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PATHOBIOLOGY OF *LOMA SALMONAE*: PROGRESSION OF INFECTION AND MODULATING EFFECTS OF INTRINSIC AND EXTRINSIC FACTORS.

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Doctor of Philosophy

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, PE

June, 2000

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ABSTRACT

Loma salmonae is a microsporidian parasite of salmonid fishes, which forms xenoparasitic complexes (xenomas) in the gills of fish. Rupture of xenomas to release the infective spores results in branchial inflammation and, in some cases death. In this study, the polymerase chain reaction (PCR) and *in situ* hybridization (ISH) were used to monitor the progression of *L. salmonae* infection under typical and atypical conditions. At 15°C, after germination in the intestine, the parasite arrived in the heart 3 days post-exposure (PE), and in the gills by 2 weeks PE, where xenomas were visible by week 4 PE. ISH allowed the detection of the parasite during its first contact with the gut mucosal epithelium and its localization in the lamina propria of the intestine within 24 h PE. ISH detected infected cells harbouring *L. salmonae* in its transfer from the intestine to the heart (5 days PE), and in the gills of infected fish 2 weeks PE, when other methods failed to detect the parasite. Use of PCR also demonstrated that extreme temperatures have a strong suppressive effect in the progression of infection. Low temperature (5°C), delays the parasite arrival in the heart, and although arrival in the gills is not delayed, by week 4 PE it was no longer detected and xenomas failed to form. High temperatures (21°C) drastically upset the progression of infection with *L. salmonae*. Parasite DNA can be detected in the heart, gills, and spleen by 3 and 7 days PE, but further development is blocked and the parasite is no longer detected after this time. Thus temperature can be used effectively for the control of infections with *L. salmonae*. Further studies addressed the effects that acquired immunity has in the progression of infection with *L. salmonae*. In fish that are passively immunized, the arrival of the parasite in the heart is delayed for 7 days, but the disease progresses and xenomas form in the gills. In contrast, fish that have acquired immunity by previous exposure to the parasite block the passage of *L. salmonae* from the heart to the gills (and xenoma formation), after an extended delay to reach the heart. As comparison, *L. salmonae* is unable to complete its life cycle in Atlantic salmon and brook trout, although the parasite is able to infect the fish, it is cleared from the host system after 2 weeks PE. To probe the efficacy that vaccination against *L. salmonae* may have on salmonids, naive rainbow trout were exposed to *L. salmonae* SV, a variant of *L. salmonae* isolated in the laboratory with low-virulence for salmonids of the genus *Oncorhynchus* (rainbow trout, Chinook salmon, coho salmon), and a strong preference for brook trout and Arctic char (hence SV = *Salvelinus* variant). When the exposed rainbow trout juveniles were subsequently challenged with typical spores from *L. salmonae* 16 weeks after first exposure to *L. salmonae* SV, they were protected, presenting 14 times fewer xenomas per filament than the naive controls. The marked degree of reduction in numbers of xenomas that formed after challenge suggests that use of the attenuated strain should be further considered as a means to protect fish in regions where the parasite is endemic. These results suggest that a management control for disease with *L. salmonae* aimed at inducing resistance in fish, could comprise of exposure of the fish to the parasite, followed by a period at high or low temperature. Furthermore, vaccination of susceptible salmonids with the low virulence *L. salmonae* SV during hatchery rearing and before exposure to virulent *L. salmonae*, may protect them during grow out periods.

DEDICATION

A mis padres, Genaro† y Josefina, por su amor, apoyo y comprensión. Gracias.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	-	Acquired immunodeficiency syndrome
AP	-	Alkaline phophatase
BCIP	-	Bromo-chloro-indolyl-phosphate
bp	-	Base pairs
BSA	-	Bovine serum albumin
°C	-	Degrees Celsius
cm	-	Centimetre
dATP	-	Deoxyadenosine triphosphate
dCTP	-	Deoxycytosine triphosphate
DIG	-	Digoxigenin
DIG-11-UTP	-	Digoxigenin-11-deoxyuridine triphosphate
dGTP	-	Deoxyguanine triphosphate
DNA	-	Deoxyrribonucleic acid
dNTP	-	Deoxynucleotide triphosphate
dTTP	-	Deoxythymidine triphosphate
EDTA	-	Ethylenediaminetetraaetic acid
ELISA	-	Enzyme linked immunosorbent assay
Fab	-	Fragment antigen binding
g	-	Grams
GC	-	Guanine - Cytosine
h	-	Hours
H&E	-	Haematoxylin and eosin
HIV	-	Human immunodeficiency virus
IFAT	-	Indirect fluorescent antibody test
ITS	-	Internal transcribed spacer region
ISH	-	*In situ* hybridization
L	-	Litres
LS1	-	*Loma salmonae* primer 1
LS2	-	*Loma salmonae* primer 2
LSU	-	Large subunit
NBT	-	Nitroblue tetrazolium
MAb	-	Monoclonal antibody
mg	-	Milligrams
min	-	Minutes
ml	-	Millilitres
mm	-	Millimetre
mM	-	Millimolar
µl	-	Microlitre
µm	-	Micrometre
nm	-	Nanometre

PAS	-	Periodic acid-Schiff
PBS	-	Phosphate-buffered saline
PE	-	Post exposure
PCR	-	Polymerase chain reaction
pmol	-	Picomole
RER	-	Rough endoplasmic reticulum
RNA	-	Ribonucleic acid
RNAse	-	Ribonuclease
rRNA	-	Ribosomal ribonucleic acid
rTaq	-	Recombinant Taq
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	-	Seconds
SER	-	Smooth endoplasmic reticulum
ssDNA	-	Single stranded DNA
SSU	-	Small subunit
SV	-	*Salvelinus* variant
Ta	-	Annealing temperature
Taq	-	*Thermus aquaticus*
TBE	-	Tris borate EDTA
TBS	-	Tris-buffered saline
TEM	-	Transmission electron microscopy
Tm	-	Melting temperature
X-Gal	-	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

1 GENERAL INTRODUCTION

Loma salmonae is an economically significant microsporidian parasite of seawater netpen cultured Chinook salmon in British Columbia (Scholz, 1999). Little is known about the basic biology of microsporidians, and even less is known about the host-parasite relationships that develop during infestation. Not surprisingly, this paucity of understanding of basic biological issues has led to the current lack of applied methods of controlling the disease attributed to *L. salmonae* infections.

This thesis was conceived with the objective of defining the progression of infection and the initial developmental stages of *L. salmonae*, through the development and use of molecular biology (polymerase chain reaction [PCR] and *in situ* hybridization [ISH]) techniques combined with an *in vivo* model of infection. Secondary objectives were to determine the modulating effects that intrinsic (host) and extrinsic (environmental) factors have in the progression or termination of infections with this parasite.

The obstacles faced by a researcher at the start of a research program are varied. Similarly, readers of this thesis may find themselves at a similar starting point as the researcher. Consequently, the introduction, and the topics contained, are selected and arranged to assist the reader in gaining an appreciation of the problem, the research obstacles that we perceive to be present, and the importance of the objectives that we hoped to resolve.

1.1 Microsporidian parasites: A general introduction

Microsporidia are obligate intracellular protozoan parasites that produce spores (Dyková 1995; Müller 1997), and lack mitochondria, peroxisomes and a typical Golgi apparatus (Canning 1990; Müller 1997; Chalifoux et al. 1998). Microsporidial spores possess an elaborate extrusion apparatus where the principal part is an extrudible hollow polar tube through which the infective stage or sporoplasm is literally injected into a host cell (Lom and Dyková 1992; Dyková 1995). Microsporidia infect many animal species, although they are most commonly found to affect invertebrates (Canning et al. 1986). Moreover, they are also important parasites of fish and are widely distributed in freshwater, marine and estuarine habitats and can cause severe disease problems in aquaculture (Canning et al. 1986; Leiro et al. 1993; Bruno et al. 1995; Dyková 1995; Speare et al. 1998a). In human beings, microsporidia have been recognized as a serious health risk for immunosuppressed people (Canning and Hollister 1991; van Gool et al. 1993), leading to an increase in the research done in this group of parasites (Didier et al. 1991; Visvesvara et al. 1991). Despite this, much is still unknown about the pathogenesis and control of microsporidian infections.

1.2 Biological features

Microsporidia belong to the phylum Microspora, and represent one of the earliest lineages of eukaryotes (Canning 1990; Leiro et al. 1993; Dyková 1995; Hung et al. 1996; Kim et al. 1996; Solter et al. 1997). All microsporidia are intracellular parasites, but some such as *Enterocytozoon (Nucleospora) salmonis* can be intranuclear (Chilmonczyk et al.

1991; Antonio and Hedrick 1995). Microsporidial infections typically begin when the microsporidian spores (Fig. 1.1) are ingested by the host and germinate in the gut (Solter et al. 1997). This group of organisms has a spectacular mechanism of infection in which a polar filament, which lies coiled inside the spore, is everted through the spore wall with force, piercing the cell membrane of a host cell, injecting the infective sporoplasm, and initiating a new infection (Weidner 1976; Weidner 1982; Pleshinger and Weidner 1985; Canning et al. 1986; Dyková 1995). After the initial transfer of genetic material, the sporoplasms do not necessarily develop at the site of infection, and they may be transported to and develop in tissues other than the site of infection in the gut. Little is known about the transport of the early stages after infection, but it is speculated that undifferentiated mesenchymal cells, macrophages and body fluids are used in their distribution (Canning et al. 1986; Dyková 1995; Kim et al. 1996). Some insect-infecting species produce "primary spores" between 24-60 h after ingestion and infection (Solter et al. 1997); these primary spores are not infective *per os* but germinate spontaneously within the already-infected host cell and serve for the cell to cell dissemination of the parasite within an infected animal (Sagers et al. 1996). In typical microsporidia, development of the parasite inside the host cell begins immediately after infection, and there are two phases of development: merogony or schizogony and sporogony (Dyková 1995) (Fig. 1.2). In poikilothermic hosts, such as fish, microsporidian development is greatly influenced by temperature (Olson 1981; Antonio and Hedrick 1995).

Merogony is a phase of intense division and proliferation that serves to produce many parasite stages called meronts (Dyková 1995), and occurs usually in direct contact with host

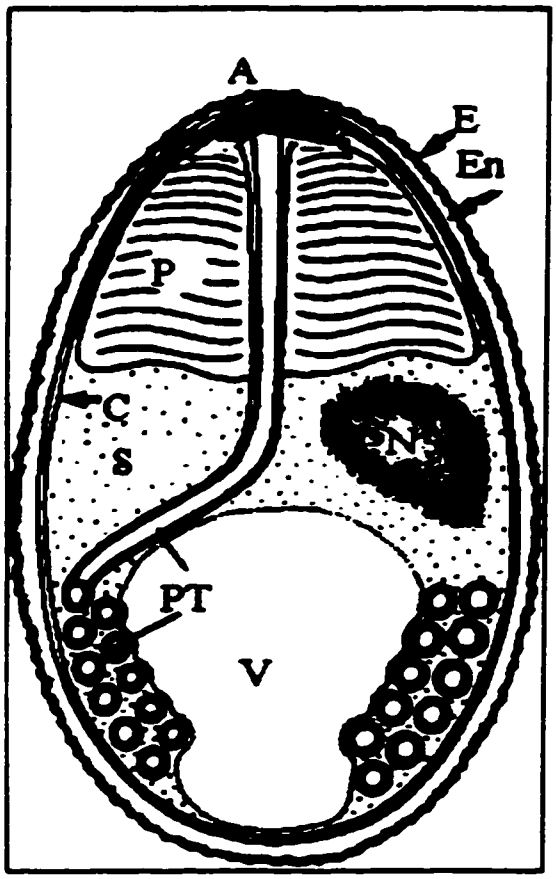

Fig. 1.1 Microsporian spore ultrastructure. A = anchoring disc; E = exospore; En = endospore; P = polaroplast; PT = polar tube; C = cell membrane; V = posterior vacuole; S = sporoplasm; N = nucleus. (From: Lom, J., Dyková, I. (1992) Protozoan parasite of fishes, Elsevier).

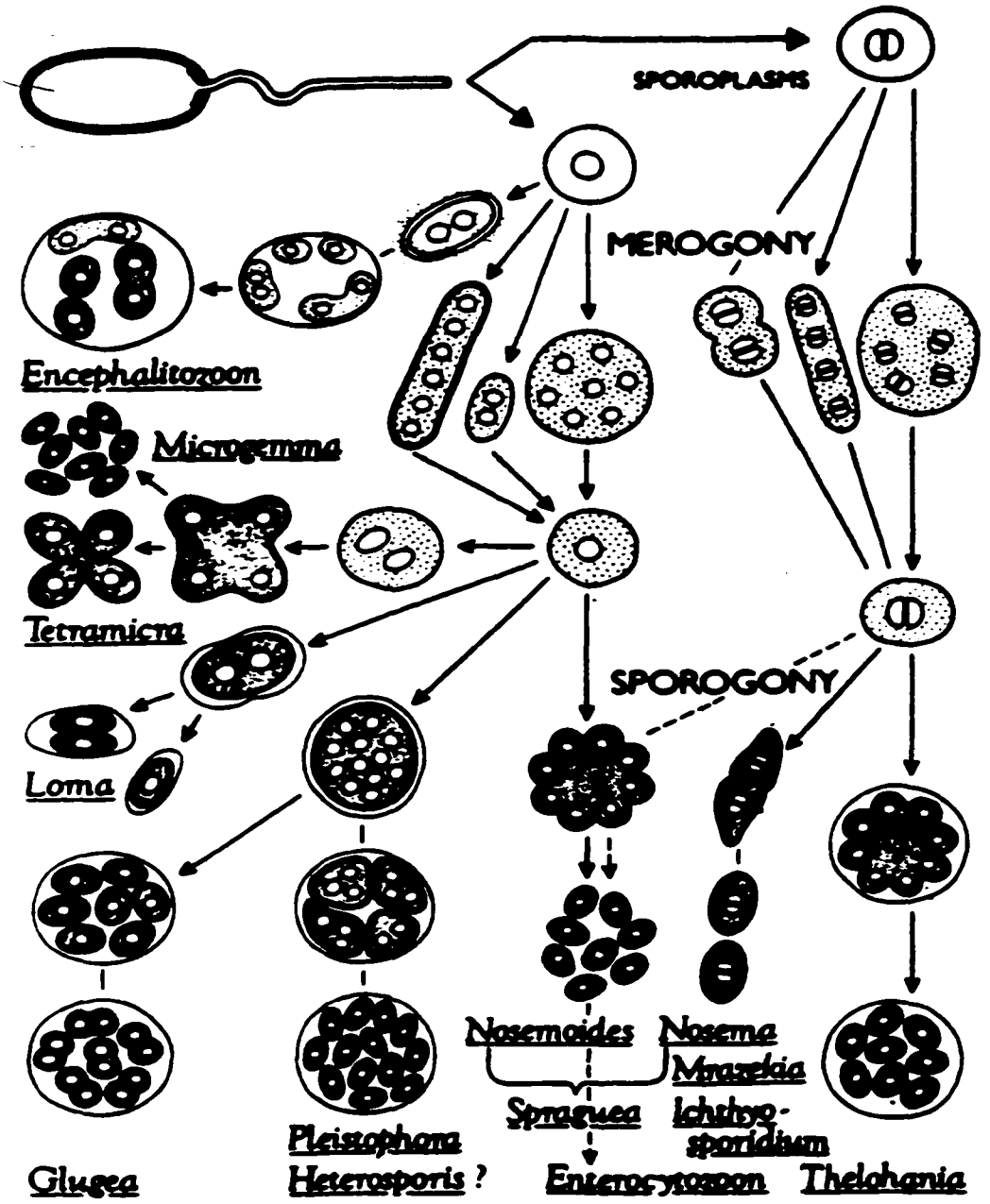

Fig. 1.2 Developmental stages of selected microsporidia. (Taken from Canning, E.U., Lom, J. and Dyková, I. (1986) The Microsporidia of Vertebrates. Academic Press, London).

cells' cytoplasm. Meronts are variously shaped, and may have one or two nuclei. They divide by binary or multiple fission or by plasmotomy, and with some exceptions (*Glugea*), have a simple plasma membrane (Canning et al. 1986).

