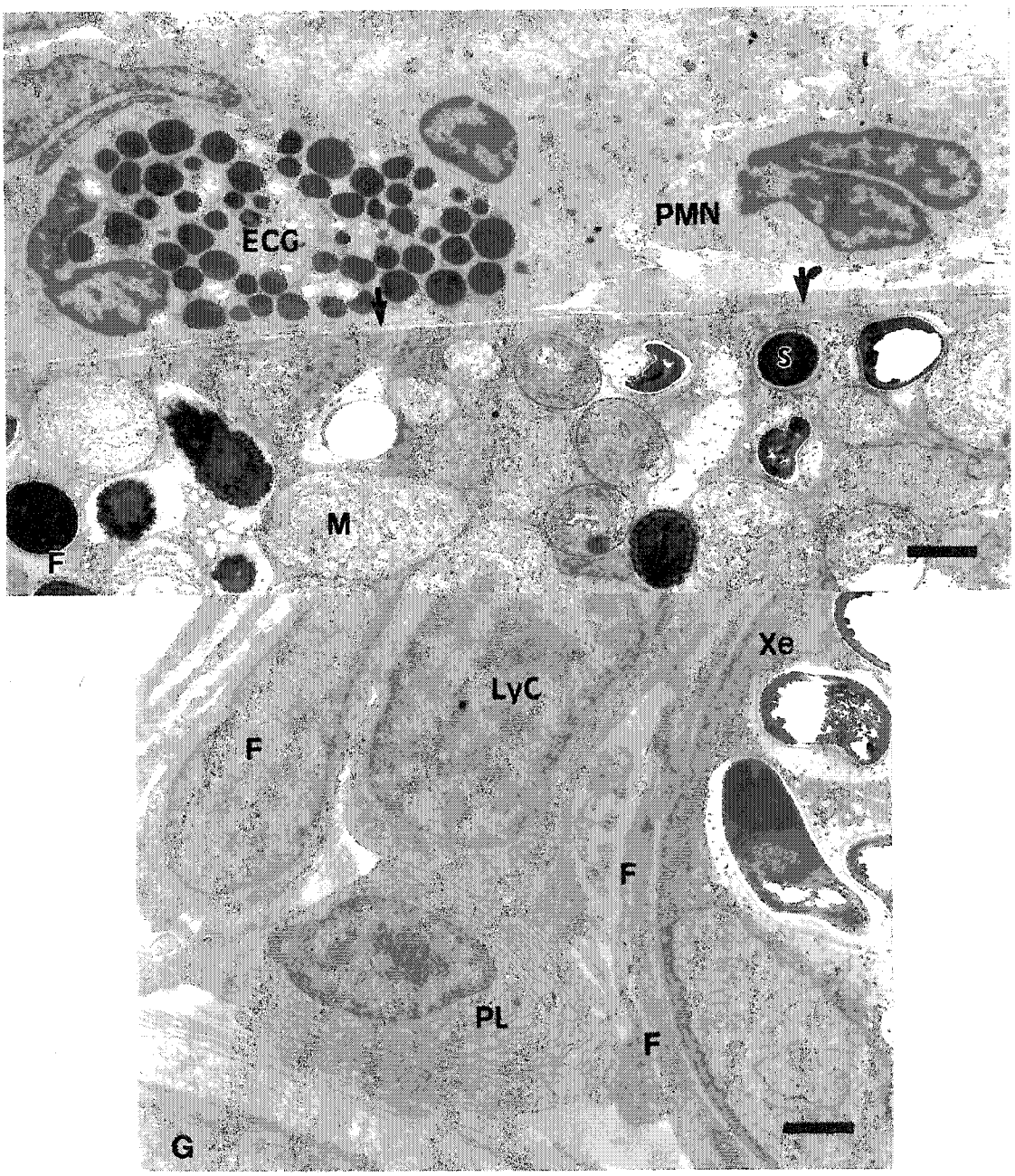


Fig. 4.11. TEMs of *Loma salmonae,* wk 7 PE. **F.** Round xenoma in connective tissue surrounded by inflammatory cells. EGC = eosinophilic granular cells; PMN = neutrophils; arrows = xenoma wall; S =spores; M =meronts. Bar = 1.6 μm. **G.** Plasma cell (PL) and lymphocyte-like cell (LyC) close to a xenoma (Xe). F = fibroblast-like cells. Bar = 1.2 μm.



irregular external contour and lobed nucleus. Also, their cytoplasm contained round and rod-shaped granules filled with an electro-dense fibrillar material that run parallel to the long axis of the granule, glycogen granules, a few ribosomes, RER, and mitochondria. Plasma cells were distinguished by their well-developed RER (Fig. 4.11G). Some other leukocytes that were surrounding the xenoma were recognized as neutrophils, and lymphocyte-like cells (Fig. 4.11G). Lymphocytes were small and round cells with a smooth cell membrane and a large nucleus located in a central position and with an abundant chromatin. The cytoplasm contained just a few ribosomes, RER, mitochondria and vesicles.

Localization and morphology of xenomas at wk 8 PE. Leucocytes, apparently neutrophils, were present inside round xenomas in filament connective tissue engulfing parasites (Figs. 4.12A, B). Individual or groups of intact and empty spores were contained in vesicles inside neutrophils with no evidence of fusion with lysosomes. Empty spores consisted of the chitinaceous electron-lucent endospore and remnants of a granular material with a few electron-dense membranes and some vesicles. Intact spores were observed within some round xenomas adjacent to areas were the xenoma plasmalemma had disappeared (Fig. 4.12C).

Spores located outside of the xenoma were ingested mainly by macrophages and appeared to be in different stages of degradation (Fig. 4.12D). Macrophages were recognized by their irregular cell membrane and their cytoplasm containing lysosomes, numerous ribosomes, RER, mitochondria, and a well-developed Golgi apparatus. Some degenerated spores were filled with a granular material and several electron-dense granules, and were

Fig. 4.12. TEMs of *Loma salmonae,* wk 8 PE. **A.** Neutrophil (*) within a xenoma ingesting spores (S), some of which look empty (ES). Membranous remnants are observed within empty spores (arrow). Both intact and empty spores show no evidence of destruction. N = nucleus of the neutrophil. Bar = 1 μm. **B.** Intact (S) and empty spores (ES) without evidence of destruction within neutrophils (*) in a xenoma. The xenoma plasmalemma (arrows) looks intact.(V) = vesicle with granular material of unknown origin. Neutrophil outside the xenoma (* *). Bar = 1 μm. **C.** Xenoma margin showing complete absence of its plasmalemma (arrows). Xe = xenoma; S = spore. Bar = 1 μm.

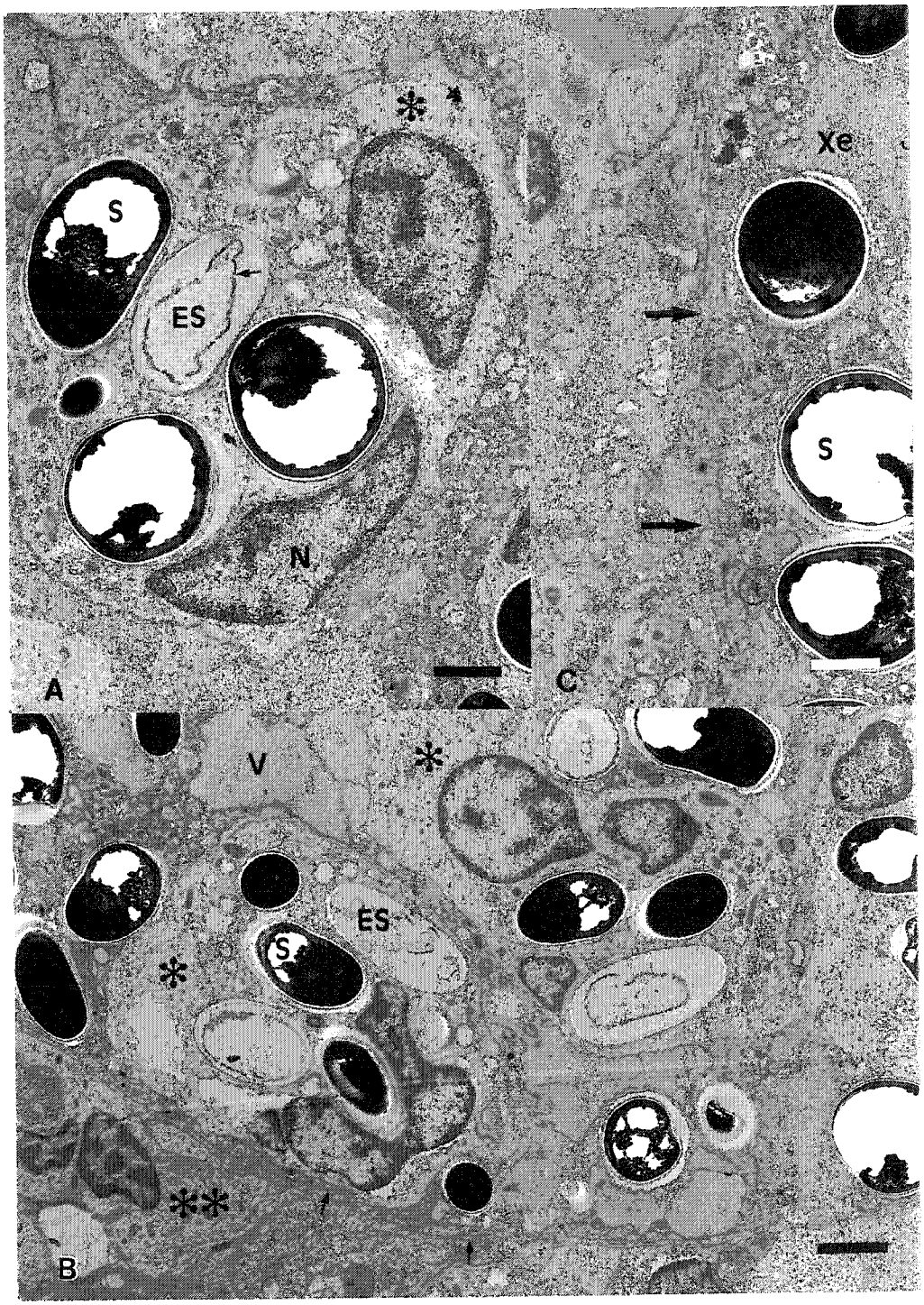
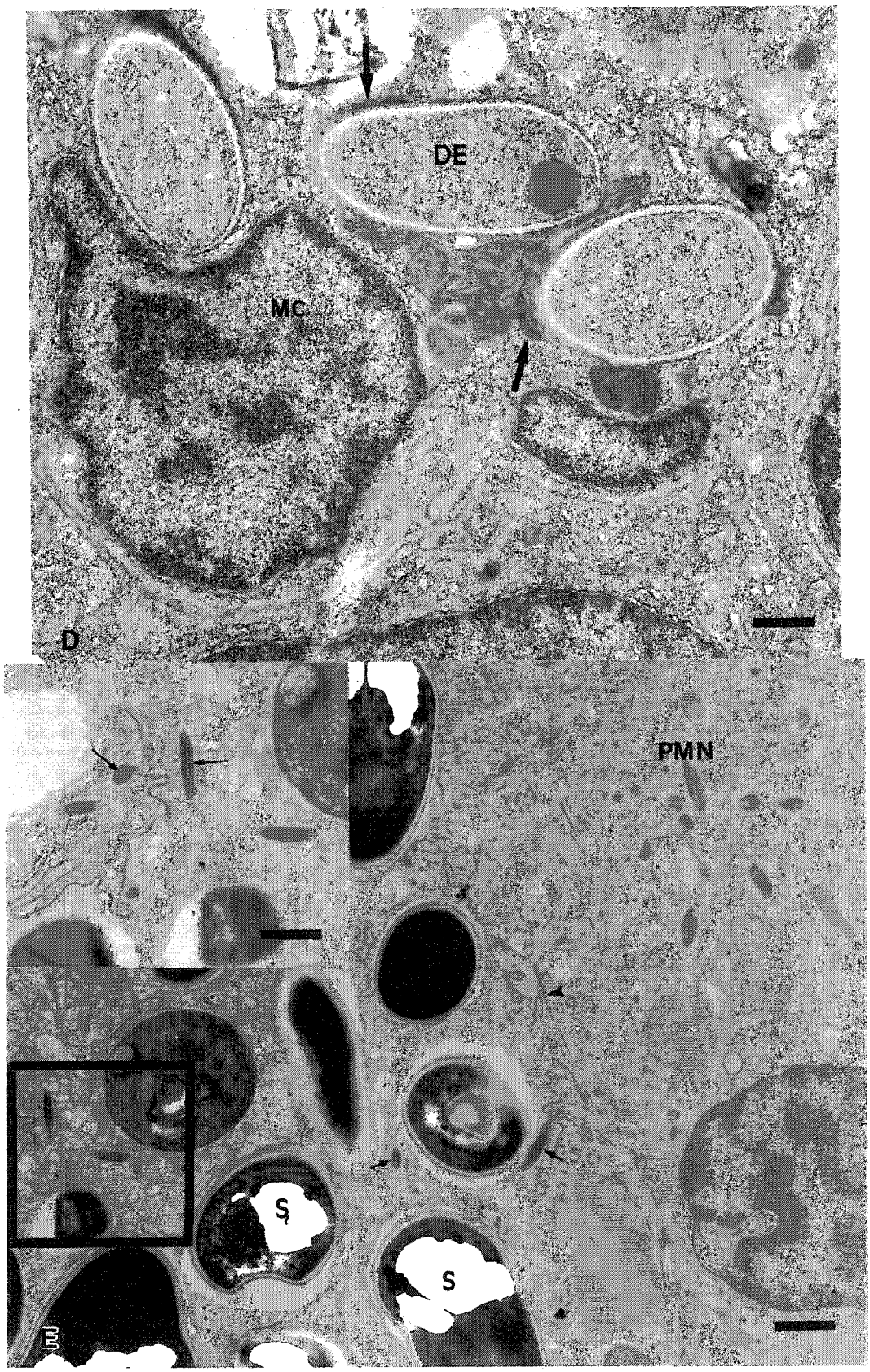