Sporogony is the second phase in the microsporidian development and is responsible for the production of spores (Canning et al. 1986). During sporogony the meronts become sporonts and later divide into sporoblasts. Sporoblasts have an electron dense coat at their surface that will become the exospore layer of the spore wall (Canning 1990). Sporonts may be uni- or binucleated and divide directly into sporoblasts by binary fission (Morrison and Sprague 1983). Sporonts may also progress into a multinucleated stage that then undergoes two sequential stages of division; the cells undergoing the second division are called sporoblast mother cells (Dyková 1995). Sporoblasts are ovoid and their development culminates in the production of spores. During the spore maturation there is an increase in host cell smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER), while the host cell Golgi vesicles secrete the proteins for the polar tube. When maturation is complete, the obsolete Golgi apparatus forms the posterior vacuole of the spore (Canning et al. 1986; Dyková 1995). Microsporidia can be divided into two groups (Apansporoblastina and Pansporoblastina) based on the sporogonic processes in which the spores are either packaged within sporophorous vesicles (a membrane layer that develops around the sporonts, in addition to the electron dense surface coat secreted during the transition from merogony to sporogony) or dispersed freely in the host cell cytoplasm (Canning et al. 1986; Canning 1990). The spores can be released from the skin, the faeces, urine or from other tissues on the death of the host or after being eaten, and are highly

resistant to external conditions (Canning et al. 1986; Canning 1990; Dyková 1995).

Microsporidian spores (Fig. 1.1) are usually ovoid and vary greatly in size among species, from 2.5 x 2.5 μm in *Encephalitozoon cuniculi* (pathogen of rabbits) to 20 x 6 μm in *Mrazekia piscicola* (found in cod). The spore wall is made of two layers, the external proteinaceous layer, the exospore, is between 15 and 100 nm thick, whereas the inner, chitinous layer, the endospore, is about 150-200 nm thick (Canning et al. 1986; Weidner 1989; Lom and Dyková 1992; Dyková 1995). Inside the spore is an extrusion apparatus consisting of the polar tube, the anchoring disc, and a complex stack of membranes known as the polaroplast, the infective agent or sporoplasm, and a posterior vacuole (Weidner 1989). The polar sac or anchoring disc is found at the anterior end of the spore wall and the base of the polar tube arises from its centre. The polar sac is easily observed microscopically because it stains red with PAS (periodic acid-Schiff). The polar tube runs backwards towards the periphery of the spore and is coiled around the sporoplasm in the peripheral cytoplasm. A species-specific number of turns of the polar tube is typically observed in microsporidian spores (Canning et al. 1986). The part of the polar tube just below the polar sac is surrounded by the polaroplast. The remaining space within the spore is occupied by the posterior vacuole (Weidner 1989; Lom and Dyková 1992; Dyková 1995).

After entry into the cell that will ultimately permit sporogony some genera of microsporidia stimulate the infected cell to undergo a marked hypertrophy, with hypertrophic nuclei and surface modifications. These enlarged cells are called xenomas, and form a separate entity, made of the morphological and physiological integration of the host cell and the parasite (Bruno et al. 1995; Dyková 1995). The xenoma wall creates a boundary between

the microsporidian infected cell and the host (Dyková and Lom 1978), ensuring that free spread of the parasite does not take place (Canning et al. 1986), and frequently results in disfigurement, occlusion of body cavities and mortality (Matthews and Matthews 1980).

1.3 Microsporidia as pathogens

Microsporidia occur in all classes of vertebrates and most invertebrates (Aldras et al. 1994; Baker et al. 1995), especially insects (Dyková 1995; Becnel and Andreadis 1998; Solter and Maddox 1998b). Pathogenicity of microsporidia in insects, suggests that these parasites may form the basis of alternative or biological control of some insect plagues (Sagers et al. 1996; Bauer et al. 1998). Microsporidia have also been detected in humans, nonhuman primates, dogs, cats, blue foxes, foxes, pigs, goats, sheep, horses, rabbits, mice, (Didier et al. 1998), fish (Morrison and Sprague 1983) and birds (Black et al. 1997). Mammals are mostly affected by microsporidia species within the genera *Enterocytozoon, Encephalitozoon, Pleistophora, Trachipleistophora, Nosema, Vittaforma, Thelohania,* and *Microsporidium* (Didier et al. 1998), although other genera of microsporidia may also be infective. Microsporidial infections are the cause of significant economic losses in the silkworm, honey bee, fish and fur industries (Canning et al. 1986). Furthermore, *Enterocytozoon bieneusi, Encephalitozoon hellem* (Didier et al. 1991) and *E.(Septata) intestinalis* (Aldras et al. 1994; Hartskeerl et al. 1995; Bigliardi et al. 1996) have become a serious threat to people with acquired immune deficiency syndrome (AIDS) resulting from infection with the human immunodeficiency virus (HIV). This threat has added to the urgency of better understanding of this group of obligate intracellular parasites (Canning and

Hollister 1991; Baker et al. 1995; El Fakhry et al. 1998).

1.4 Microsporidia in fish

Microsporidian infections are common in many species of fish, and may cause severe mortalities (Pulsford and Matthews 1991), especially when conditions that favour transmission are present in hatcheries or culture sites (Leiro et al. 1994; Leiro et al. 1996). Microsporidian infections in farmed fish (which are generally stocked at high densities) are dangerous when they are allowed to persist (Estevez et al. 1992). In valuable species like salmonids (Morrison and Sprague 1983; Elston et al. 1987; Bruno et al. 1995), flatfishes (Laudan et al. 1989; Leiro et al. 1994), and eels (Buchmann et al. 1992), this may lead to great mortality and economic losses (Constantine 1999). There are eleven genera of microsporidia known to infect fish: *Microsporidium, Enterocytozoon (Nucleospora), Glugea, Heterosporis, Ichthyosporidium, Loma, Microfilum, Microgemma, Pleistophora, Spraguea,* and *Tetramicra* (Dyková 1995).

Several species of fish infected with microsporidia can mount an immune response and develop antibodies against the parasite (Buchmann et al. 1992; Enriquez et al. 1998), and may also become resistant to reinfection, as happens with fish exposed to some protozoan parasites like ciliates, flagellates, and myxosporidians (Leiro et al. 1993). However, it has been noted that microsporidian infections can also debilitate the fish and suppress the host's immune response to foreign antigens (Laudan et al. 1987; Laudan et al. 1989; Antonio and Hedrick 1995; Wongtavatchai et al. 1995a).

1.4.1 Transmission

Transmission of microsporidia usually takes place when spores are ingested (Shaw et al. 1998). After ingestion, the spores germinate in the gut lumen (Weidner 1976; Weidner 1982; Weidner 1989) and enter epithelial cells (Canning et al. 1986; Laudan et al. 1989). Microsporidia may develop at the site of infection or they may be transported to other sites of development possibly within monocytes (Canning et al. 1986; Dyková 1995; Kim et al. 1996). Direct horizontal transmission between fish is probably the most common way of transmission of microsporidia, and occurs after xenomas rupture and liberate the spores in the water column (Dyková 1995). Experimentally, microsporidian infections can be transferred from fish to fish by intramuscular injection of spores (Matthews and Matthews 1980; Dyková 1995; Shaw et al. 1998).

1.4.2 Diagnosis

Diagnosis and identification of microsporidia genera has largely depended on the identification of the morphological characteristics of the spores or the merogonic and sporogonic stages (Canning et al. 1986; Canning 1990; Dyková 1995; Wongtavatchai et al. 1995b); however, the small size of the spores makes this method difficult (Didier et al. 1995). Conclusive identification has usually only been accomplished by transmission electron microscopy (TEM) (Markey et al. 1994; Wright and Lynn 1995) which has been the traditional "gold standard" for microsporidia identification (Didier et al. 1996). Until recently, TEM was the only method to diagnose fish microsporidia (Dyková 1995), and species were recognized by the number of coils of the polar tube in the spore (Didier et al.

1996).

Histochemical methods developed for microsporidian detection rely on the use of chitin-staining fluorochromes, PAS, Giemsa, and modified trichrome staining (Visvesvara et al. 1991; van Gool et al. 1993; Didier et al. 1995; Dyková 1995; Didier et al. 1996). Live spores are easy to identify because of the large posterior vacuole, and the polar cap reacts positively to PAS and appears as an intense red dot. In tissue the spores appear dark blue with Giemsa stain.

Serological methods that use polyclonal and monoclonal antibodies, including the indirect fluorescent antibody test (IFAT), and the enzyme linked immunosorbent assay (ELISA) have been used successfully to detect subclinical microsporidian infections in humans (Niederkorn et al. 1980; Canning and Hollister 1991; Zierdt et al. 1993; Aldras et al. 1994; Didier et al. 1995) and other mammals (Halánová et al. 1999). These may become important tools in fish microsporidian diagnosis (Pomport-Castillon et al. 1997a; Enriquez et al. 1998). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been successfully used to differentiate microsporidian species, due to species-specific differences in spore protein profiles (Canning and Hollister 1991; Visvesvara et al. 1991; Leiro et al. 1994).

However, most of the methods currently available are often inadequate for species identification. Therefore, molecular methods, especially the polymerase chain reaction (PCR) have been developed for the identification and diagnosis of microsporidian infections, particularly in humans (David et al. 1996; Didier et al. 1996; Fedorko and Hijazi 1996; Weiss and Vossbrinck 1998) but also in economically important animals (Kent et al. 1996;

Black et al. 1997; Docker et al. 1997; Pomport-Castillon et al. 1997b; Bell et al. 1999).

1.4.3 Treatment

There are no approved chemotherapeutic agents in Canada for the treatment of microsporidian infections of farmed fish (Mullins et al. 1994; Speare et al. 1998d). The effects of several chemotherapeutants, such as triazine derivatives (Schmahl and Senaud 1996), toltrazuril (Schmahl et al. 1990), and quinine hydrochloride (Speare et al. 1998d), in the development of microsporidian infections have been studied with varying results. However, fumagillin, which acts by inhibiting DNA or RNA synthesis (Kent and Dawe 1994; Schmahl and Benini 1998), has been successful in treating experimental microsporidian infections in fish (Kent and Dawe 1994; Brocklebank et al. 1995) and in humans (Didier et al. 1996; Molina et al. 1997). Although fumagillin was toxic when used in fish at high doses (Kent and Dawe 1994), the fumagillin analog TNP-470 was efficacious in reducing microsporidian associated pathology without associated toxicity (Higgins et al. 1998). Benzimidazole derivatives (albendazole, mebendazole, and fenbendazole), which interfere with the formation of the microsporidian merogonic and sporogonic stages by preventing microtubule assembly, irreversibly damage all developmental stages of *Glugea* (Schmahl and Benini 1998), and could be a good agent for use in control of microsporidian infections.

Considering the fact that licensed therapeutics agents have not been approved for treating fish infected with microsporidian infections in Canada, the only current option to prevent disease outbreaks is the implementation of management policies that can limit the

economic effects of the infection (Speare et al. 1998a), coupled with good detection and diagnostic methods.

1.4.4 Pathology

Studies on the pathology of microsporidia infections in fish show two different patterns of tissue reaction that depend on the presence or absence of xenomas, both leading to the host cell destruction by replacing cellular contents with mature spores (Dyková 1995).

Microsporidian xenomas may be observed in cells and tissues as varied as connective tissue between the muscle fibres (Estevez et al. 1992), neurocytes, oocytes (Dyková 1995), and gills (Morrison and Sprague 1983), and may reach several mm in diameter (Dyková and Lom 1980; Dyková 1995). Tissue reaction is generally directed towards isolation of the parasite by deposition of concentric layers of collagen fibres that results in xenoma encapsulation (Dyková and Lom 1978; Dyková and Lom 1980; Laudan et al. 1989; Dyková 1995). Xenomas have 3 stages of development: early, grown, and mature. Early xenomas do not induce a host cell reaction and host tissue is damaged by pressure-atrophy and tissue displacement as the xenoma grows (Dyková and Lom 1978; Dyková and Lom 1980; Matthews and Matthews 1980; Laudan et al. 1989; Dyková 1995). Mature xenomas are filled with spores and elicit an inflammatory response with formation of granulation tissue. This inflammatory response consists of proliferating fibroblasts, histiocytes and new capillaries, and coincides in some species with changes in the xenoma wall that result in its complete disappearance (Dyková and Lom 1978; Dyková and Lom 1980; Dyková 1995). At this stage the xenoma becomes a granuloma with a spore mass in its centre. In the mature

xenoma that has lost its wall, spores are eliminated by phagocytosis and subsequent necrosis of the phagocytes (Dyková and Lom 1978; Dyková and Lom 1980). The granulation tissue matures and the granuloma diminishes until there is resolution of the tissue lesion.

However, in the *Pleistophora*-type infection there is no xenoma formation. The tissue reaction is more severe, as the infective and replicating stages can invade other muscle fibres during merogony and sporogony (Dyková and Lom 1980), destroying and replacing the contents of infected cells without inducing hypertrophic growth (Dyková and Lom 1980; Pulsford and Matthews 1991; Dyková 1995). During the proliferative stages of the parasite there is cell infiltration of the myosepta by lymphocytes and macrophages (Matthews and Matthews 1980; Dyková 1995), but phagocytosis of spores occurs only after the mature spores are released from the disintegrated muscle fibres (Dyková and Lom 1980). In some infections, groups of infected muscle tissues may become surrounded by connective tissue, forming large granulomas (Dyková and Lom 1980).

1.5 Genus *Loma*

Loma is a genus of fish-infecting microsporidia whose xenomas are found in the gills of infected fish (Morrison and Sprague 1983). Meronts are uninucleated and become multinucleate cells that grow into multinucleate cylindrical plasmodia, embedded in the host cell cytoplasm (Morrison and Sprague 1983). Sporogony is polysporoblastic and up to 8 spores are formed within a single parasitophorous vacuole (Morrison and Sprague 1983; Canning 1990). The infected cells become hypertrophied and form xenomas, with a single central and hypertrophied nucleus, where spores are mingled with developing stages, and a

xenoma wall is formed by the host cell membrane coated with layers of fibrils (Lom and Dyková 1992).

There is a growing list of species within the genus *Loma*, which include *L. dimorpha* (Arias et al. 1999), *L. salmonae* (Morrison and Sprague 1983), *L. camerounensis* (Fomena et al. 1992), *L. morhua* (Morrison and Sprague 1981a), *L. branchialis* (Morrison and Sprague 1981b), *L. fontinalis* (Morrison and Sprague 1983), *L. diplodae* (Canning et al. 1986), and *L. embiotocia* (Shaw et al. 1997).

1.5.1 Description of *Loma salmonae*

Loma salmonae belongs to the phylum Microspora, class Microsporea, order Microsporida, suborder Pansporoblastina, family Glugeidae (Canning 1990). This parasite is widespread in rainbow trout (Bruno et al. 1995; Bader et al. 1998), Chinook salmon *Oncorhynchus tschawytscha* (Hauck 1984), and coho salmon *O. kisutch* (Kent et al. 1989; Lom and Dyková 1992), where it infects the secondary lamellae of the gills (Hauck 1984) plus other vascularized tissues, including the heart, spleen, kidney, and pseudobranch, although not with the same frequency as the gills (Canning et al. 1986; Kent et al. 1989; Markey et al. 1994; Kent et al. 1995). *Loma salmonae* is found in North America (California, Georgia, British Columbia) and in Japan, and is widespread in hatchery netpen cultured, and wild salmonids (Canning et al. 1986; Markey et al. 1994; Kent et al. 1998). It has also been reported in France (Lom and Dyková 1992; Bruno et al. 1995) and in England and Scotland (Bruno et al. 1995). *Loma salmonae* was first reported in Canada in 1986 (Magor 1987). Gill disease associated with *L. salmonae* is a problem affecting the

production of Pacific salmon, especially Chinook salmon (Kent and Dawe 1994; Kent et al. 1998; Speare et al. 1998a). A recent survey of pathogens in marine fishes from Pacific water off British Columbia confirms the presence of *L. salmonae* in Chinook salmon, chum salmon *O. keta*, coho salmon, sockeye salmon *O. nerka*, pink salmon *O. gorbuscha* and some non-salmonid fishes (Kent et al. 1998). This finding suggests that feral fish can act as reservoirs for *L. salmonae* and a source of infection for farmed salmonids (Docker et al. 1997; Kent et al. 1998).

Loma salmonae was considered essentially a freshwater parasite; however, infections can persist after transfer of fish to seawater, where the inflammatory response is severe (Kent et al. 1989), and recent experiments have shown that *L. salmonae* can also be transmitted in seawater (Kent et al. 1995). *Loma salmonae* spores can be naturally transferred *per os* during cohabitation by the release of spores from infected fish, or experimentally by intramuscular and intravascular injection of spores in naive fish (Shaw et al. 1998). Fish that clear the infection become resistant to subsequent infections (Speare et al. 1998b; Beaman et al. 1999b; Kent et al. 1999).

Loma salmonae forms white xenomas up to 0.4 mm in size, with a cell wall in close association with the basement membrane of the pillar system of the lamella (Hauck 1984; Speare et al. 1989; Bruno et al. 1995), between pillar cells or in the endothelial cells of the marginal channel (Kent et al. 1989; Speare et al. 1989; Kent et al. 1995). Mature, pyriform spores can be found throughout the xenoma (Bruno et al. 1995). The polar tube coils 14-17 times around the infective sporoplasm (Morrison and Sprague 1983; Canning et al. 1986). In heavy infections, the degree of epithelial hyperplasia and filament fusion induced by the

multifocal distribution of the xenomas and the associated inflammatory responses (Kent et al. 1995), determines the extent of gill filament distortion. Gill distortion usually occurs when the xenoma wall starts to lose its integrity and begins releasing spores into the tissue (Markey et al. 1994; Speare et al. 1998c). These changes in the gill structure result in a granulomatous branchitis that reduces the surface area available for oxygen and electrolyte exchange and possibly has a negative effect in respiratory efficiency (Speare et al. 1989; Speare et al. 1998b). Xenomas of *L. salmonae* are visible in the gills about 5 weeks after infection (Speare et al. 1998a), with rupture around week 7-8 post exposure. However, the onset and development rate of *L. salmonae* xenomas after infection of a host cell is highly dependent on water temperature (Beaman et al. 1999a; Beaman et al. 1999b; Speare et al. 1999b). Extreme temperatures can halt the development of infection and block the parasite's life cycle (Speare et al. 1998b).

1.6 Disease and pathogenesis

Disease has been defined as "any deviation of the body from its normal or healthy state causing discomfort, sickness, inconvenience, or death" (Piper et al. 1982). Disease is a natural occurrence contributing to the regulation of fish populations, including salmonids (Arkoosh et al. 1998). Disease occurrence is influenced by three factors: host susceptibility, presence and virulence of the pathogen and environmental conditions (Snieszko 1973; Piper et al. 1982).

The stress associated with fish farming combined with the high density at which the fish are stocked favours the transmission of pathogens and thus of infectious disease

(Alderman 1988). Among the infectious agents causing disease are bacteria (Marquis and Lallier 1989; Roberts 1993; Nougayrede 1995; Kawula et al. 1996; Trust et al. 1996; Gudmundsdóttir et al. 1997; Hoel et al. 1998), viruses (Hedrick and McDowell 1987; Nougayrede 1995; Sano 1995; Nylund et al. 1997; Damsgård et al. 1998; Gray et al. 1999; Rimstad et al. 1999), fungi (Nougayrede 1995), and parasites (Bakke et al. 1990; Woo and Li 1990; Nougayrede 1995; Cobb et al. 1998; Beaman et al. 1999b; Leiro et al. 1999).

A knowledge of pathogen biology, including life cycle and host parasite interactions, is important to be able to develop strategies for the control of pathogens affecting farmed fish. Pathogenesis is the process by which a disease or a lesion develops, i.e., its sequence of events and mechanisms (Slauson and Cooper 1990). The pathogenesis of diverse infectious agents has been studied principally by observing the pathogen using histological methods in various tissues at different stages of infection. A knowledge of pathogenesis is particularly important in interpreting the effects of external and internal factors such as vaccination, immunomodulatory and antiparasitic therapy, temperature, adverse culture conditions or natural resistance, which may alter the process of infection.

In the study of pathogenesis, the adaptation of diagnostic techniques is commonly used. Histological methods involve the standard sectioning and staining of tissues with a dye (primary stain) that has a particular affinity with the element to be studied. To highlight the stained primary element, a secondary stain is used to counter stain the background (Bancroft and Cook 1984). Haematoxylin and eosin (H&E), giemsa, and gram stains are routinely used in histopathology for the detection of pathogenic organisms and for the morphologic assessments of tissues (Fedorko and Hijazi 1996; Higgins et al. 1998; Goodgame et al. 1999;

Grøtmol et al. 1999; Duong et al. 1998). Giemsa has also been used to follow the route of infection of myxosporidian (El-Matbouli et al. 1995) and microsporidian parasites (Fedorko and Hijazi 1996). Fluorescent stains like calcofluor white have also been used to detect microsporidia spores in stool and biopsy specimens (Goodgame et al. 1999). These stains bind to the endospore and permit the rapid detection of spores in samples examined by fluorescence microscopy (Fedorko and Hijazi 1996). More sophisticated detection methods have used transfected pathogen strains that express a foreign gene, so that the parasites can be stained with special dyes such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Buckner et al. 1999), allowing the detection of live parasites during the infection. However, microsporidia detection and study by these methods is difficult due to the small size and the modest inflammatory response elicited by these parasites.

Immunohistochemical stains also allow the detection of pathogens in tissues of infected animals, and rely on the use of specific polyclonal and monoclonal antibodies to identify parasite-specific constituents in tissue sections (Evensen and Olesen 1997). This antibody is usually labelled with an enzyme that reacts with a suitable substrate to give a coloured product (Ormerod and Imrie 1992). The avidin-biotin-alkaline phosphatase complex has been used in immunohistochemistry to study the occurrence of nodaviruses and their possible route of infection in fish, and allows the detection of a virus in single cells (Grøtmol et al. 1999). Lately, molecular techniques like PCR and *in situ* hybridization (ISH) have been used for the detection and diagnosis of pathogens in fish. The uniqueness of the DNA, makes these highly specific and sensitive techniques ideal for studying the pathogenesis of infection disease. A brief description of these two techniques follows.

1.7 Molecular methods for the study of pathogens

1.7.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was first introduced by Saiki et al. in 1985. The technique involves the *in vitro* enzymatic synthesis of millions of copies of a specific DNA segment. The extreme sensitivity of this reaction can readily detect single copy genes present in small amounts of tissue, or microorganisms present at low levels in the infected tissue (Young 1994).

PCR consists of repetitive cycles composed of three thermal steps, which allow the denaturation of the target DNA at high temperature, the hybridization of specific oligonucleotides on the target DNA and the extension of these primers flanking the target region by a thermostable DNA polymerase in the presence of a mixture of deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) (Saiki et al. 1985; Erlich et al. 1991; Xu and Larzul 1991; Young 1994). The primers are oriented so that the synthesis of DNA by the polymerase takes place in the region flanked by the two primers (Young 1994). These newly synthesized DNA strands can themselves act as template for the PCR primers in the following cycles, so that repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the specific DNA sequence flanked by the two primers to approximately 2^n, where n is the number of cycles (Saiki et al. 1985; Wright and Wynford-Thomas 1990; Erlich et al. 1991; Young 1994). The amplified PCR product or amplicon size is the sum of the distance between the two primers plus the sum of the lengths of the two primers, and can be visualized as a discrete band in an ethidium bromide-stained gel (Erlich et al. 1991; Young 1994).

The three steps involved in a PCR reaction are carried out at three different temperatures, 94°C for the denaturation of the original DNA sample, 37-65°C for the annealing step that allows the primers to anneal to the DNA template, and 72°C for the extension step, in which the thermostable DNA polymerase is stimulated to copy the DNA template (Erlich et al. 1991; Young 1994; Prichard 1997). The enzyme used to accomplish this is a thermostable DNA polymerase isolated from *Thermus aquaticus*, which gives its name to the enzyme (Taq DNA polymerase) (Xu and Larzul 1991). The changes in temperature required in a PCR reaction are rapidly and easily accomplished by commercial thermocyclers (Young 1994).

The specificity of the reaction depends on the stability of the hybridization complex resulting from the association between the primer and the template DNA, however, other factors that also affect PCR are the times and temperatures of the annealing and extension steps, the magnesium concentration in the reaction buffer, and the concentration of Taq DNA polymerase (Young 1994). The stability of the complex depends greatly on the melting temperature (Tm) of the complex, the size of the primers and the GC content (Xu and Larzul 1991). The two primers are considered as a unit, and their Tm should be similar. An optimal annealing temperature (Ta) ranges from (Tm-15) to (Tm-25)°C. Lower Ta results in nonspecific primer/template hybridization that can initiate nonspecific amplification. Very high Ta causes instability of the complex that results in a dramatic decrease of the PCR efficiency (Xu and Larzul 1991). The use of Taq DNA polymerase has greatly increased the specificity of the PCR reaction, allowing to increase the stringency of primer hybridization by increasing annealing temperature and lowering $MgCl_2$ concentrations (Erlich et al. 1991;

Ely et al. 1998). Because of the number of DNA copies that can be generated by PCR, contamination of the amplification reaction is a major concern (Prichard 1997). Contamination with products of a previous PCR reaction (product carryover), exogenous DNA, or other cellular material can create serious problems in research and diagnostics, because of the false positives that can be produced (Kwok and Higuchi 1989; Barker Jr 1994; Young 1994). The best way to avoid these contamination problems is by having good laboratory practices, such as autoclaving solutions and disposable equipment, pre-aliquoting reagents, using dedicated pipettes and filtered tips, using disposable gloves, premixing reagents, and the physical separation of the areas where PCR is done, from those where the sample is prepared and the reagents are mixed (Kwok and Higuchi 1989; Erlich et al. 1991). Having multiple negative controls in which no DNA is added to the reaction is also necessary (Kwok and Higuchi 1989). A minor problem, though surmountable is the fact that the PCR is not yet set up for use under field conditions and requires the transport of samples from the field to a central processing centre (Barker Jr 1994).

PCR has found many applications in research and diagnostics. It can be used in the production of amplified fragments to clone into vectors, in the sequencing of amplified fragments, in site directed mutagenesis studies, in DNA typing, in analysis of RNA transcription, in the development of probes for genetic studies, in the screening of cDNA libraries (Lanzillo 1990; Wright and Wynford-Thomas 1990; Xu and Larzul 1991; Young 1994; Prichard 1997), and in studies tracing the pathogenesis and pathways followed by pathogens after an infection (Schubbert et al. 1997; Rimstad et al. 1999). In diagnostics the technique's sensitivity allows the detection of small amounts of foreign DNA in

heterogeneous samples, making it ideal for the identification of infectious agents (Zhu et al. 1993; Marsh et al. 1995; Prichard 1997) and for the identification of genetic variation associated with disease (Xu and Larzul 1991; Young 1994). Among infectious agents that can be identified by the PCR are microsporidia (Docker et al. 1997). Because microsporidia are organisms for which phenotypic characteristics are lacking or changing, descriptions based on observations with light microscopy may not be complete for purposes of identification. Sequences of rRNA hyper-variable regions of the small subunit are species specific, and primers targeted at this region are used for gene amplification with PCR to distinguish organisms at or near the species level (Vossbrinck et al. 1993; Zhu et al. 1993; Visvesvara et al. 1995; Fedorko and Hijazi 1996). All these considerations, make PCR an ideal technique to study the progression of infection of microsporidian parasites of fish. However, this technique also has its limitations, because it can only detect the presence or absence of a pathogen in a given tissue at a given time, without providing any information on the host tissue-parasite relationship, an obstacle surmounted by other molecular techniques like *in situ* PCR (Murray 1993) and *in situ* hybridization (Stahl and Baskin 1993).

1.7.2 *In situ* hybridization

In situ hybridization (ISH) allows detection and localization of specific nucleic acid sequences directly within a cell or tissue (Singer et al. 1986; Stahl and Baskin 1993; Murray and Ambinder 1994), allowing the identification of cells that contain a particular DNA or RNA of interest, and providing important information about the distribution of specific sequences in individual cells (Höfler 1990).

The main principle of ISH is the hybridization of labelled DNA or RNA fragments (probes), complementary to the DNA or RNA of interest in the cells in which it occurs, under appropriate conditions (Höfler 1990; Murray and Ambinder 1994). This gives information not only on the presence or absence of a particular nucleic acid in a cell, but also provides useful information on the distribution of the nucleic acid and the cells that have it. A main concern for ISH is the optimal preservation of morphological detail, while simultaneously the tissue is permeabilized to allow penetration of the probe without losing target DNA or RNA (Höfler 1990). Several conditions have to be met to have a successful ISH. Fixation has to preserve the morphology of the tissue while reducing the loss of nucleic acids so that the DNA or RNA of interest can be detected, without inhibiting the probe penetration by excessive protein cross linking (Singer et al. 1986; Leitch et al. 1994). Hybridization of the probe to the nucleic acid of interest can be increased by the permeabilization of the fixed cellular matrix by using protease (proteinase K), acid (HCl), detergents (Triton X-100), or heat denaturisation (Singer et al. 1986; Höfler 1990; Murray and Ambinder 1994), which help to unmask the nucleic acid from associated proteins (Leitch et al. 1994).