Fig. 4.12. TEMs of *Loma salmonae,* wk 8 PE. **D.** Macrophage (MC) in connective tissue digesting spores (DE). Pansporoblastic matrix (arrows) covers the destroyed spores. Bar = 0.6 μm. **E.** Everted polar tubes dispersed through the xenoma cytoplasm (arrows). The plasmalemma of the xenoma has completely disappeared (*), but spores (S) remain in place within the xenoma. A neutrophil (PMN) is observed in association with this area. **Insert:** Magnification of the boxed area showing different sectional planes of the polar tubes (arrows). Bar = 0.5 μm

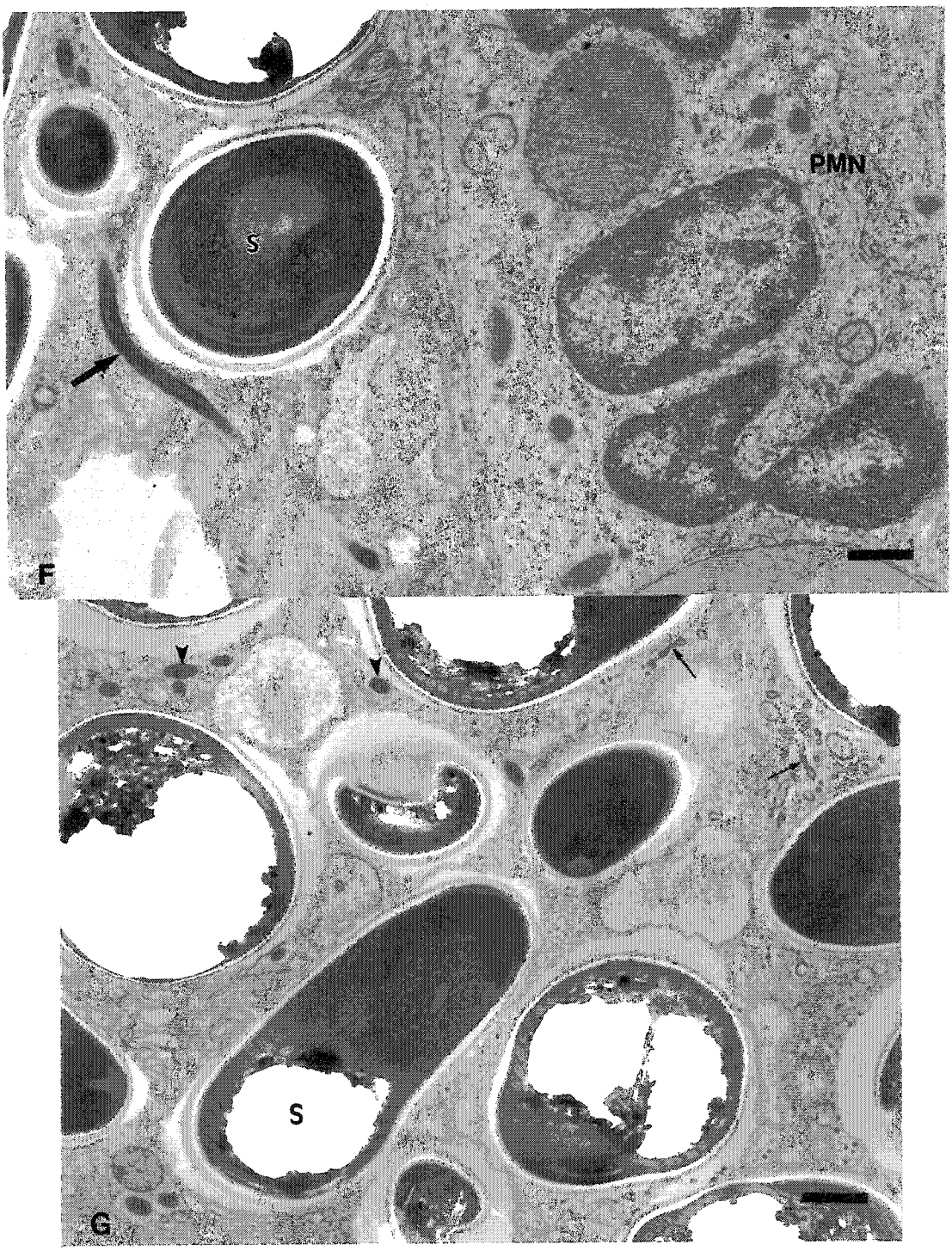


surrounded by the electron-dense pansporoblastic matrix. Other spores appeared wrinkled, collapsed or invaginated, with an electron-dense interior.

An important finding was the presence of everted polar tubes that were scattered in the cytoplasm of one of the round type xenomas (Fig. 4.12E,F). The polar tubes were not observed outside of the xenoma boundaries. Longitudinal and cross-sections of polar tubes were in close association with mature spores but none of the spores showed the characteristic emptiness that follows germination. The nucleus of the host cell was not observed, the RER was dilated, and mitochondria were not as numerous as those seen in wk 6 and 7 PE. Some small bulb-like structures observed inside SPOV and next to the spores resembled tubular appendages type I (Takvorian and Cali 1983, Cali and Takvorian 1999) (Fig. 4.12G). Other vesicular structures with a granular interior could not be identified as normal components of the host cell or from the parasite (Fig. 4.12G). Some of the xenomas located in the connective tissue of the filament showed areas of interruption in the integrity of their plasma membrane (Fig. 4.12E). Indeed, just small fibres and vesicles with granules were observed in these areas. Despite this, spores remained inside the xenoma and, simultaneous, the areas devoid of the xenoma membrane were adjacent to inflammatory cells (Fig. 4.12E). Inflammatory response characterized by numerous leucocytes was observed adjacent to and inside the xenoma. Many of the leucocytes were actively ingesting spores.

Flattened xenomas were located beneath the endothelium within the basement membrane of lamellar arteries close to the marginal capillary channel (Fig. 4.12H). These flattened xenomas had one surface that was parallel to the endothelium and another parallel to the lamina densa of the basement membrane. Mature spores appeared to be aligned within

Fig. 4.12. TEMs of *Loma salmonae,* wk 8 PE. **F.** Everted polar tube (arrow) close to an intact spore (S) in a round xenoma undergoing inflammatory reaction. PMN = neutrophil. Bar = 0.5 μm. **G.** Tubular appendages with bulb-like appearance (arrows) and everted polar tubes (arrowheads) within a round xenoma. S = spore. Bar = 0.7 μm.



the xenoma and occupied almost the complete cytoplasm stretching the host cell membrane in the points of contact with the parasite. Spores constituted the main parasitic stage observed within this structure although a few meronts were seen as well. The cytoplasm of the xenoma contained a few mitochondria, vesicles and typical type II tubules were adjacent to the spores. The xenoma was surrounded by a fine mesh of fibrils (Fig. 4.12H-J). The fibrils isolated the xenoma between the lamina densa and the endothelium (Fig. 4.12 I). These fibrils were not observed in the rest of the lamellae basement membrane nor in basement membrane of gill samples from the control group. Vesicles with electron-dense content were also observed between endothelial cells and the flattened xenoma (Fig. 4.12 I), and were most abundant toward the poles of the xenoma. Inflammatory reaction against this type of xenoma appeared absent. Serial sections from this xenoma showed that it had a spindle shape (Fig. 4.12J), and ended in a round tip occupied by a single spore (Fig. 4.12J).