The probe size has a major influence in its diffusion into the cell, and the optimal length is about 50-300 bp (Höfler 1990). The probes can be double stranded DNA, synthetic oligonucleotides, single stranded cDNA probes, and cloned single stranded RNA (Murray and Ambinder 1994). Labelling consists of the incorporation of radioactively or non-radioactively labelled nucleotides into the probe (Höfler 1990; Murray and Ambinder 1994). Isotopic probes can be labelled with ^{3}H, ^{32}P, ^{33}P, or ^{35}S (Stahl and Baskin 1993; Wilcox 1993) while non-isotopic probes may be labelled with biotin, peroxidase or digoxigenin (Singer

et al. 1986; Murray and Ambinder 1994). Labelling methods include nick translation, random primer extension, in vitro transcription, and primer extension *in situ* (Ballard and Ward 1993; Leitch et al. 1994). The PCR can be used to generate labelled probes, producing oligonucleotide probes of very high specific activity (Stahl and Baskin 1993).

Hybridization is accomplished by the application of the labelled probe to the permeabilized tissue or cell smear, under favourable conditions that facilitate hydrogen bonding of homologous probes, while preventing the matching of heterologous probes (Leitch et al. 1994; Murray and Ambinder 1994). A balance in the conditions can be found by varying the amount of salt and the temperature at which the hybridization takes place. After hybridization has taken place, the tissues are washed to remove excess non hybridized probe.

Radiolabelled probes are detected by autoradiography, using a photographic emulsion sensitive to radiation for the detection of bound probe (Leitch et al. 1994; Murray and Ambinder 1994), while non-radioactive probes are detected by immunohistochemistry with antibodies (conjugated to either peroxidase or alkaline phosphatase) directed against the probe label, or by using avidin-biotin systems (Murray and Ambinder 1994). The sensitivity of non-isotopic methods is comparable to that of isotopic detection (Stahl and Baskin 1993; Panoskaltsis-Mortari and Bucy 1995).

ISH is an important tool in research, studying malignancy in histopathological material, chromosomal arrangements of genes, studies of intra-nuclear chromosomal organizations and studies of gene expression in different sites and circumstances (Wilcox 1993; Fleming et al. 1994). ISH has found multiple applications in the detection of many

viral, bacterial and protozoan infections (Murray and Ambinder 1994), which makes it an attractive diagnostic option.

1.8 Rationale for research and objectives

Aquaculture is an important industry, but conditions found in the farming sites favour the appearance of disease that may lead to economic losses. The impact of microsporidia on farmed fish is well recognized, however, very little information exists regarding the pathogenesis of microsporidia-induced disease. Therefore, a study of the pathogenesis of *L. salmonae* in a normal salmonid host using a *L. salmonae*-specific PCR to monitor the parasite in the body of the fish host after infection was conducted. Another objective was to develop an *in situ* hybridization protocol with which to clarify several aspects of the initial stages of infection.

Considerable information has been gathered regarding the role of temperature in the modulation of xenoma formation in *L. salmonae*, and the development of resistance after exposure to this parasite. However, it is unknown how temperature, or acquired or innate resistance influence the progression of infection with *L. salmonae*. To help understand how these different host and environmental conditions affect the progression of the infection, a series of studies on the pathogenesis of *L. salmonae* under different temperatures and host conditions were conducted. The objective of these studies was to understand the effects of different factors on microsporidian pathogenesis to help in the development of strategies for the control of this emerging parasite of salmonid fish. During these studies, a novel variant of *L. salmonae*, highly infective to brook trout but not to rainbow trout was isolated. Studies

on this variant, its biology and host range were therefore included. The following specific objectives of the thesis were delineated:

Objective 1. Use PCR to examine the progression of infection with *L. salmonae*, in rainbow trout to understand the pathogenesis of this parasite in a normal salmonid host.

Objective 2. Use PCR to compare the normal pathogenesis of *L. salmonae*, with the pathogenesis observed under different temperatures (5° and 21°), different hosts (brook trout, Atlantic salmon), and different immune status (resistant vs. passively immunized vs. naive).

Objective 3. Develop an ISH protocol to study the early stages of infection of *L. salmonae* in a susceptible fish species.

Objective 4. Compare the pathogenesis of a recently discovered strain of *L. salmonae* among different hosts.

Objective 5. Study the immunoprophylactic use of the new *L. salmonae* variant in rainbow trout.

2 THE INFLUENCE OF TEMPERATURE ON THE PROGRESSION OF INFECTION OF *LOMA SALMONAE* IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS)*

2.1 ABSTRACT

Temperatures above 20°C or below 9°C interrupt the life cycle prior to sporogony of the intracellular microsporidian parasite *Loma salmonae*, inhibiting the production of xenomas in gill lamellae. Juvenile rainbow trout (*Oncorhynchus mykiss)* were experimentally infected with *L. salmonae* spores and the progression of infection, as determined by PCR, was compared at water temperatures of 5°C, 15°C and 21°C. At 15°C, parasite DNA was first detected in the heart (3 days post-exposure [PE]), and then in the gills and spleen (2 weeks PE). Xenomas were visible in the gills by week 4 PE. In contrast, at 5°C, the parasite was first detected in the heart at 7 days PE. However, even though parasite DNA was detected in the gills at 7 days PE, xenomas failed to form in the gill and by week 4 PE parasite DNA was no longer detected. In fish held at 21°C, parasite DNA was detected in the heart, gills, and spleen by 3 days PE and again at 7 days PE. Xenomas also failed to form in these fish and parasite DNA was no longer detected by week 2 PE. Therefore spore germination and movement of parasite DNA into the host presumably through the intestinal wall was not blocked by the temperatures used in this study. At 15°C the parasite reached the heart in three days and, following a two-week delay, reaches the gill. Migration to the heart and gills also occurred at the other temperatures, but at aberrant periods of time. We

speculate that the normal life cycle of *L. salmonae* depends on completion of an unknown developmental process within the heart, prior to reaching the gill. This development may be adversely affected by temperature, and may explain the thermal limits of this parasite.

2.2 INTRODUCTION

Loma salmonae is an obligate intracellular microsporidian associated with gill disease in *Oncorhynchus* species of salmonids, particularly Chinook salmon (*O. tshawytscha*) (Morrison and Sprague 1983; Kent et al. 1989). During sporogony this parasite forms large spore-filled branchial xenomas and sporadically, xenomas in other vascularized tissues (Kent et al. 1989; Docker et al. 1997). Disease in infected fish is related to the inflammation that occurs after xenomas rupture (Speare et al. 1989; Markey et al. 1994), which can lead to severe economic losses for the Chinook salmon aquaculture industry in Canada (Kent et al. 1989; Kent et al. 1998; Constantine 1999).

Relatively little is known about the life cycle of *L. salmonae*. The infection begins after ingestion of spores (Kent et al. 1995; Shaw et al. 1998; Speare et al. 1998a), and following a temperature-dependent time course (Beaman et al. 1999a; Speare et al. 1999b), xenomas begin to form and grow on the gill 4-8 weeks later. Two previous reports have documented basophilic structures in the intestine of infected fish, which may represent early stages of the parasite (Markey et al. 1994; Shaw et al. 1998), but little is known about the transport of the parasite from the gut to the gill, and whether the parasite undergoes development during transport. A better understanding of parasite transport and development would permit strategies for control. In a recent study (Beaman et al. 1999a), water

temperature was shown to modulate *L. salmonae* development rate, and successful entry into sporogony.

Temperature is well known to have an effect on the developmental rate of parasites including microsporidial pathogens of fish (Olson 1981; Antonio and Hedrick 1995; Kim et al. 1997). Temperature manipulation has therefore been proposed as a non-pharmacological method to interfere with the growth of microsporidian species like *Glugea plecoglossi* (Takahashi and Ogawa 1997) and *L. salmonae* (Speare et al. 1998b), while permitting the fish to develop immunity against these parasites (Kim et al. 1996). Recent work has described the polynomial relationship between water temperature and the rates of xenoma formation in trout following experimental infection with *L. salmonae*, and also has established the upper (20°C) and lower (9°C) critical water temperatures above or below which xenomas fail to form (Speare et al. 1999b). However, the biological mechanisms by which temperature affects the life cycle kinetics, or limits the ability of the *L. salmonae* to complete its life cycle are unknown.

The purpose of this study was to identify the presence of *L. salmonae* using a recently developed polymerase chain reaction (PCR) technique (Docker et al. 1997), to gain an understanding of the route by which the parasite typically reaches the gill when fish are held at water temperature optimal for parasite development (Speare et al. 1998a). Additionally we wished to determine the fate of the parasite in fish held at water temperatures above or below the permissive range for xenoma formation.

2.3 MATERIALS AND METHODS

2.3.1 Sample population

Naive, size-graded, diploid juvenile rainbow trout (average weight 20 ±1 g and average length 10 cm) were purchased from a commercial hatchery in Prince Edward Island, Canada, with no previous history of *L. salmonae*. The fish were acclimated to the temperature of their treatment for one week before infection. All procedures were performed according to the guidelines of the Canadian Council on Animal Care (Anonymous 1993).

2.3.2 Experimental design and fish allocation

Trial 1a: Progression of L. salmonae infection in rainbow trout under optimized conditions. Sixty rainbow trout were used in this study. Five naive fish were sampled before infection and used as negative controls. The fish were kept at 15°C in one 100-L circular fibreglass tank. This trial was conducted over a period of 10 weeks.

Trial 1b: Progression of L. salmonae infection in rainbow trout under optimized conditions during the first week of infection. Because *L. salmonae* was first detected in the heart of infected fish 1 week after infection (see results Trial 1a), a second experiment was designed to identify the time when *L. salmonae* first appeared in the heart. Thirty-five juvenile rainbow trout were used for this purpose. The fish were kept at 15°C in one circular fibreglass tank with a habitable volume of 78 L. This trial was conducted over a period of seven days.

Trial 2: Effect of water temperature on the progression of infection with L. salmonae. Sixty fish were randomly allocated to each of three identical 100-L circular fibreglass tanks.

The habitable volume of each tank was 78 L. The temperatures in each tank were 5°C, 15°C and 21°C respectively. The 15°C group was used as a positive control, because the development of disease at this temperature is well documented (Speare et al. 1998a), and it also allowed comparison of results with trials 1a and 1b. This trial was conducted over a period of 8 weeks.

2.3.3 Temperature control

Water temperature in fish and header tanks was monitored using a Campbell Scientific Datalogger. One header tank supplied ambient well water at approximately 11°C, and another supplied water heated at 27°C. To prevent gas supersaturation, heated water was passed through an aeration/degassing column prior to mixing just before entering each of the tanks at the desired temperature (15°±0.5° or 21°±0.5° C). In the case of the 5°±0.5° C tank, a chiller was used to cool water in the header tank to 4.5°±0.5° C. Water from a second header tank containing ambient well water was mixed with chilled water just before entering the tank to obtain the desired temperature.

2.3.4 Infection method

On the day of infection, gills from ten fish previously infected with *L. salmonae,* (derived from Chinook salmon and extensively passaged in rainbow trout) and selected on the basis of having abundant xenomas (Speare et al. 1998a) were harvested (the fish were killed with an overdose of benzocaine (150 mg/L) and the opercula were removed to expose the infected gill arches). Each gill arch was excised and the cartilage cut transversely every

1 mm. Food had been withheld from naive fish for 24 h before experimental infection. The infected gill inoculum was divided into equal portions of approximately 2 g, each of which was mixed with tank water and then poured into the tank. During this procedure, water flow was shut off to permit monitoring of feeding, and to prevent loss of infective tissue into the outflow drain. Consumption of inoculum was carefully monitored.

2.3.5 Sampling and assessment of infection development

Trial 1a: Random sampling of five fish took place once a week after infection and continued for 10 weeks. Samples of gill, liver, heart, spleen, head kidney, and intestine were obtained from each fish. The small size of the fish allowed for the processing of at least 50% of the total mass of each organ.

Trial 1b: Five fish were sampled each day after infection for 7 days. Organs sampled were heart, gills, stomach, pyloric caeca and intestine.

Trial 2: Five fish from each tank were sampled at 3 days post infection, then weekly for 8 weeks.

Fish were euthanized with an overdose of benzocaine (150 mg/ml) before sampling. In all trials, each freshly harvested organ (intestine was not rinsed of its contents) was incubated in separate 1.5 ml microcentrifuge tubes containing 500 µl of proteinase K buffer (10 mM Tris pH 8, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 1%SDS, 150 mM NaCl, 200 µg/ml proteinase K [Sigma]) for 20-24 h at 37°C (Docker et al. 1997). After digestion, the homogenate was treated with RNAse A and extracted once with 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma). The aqueous phase was then transferred to a

new microcentrifuge tube and mixed with 1/10 volume of 3M sodium acetate, pH 7 (Sigma), and two volumes of ice-cold ethanol or 1 volume of room temperature isopropanol. The precipitate was then immediately centrifuged for 3 min at 16 000 g, and the pellet was air dried for 1 h. The pellet was resuspended in 50 µl of sterile deionized water and stored at 5°C prior to use.

2.3.6 Polymerase chain reaction (PCR)

Two *L. salmonae*-specific oligonucleotide primers that amplify a 272 bp fragment specific for *L. salmonae* were used (Docker et al. 1997): LS1 (5'-CTGGATCAGACCGATTTATAT-3') and LS2 (5'- ATGACATCTCACATAATTGTG-3'). The PCR reaction was carried out in 50 µl and consisted of 50 pmol of each primer (LS1 x LS2), 1 x PCR buffer (Pharmacia-Biotech), 0.2 mM deoxyribonucleotides (dNTPs) (Pharmacia-Biotech), 1.25 units of Taq DNA polymerase (Pharmacia-Biotech). Five µl of the extracted DNA were added to this mix, as well as deionized water up to a volume of 50 µl. The reactions were run in a thermocycler (PTC-200 DNA Engine, Peltier Thermal Cycler, M.J. Research, Watertown, Mass.) for 30 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 3 min extension at 72°C. The cycles were preceded by an initial denaturation at 95°C for 3 min, and followed by a 10 min final extension at 72°C. Negative controls which used distilled water instead of DNA were included in each series of reactions to screen for possible contaminants. The PCR reactions were screened for correct amplicon size by electrophoresis in a 1 % agarose gel in TBE buffer.

2.4 RESULTS

Trial 1. Progression of L. salmonae infection at optimal temperature (15°C).

In fish sampled prior to infection, *L. salmonae* was not detected in any of the organs sampled. The data (Table 2.1) suggested a general trend in the detection of the parasite in the heart (4/5 fish) and intestine (1/5 fish) of infected fish at one week PE. By week two PE, parasite DNA was detected in all organs with the exception of the kidney. *L. salmonae* was only detected once in the kidney of one fish at 5 weeks PE. The heart, gills and spleen were sites where the parasite occurred more frequently until week 6 PE, although it was also detected in other organs. Subsequently, detection of the parasite diminished in all tissues until it was no longer detected in any organ by week 10 PE.

Table 2.1 Presence of *Loma salmonae* DNA in selected rainbow trout organs at different times following exposure to the microsporidian parasite at 15°C (# positive / # examined).

	Number of trout positive for *L. salmonae* by PCR									
Organ	Week1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
Heart	4/5	4/5	5/5	5/5	5/5	5/5	4/5	3/5	4/5	0/5
Gills	0/5	3/5	3/5	4/5	3/5	5/5	3/5	3/5	0/5	0/5
Spleen	0/5	2/5	2/5	5/5	5/5	4/5	1/5	1/5	0/5	0/5
Liver	0/5	3/5	0/5	1/5	3/5	1/5	2/5	1/5	0/5	0/5
Intestine	1/5	2/5	1/5	0/5	3/5	0/5	2/5	0/5	0/5	0/5
Kidney	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Total	4/5	4/5	5/5	5/5	5/5	5/5	4/5	3/5	4/5	0/5

The results of Trial 1b (Table 2.2), showed that *L. salmonae* could invade the fish and be transported to the heart as early as 1 day PE, and was present in the heart of most

infected fish by day 3 PE. It was not detected in gills during this period.

Table 2.2 Presence of *L. salmonae* DNA in selected rainbow trout organs at different times following exposure at 15°C (# positive / # examined).

Number of trout positive for *L. salmonae* by PCR

Organ	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Heart	1/5	2/5	5/5	3/5	3/5	5/5	3/5
Gills	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Stomach	0/5	0/5	0/5	0/5	0/5	1/5	0/5
Pyloric caeca	0/5	1/5	0/5	0/5	0/5	1/5	0/5
Intestine	1/5	1/5	0/5	3/5	3/5	2/5	0/5
Total	1/5	2/5	5/5	3/5	3/5	5/5	3/5

Trial 2. Progression of L. salmonae infection at 5° and 21°C

Samples collected from control rainbow trout at 15°C showed that the parasite reached the heart by 3 days PE and did not appear in the gill or other organs until week 2 PE.

When the rainbow trout were exposed to *L. salmonae* at 5°C, parasite DNA was not detected in the heart or any other tissue sampled at 3 days PE (Table 2.3). At seven days PE the parasite was detected in the heart, gills and spleen of infected fish. The fish remained positive for *L. salmonae* until week 3 PE after which all the fish sampled were negative.

At 21°C *L. salmonae* DNA was detected in the heart, gills and spleen of fish sampled 3 and 7 days PE (Table 2.4). Parasite DNA was not detected in any tissue in subsequent samples, and xenomas were never detected.

Table 2.3 Presence of *L. salmonae* DNA in selected rainbow trout organs at different times following exposure to the parasite at 5°C (# positive / # examined).

Number of trout positive by PCR

Organ	Day 3	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
Heart	0/5	5/5	4/5	4/5	0/5	0/5	0/5	0/5	0/5
Gills	0/5	3/5	1/5	3/5	0/5	0/5	0/5	0/5	0/5
Spleen	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Intestine	0/5	3/5	0/5	3/5	0/5	0/5	0/5	0/5	0/5
Total	0/5	5/5	4/5	4/5	0/5	0/5	0/5	0/5	0/5

Table 2.4 Presence of *L. salmonae* DNA in selected rainbow trout organs at different times following exposure to the parasite at 21°C (# positive / # examined)

Number of trout positive for *L. salmonae* by PCR

Organs	Day 3	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
Heart	4/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Gills	2/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	3/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Intestine	2/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Total	4/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

2.5 DISCUSSION

Relatively few studies have examined the pathways by which microsporidian parasites migrate within host tissue. The present study utilized a combination of a highly reproducible infection model (Speare et al. 1998a), and the availability of *L. salmonae*-specific PCR primers (Docker et al. 1997) to monitor the progress of the parasite in various

tissues.

Whereas many parasites have well defined routes of infection and utilize multiple organs for different developmental stages to complete their life cycle, this has yet to be described for many microsporidia. Detailed descriptions of the life cycle of the human pathogen *Enterocytozoon bieneusi* show that this pathogen completes its life cycle within a single enterocyte (Desportes-Livage et al. 1996), and hence studies of its development in different tissues have not been done. *Loma salmonae* however appears to enter the body exclusively through the gut (Shaw et al. 1998) and is not visually detected in the dissecting microscope as branchial xenomas until 5 weeks post exposure (Speare et al. 1998b), which suggested that pre-gill stages may occur in other organs. A recent finding (Chapter 3) has illustrated an as yet undefined developmental stage of *L. salmonae* in the heart, appearing at 5-6 days post infection. In contrast, xenomas are relatively rare in the heart (Hauck 1984). Our findings at 15°C suggested that under normal conditions, the parasite enters the fish through the gut, is transported to the heart where it undergoes a stage of development, and then subsequently is transported to the gill where it undergoes sporogony. Specifically, under conditions known to be optimal for the parasite with respect to xenoma formation, the parasite reaches the gill only after a period of time spent in the heart.

A second objective of this study was to examine the temperature sensitivity of *L. salmonae* development and to try to determine points where parasite development is blocked. Previous studies have shown that the parasite can infect fish at temperatures of 5°C, but fails to undergo sporogony unless water temperature is subsequently brought up to a permissive temperature (above 11°C). The parasite remains latent, without completing its life cycle,

when kept at 5°C for up to 4 weeks (Speare et al. 1999b). The present findings strongly support these earlier findings in that spore activation is not constrained at 5°C and parasite DNA was detected in the fish for up to 3 weeks PE. Furthermore, in contrast to the sequence of tissue localization at 15°C, at 5°C there appeared to be minimal tissue tropism, as the parasite was found in gills, heart and spleen as early as week 1 PE. This lack of early tissue tropism was also apparent in the study conducted at 21°C. Taken together, the results of these trials help select among the following general hypotheses explaining the life-cycle and temperature sensitivity of *L. salmonae* development.

Hypothesis 1: At optimal temperatures, the parasite, after entry via the gut, seeds randomly to many organ sites, only some of which are permissive to the life cycle. Our data rejects this, because of the absence at 15°C of the parasite in the gill until week 2 PE. If distribution was random we would expect to see it in the gill as early as we did in the heart.

Hypothesis 2: The parasite, after entry via the gut, seeds randomly to many organ sites, but only some (the heart) are permissive to a pre-sporogony phase. Our data also largely reject this for the same reason as noted for rejecting hypothesis 1.

Hypothesis 3: The parasite, after entry via the gut, rapidly expresses tissue tropism causing it to selectively invade the heart. Subsequently, development takes place within the heart, possibly in association with the macrophages lining the ventricular endocardium, yielding a stage with characteristics permitting its transport to other organs (spleen, gill and to some extent liver). However, despite non-selective uptake at this second stage, further development to sporogony is selectively permitted by factors found mainly in the gill. Our data supports this for 15°C, but not at temperatures of either 5° or 21°C, making hypothesis

3 valid only for optimal water conditions. The favourable condition in the gill, permitting sporogony, may simply be the presence of the pillar cell - a cell type found only in the gill.

Hypothesis 4: At 5° and 21°C it is possible that the typical host-pathogen interaction fails, such that successful development in the heart does not occur. Our data supports this by showing the parasite not only in the heart but also in the gills and spleen a week earlier than what occurs at optimal temperature.

This study is the first to demonstrate typical and altered patterns of tissue distribution for *L. salmonae* following experimental infection at optimal and sub-optimal temperatures. Whereas hypothesis 4 tries to explain these aberrations from the viewpoint that errors in, or lack of, early parasite development may have caused the parasite to deviate from its early cardiac tropism, an alternate possibility considers the effect of water temperature on the parasite, the host immune system or both. For example, water temperature may have a direct effect on the development of the parasite, as evidenced by studies on *Nucleospora salmonis* (Antonio and Hedrick 1995) and *Glugea stephani* (Olson 1981), and it can additionally affect the kinetics of the immune response of the fish (Carlson et al. 1995), and adversely affect the primary antibody response (Le Morvan et al. 1998), T helper cell-mediated immune response (Le Morvan et al. 1998), and macrophage migration (Finn and Nielsen 1971). It is possible then that failure of *L. salmonae* to establish itself in the heart at 5°C and 21°C, was due to a temperature-mediated failure on the interaction of the endocardial macrophages and the *L. salmonae*-infected cells, thus allowing the parasite to remain in circulation and be taken up non-selectively by any organ site with reticulo-endothelial function.

Failure of *L. salmonae* to localize in the heart at the typical times at low and high

temperatures may be mediated by parasite or host factors, but the parasite always reaches the heart. At high and low temperatures, parasite development within the heart may not follow normal patterns, such that intermediate stages with attributes permitting them to subsequently colonize the gill, are not formed. Alternatively, these stages may form within the heart, however, the high and low water temperatures may alter host-parasite interaction at the level of the gill causing further development to fail. However, this last possibility is partially refuted by an earlier finding showing that even a very brief exposure (days) of the fish to *L. salmonae* spores at 15°C, allowed the parasite to complete its life cycle even when subsequent water temperature was sharply reduced (Speare et al. 1999b). Linking that study with results of the present study would therefore suggest that the temperature-sensitive event is more likely to be the development of an early conditioning of the parasite in the heart. A fifth alternative is that aberrant exposure of the gill to the early stage (an event which our data suggests occurs at both high and low water temperatures) causes the gill to become refractory to being later colonized by stages released after maturation in the heart. This remains a viable possibility to consider, in light of the profound immunity which develops in trout following *L. salmonae* infection (Speare et al. 1998b).

Although providing the first findings relating to typical and atypical tissue temporal distribution of *L. salmonae* DNA, PCR was unable to rule out some of the hypotheses advanced to explain the findings. Further work may point to proteins expressed at different stages of the parasite's life cycle, leading to the development of probes with which to further define the aberrant development.

Findings in this study are important with respect to the viability of Chinook salmon

farming in Canada, given the marked impact of *L. salmonae* on production sites (Kent et al. 1995; Constantine 1999). Currently there are neither licenced effective pharmacological agents (Speare et al. 1999a) nor licensed vaccines. Prior data, demonstrating that fish develop protective immunity after exposure to *L. salmonae*, even when the primary exposures have been conducted at low water temperatures, non-permissive for xenoma formation, suggest that this may be an effective interim approach for controlling the effects of this disease (Speare et al. 1998b). Similar studies have been undertaken with ayu (*Plecoglossus altivelis*) housed in high temperature water after exposure to *G. plecoglossi*, with promising results (Takahashi and Ogawa 1997). However, thermal manipulation raises concerns about parasite latency, which are not easily placated. Prior findings had suggested that the parasite could remain latent for up to, but not more than 4 weeks if fish carrying the parasite were continuously kept in cold water after exposure (Beaman et al. 1999a). The current findings support this, in that the clearance rate of parasite DNA at the cold water temperature is within 4 weeks. Future studies addressing the progression of infection with *L. salmonae* in fish previously exposed to this pathogen, and its effects on the parasite's development are necessary for the design of a more specific strategy for the non-pharmacological control of infections with *L. salmonae*.

3 DEVELOPMENT OF AN *IN SITU* HYBRIDIZATION (ISH) PROTOCOL AND STUDIES ON THE INITIAL DEVELOPMENTAL STAGES OF *L. SALMONAE*

3.1 ABSTRACT

Loma salmonae, a microsporidian parasite of salmonids of the genus *Oncorhynchus*, causes significant economic losses in pen-reared Chinook salmon (*O. tschawytscha*). Final stages of *L. salmonae* infections are easily recognized as xenomas that form in the gills during sporogony. However, early pre-xenoma stages of infection (three weeks or less after infection) are difficult to detect in histological slides in both gills and other tissues. In order to visualize the early systemic stages of the parasite and pre-xenoma stages in the gill of experimentally infected rainbow trout, an *in situ* hybridization (ISH) method, using a *L. salmonae*-specific digoxigenin-labelled single-stranded DNA probe was generated. This method allowed the detection of the parasite during its first contact with the gut mucosal epithelium and its localization in the lamina propria of the intestine within 24 h of infection. ISH also detected dividing merogonic stages in infected cells in the heart 5 days PE, providing the first evidence of parasitaemia and hematogenous distribution of this parasite; however, the identity of the cells harbouring the parasite has yet to be determined. The ssDNA probe was also able to detect *L. salmonae* in the gills of infected fish as early as two weeks post exposure. This method proved to be sensitive and specific for the detection of *L. salmonae* during the early stages of infection, and provided additional information regarding the localization of the parasite through its passage in the host's body.

3.2 INTRODUCTION

Loma salmonae, an obligate intracellular parasite of the gills of salmonid fish of the genus *Oncorhynchus* (Morrison and Sprague 1983), is the causal agent of microsporidial gill disease in Chinook (*O. tshawytscha)* and coho (*O. kisutch*) salmon, and in rainbow trout (*O. mykiss)* (Hauck 1984; Magor 1987; Kent et al. 1989; Speare et al. 1989; Markey et al. 1994; Bruno et al. 1995). *Loma salmonae*-infected salmon experience high mortality rates and increased susceptibility to other infectious diseases (Speare et al. 1998a). Infections with *L. salmonae* usually result in the development of abundant characteristic xenomas in the gills (Morrison and Sprague 1983). Xenomas are parasite-infected cells that undergo hypertrophy as the pathogen divides and produces spores (Morrison and Sprague 1983; Docker et al. 1997). The presence of xenomas leads to granuloma formation (Dyková and Lom 1980) which induces an epithelial hyperplastic response that reduces the respiratory surface of the gills, contributing to fish death. Rainbow trout are susceptible to *L. salmonae* but clinical signs of infection or mortality are rare, making this species an ideal model for the study of the development of this disease (Speare et al. 1998a).

Infection with *L. salmonae* occurs following germination of spores in the gut and the invasion of a gut epithelial cell (Shaw et al. 1998). Until recently very little was known of those stages of the parasite's life cycle prior to the formation of xenomas. Recent studies (Chapter 2) using the polymerase chain reaction (PCR), have shown that *L. salmonae*, is rapidly transported (3 days post exposure [PE]) from the intestine to the heart, appearing in the gills of infected fish 2 weeks PE at 15°C. However, it has been difficult to visualize these early stages of infection by routine microscopy.

The lack of approved drugs to control *L. salmonae* infections in the aquaculture industry in Canada (Mullins et al. 1994; Brocklebank et al. 1995; Shaw et al. 1999), makes routine screening of fish stocks for the early detection of the parasite, imperative for disease management practices, which are currently limited to early harvest in advance of mortalities (Speare et al. 1999b). Current techniques used for routine screening of *L. salmonae* include the use of wet mounts, histology, and immunohistochemistry with monoclonal antibodies (MAbs) (Kent et al. 1999). However, the MAbs used for the detection of *L. salmonae* by immunohistochemistry are not able to detect pre-sporogonic stages (before 4 weeks PE at 15°C), and by the time the characteristic xenomas can be observed, it might be too late to take any preventive action. Thus there is a need to develop a technique capable of identifying early stages of the parasite in the gills which cannot be detected by immunohistochemistry. This technique may also help to clarify early events of infection and help us understand the disease mechanisms as a basis for control of microsporidiosis with *L. salmonae*.