Irregular-shaped xenomas were observed in the connective tissue of the filament, just below the lamellae and were filled with parasites in different stages of development (Fig. 4.12K). The plasma membrane of this type of xenoma was irregular and emitted several projections of different length in opposite directions. The larger projections were rich in mitochondria and the tips of some projections were dilated and contained one or more mature spores (4.12L). Some projections appeared to embrace the base of the lamellae. The xenoma did not show any indication of disruption of its cell membrane and it was impossible to recognize the identity of the original host cell. Tissue reaction against this type of xenoma was not observed.

Fig. 4.12. TEMs of *Loma salmonae,* wk 8 PE. **H.** Higher magnification of a portion of Fig.4.10. Flattened xenoma underneath lamella endothelium (arrows) and within the basement membrane (arrowheads). Spores (S) are aligned within this xenoma. RBC = red blood cell. Bar = 1.2 μm. **I.** Detail of one extreme of the flattened xenoma. It is surrounded by a fine mesh of fibrils (*) which isolated this xenoma from the endothelium (arrows) and the lamina densa (LD). Vesicles with electron-dense core (arrowheads) were localized underneath endothelium in the extremes of the xenoma. Bar = 3.8 μm. **J.** Single spore (S) observed in the last specimen of a serial section of the flattened xenoma (Xe). En = endothelium; F = basement membrane fibrils; LD = Lamina densa. Bar = 3.9 μm.

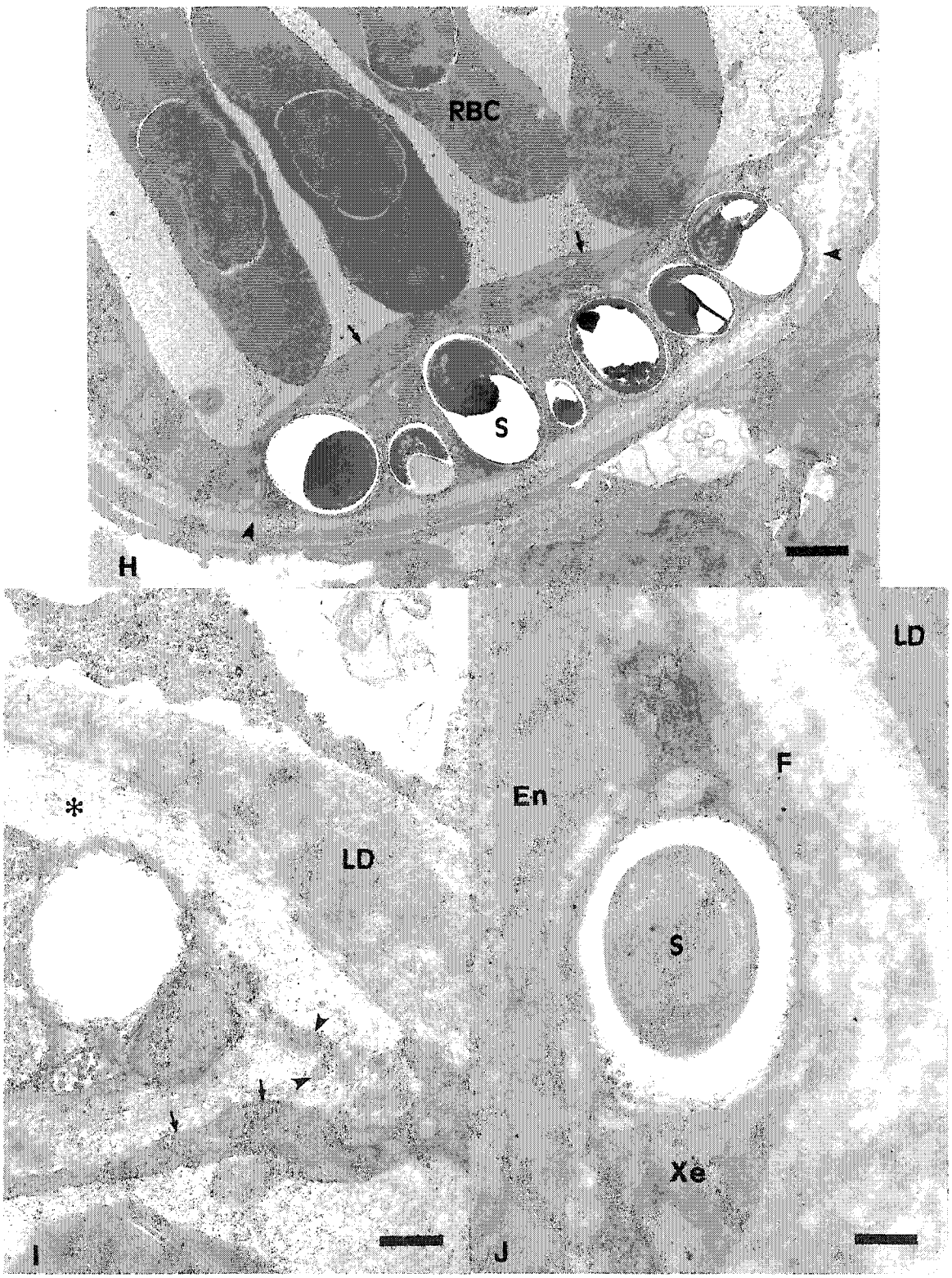
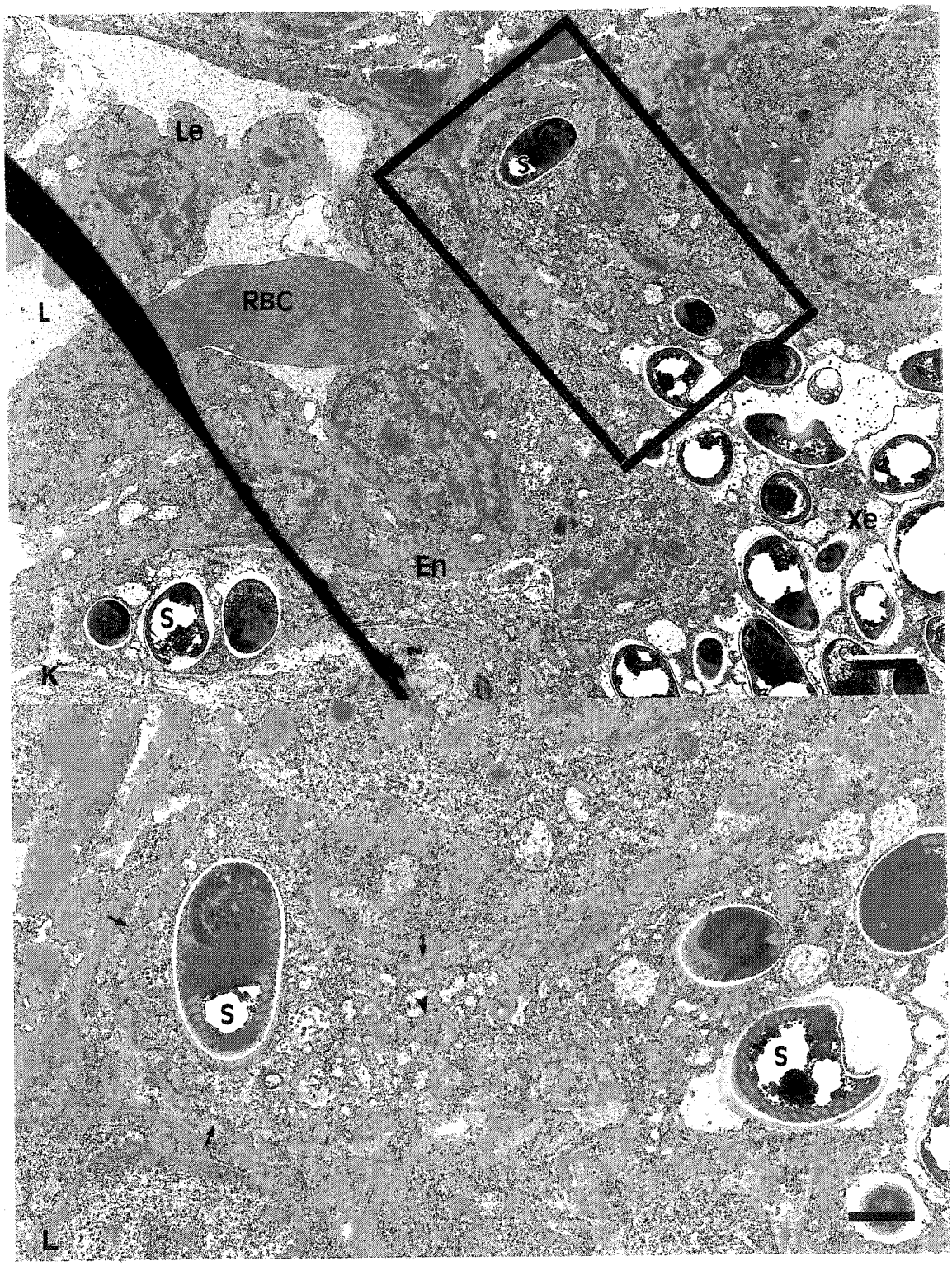


Fig. 4.12. TEMs of *Loma salmonae*, wk 8 PE. **K.** Irregularly shaped xenoma (Xe) located in filament connective tissue beneath endothelium (En) of lamella artery emits projections that contain spores (S). RBC = red blood cell; Le = leucocyte; L = Lumen. Bar = 2.5 μm. **L.** Detail of the boxed area in Fig. 4.12K. The plasmalemma of the xenoma projection (arrows) looks irregular and the cytoplasm contains spores (S) and mitochondria (arrowhead). Bar = 1.1 μm.



4.5 DISCUSSION

There are only a few light microscopic studies that have attempted to analyse the route of infection that *L. salmonae* uses for its establishment in the gills (Shaw et al. 1998, Sanchez et al. 2000). Most of the information regarding *Loma* infecting gills is related to the description of the morphological characteristics of different species of the parasite (Morrison and Sprague 1981a, Morrison and Sprague 1981b, Morrison and Sprague 1983) or the tissue reaction provoked in response to the microorganism (Markey et al. 1994, Kent et al. 1995). The present investigation represents the first ultrastructural study of gills during the first 8 wk of infection with *L. salmonae* and is an extension of previous studies which have attempted to follow the early stages of the parasite infection in fish (Speare et al. 1998b, Sanchez et al. 2000). In this study, the presence of cells in the gills containing 1 or more meronts is first described by wk 3 PE. The term infected cells has been used to describe those host cells harboring only and exclusively the merogonic stages of *L. salmonae* with no morphological evidence of sporogony and leaves the designation of xenoma (Weissenberg 1968, Weidner 1976, Sprague and Hussey 1980) to those complex structures containing parasites in all stages of development, which in this study were first recognized at wk 4 PE.