DNA-based diagnostic methods such as PCR (Docker et al. 1997), are especially suited for the detection of pathogens because of their specificity and sensitivity. The uniqueness of the DNA sequences of the host and the pathogen, allow recognition of the specific target (pathogen) DNA in the host regardless of the stage present, or if the infection is subclinical (Stokes and Burreson 1995). Recently there has been an increase in the use of PCR in the detection of pathogens that affect aquatic animals (Stokes and Burreson 1995). *Loma salmonae*-specific primers have been developed for the detection of this parasite in infected fish (Docker et al. 1997). These primers recognize the internal transcribed spacer

regions (ITS), and portions of the gene encoding the small subunit (SSU) and the large subunit (LSU) of the ribosomal DNA sequence of *L. salmonae*, and do not cross-react with DNA from other microsporidian species (Docker et al. 1997). In a previous study, the primers were used to assess the influence that temperature has in the progression of the infection with *L. salmonae* through the body of experimentally infected fish (Chapter 2). However, although conventional PCR does not have the ability to reveal stages of this or other parasites (Antonio et al. 1999) in the host tissues and cells, this problem can be overcome by the use of *in situ* PCR (Murray 1993) or *in situ* hybridization. *In situ* hybridization (ISH) involves the anatomical localization of labelled RNA or DNA molecules that hybridize with complementary target RNA or DNA sequences in the cell (Stahl and Baskin 1993), allowing the direct detection of infectious agents (Murray and Ambinder 1994).

In this study, we used the *L. salmonae* specific primers LS1 and LS2 to generate a *L. salmonae*-specific DNA probe, with which to develop an ISH protocol for the detection of the very early stages of the parasite in both the intestine and gills of experimentally infected fish.

3.3 MATERIALS AND METHODS

3.3.1 Sample population and experimental design

Naive, size-graded, diploid juvenile rainbow trout (~20 ± 1 g) were purchased from a commercial hatchery in Prince Edward Island, Canada, with no previous history of *L. salmonae*. All procedures were performed according to the guidelines of the Canadian

Council on Animal Care (Anonymous 1993).

This study was conducted in three phases:

1.- Development of a digoxigenin-labelled single stranded deoxyribonucleic acid (ssDNA) probe for the detection of L. salmonae in gills of infected fish.

Loma salmonae spore DNA was used in a PCR reaction to produce an amplicon that served as template in a second PCR reaction that labelled the probe with digoxigenin.

2.- Testing of the ssDNA probe in gills of infected fish.

After the probe was developed, it was tested in gills from infected fish, and the ISH protocol was developed. Forty juvenile rainbow trout were kept in one 100-L circular fibre glass tank at 15°C. This trial lasted 8 weeks.

3.- Study of the early stages of infection of L. salmonae in the gastrointestinal (GI) tract and heart of rainbow trout.

One hundred juvenile rainbow trout were used. The fish were kept in one 100-L circular fibre glass tank at 15°C. This trial lasted one week.

3.3.2 Fish infection and sampling.

All fish were infected by gastric intubation with finely cut xenoma-laden gills harvested from rainbow trout euthanised with an overdose of benzocaine. This *L. salmonae* strain was originally derived from Chinook salmon and extensively passaged in rainbow trout. The cartilaginous gill arch was removed and the remaining gill filaments were finely cut until only a pulp remained. The pulp was mixed with an equal volume of distilled water, and taken up into a 1 ml syringe fitted with a 5 cm piece of tubing of approximately 5 mm

diameter. Each fish was orally intubated with 0.1 ml of the pulp.

The first left gill arch from five infected fish were sampled once a week for 8 weeks PE. These gills were used in the development of an *in situ* hybridization protocol for the detection of *L. salmonae*. Gills from naive non-infected rainbow trout were included as negative controls.

For the study of the early stages of infection of *L. salmonae*, samples from the stomach, small and large intestine and heart were taken from five fish at 1, 2, 4, 8, 12, 18, 24, 30, 36, 42, 48, 60, and 72 hrs after infection, and 4, 5, 6, and 7 days post-infection. Tissue from naive non-infected rainbow trout harvested at the end of the trial were used as negative controls.

All organ samples were fixed in 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) for 40 minutes at room temperature, and then stored at 5°C in 70% ethanol until further processing. The tissues were dehydrated in ethanols and embedded in paraffin in an automatic tissue processor. The stomach, small and large intestine were placed longitudinally, to allow examination throughout their length. Three sections 6 μm thick were cut and placed on slides that had been pre-treated with 3-aminopropyltriethoxysilane (Sigma) and dried for 2 h at 40°C.

3.3.3 Template preparation for DNA labelling

Template was prepared in two steps. First, *L. salmonae* DNA from 1×10^6 purified spores was obtained by proteinase K digestion (10 mM Tris, pH 8.0; 10 mM EDTA; 1% SDS; 150 mM NaCl; 200 μg ml^{-1} proteinase K) at 37°C overnight, followed by phenol

chloroform extraction and precipitation. Spore DNA was then amplified in a PCR reaction that used the *L. salmonae* specific primers LS1 and LS2 (Docker et al. 1997) to produce a template DNA for a second amplification reaction. Briefly, 3 µl of extracted spore DNA were mixed with 27 µl of PCR mastermix, consisting of 13.4 µl of deionized water; 3 µl of 10x PCR buffer (Pharmacia) to obtain a final concentration of 1x; 5 µl of both primers, LS1 and LS2 (Genosys Custom Biopolymers), from a stock of 10 pmol/µl to a final concentration of 50 pmol of each primer per reaction; 0.3 µl of DNA polymerization mix (Pharmacia), to get a final concentration of 0.2 mM for each dNTP; 0.3 µl of Taq or rTaq DNA polymerase (1.5 U per reaction)(Pharmacia). One drop of light mineral oil was added on top of the reaction to seal the reaction mixture. The reactions were run in a thermocycler (PTC-200 Peltier Thermal Cycler, M. J. Research) using the following profile: 30 cycles consisting of three steps, denaturation for one minute at 94°C, annealing at 50°C, and extension for 3 minutes at 72°C. The 30 cycles were preceded by an initial 3-minute denaturation at 95°C and a final 10-minute extension at 72°C. The amplicon obtained from this reaction was purified using a High Pure PCR product purification kit (Boehringer Mannheim)

3.3.4 Single stranded DNA probe production and labelling

The probe for ISH was obtained by a second PCR amplification reaction that used 50 ng of the amplified fragment as a template. The conditions were the same as for the first PCR, with the following changes. First, to generate a ssDNA probe, only the LS1 primer was used (Finckh et al. 1991). Second, deoxythymidine triphosphate (dTTP) was partially replaced by a homologue, digoxigenin-11-deoxyuridine triphosphate (DIG-11-UTP) (PCR

DIG Probe Synthesis Kit, Boehringer Mannheim) to label the probe with digoxigenin. The PCR reagents used in this PCR were 0.75 µl of Expand™ High Fidelity Enzyme mix (3 U/µl, Boheringer Mannheim); 5 µl of 10x PCR buffer with $MgCl_2$ for a final concentration of 1x; 5 µl PCR DIG probe synthesis mix (2 mM dATP, dCTP, dGTP each, 1.3 mM dTTP, and 0.7 mM DIG-11-dUTP) for a final concentration of 200 µM; sterile deionized water up to 50 µl. The labelled product (272 bp) was purified with a High Pure PCR product purification kit (Boehringer Mannheim).

3.3.5 Tissue permeabilization before hybridization

Dried gill sections (section 3.3.2) were deparaffinized in xylene for 7 minutes, rehydrated in a descending ethanol series (100, 95 and 70% for 5, 2 and 2 minutes respectively), and washed in distilled water. The tissues were then equilibrated in TES (50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, pH 7.4) for 10 minutes before treatment with proteinase K (15µg/ml in TES) for 15 min at 37°C. Proteolysis was halted by washing in two changes of PBS with 2 mg/ml glycine (Fisher Scientific) for 5 min each. Tissue sections were then immersed in aqueous 20% acetic acid at 4°C for 15 sec, after which they were washed in two changes of distilled water for 10 min each. Tissues were briefly post-fixed in 0.4% paraformaldehyde in PBS for 5 min, rinsed in distilled water and air dried.

3.3.6 *In situ* hybridization

Hybridization was performed by applying 100 µl of the hybridization buffer (5µl 100x Denhardt's solution [2% Ficoll 400 (Pharmacia), 2% polyvinylpyrrolidone (BDH), 2%

BSA (Fisher Scientific)], 50µl dextran sulfate 50% [Sigma], 10 µl sperm DNA 100 mg/ml [Boehringer Mannheim], 100 µl 20X SSC [3M NaCl (Sigma), 0.3M sodium citrate (Sigma) pH 7.0], sterile deionized water to 250 µl, and 250 µl formamide [Sigma]) with 30 ng of digoxigenin-labelled probe onto the permeabilized tissue sections. The slides were covered with hybri-slips [Sigma], placed onto a preheated baking tray and incubated at 90-95°C for 3 min to denature the target DNA. The slides where then transferred to a humidified box and incubated at 40°C overnight.

Negative controls used in the development of the ISH protocol included: naive fish tissue hybridized with the labelled probe; infected fish tissue hybridized with unlabelled probe or with hybridization buffer without probe; infected fish tissue hybridized with the labelled probe, but without incubation with the AP-conjugated anti-DIG Fab fragments.

3.3.7 Hybridization detection

After the overnight incubation, the sections were washed twice with 4x SSC for 5 minutes at room temperature. The slides where then equilibrated in tris buffered saline [TBS: 50 mM Tris-HCl (Fisher Scientific), 100 mM NaCl (Sigma), pH 7.2] for 5 min and then blocked with TBT [TBS + 3% BSA (Fisher Scientific), 0.5% Triton X-100 (Fisher Scientific)] at room temperature for 10 min. After equilibration, the slides were then transferred to a slide incubation tray and the sections incubated with alkaline phosphatase (AP) conjugated anti-digoxigenin Fab fragments [Boehringer Mannheim] diluted 1:600 in TBT for 1 h. The slides were then washed in TBS for 5 min, twice. The signal (purple precipitate) was developed by applying 200 µl of a buffer containing 45 µl nitroblue

tetrazolium (NBT) [Boehringer Mannheim] and 35 µl bromo-chloro-indolyl-phosphate (BCIP) [Boehringer Mannheim] in 10 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [Sigma], pH 9.5, and incubating the slides for 5 h in the dark. Colour development was terminated by washing in distilled water for 5 min. The slides were then counterstained with 10% methyl green, air dried and mounted with Permount (Fisher). Sections were viewed and photographed with an Olympus BX50 photomicroscope.

3.4 RESULTS

A ssDNA digoxigenin-labelled probe was obtained. Hybridization of the probe to paraffin sections of fish tissue infected with *L. salmonae* yielded strong colour development with no hybridization to normal fish tissue. Hybridization signals were observed as purple precipitates in target tissues, indicating the binding of the labelled probe to *L. salmonae* DNA, and thus revealing the presence of the parasite. The probe was able to detect *L. salmonae* in the gills of infected fish as early as 2 weeks post infection. Small *L. salmonae*-infected cells (pre-xenomas) were detected in the filaments or on the lamellae of infected fish (Fig. 3.1). These infected cells are easily identified by the intense colour of the precipitate. In some of these cells, in more advanced stages of infection (3 weeks PE) dividing stages or meronts could be discerned within the immature xenomas (Fig. 3.2). In mature xenomas (5 weeks PE or more), the probe hybridized only to portions of the xenoma, possibly to spores that were sectioned or to replicating stages interspersed among the spores, giving a mottled appearance (Fig. 3.3).

Negative controls showed no hybridization signals in the sampled organs (Figs. 3.4

and 3.5), validating the probe specificity in infected fish, and that background or non-specific binding of reagents did not occur.

The parasite was identified in stomach contents as early as 2 h PE, and in close association with the mucosal epithelium of the stomach at 4 h PE (Fig 3.6). Other than these observations, no other stages were ever detected in the stomach region.

Early parasite stages (possibly sporoplasm) could be detected in epithelial cells and lamina propria of the small intestine as early as 12 h PE (Fig. 3.7), and again at 24 h PE (Figs. 3.8). In the large intestine sporoplasms (or meronts) could be observed as early as 12 h PE in the lamina propria (Fig. 3.9), and were still visible at 24 h PE (Fig. 3.10). Early stages of the parasite were not detected in sites other than the gastrointestinal mucosa.

Five and six days post infection, cells containing merogonic stages were detected in the heart ventricle, associated with blood cells (Fig. 3.11) or the endocardial epithelium (Fig. 3.12).