4.5.1 Early Stages

Weeks 1 and 2 PE. Gill samples from fish at wk 1 and 2 PE analyzed using HRLM did not reveal any structures that could be identified as intracellular parasites. This was not unexpected because previous studies demonstrated that *L. salmonae* DNA only began to be

detected in gills at wk 2 PE using PCR and *in situ* hybridization procedures in experimentally infected fish kept at 15°C (Speare et al. 1998a, Sanchez et al. 2000).

Loma salmonae could be circulating in the blood to, and through, the gills during the first 2 wk of infection inside a blood cell as a parasitic stage that was not resolved with HRLM. Another possibility is that the concentration of infected blood cells containing only a single meront in their cytoplasm was very low and, therefore, the probabilities of being observed using HRLM were low. The size of the gill sections analyzed for HRLM were much smaller and thinner compared with the paraffin sections of the entire gill arch used for the *in situ* hybridization study and represented much smaller amounts of gill tissue than that utilized for PCR. The *L. salmonae* DNA identified in gills at wk 2 PE could have been within infected blood cells free in the blood vessels and these cells can be easily lost during processing of the tissue for HRLM and TEM. Subsequently, between wk 2 and 3 PE, *L. salmonae* may have undergone a series of merogonic divisions within infected blood cells. These infected cells may be large enough to be slowed and trapped in blood vessels of the gills or displayed surface receptors that enable their phagocytosis by tissue cells or migration from the blood spaces in gills.

Week 3 PE. The presence of implanted infected cells containing only meronts in the connective tissue of the gills at wk 3 PE, suggests that, prior to this period of infection, *L. salmonae* was only undergoing merogonic divisions in the cytoplasm of a host cell free in the circulation. Sporogony stages were observed after the infected cells established residence in the gill.

The identity of the wandering infected cell remains unknown, but some blood cells may participate in the transport of microsporidia from the gut to the site of development (Canning et al. 1986, Dyková 1992). Sporoplasm-like structures have been found associated with epithelial cells of the gastrointestinal tract of coho salmon (*O. kisutch*) within 24 hr of oral exposure to spores of *L. salmonae* (Shaw et al. 1998). However, attempts to track the movement of *L. salmonae* from the gastrointestinal tract to the gills have failed (Shaw et al. 1998, Sanchez et al. 2000). Previous *in situ* hybridization studies have suggested the participation of intraepithelial cells in the transport of *L. salmonae* (Sanchez et al. 2001a, Sanchez et al. 2001b). Possibly the most viable cell candidates for the reception and subsequent transport of *L. salmonae* to the gills would be intraepithelial leucocytes, i.e., macrophages and lymphocytes. These cells are part of the cell population in the fish gut (Kaattari and Piganelli 1996, Rombout et al. 1998) and can migrate to interstitial tissue and blood. During its interaction with the epithelium of the gut, *L. salmonae* could specifically interact with these leucocytes and trigger and direct the eversion of the polar tube into those cells. A specific ligand-receptor interaction between a microsporidia and its target cell before the eversion of the polar tube, has been demonstrated (Foucault and Drancourt 2000). Further studies are needed to elucidate the specific cell type that is infected by spores of *L. salmonae* during their passage through the gut.

It has been suggested that the favourable conditions in the gills which allow sporogony may simply be attributed to the presence of pillar cells (Sanchez et al. 2000); however, this does not explain the occasional presence of xenomas in other organs. The present results indicate that the presence of pillar cells may not be the only condition for

initiating spore maturation since infected cells containing meronts and xenomas were observed at wk 3 and 4 PE, respectively underneath the endothelium of lamellar arteries, which is outside the normal limits of the pillar cells. The explanation for the predilection of *L. salmonae* for a certain site is unknown, and perhaps the first signals to remain in gills are interactions with some yet to be determined biochemical signals only encountered in this tissue.

In gill lamellae, the infected cells and xenomas were localized beneath pillar cells and the endothelium of lamellar arteries. Pillar cells are considered a specialized type of endothelial cell in the gill (Olson 1991). Evidence from this study suggests that infection of pillar cells by *L. salmonae* is the result of a secondary infection.

Infected cells in gills were confined to an interstitial position and no evidence of direct damage to them was observed by wk 3 PE, although scarce EGCs were observed in closed proximity to infected cells. This suggests that during this crucial time of localization in gills, the infected cells did not generate proinflammatory signals. A first step in the Ag recognition is the acidification of the phagosome, followed by fusion of the lysosomes and ultimate destruction of the parasites. The processed Ags are then expressed on the plasma membrane of the host cell (Watts 1997, Watts and Powis 1999). Acidification-blocking mechanisms have been known to occur with other microsporidians, i.e., *Glugea hertwigi* (Weidner and Sibley 1985, Weidner 1975), and with intracellular protozoans, including *Toxoplasma gondii* and *Trypanosoma cruzi* (Garcia-del Portillo and Finlay 1995). This could be the mechanism by which *L. salmonae* observed within pillar cells at wk 3 PE can survive phagocytosis. An interesting finding was the presence of EGCs near the infected cells and

xenomas in fish at wk 3 and 4 PE respectively, but not in control fish. An inflammatory response associated with EGCs in the gills of gilthead seabream (*Sparus aurata*) has been reported to occur after the intraperitoneal injection with extracellular products from *Pasteurella piscida* (Noya and Lamas 1997). Likewise, EGCs were described in the lamina propria of the intestine of a sergeant major fish (*Abudefduf saxatilis*) in response to the presence of xenomas of *Glugea* sp.(Reimschuessel et al. 1987). In the present study, the role of the EGCs in the gills of rainbow trout infected with *L. salmonae* is unknown. The close proximity of the EGCs with the blood vessels suggests their presence in the gills is by migration through the endothelium, as reported previously (Powell et al. 1990). Further study is necessary to elucidate the function of the EGCs during *L. salmonae* infection.

Week 4 PE. It is possible that at wk 4 PE, and during the process of sporogony, which is mainly characterized by the formation of an electron-dense chitinous coat, some xenomas have been recognized as foreign and have initiated an inflammatory response. The present findings substantiate previous research in which severe branchitis associated with early xenoma destruction was detected in experimentally infected rainbow trout at wk 4 PE (Speare et al. 1998b).

4.5.2 Late Stages

Weeks 5 and 6. Evidence given here and in previous studies indicate that immature forms of *L. salmonae* have a peripheral distribution within the host cell at wk 3 PE (Rodriguez-Tovar et al. 2002). At wk 5 and 6 PE, mature spores were peripherally distributed within the xenoma. In an ultrastructural study of *G. stephani* in the digestive tract (Weidner 1976), and

G. anomala in epidermal cells (Canning et al. 1982, Vávra and Ronny-Larson 1999), immature parasites were observed in the peripheral area within the xenomas, whereas mature parasites were localized in the centre of the disintegrated host cell cytoplasm. The peripheral localization of the immature parasites was suggested to occur in close association with host cell mitochondria as a response of an active parasite division (Weidner 1976, Vávra and Ronny-Larson 1999), although the time of the infection was not indicated in these studies. In the case of *L. salmonae*, only during the third wk PE did meronts appear to occupy the marginal area within the host cells, and by wk 5 and 6 PE, that area in xenomas was eventually occupied by mature spores. It can be hypothesized that the type of infected host cell or its proximity to the blood supply (O_2 concentration) could have an influence on the location of the parasite within the xenoma. The motive for infecting a host cell in the gills is still unknown; however several reasons can be suggested, e.g. infected blood cells containing immature forms (meronts) of *L. salmonae* could be ingested by the pillar cells, allowing the internalization and intracellular parasite development, as was hypothesized previously (Rodriguez-Tovar et al. 2002), and in this study meronts were observed in the cell body and flanges of pillar cells. Pillar cells are numerous in the capillary channels of the lamellae (Karlsson 1983, Olson 1991), and form a major component of the water-blood barrier, the site of gas exchange in fish. It would be advantageous to the intracellular parasite to have such immediate access to a rich O_2 supply, since microsporidia are without mitochondria (Vávra and Ronny-Larson 1999), and depend on their host for oxidative phosphorylation.

Another common finding at wk 6 PE was the abundance of inflammatory cells outside the xenoma. These leucocytes surrounding the xenoma were identified as neutrophils, macrophages, EGC, lymphocytes and plasma cells. Although there was no morphological evidence of damage to the xenoma plasmalemma during wk 5 and wk 6 PE, the close proximity of these inflammatory cells to the xenomas could indicate that some kind of inflammatory signal was being generated by these structures. Despite this, leucocytes were not observed attacking the xenomas, suggesting either a weak inflammatory signal from the xenoma membrane or the production of a substance by the parasite that temporally inhibited the leucocyte activity against xenomas. The xenoma plasma membrane and wall were not continuously covered by neighbouring fibroblast-like cells, but it was frequently exposed to the surrounding connective tissue. It is possible that antigenic parasite metabolites were expelled from the xenoma through these exposed areas and recognized at the beginning of the xenoma formation. This process was more pronounced at wk 6 and 7 PE. Similar diffusion mechanism of Ag through parasitic cysts has already been described for *T. gondii* (Reiter-Owona et al. 1996). This would explain the large number of leucocytes that were observed firstly surrounding, and then attacking the xenomas during the late stages of the infection in the present study.

Week 7 PE. Some round xenomas at wk 7 PE showed meronts that were apparently undergoing degenerative changes. The meaning of this remains unknown, but it could be due to the exhaustion of nutritive materials necessary to sustain the xenoma development. However, it could be an artifact of fixation and processing for TEM, although the adjacent

mature spores (which are more impermeable to fixatives), and even host cell mitochondria (the organelle which is used as an indicator of poor fixation) looked perfectly well preserved.

In some gill samples, mature spores found outside of the xenoma at wk 7 PE were captured and digested mainly by macrophages. Probably those spores were released from ruptured xenomas (ripe cysts) that underwent the attack of inflammatory cells (Morrison and Sprague 1981a). Ruptured xenomas probably were not observed in the specimens because they were either completely disintegrated or the xenoma was not present in the embedded piece of gill tissue or it was too far from the level of the cut section to be observed in TEM, since no ruptured xenomas were detected in subsequent serial sections. The ingested parasites showed the typical electron-dense coat (pansporoblastic matrix) and multiple levels of degradation as was previously described (Rodriguez-Tovar et al. 2002) at wk 4 PE. However, mature spores localized within the xenoma appeared to be ingested mainly by neutrophils. It has been reported that the migratory activity of the neutrophils in response to chemotaxis in some teleosts, like the carp (*Cyprinus carpio*), is higher than that described for macrophages, thus their arrival at the site of the infection occurs before the arrival of the macrophages (Neumann et al. 2001). If this was the case in our study, it would explain why the presence of neutrophils within some xenomas was more numerous than macrophages. However, the inflammatory cells may not be only responding to the xenomas to ingest and destroy the parasite, but in addition, it may be that at the end of the parasite's development, *L. salmonae* releases chemotactic factors that attract neutrophils and macrophages as an attempt to use them as host cells for autoinfection and proliferation. The secretion of mediators that recruit inflammatory cells to the site of the infection has been reported in

other infections by microsporidians (Didier and Bessinger 1999), however, there is no information available about any metabolite with chemoattractant properties released by *L. salmonae* and further studies should be addressed to elucidate the probable production of those substances by this parasite. Within some xenomas, a few ingested parasites did not show evidence of degradation and destruction, so it is possible that *L. salmonae* avoided the acidification process within leucocytes and could survive the phagocytic process, as was indicated with another fish microsporidian, *Glugea sp* (Weidner and Sibley 1985).

Week 8 PE. Inflammatory reactions to some of the round xenomas located in connective tissue were more severe at wk 8 and were evidenced by the disruption of the xenoma membrane. This damage coincided with the presence of inflammatory cells, mainly neutrophils and macrophages, in the areas where xenoma cell membrane damage was observed. Nevertheless, the stimulus that triggered the xenoma destruction is still unknown. Based on the results of this study, it is possible that the plasmalemma of those xenomas underwent antigenic variation that permitted direct attack by macrophages and PMN neutrophils. This phenomenon was previously hypothesized to occur in infections with other fish microsporidian (Dyková and Lom 1980, Dyková et al. 1980). In addition, the possibility that a toxic metabolite from the intracellular parasite caused damage to the host cell membrane to ensure its release from the xenoma cannot be excluded (Canning et al. 1986). *Loma salmonae* could have induced the production of a local leucocyte inhibitory substance during the xenoma rupture. This is based on the ultrastructural observation, at wk 8 PE, of neutrophils near areas with xenoma membrane damage and to mature spores (which appeared unaffected), regardless of the nearness of the phagocytic cells. Certain fish microsporidians

are able to induce the production of prostaglandins and leukotrienes from macrophages and cause immunosuppression and thus survive in the host (Shaw and Kent 1999). In the present study, macrophages were a main component of the inflammatory reaction against xenomas. It may be that, macrophages, under the effect of the microsporidia infection (as an evasive mechanism), could have secreted locally immunosuppressant substances. In contrast to phagocytic evasion strategies in prokaryotes, there is no information available on leucocyte suppressive substance produced by *L. salmonae*. Thus, additional studies for the future will be focused on the biochemical arsenal that this parasite could use for its defence.

A significant observation in some round xenomas at wk 8 PE was the presence of two types of tubular appendages in the same xenoma. In addition to the typical type II tubules (Cali and Takvorian 1999), type I tubules were also seen in round xenomas undergoing inflammatory reaction. This observation is important considering that there is only one study reporting the presence of 3 types of tubules in another fish microsporidian, *G. stephani* (Takvorian and Cali 1983, Cali and Takvorian 1999). Previous work reports the presence of only one type of tubule for *Loma* (Morrison and Sprague 1981a). The present study reports for the first time the simultaneous presence of 2 different tubular appendages during the infection by *L. salmonae*. Current information regarding the role of the tubules during the microsporidia development is merely speculative. Tubular appendages are thought to be formed by the spore during formation (Moore and Brooks 1992) as a connection between the host cell cytoplasm and the parasite to facilitate the interchange of substances. Also, they may increase the parasite surface area for active transport and disappear once the spore reaches full maturity (Morrison and Sprague 1981a, Takvorian and Cali 1983, Cali and

Takvorian 1999). In this study, the presence of both types of tubules was observed only in round xenomas accompanied by inflammatory reactions. It may be that under the host attack, transport between the immature spores and the host cytoplasm needs to be increased to complete their development. The presence of two types of tubular appendages may reinforce either the nutrition or maintenance of the sporogonic process. More studies are necessary to elucidate the role of these structures during parasite development and under the influence of inflammatory reaction.

Another remarkable finding at wk 8 PE was the observation of numerous everted polar tubes scattered through the cytoplasm of a round xenoma located in the filament connective tissue. For most microsporidia, the usual site for introduction of the infection is the host gut (Didier et al. 2000, Bigliardi and Sacchi 2001), although there are some microsporidians that can produce autoinfective spores and germinate within neighbouring cells of the same host in which they were generated (Cali and Takvorian 1999, Dunn and Smith 2001). The changes which cause the eversion of the polar tubes are multiple (Bigliardi and Sacchi 2001), involving alteration in the hydrostatic internal pressure of the parasite and/or the rapid catabolism of stored trehalose into smaller molecules (Undeen 1990, Cali and Takvorian 1999). In the present study, the close proximity of the spores to the leucocytes could have stimulated their germination in association with the phagocytic event (Didier and Bessinger 1999). Although several mature spores within xenomas were engulfed by leucocytes and looked empty, they did not show any evidence of cellular destruction. The presence of empty intact spores within leucocytes suggest these spores had everted their polar tubes expelling sporoplasms. The morphological features of the empty spores coincided

with those observed in previous studies about the germination mechanisms (Weidner et al. 1984, Cali and Takvorian 1999). Two possibilities could have occurred to explain the eversion of the polar tubes inside phagocytes within the xenoma: 1) Matures spores were ingested by the phagocytes within round xenomas and by action of the acidification phagocytic event, the polar tubes were everted (Didier and Bessinger 1999), or during the phagocytic process the polar tubes were extruded as a result of interacting with the host cell membrane (Magaud et al. 1997, Desportes-Livage 2000, Bigliardi and Sacchi 2001). Some authors consider this event to be a ligand-receptor dependant mechanism where spores first establish physical contact with the phagocytic cell membrane and after that, eversion can occur (Foucault and Drancourt 2000, Vivarès and Méténier 2001). 2) Germinated spores were ingested by phagocytes as part of the clearance mechanism. This possibility cannot be excluded either, since empty and intact spores were also observed in the xenoma cytoplasm and they eventually could be ingested by phagocytes. Several serial sections were made in an attempt to reveal more of the empty intact spores within leucocytes and xenoma cytoplasm. Although empty and intact spores were observed in direct contact with the phagocyte plasma membrane, no polar tubes were observed within leucocytes and no everted polar tubes attached to spores were seen in the xenoma cytoplasm. In addition, the possibility that some (to be determined) metabolites produced inside the xenoma could have activated the eversion of polar tubes before spores were ingested cannot be disregarded. Further ultrastructural studies will be necessaries to elucidate the eversion mechanisms inside the xenoma.

As part of the *L. salmonae* life cycle, autoinfection could be a way to perpetuate the disease in the susceptible host, as suggested for other microsporidia which use leucocytes for their replication (Matthews and Matthews 1980, Bekhti and Bouix 1985b, Didier and Bessinger 1999). With *L. salmonae*, uptake by phagocytes was thought to be involved with dissemination of the organism within the susceptible host (Hauck 1984, Shaw et al. 1998, Shaw and Kent 1999). Results from this study substantiate this hypothesis, although direct evidence of polar tubes piercing cells beyond the xenoma margins was not observed. However, from another ultrastructural study not related to this investigation, it was noticed in the gills of an Atlantic cod (*Gadus morhua*) infected with *Loma sp.* some leucocytes surrounding xenomas contained everted polar tubes as did the cytoplasm of xenomas (unpublished observations). This suggests that in *Loma,* infected leucocytes could be candidates for propagating the disease in the host by autoinfection.

Xenomas are usually described from microscopical studies as complex structures formed by hypertrophic host cells containing parasites in multiple stages of development (Weissenberg 1968, Weidner 1976, Sprague and Hussey 1980), and usually take a cystic shape. However, in the present study two unique features of xenoma morphology were observed at wk 8 PE. The flattened xenoma found under the gill endothelium confined within the basement membrane suggests the possibility that the parasite established there in order to evade the host attack and probably developed directly from the small infected cells containing only meronts that were observed beneath endothelium at wk 3 PE (Rodriguez-Tovar et al. 2002). Through the analysis of serial sections it was possible to determine that this flattened xenoma had a spindle shape and was mainly occupied by aligned mature

spores. The flattened form of this xenoma could have been caused by the external constraint of the lamina densa of the basement membrane on one side and from the endothelium on the other side, restricting the expansion of the developing xenoma, as typically occurs in other xenomas. The identity of the fibrils that surrounded the flattened xenoma is unknown, although their proximity to the endothelium could suggest that they are collagenous. Since these fibrils were not observed in the controls, it may be that the parasite multiplication underneath the lamellae endothelium promoted the formation of a new layer of extracellular matrix material to act as a barrier against the host defence mechanisms, or to strengthen the basement membrane confining the xenoma.

As was implied in the early study, a blood cell could be one possible candidate for harboring the parasite. Leucocytes have the ability to transmigrate through extravascular spaces by using enzymes (metalloproteinases), that selectively degrade components of the basement membrane (Madri and Graesser 2000), and by using cell-to cell interaction with the endothelial cells (Luscinskas et al. 2002). If this happened in the present study, then the flattened xenomas could have developed from infected leucocytes confined within the basement membrane underneath the endothelium while transiting from the blood vessels. Some of these infected cells successfully transmigrated through the basement membrane and reached the connective tissue and formed the xenomas observed in the filament, whereas others which were no longer capable of passing beyond the limit of the basement membrane becoming implanted within this barrier. This event of pathogen transportation and dissemination through blood cells has been well documented for *Tetramicra brevifilum* (Matthews and Matthews 1980), *Trypanosoma cruzi* (Bogdan and Rollinghoff 1999, Tyler

and Engman 2001), and *Mycobacterium* (Bermudez and Sangari 2001, Raupach and Kaufmann 2001). Nevertheless, in the case of *L. salmonae* more studies are necessary to identify which blood cell(s) is involved in the dissemination of the parasite in the host.