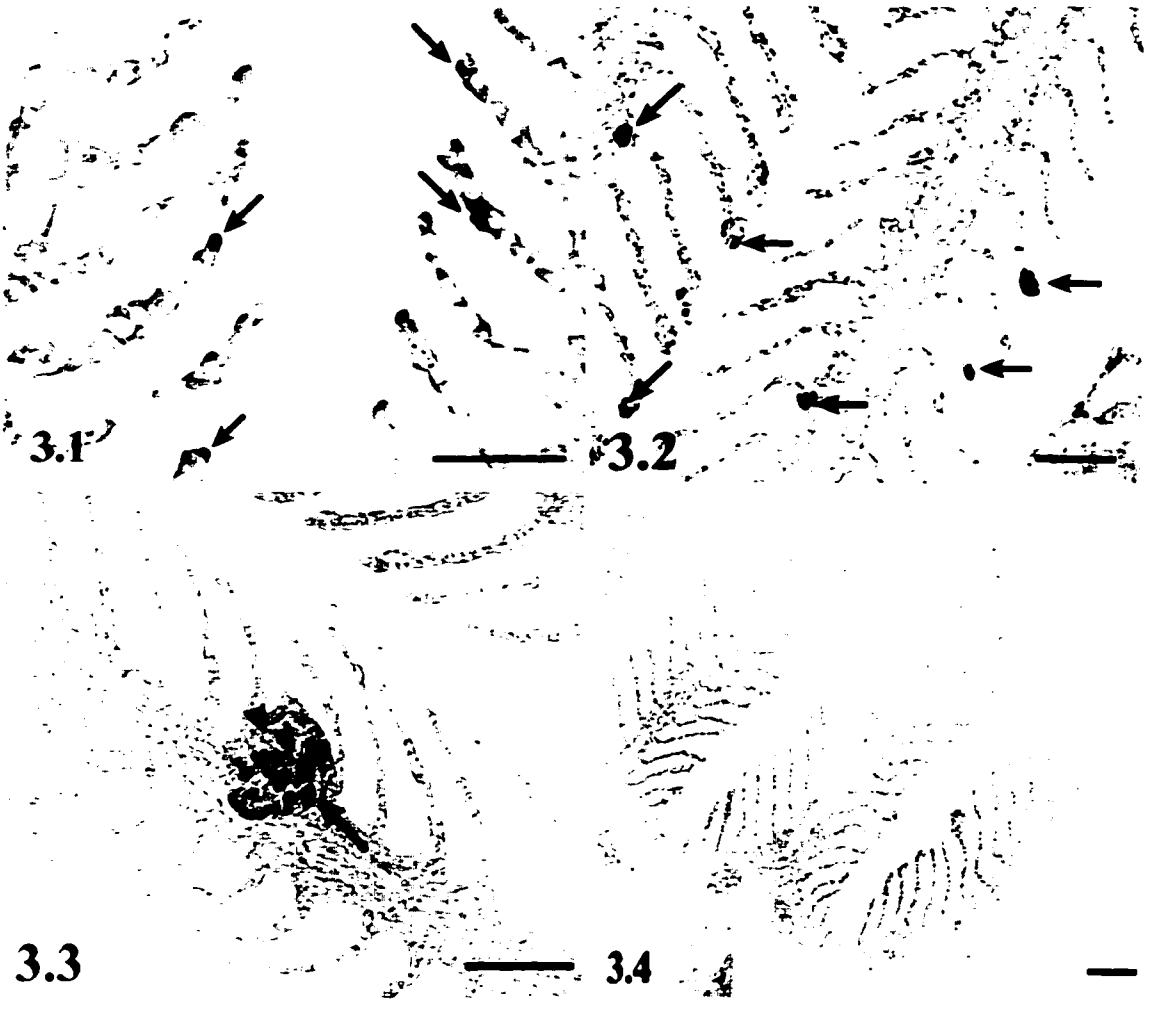

Fig. 3.1 Rainbow trout (*Oncorhynchus mykiss*) gill filament 2 weeks post-exposure (PE) to *Loma salmonae* spores. Very early stages of infection can be seen (arrows) at the tip and sides of the lamellae. Fig. 3.2 Rainbow trout gill filament 3 weeks PE to *L. salmonae* spores. Meronts can be observed at the tips or base of lamellae (arrows). Fig. 3.3 Rainbow trout gill filament 6 weeks PE to *L. salmonae* spores. A mature xenoma is seen, intact spores (arrowhead) do not hybridize to the ssDNA probe and can be seen in the clear zones of the xenoma; merogonic stages or sectioned spores (arrow) hybridize with the ssDNA probe. Fig. 3.4 Naive rainbow trout gill not infected with *L. salmonae*. No hybridization signals can be detected. *In situ* hybridization with *L. salmonae* ssDNA digoxigenin (DIG)-labelled probe and alkaline phosphatase (AP)-conjugated anti-DIG Fab fragments. Methyl green counterstain. Bar = 100 μm.

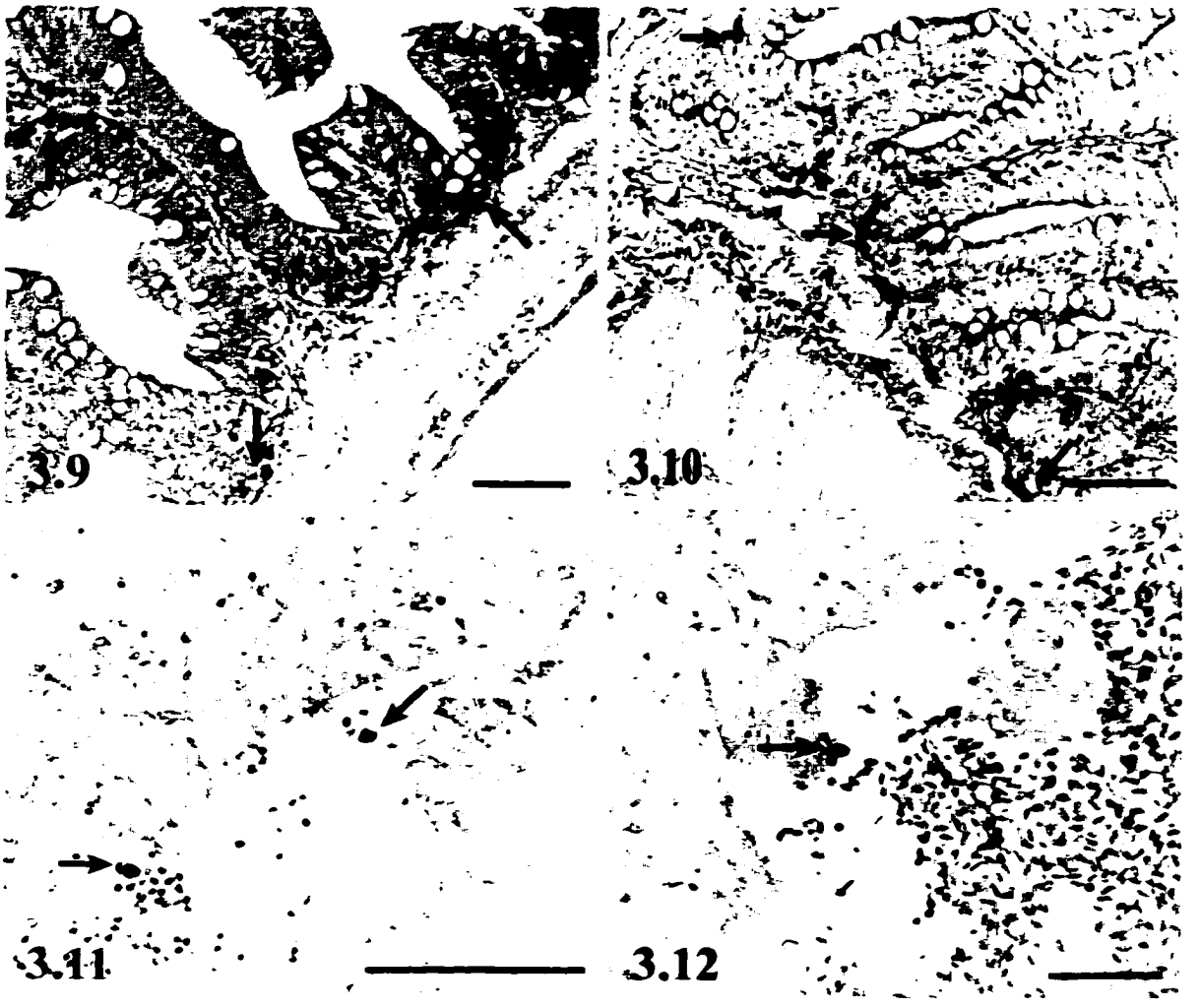

Fig. 3.9 Rainbow trout large intestine 12 h PE to *L. salmonae* spores. Numerous early stages (sporoplasms) can be seen (arrows) at the lamina propria level. Fig. 3.10 Rainbow trout large intestine 24 h PE to *L. salmonae* spores. Many early stages (arrows) can be seen at the lamina propria level. Fig. 3.11 Rainbow trout heart (ventricle) 5 days PE to *L. salmonae* spores. Infected cells with early merogonic stages of *L. salmonae* can be observed among non-infected cells in the blood. Fig. 3.12 Rainbow trout heart (ventricle) 6 days PE to *L. salmonae* spores. An infected cell can be observed in association with the ventricular endocardial epithelium. ISH with *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments. Methyl green counterstain. Bar = 100 μm.

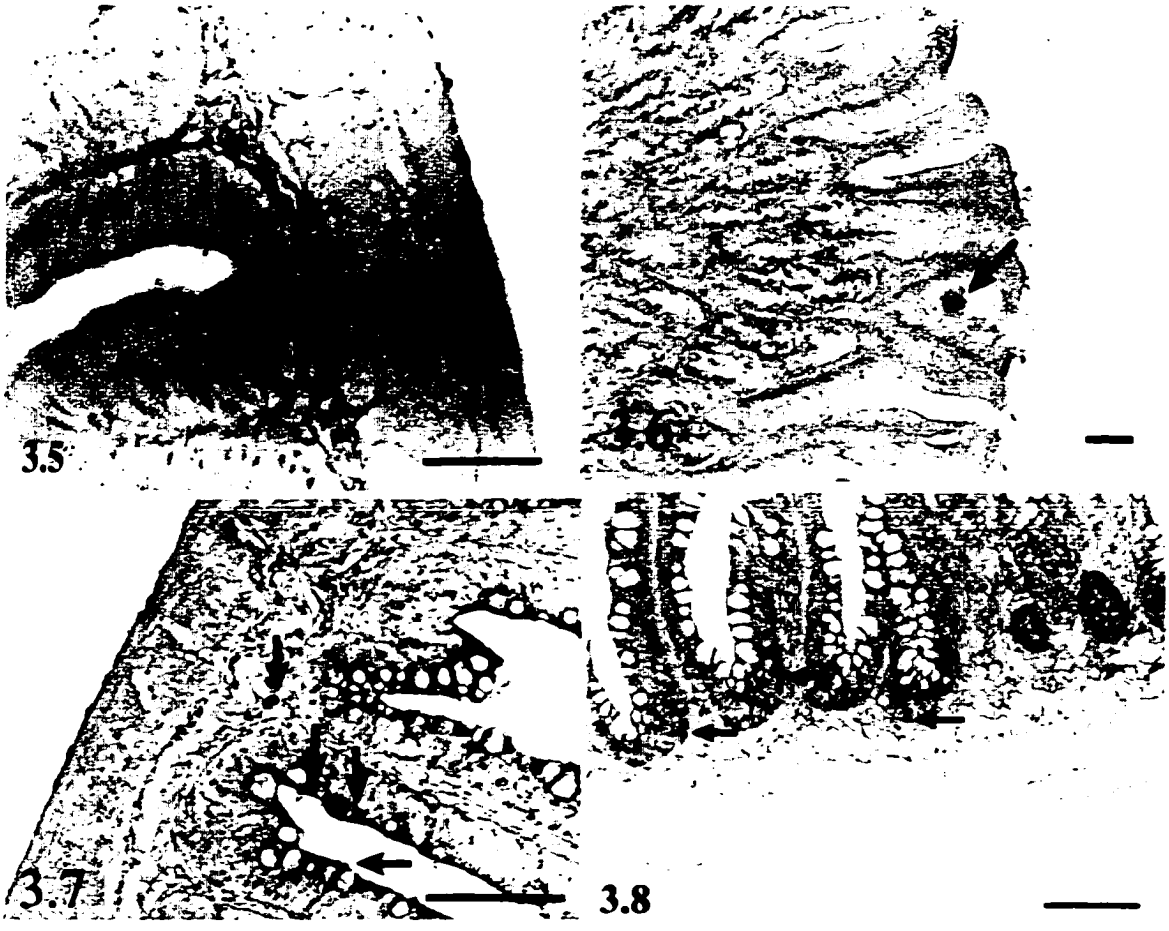

Fig. 3.5 Naive rainbow trout small intestine. No hybridization signals can be detected. Fig. 3.6 Rainbow trout stomach 4h PE *to L. salmonae* spores. An early stage, possibly a sporoplasm, can be seen in association with the mucosal epithelium of the stomach. Fig. 3.7 Rainbow trout small intestine 12 h PE to *L. salmonae* spores. Early stages of infection (sporoplasms) can be seen (arrows) at the lamina propria level or in epithelial enterocytes close to the intestinal lumen. Fig. 3.8 Rainbow trout small intestine 24 h PE to *L. salmonae* spores. A large number of early stages of infection (arrows) can be seen associated with the lamina propria or at the base of enterocytes. ISH with *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments. Methyl green counterstain. Bars = 100 μm.

3.5 DISCUSSION

Most molecular techniques used for the detection of microsporidian pathogens in humans or animals rely extensively on PCR (Fedorko and Hijazi 1996). Although conventional PCR can accurately detect the presence or absence of a given pathogen in an organ, it does not provide information on the cellular localization of the target pathogen. This additional information can be obtained with the use of *in situ* PCR (Murray 1993) or ISH (Wilcox 1993), which are exquisitely sensitive and can detect the presence of a pathogen within a single cell among tens of thousands of uninfected cells. The cell structure of the infected tissues is maintained, allowing the detection of a pathogen in its precise location at the tissue, cellular, and subcellular level (Fleming et al. 1994; Murray and Ambinder 1994; O'Leary et al. 1995). Hybridization results can then be correlated with structural features. ISH has been used for the differentiation of viral strains in crustaceans (Durand et al. 1998), the detection of early stages of infection of myxosporidia in fish and in their tubificid hosts (Antonio et al. 1999), the diagnosis of microsporidia like *E. bieneusi* in immunosupressed humans (Velásquez et al. 1999), and to track the transport of phage DNA through the intestinal wall of rodents (Schubbert et al. 1997).

In the present study, a ssDNA, DIG-labelled probe suitable for use in ISH was developed. Single stranded DNA probes have the advantage of hybridizing more efficiently than double stranded probes without self-annealing (Murray and Ambinder 1994), while being easier to handle and less complex to synthesize than RNA probes (Stahl and Baskin 1993). Other approaches for the production of ssDNA probes by PCR include the use of unequal molar amounts of the amplification primers (Gyllensten and Erlich 1988), but the

use of only one primer (Finckh et al. 1991) is sufficient for the generation of ssDNA probes. The primers used in the generation of the probe are specific for *L. salmonae* and do not cross-react with other microsporidia (Docker et al. 1997), therefore this probe was considered to be specific for *L. salmonae*. This is the first time that *in situ* hybridization has been used in the localization of a fish-infecting microsporidian.

PCR offered a convenient way of labelling the DNA with DIG, while at the same time synthesizing a ssDNA probe. Among the advantages of non-isotopic ISH are the almost unlimited shelf-life of the probe, the avoidance of the biohazards linked to the use of radioisotopes, the speed of the method (Stahl and Baskin 1993), and the optimal cellular and subcellular resolution (Höfler 1990; Bloch 1993; Wilcox 1993; Panoskaltsis-Mortari and Bucy 1995).

Direct detection of *L. salmonae* in tissue sections is an attractive diagnostic option, as it identifies the precise cellular location and the extent of infection. Conditions used in the ISH protocol developed in this study, resulted in optimal visualization and thereby localization sites of the parasite, in the gills, stomach lumen, the intestines and heart of the infected fish. Although ssDNA probes do not need denaturation prior to hybridization, the fact that it was added before tissue denaturation, may have resulted in the strong signals observed, as has been reported by other authors (Antonio et al. 1998). However, the technique has its limits, for example, the use of paraffin sections and light microscopy does not allow the resolution needed to identify the specific cell types that are host to the parasite, especially when the hybridization signal is so intense that it precludes the visualization of the morphology of the infected cell, and the morphological details of the parasite.

The *L. salmonae* probe generated in this study revealed the presence of pre-sporogonic stages (most likely sporoplasms and meronts, because of the time of detection) not previously visualized with conventional histological methods. This study demonstrated that ISH can detect early stages of *L. salmonae* in the gills of infected fish as early as two weeks PE, which agrees with results obtained previously with PCR (Chapter 2). However, when the ISH was applied to mature xenomas (5 weeks PE onwards), the probe was only able to hybridize to parts of the xenomas, yielding a mottled appearance. This may indicate that although the DNA probe was able to hybridize well with *L. salmonae* DNA in the early and immature stages, it hybridizes poorly or not at all with DNA in mature spores in paraffin sections. Similar results have been reported with mature spores of other pathogens which remain intact even in 5 μm thick paraffin sections (Stokes and Burreson 1995), and could be due to the inability of the anti-digoxigenin antibody or the probe to penetrate the thick spore wall, even after proteinase K treatment. However, the lack of hybridization with mature, intact spores did not have a significant effect in the final interpretation, as pre-sporogonic stages of *L. salmonae* reacted with the probe and could be found interspersed between areas which presumably represented the spores.