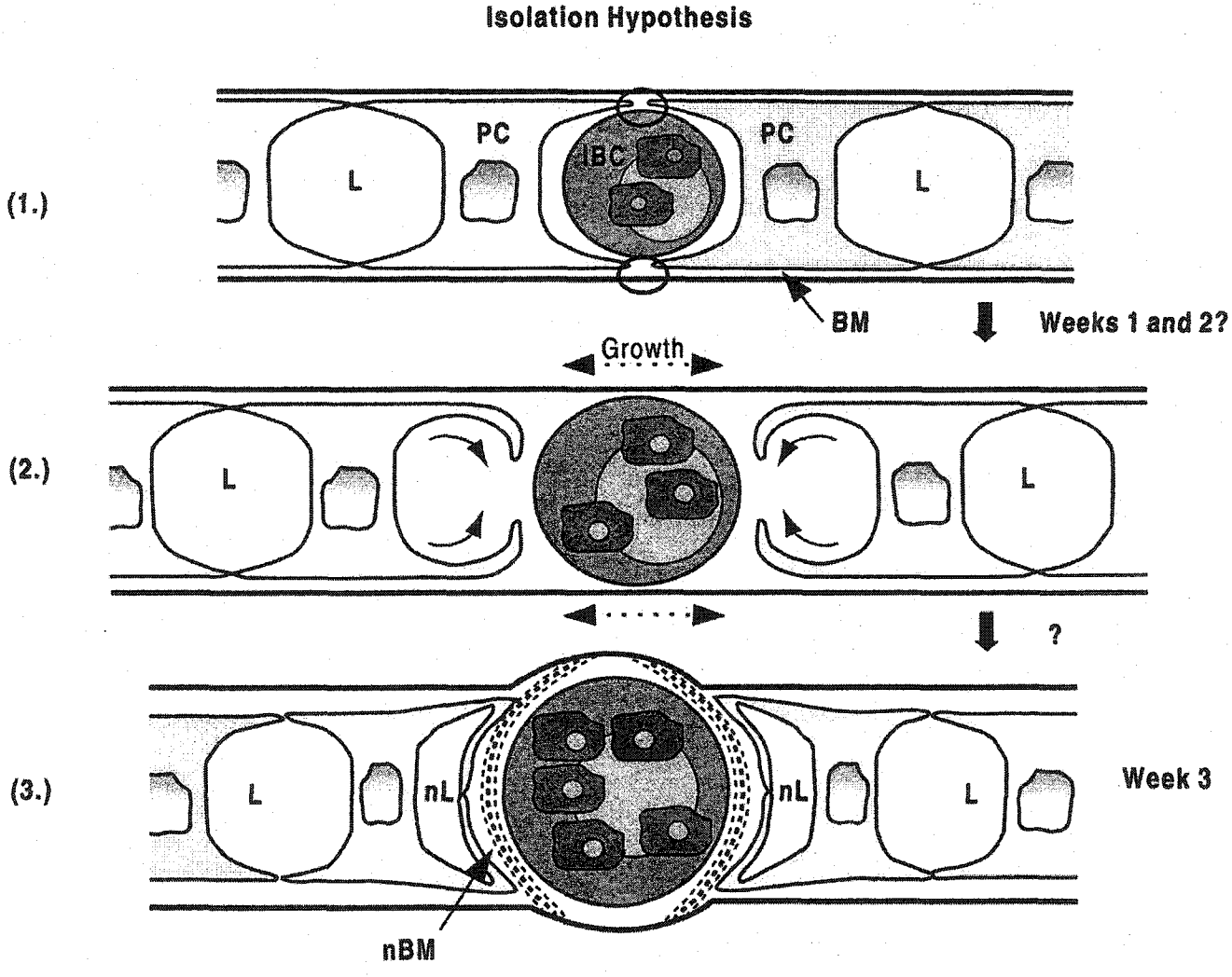
Additionally, small vesicles of variable diameters were observed only underneath the endothelium adjacent to the flattened xenoma and were more numerous towards the extremes of it. These vesicles were neither observed in the rest of the gill filaments nor in control fish. It is reported that under certain angiogenic pathological stimulation, such as inflammation, diabetes and tumours, the endothelial cells are able to shed vesicles containing matrix-degrading metalloproteases that help the wound repair (Foda and Zucker 2001, Taraboletti et al. 2002). In the case of *L. salmonae*, it can be hypothesised that under the parasite influence, endothelial cells shed vesicles containing those enzymes in attempt to degrade the extracellular matrix (basement membrane) and release the parasites from beneath endothelium allowing for spore dispersion in the host. However, the possibility that endothelium could be attempting to destroy the parasite cannot be disregarded. Further studies focused on the endothelial events that occur during the *L. salmonae* infection will elucidate the nature of those vesicles and their role in the elimination of the parasite.

The irregular xenoma type observed in the filament connective tissue, located at the base of the lamella and embracing it through large projections could be a way by which *L. salmonae* uses the host cell to spread within the gill tissue or to reach the capillary channels of the lamellae or both. The presence of spores in the tip of those projections could suggest that the parasite uses these structures to gain proximity to a blood vessel or potential host cells to infect them. Certain intracellular microorganisms are able to modify the cytoskeleton

(actin), of the host cell for spreading and infecting neighbour cells or for being transported to the exterior (Temm-Grove et al. 1994, Higley and Way 1997, Cossart and Bierne 2001, Suzuki and Sasakawa 2001). Although there was no morphological evidence of actin polymerization in this research, it does not mean that actin filaments were not present. However, the presence of numerous mitochondria in these projections could suggest some energy-dependant transport mechanism to the exterior. Further studies regarding modifications in the xenoma morphology will elucidate the role of this type of xenoma during the infection caused by *L. salmonae.*

Based on the morphological findings in the present study, 3 hypotheses are proposed to explain the localization of infected cells close to the blood channels of gill lamellae and lamellar arteries. In the first, the *Isolation Hypothesis* (Fig.4.13A), infected blood cells may arrive within the lumen of the lamellae and, because of their enlarged size they are stopped there, like emboli. This tissue selection could be mediated by specific receptors. Then, by direct effect of the parasite development, the continuous growth of the infected host cell could cause separation and retraction of the flanges of the pillar cells adjacent to it, after which the flanges would be reoriented inwards to cover and isolate completely the infected cell from the blood. The identity of the isolated host cell in the wall of the capillary channel is unknown. Its close proximity to the blood space suggests the participation of blood cells (possibly lymphocytes) in the transport and subsequent development of the xenoma in the wall of the blood channel.

Fig. 4.13A. Proposed hypothesis explaining the localization of *L. salmonae* in cells within the gill. Isolation Hypothesis (1.) During the first 2 wk of the infection pillar cells (PC) respond to a blood cell (IBC) infected with meronts within the capillary lumen (L) of a lamella by separating their connections (open circles). Separated pillar cells retract their flanges (curved arrows) and reorient them (2.) to isolate the infected cell from the lumen creating (3.) new basement membrane (nBM) and presumably a new blood space (nL). Infected blood cell eventually will develop into a xenoma; BM= basement membrane.



In the second, the *Internalization Hypothesis* (Fig. 4.13B), pillar cells acting as phagocytic cells may be secondarily infected by ingestion of infected blood cells. This infection of the pillar cell would cause disruption of its connections with the neighbouring pillar cells, which retract and rearrange their flanges to isolate the infected pillar cell from the wall of the blood space. The elimination of foreign material from the blood and the high phagocytic capacity of the pillar cells has been reported previously (Chilmoncsyk and Monge 1980). Although there was no evidence of cellular debris in the cytoplasm of the infected pillar cells, by wk 3 PE, the entire blood cell could be completely degraded. Furthermore, its intracellular clearance could have already occurred leaving behind the infective intracellular meronts which are resistant to degradation.

In the third, the *Evasion Hypothesis* (Fig. 4.13C), infected blood cells in the blood stream migrate through the endothelium of gill lamellar arteries and capillary channels and may become established beneath the endothelium within the confines of the basement membrane or migrate through the basement membrane and become established within the adjacent filament connective tissue tissue. The reasons for the migration of the infected cell from the blood are unknown, but this mechanism could allow the parasite to evade the attack of the host defence. Similar mechanisms of infection and surviving within leucocytes, and subsequent establishment in tissues, occur in other microsporidians (Weissenberg 1968, Weidner 1970). For example, phagocytic blood cells from turbot (*Scophthalmus maximus*) serve as vehicles for the microsporidian *Tetramicra brevifilum* migrating through the endothelium and eventually transforming into xenomas in connective tissue (Matthews and Matthews 1980).

Fig. 4.13B. Internalization Hypothesis. (1.) Pillar cells (PC) are secondarily infected apparently by phagocytosis of infected blood cells (IBC). This event occurs during the first 2 wk of the infection. (2.) The resulting infected pillar (IPC) cell enlarges and loses its connections with the neighboring pillar cells (open circles) resulting in (3.) its isolation from the capillary lumen (L) of the lamella. Intracellular meronts subsequently will divide into multiple parasites originating a xenoma; BM= basement membrane; nBM = new basement membrane.

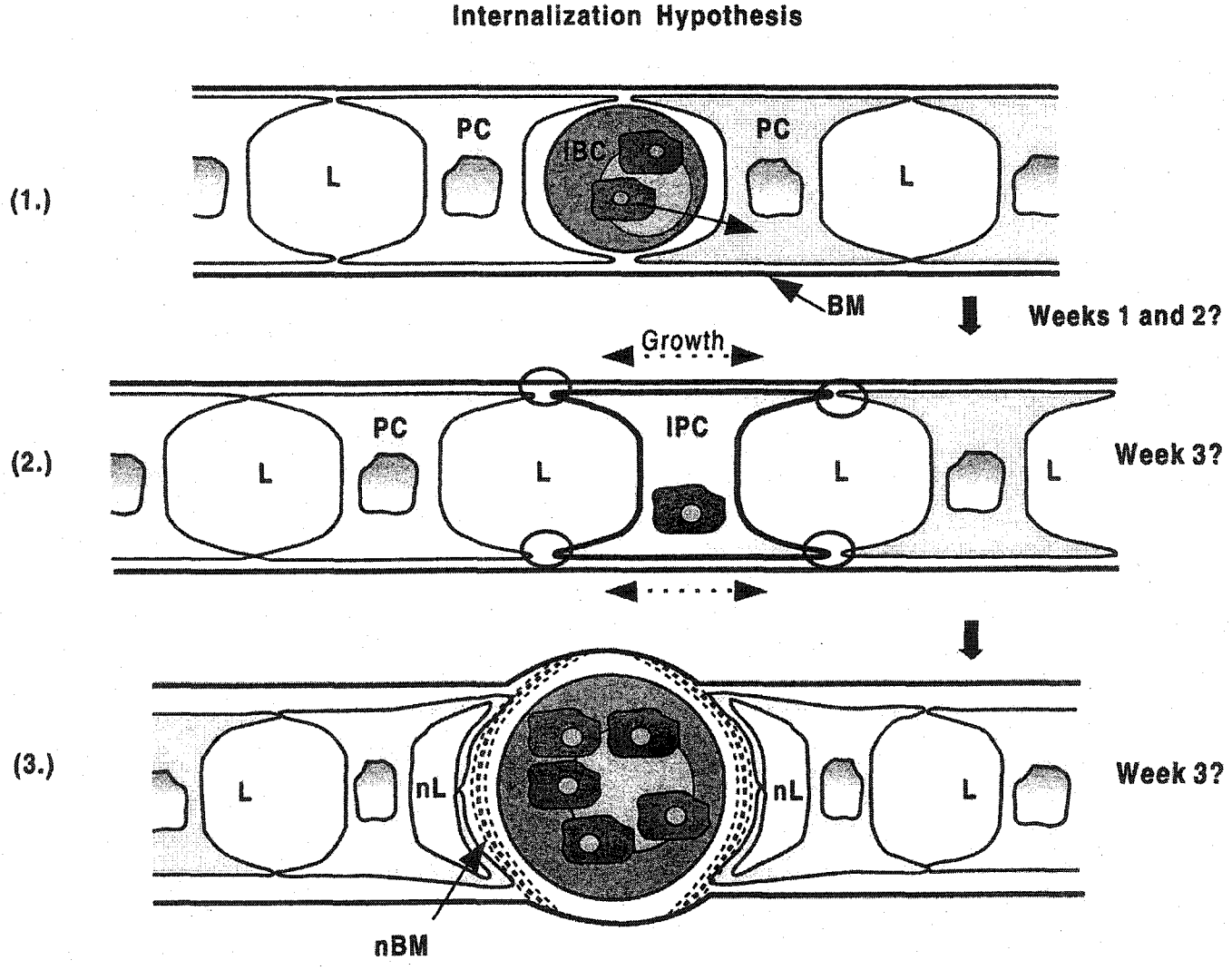
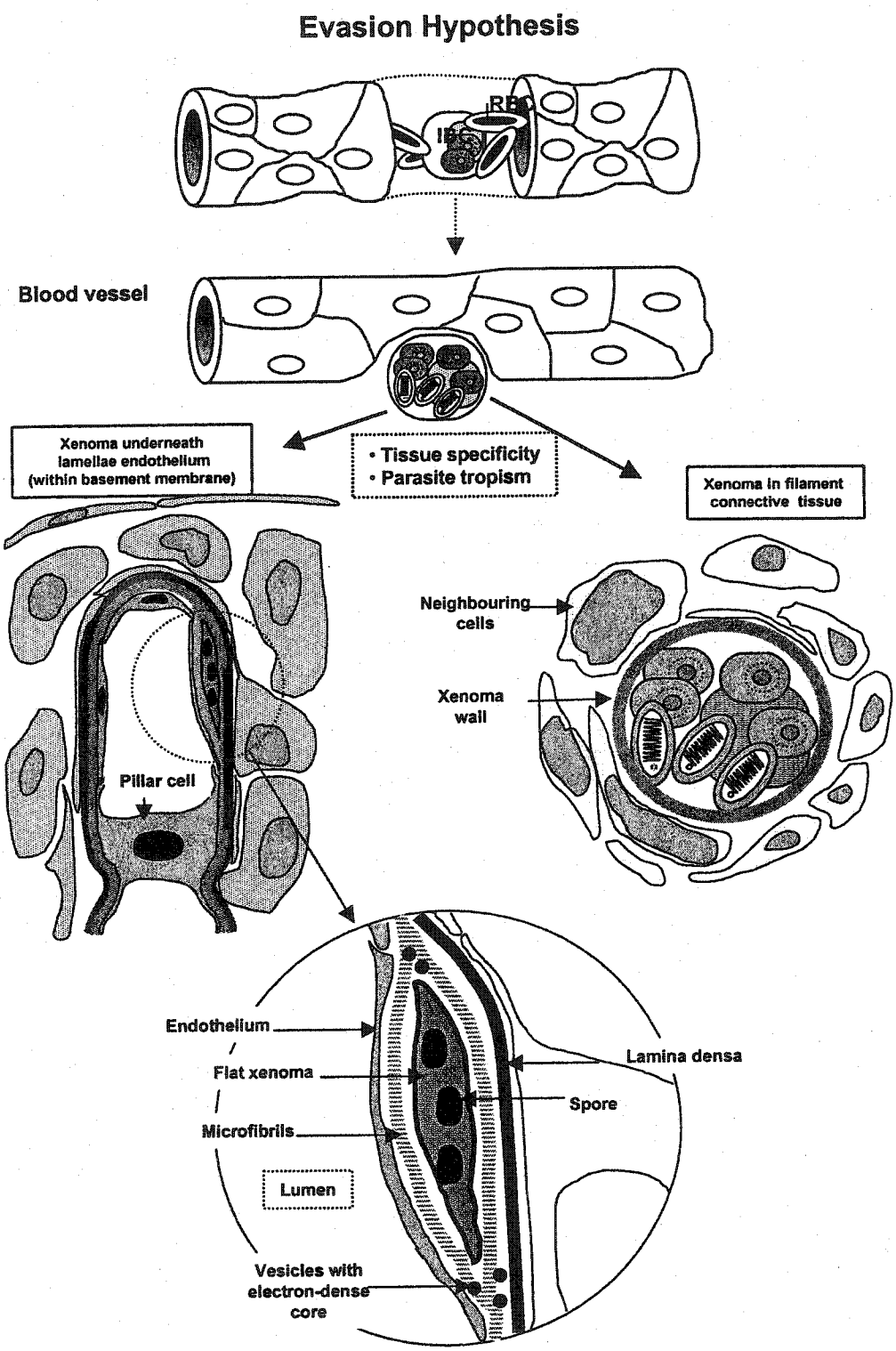


Fig. 4.13C. Evasion Hypothesis. Traveling infected blood cells (IBC) migrate out of the blood stream by wk 3 PE at the level of the lamellar arteries (LA) residing either underneath endothelium or in the adjacent filament connective tissue and then develop into a xenoma.



In summary, results from the present study reveal new information about the events that occurred during the *L. salmonae* infection. This research represents the first report of the ultrastructural changes that occur during the localization of *L. salmonae* in the gills of experimentally infected rainbow trout. Meronts are the earliest parasitic stages recognizable by the third week of infection. Pillar cells are secondarily infected with *L. salmonae* through a mechanism that could involve the phagocytosis and subsequent degradation of an infected blood cell. New vaccines or drugs against *L. salmonae* could take advantage of the phagocytic activities of pillar cells to avoid or block the establishment of this parasite. Endothelial cells appear not to be infected by the parasite. Weeks 5 and 6 PE were characterized by the presence of mature parasites in the peripheral areas within some xenomas and although inflammatory cells were observed around xenomas at wk 6 PE, they remained unaffected. Degenerative changes in meronts and inflammatory reaction to mature spores occurred by wk 7. At wk 8 PE two types of tubular appendages, I and II, were observed within round xenomas undergoing inflammatory reaction, although their role remains unknown. Round, flattened and irregular xenoma types were observed during the infection by *L. salmonae* and the observation of the last two types of xenomas could change completely the concept about the xenoma morphology.

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5 GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

5.1 GENERAL DISCUSSION

Loma salmonae is an intracellular parasite that infects the gills of fish of the genus *Oncorhynchus*. It is widespread in the wild Pacific salmon population in both fresh and marine habitats in the west coast of Canada (Kent et al. 1986, Constantine 1999). *Loma salmonae* provokes severe mortalities in coho (*Oncorhynchus kisutch*) and chinook salmon (*O. tshawytscha*) in British Columbia, causing economic losses (Magor 1987, Kent et al. 1986, Shaw and Kent 1999). Presently, there is no approved treatment for controlling the infection with *L. salmonae*. Some drugs (Kent and Dawe 1994, Speare et al. 1998b) and chemical agents (Speare et al. 2000) have been tested with good results under experimental conditions, and an experimental vaccination using an attenuated *L. salmonae* strain has given promising results in significantly reducing the parasitosis in rainbow trout exposed to live spores (Sanchez et al. 2001b). Noticeable advances in the systemic distribution of the parasite in the host during the course of the disease have been made using immunological and molecular techniques (Shaw et al. 1998, Sanchez et al. 1999, Sanchez et al. 2000, Sanchez et al. 2001c). However, the mechanisms that provide the resistance to this parasite and a complete understanding of its life cycle at the ultrastructural level have not been fully elucidated. Obviously, in fish culture, the control of *L. salmonae* is accomplished by avoiding infections and raising resistant fish for stocking. Thus, as part of an efficient treatment strategy or vaccination program to create resistance to *L. salmonae*, the

acquirement of such knowledge is crucial to develop strategies to eradicate this parasite from areas where it is enzootic.

The main goal of this thesis was to use a rainbow trout (*O. mykiss*) model, which has proven to be suitable for *L. salmonae* research (Speare et al. 1998a), to determine the temporal development of resistance to this parasite, to identify parameters of cell-mediated immunity and relate this to resistance and to describe how the parasite localizes and develops in the gills.

The exposure and re-exposure model (ERE) was used in this experiment because has proven to be a suitable method for studying the resistance mechanisms with many fish parasitic diseases, such as *Ceratomyxa shasta* (Bartholomew 1998), *Cryptobia salmositica* (Woo 2001), *Ichthyophthirius multifiliis* (McCallum 1986, Burkart et al. 1990, Buchmann et al. 2001), *Trypanosoma danilewskyi* (Islam and Woo 1991), and *Sphaerospora truttae* (McGeorge et al. 1996). In the majority of the studies relating resistance to fish microsporidiosis, like *L. salmonae*, several experiments involving ERE models have been carried out in fish exposed orally or intraperitoneally (IP) to either live or dead spores to determine the onset of xenoma development and resistance (Speare et al. 1998c, Kent et al. 1999). After xenoma formation in the gills, the fish recovering from disease remain resistant to subsequent re-challenges to the live inoculum. Thus, strong protection to L. *salmonae* is established after infection (Speare et al. 1998c, Kent et al. 1999), suggesting the establishment of immunological memory.

Previous ERE studies in fish considered the intensity of the infection and the prevalence of the disease in fish (Speare et al. 1998c, Speare et al. 1998d, Kent et al. 1999).

In the Chapter 2, the ERE model in combination with an efficient method for measuring sphere-like structures (Löfstedt and Ireland 2000) proved to be useful tools for the determination of the onset of resistance in rainbow trout re-exposed to live spores following an initial exposure with live or dead spores. For the first time the association between the growth of the xenoma and the time of its development in fish was demonstrated. The simple linear regression and the regression equation were appropriate tools for predicting the size of the xenomas based on the time of the exposure in naive fish exposed to live *L. salmonae* spores. The best period for detecting small xenomas under light microscopy was at week (wk) 4 postexposure (PE), and this time was the starting point to distinguish small from large xenomas, considered as originating from the initial exposure. This model proved to be highly predictive between wk 4 to 9 PE, and showed that the xenoma development had a consistent rate of diameter growth of 16 μm per wk. The onset of partial resistance after re-exposure to live spores occurred at wk 2 PRE, coinciding with the first detection of *L. salmonae*-DNA in gills (Sanchez et al. 1999, Sanchez et al. 2000, Sanchez et al. 2001c), whereas the onset of complete resistance occurred at wk 8 PRE, coinciding with an inflammatory response to the released spores. The inflammatory reaction was also associated with a delay in the xenoma growth at wk 5 and 6 PRE, and could be responsible for the early dissolution of xenomas at wk 4 PRE.

The inoculation of fish with dead spores IP induced an earlier and stronger refractory response to subsequent exposures with live spores by blocking the development of xenomas in gills at wk 6 PRE. The resistance of fish IP inoculated with dead spores suggested the presence of immunological memory against shared antigens (Ag) present in the live parasite,

and dead spores could be considered as a potential vaccine (Kent et al. 1999). Comparable results in the induction of resistance to inactivated parasites in fish following IP inoculation have been reported in rainbow trout (Stables and Chappell 1986, Whyte et al. 1990) and in channel catfish (Burkart et al. 1990). In the present study, development of cell-mediated immunity very likely contributed to the resistance in fish not only in those exposed to live spores, but to dead spores as well. Although the humoral response occurs in the fish infected with *L. salmonae* (Sanchez et al. 2001a), and in many microsporidia infections (Leiro et al. 1993, Hung et al. 1996), it is not effective for controlling or stopping the disease (Didier 2000). This likely is due to the intracellular habitat of this parasite, and possibly to the development of immunosuppression (Laudan et al. 1986a,b, Laudan et al. 1987, Pomport-Castillon et al. 1999). Thus, cell-mediated immunity is thought to contribute most to resistance mechanisms against microsporidia in mammals (Khan and Moretto 1999, Khan et al. 1999, Braunfuchsová et al. 2001), and in fish (Wongtavatchai et al. 1995). However, with *L. salmonae*, the occurrence of cell-mediated immunity after exposure to live and dead spores had not been demonstrated and its relation to protection was not known. Those issues were addressed in Chapter 3, in which the progression of the cell-mediated immunity was measured using *in vitro* lymphocyte proliferation. This method has been proved to be an useful tool to identify cell-mediated immunity in fish to different parasites (Taylor and Hoole 1994, Nie et al. 1996, Richards et al. 1996) and mitogens (Koumans-van Diepen et al. 1994). Based on the results of this study, the effector mechanisms of resistance to *L. salmonae* are probably more dependant on T cells than B cells. For example, it was shown that lymphocyte proliferative responses to the single exposure to live spores generated a maximum

stimulation index (SI) at wk 8 PE, when cultures were challenged with the crude soluble extract and dead spores from *L. salmonae*. As was observed in Chapters 2 and 4, this time coincided with a severe inflammatory response to the spores. However, when the anamnestic response was evaluated in recovered fish, re-exposed to live spores, a significant increase in SI occurred 1 wk after re-exposure but returned to levels seen with a single exposure in subsequent weeks. The SI observed in fish exposed to a single exposure with dead spores was considered higher and more consistent than that seen with fish exposed to the living parasite. The IP inoculation induced better proliferative response than the oral exposure to live spores, perhaps because of the high concentration of the inoculum deposited in the peritoneal cavity exposing a large population of APCs to spore Ag. Another reason for the high SI in this group could be in one structural component of the spore shell, chitin, abundant in microsporidia (Méténier and Vivarès 2001), and known to possess immunomodulatory properties (Sakai et al. 1992, Sakai 1999, Esteban et al. 2000, Seferian and Martinez 2000, Esteban et al. 2001).

Although rainbow trout initially inoculated with dead spores and then re-exposed to live spores had the higher SI, only partial protection was observed. Some fish injected with killed spores did show some xenoma development but at much reduced levels compared to naive fish challenged with live spores at the same time. However, a considerable reduction, ("nearly complete immunity") (Burkart et al. 1990), in the number of parasites in infected fish when compared with naive fish has been considered an acceptable criterion for protection by others (Burkart et al. 1990, Woo and Li 1990, Woo 1997). The difference in protection between live and dead spores suggest that the immunogen (s) may be either a

molecule that is rapidly turned over in the live spores (membrane proteins?) or an Ag that was denatured by the freezing procedure for killing the spores (Burkart et al. 1990). Besides, since live spores develop rapidly into merogonic and sporogonic stages in the host cell, structural constituents or byproducts of the different parasite stages (not found in the dead spore), could be a stimulus for triggering the protective immune response observed in the recovered fish. Hypothetically, during *L. salmonae* infection, the parasites are transported by leucocytes from the gut to the gills, where they are implanted and form xenomas (Shaw and Kent 1999, Sanchez et al. 2001c). However, the development of this parasite in gill tissue has not been investigated and an understanding this development is essential to create strategies for avoiding or stopping the parasite development in the susceptible host. The main aims of Chapter 4 were to describe the ultrastructural development of *L. salmonae* and to determine, what cell(s) harbors the parasite and to evaluate the tissue reaction generated in gills in fish exposed to live spores. In addition to the original ultrastructural descriptions of this parasite (Morrison and Sprague 1981b, Morrison and Sprague 1983), new information regarding the implantation of the parasite in gills and new features observed at wk 8 PE have been gathered in this study. After oral exposure, the parasite is first found infecting cells at wk 3 PE. Only meronts were observed inside infected cells associated with the capillary channels of gill lamellae and the lamellar arteries, suggesting that prior to this period of infection, *L. salmonae* was only undergoing merogonic divisions in the cytoplasm of a host cell free in the circulation. The inflammatory reaction against these immature parasites was absent, implying a low antigenic expression during merogony. An early inflammatory reaction to spores at wk 4 PE was observed and coincided with the early xenoma dissolution

described in Chapter 2 and by others (Speare et al. 1998d, Rodriguez-Tovar et al. 2002). Also, the proliferative response to ConA was reduced during this wk (Chapter 3). Apparently, this early host response to xenomas activated a surveillance mechanism since inflammatory cells were more frequently observed around xenomas at wk 5 and 6 PE, and this time coincided with the observation of intermediate xenomas in Chapter 2. Eventually, the inflammatory response to the parasite becomes more evident at wk 7 PE, and at wk 8 remarkable new findings probably associated with the inflammatory reaction were reported for first time in *L. salmonae* infection. As was described in Chapters 2 and 3, wk 8 PE has been characterized by an intense host response to the spores. In addition to the tubular appendage type II, the type I was also observed in xenomas under inflammatory reaction. It was suggested that the presence of tubular appendages could be to increase the transport of nutrients to the still immature parasites (Morrison and Sprague 1981a, Takvorian and Cali 1983, Cali and Takvorian 1999). In these same xenomas, everted polar tubes were observed associated with empty and intact spores within these xenomas maybe in response to the inflammatory reaction (Didier and Bessinger 1999). Additionally, two new morphological forms of xenomas, flattened and irregular, were observed beneath lamella endothelium and within connective tissue respectively. Flattened xenomas were suggested to occur when infected leucocytes were confined within the basement membrane beneath the endothelium while transmigrating to the connective tissue. The irregular xenomas observed in the filament connective tissue were suggested to occur as a spreading mechanism for infecting neighbouring cells. Finally, three hypotheses, Isolation, Internalization and Evasion, are proposed to explain the localization of the parasite in the gills.

In this study, some aspects of the biology of *L. salmonae* infection were investigated, which involved the study of the onset of resistance in naive and recovered fish exposed to live or dead spores. Additionally, the role of the cell-mediated immunity and its relationship with resistance in naive and exposed fish to live and dead spores and *in vitro* challenged with soluble and particulate *L. salmonae* Ag was investigated, and finally an ultrastructural study of the localization and development of this parasite in experimentally infected fish.

5.2 CONCLUSIONS

The ERE model in combination with the simple linear regression analysis were appropriate to determine the onset of resistance in rainbow trout exposed to *L. salmonae*. The onset time for partial and a complete resistance in fish exposed to live spores occurred at wk 2 and 8 PRE, respectively. In fish exposed to inactive spores, this period was observed at wk 3 and 6 PRE respectively. The use of inactive spores seems to be an excellent option for preventing and/or reducing the infection in gills. Moreover, an increase in the lymphocyte proliferation to the particulate and the crude soluble extract in the re-exposed and exposed groups compared to the control groups was evident. This suggests that there is a specific antigenic component in the response. The anamnestic response from recovered fish was not as high as that observed in fish exposed to dead spores. Fish exposed to dead spores developed the higher proliferative response, but protection in those fish was partial. Finally, it was demonstrated that meronts are the earliest parasitic stages recognizable by the third week of infection. Pillar cells are secondarily infected with *L. salmonae* through a mechanism that could involve the phagocytosis and subsequent degradation of an infected

blood cell. Endothelial cells appear not to be infected by the parasite. Weeks 5 and 6 PE were characterized by the presence of mature parasites in the peripheral areas within some xenomas and although inflammatory cells were observed around xenomas at wk 6 PE, they remained unaffected. Degenerative changes in meronts and inflammatory reaction to mature spores occurred by wk 7. At wk 8 PE two types of tubular appendages, I and II, were observed within round xenomas undergoing inflammatory reaction, although their function remains unknown. Round, flattened and irregular xenoma types were observed during the infection by *L. salmonae* and the observation of the last two types of xenomas could change completely the concept about the xenoma morphology.

5.3 FUTURE DIRECTIONS

This study has added new information about the onset of resistance to *L. salmonae* in rainbow trout exposed to *L. salmonae* live and dead spores at 15°C. However, it would be interesting to determine if exposure and re-exposure methods in different *Oncorhynchus* species would generate similar results. If so, future treatment or vaccination programs could be applied similarly for every salmonid species. Likewise, it would be important to determine if the rate of xenoma growth changes when fish are kept at different temperatures. As was suggested, temperatures beyond the permissive growth range for *L. salmonae* interrupt the life cycle of the parasite (Beaman and Speare 1999, Speare et al. 1999). Thus, it would be interesting to determine if that interruption is caused or related to some alteration in the xenoma growth. There is a need for extending the observations of the culture of lymphocytes using protein fractions derived from *L. salmonae* spores. The results obtained

from such experiments would better define which *L. salmonae*-Ag are responsible for inducing favorable responses and relate to protection. Also, it would be interesting to determine the "waves" of cytokines produced during the infection with this parasite. The information obtained would clarify the mechanisms that regulate the immune response during the *L. salmonae* life cycle. Finally, it is essential to recognize the cell(s) responsible for transporting the parasite from the gut to the gill. Transmission electron microscopy combined with molecular biology techniques (*in situ* hybridization) will definitively clarify this enigma. The knowledge acquired from this study will help to develop strategies to block the *L. salmonae* life cycle either before the transport of the parasite to the gills or before the parasite is implanted in the gills.

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