The ssDNA probe generated in this study may prove useful as a diagnostic tool for *L. salmonae*, as even one or two meronts can be detected in individual host cells, and can be used in the future in the identification of *L. salmonae* stages that are not readily visible by routine histology and immunocytological methods, especially because the MAbs that have been developed against *L. salmonae*, might only be useful after sporogenesis, limiting the detection threshold of the parasite, as has also been reported for other fish parasites (Morris

et al. 1999).

The usefulness of the ISH assay for the detection of *L. salmonae* in the very early stages of infection in the intestine and heart was tested. By combining the advantages of morphological observation with molecular information at the level of nucleic acid sequences (Velásquez et al. 1999), this method allowed the visualization of infected host cells in the heart that had not been observed before, which to our knowledge are the first that show a fish microsporidian, in this case *L. salmonae*, moving through the host after penetration through the intestinal wall.

Putative early developmental forms of *L. salmonae* had been previously reported from the intestine of infected Chinook salmon by routine histology and haematoxylin-eosin (H&E) staining (Shaw et al. 1998), therefore it had been surmised that the portal of entry of *L. salmonae* was the intestinal wall, however the passage of the parasite through the intestinal wall had never been clearly demonstrated. In studies of intestinal microsporidia of humans, where comparisons between ISH and other more conventional histological methods have been done, ISH stained a variety of parasite stages that were missed when the sections were stained with H&E or Giemsa (Mansfield et al. 1996; Tzipori et al. 1996; Kondova et al. 1998; Mansfield et al. 1998). It is possible that the structure seen in the intestine of Chinook salmon and believed to be a sporoplasm (Shaw et al. 1998), was in fact another structure, as observation of sporoplasms or meronts by conventional techniques in the host tissue is very difficult, unless it has entered the sporogonic phase. The development of ISH in this study has allowed for the identification of the initial stages of *L. salmonae* in the stomach lumen as early as 2 h PE in close association with the epithelial surface of the

stomach at 4 h following exposure to mature spores although no other early stages were seen inside the cells after this time in the stomach.

Loma salmonae sporoplasms that penetrate the gut epithelium of the fish host appear to migrate to the lamina propria, and the results suggested that there was not a single preferential site for infection, since sporoplasms were detected in both the small and large intestine. From this site, sporoplasms presumably access the blood flow and are transported to other parts of the body of the fish. Although infection begins as the sporoplasm penetrates the mucosal epithelium of the intestine (Shaw et al. 1998), it is not known with certainty whether the sporoplasms or the meronts are capable of moving between cells. Movement of microsporidia between cells (Dyková 1995) could explain the detection of early stages of *L. salmonae* in the lamina propria. This study unequivocally demonstrate for the first time the passage of *L. salmonae* through the gut. *Loma salmonae* moves to the lamina propria, from where it seems to be transported to other parts of the body inside infected cells. The putative transport cells have not yet been identified, but, as in the case of *Eimeria*, these carrier cells could be intraepithelial lymphocytes (Daszak 1999) or T cells (Riley and Fernando 1988; Jeurissen et al. 1996). It has been hypothesized that microsporidia might infect migratory cells such as monocytes (Matthews and Matthews 1980). How these cells become infected is not known, but it is proposed that they might become infected during phagocytosis of the parasite in the lamina propria of the intestine (Didier et al. 1998). Microsporidia (*Enterocytozoon bieneusi*) observed in the lamina propria of infected monkeys had the appearance of being within macrophages, suggesting the possibility of hematogenous spread of the parasite (Tzipori et al. 1996). This hypothesis had not been experimentally

demonstrated, but our results provide the first evidence of a microsporidian parasite of fish moving through the body of the host fish within infected cells found in the blood.

The results of this study suggest that in *L. salmonae,* as with some myxosporidians (Moran et al. 1999) and other microsporidians such as *Encephalitozoon cuniculi* (Cox et al. 1979), merogony (the proliferative phase that leads to the formation of high number of meronts (Didier et al. 1998), begins in sites other than the site of xenoma formation. The infective stage seems to have initiated the early dividing (merogonic) stages in the infected host cells by the time they were detected in the heart (5 days PE), although the proliferative phase is completed at the site of sporulation in the gills. This suggests that *L. salmonae* is transported from the site of germination to the site of sporulation inside infected cells. Whether these cells are the original infected enterocytes, lymphocytes or monocytes infected by the movement of the meronts from cell to cell is not known. Spore passage from the intestine to the blood circulation by the action of some phagocytic cell has also been pointed out by Cox et al. (1979) for *E. cuniculi*, and it was proposed that the first cycle of growth occurred in these phagocytic cells. This allows the movement of the parasite to organs with a high blood flow, such as brain in rabbits. Our results confirm this for *L. salmonae*, as cells harbouring the dividing parasite were detected as they passed through the heart of the infected fish. Evidence of these stages in transport cells had not been observed before. The present study corroborates previous findings (Chapter 2), especially by visualizing the merogonic stages passing through the heart. Studies to determine the specific identity of the infected cells should be undertaken. This ISH technique, adapted for transmission electron microscopy (TEM), with the aid of a MAb specific for different fish leukocyte

subpopulations, could help to better localize these early stages of development to study their relationship with other cells, and more importantly to identify the cells that are used as host and for transportation.

Loma salmonae had been considered previously as relatively non-pathogenic to salmonid fish in fresh water, but is now recognized as a cause of high morbidity and mortality in Pacific and Chinook salmon in Canada (Scholz 1999). The generation and use of a *L. salmonae*-specific probe proved useful in defining various aspects of its life cycle that have been difficult to visualize until now, because of the lack of sensitive methods not only of detection but also of localization, so that the initial stages of the infection could be elucidated. ISH proved to be a sensitive and specific indicator of microsporidial infection in fish and revealed stages of the parasite that would have been difficult to appreciate by conventional histochemical techniques. This higher sensitivity and specificity of ISH over conventional histological techniques is not unexpected, because, whereas conventional techniques rely in specific anatomical structures, such as the spore wall, ISH is capable of detecting the DNA of the parasite, which is present in all life stages. While conventional techniques could detect empty spores, ISH will only detect viable stages of the organism. Although, ISH has been utilized to investigate the pathogenesis of human microsporidia (Mansfield et al. 1996), its use in studies of microsporidial infections in fish had not been reported. The development of this ssDNA probe specific for *L. salmonae* will allow the timely detection of the parasite in paraformaldehyde-fixed, paraffin-embedded tissue samples, and will also be useful for further research in the pathogenesis of this important parasite of fish.

4 PROGRESSION OF *LOMA SALMONAE* INFECTION IN IMMUNE RAINBOW TROUT AND IN INNATELY RESISTANT FISHES (ATLANTIC SALMON AND BROOK TROUT).

4.1 ABSTRACT

This study compared and contrasted the development in various tissues of experimental infections of the microsporidian *Loma salmonae*, a branchial pathogen of salmonids within the genus *Oncorhynchus*, following exposure of (1) naive susceptible rainbow trout as a control group, (2) naive rainbow trout passively immunized with sera from previously exposed rainbow trout, (3) previously exposed and resistant rainbow trout, and (4) two innately resistant species, Atlantic salmon and brook trout. The fish were infected *per os* with viable *L. salmonae* spores. The infection was followed in the fish by detection of parasite DNA by PCR in selected organs, at several times post exposure (PE). In controls, parasite DNA was detected in the heart by day 3 PE, and in the gill at 2 weeks PE. Visible xenoparasitic complexes (xenomas) were detected in the gill by week 4 PE. In the passively immunized fish, although the parasite was delayed in reaching the heart, first detection of DNA and of xenomas was otherwise similar. A delay in the arrival in the heart was also detected in rainbow trout which had been previously exposed, and which had recovered from *L. salmonae* infection. In this group, although the parasite was able to reach the heart by week 3 PE, it failed to reach the gill and form xenomas. However, failure of the parasite to reach the gills does not explain the failure of xenoma formation in Atlantic salmon and brook trout. In both Atlantic salmon and brook trout, the parasite reached the

heart and gills quickly, where it remained for 2 weeks before being cleared. Collectively, the results suggest that *L. salmonae* spores are able to germinate and invade permissive and non-permissive host species. Acquired resistance appears to involve mainly cellular responses, but humoral factors may have a limiting effect in the initial stages of infection, whereas innate resistance could involve genetic factors.

Spore germination and passage of the parasite's DNA through the host intestinal wall was not blocked in the naive fish or the non-susceptible species, but it was delayed to different degrees in both the resistant fish and the passively immunized fish. Migration to the heart and gills occurred in all groups of fish (except for resistant fish), but at irregular periods of time when compared with the controls.

4.2 INTRODUCTION

The microsporidian *Loma salmonae,* which infects salmonids of the genus *Oncorhynchus* (Morrison and Sprague 1983; Magor 1987; Kent et al. 1989; Speare et al. 1989; Kent et al. 1995), is one of few species of microsporidia that infect the gills of economically important salmonids in coastal British Columbia (Hauck 1984; Kent et al. 1998). Infection with *L. salmonae* culminates with the production of xenoparasitic complexes (xenomas) in the gills of the infected fish (Hauck 1984). The associated branchitis that results from xenoma dissolution contributes significantly to the death of the infected fish (Speare et al. 1989; Kent et al. 1995). *Loma salmonae*'s host range (Speare et al. 1998a), the influence of temperature on the parasite's life cycle (Beaman et al. 1999a; Speare et al. 1999a), the induction of protective immune responses (Speare et al. 1998b; Kent

et al. 1999), and tissue distribution patterns in fish following experimental infection have been determined (Chapter 2). However, in spite of all the studies done with *L. salmonae,* effective disease control methods remain elusive. Results from drug trials on microsporidial spores have been disappointing (Shaw et al. 1999), and even though some pharmacological agents have been shown to reduce *L. salmonae* infection (Kent and Dawe 1994; Coyle et al. 1998; Higgins et al. 1998; Speare et al. 1998d), slow licensing of drugs for use in commercial aquaculture suggests difficulty in approving their use in a timely manner. Furthermore, the disease is unlikely to be eradicated by test and slaughter practices, particularly since feral Pacific salmon may act as reservoirs of the parasite (Kent et al. 1998). It is therefore critical to increase our understanding of host-parasite relationships with *L. salmonae* to uncover alternative biological approaches for disease control.

Recent advances in the understanding of the host-parasite relationship of *L. salmonae* with susceptible hosts such as rainbow trout (*O. mykiss*) provide several appealing possibilities for disease control. Previous work on the progression of infection of *L. salmonae* under optimal temperatures (15°C) indicated that the parasite passes through the intestinal wall following the ingestion of spores (Chapter 2). Once inside the host, the parasite migrated to the heart where it localized (and underwent further development) for two weeks before appearing at its final location in the gill. Under sub-optimal temperatures, e.g. at 5° or 21°C, parasite development was halted even though it was able to reach the gills. Recent studies have demonstrated that infection with *L. salmonae* confers resistance to reinfection in rainbow trout (Speare et al. 1998b) and Chinook salmon (*O. tschawytscha*) (Kent et al. 1999) that recover from the disease. In RT, this resistance developed even when

the parasite's life cycle was not completed following exposure to *L. salmonae* at high or low temperatures (Beaman et al. 1999a). It is unknown whether immune fish block early stages of the infection (penetration of the sporoplasm through the gut wall), intermediate stages (cardiac phase), or later stages (branchial phase). Nor is it known whether parasites remain latent in immune fish, causing them to become reservoirs. Furthermore, it is also unknown whether resistance is due to humoral or cell mediated mechanisms of defense, or whether immunity can be passively transferred to naive fish.

Studies on the host range of microsporidian parasites are important, since many microsporidian parasites are able to infect sympatric and allopatric species (Terry et al. 1997). Interestingly, *L. salmonae* has a very restricted host range, infecting only species of the genus *Oncorhynchus*. Previous attempts at experimentally infecting other species such as Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), and Arctic char (*S. alpinus*) with *L. salmonae* as determined by xenoma formation have failed (Speare et al. 1998a). However, there are reports that brook trout has been experimentally infected with *L. salmonae* using spores from naturally infected Chinook salmon (Shaw et al. 2000). It is curious that *L. salmonae* exhibits such a restricted host range, particularly when compared to other vertebrate microsporidia such as *Encephalitozoon cuniculi* which is capable of infecting widely different hosts such as rabbits (Mathis et al. 1997), cows (Halánová et al. 1999), and humans (Rossi et al. 1998), or *Enterocytozoon bieneusi* which is capable of causing infection in humans (del Aguila et al. 1997) and macaques (Tzipori et al. 1996; Mansfield et al. 1998). However, it is not known whether apparently refractory species of fish such as Atlantic salmon and brook trout resist infection per se by blocking infection, or

by inhibiting intermediate or prexenoma stages. Knowledge of the resistance mechanisms may suggest methods for enhancing similar mechanisms in otherwise susceptible species.

The purpose of this study was to compare and contrast the progression of infection with *L. salmonae* in a susceptible host species rainbow trout, against that in: a) previously infected rainbow trout that have developed resistance to re-infection, b) naive rainbow trout passively immunized with sera from resistant rainbow trout, and c) naturally resistant, non-susceptible hosts (Atlantic salmon and brook trout). This study takes advantage of the use of *L. salmonae*-specific PCR primers (Docker et al. 1997) and PCR for the monitoring of the progression of the infection, by examining key organs (intestine, heart, spleen, gill) of the experimental fish at different times post exposure (PE).

4.3 MATERIALS AND METHODS

Two trials were designed to test the following hypotheses: 1) Previous infection with *L. salmonae*, does not inhibit reinfection; and 2) Injection of naive fish with serum from "resistant" fish does not inhibit infection with *L. salmonae* as compared to naive fish.

A second trial was conducted simultaneously to test for differences in the progression of infection with *L. salmonae* in naturally "resistant" species from that of susceptible rainbow trout.

4.3.1 Sample population

Size graded juvenile rainbow trout, Atlantic salmon and brook trout were obtained from a certified disease-free commercial hatchery on Prince Edward Island, Canada, with no

previous history of *L. salmonae* infection. The fish were acclimated in their tanks for one week before the beginning of the trial. All procedures were carried out according to the guidelines of the Canadian Council on Animal Care (Anonymous 1993).

4.3.2 Experimental design

Resistance and passive immunization trial: Three circular 100-L fibreglass tanks were used. The habitable volume of each tank was 78.0 L. Sixty naive juvenile rainbow trout (15-20 g) were allocated to each of two tanks. Forty rainbow trout that had been previously infected with *L. salmonae* and had cleared the disease as determined by the absence of xenomas were allocated to the third tank. The water temperature in the tanks was 15°±0.5°C. This trial lasted 8 weeks.

Each of the three tanks was considered as a separate group:

Group I consisted of "resistant" fish, i.e. fish that had been infected with *L. salmonae*, had cleared the disease, and were considered to be immune to reinfection. Fish had been infected more than 10 weeks before the beginning of this trial and had cleared the disease (xenomas). Absence of parasites was corroborated by randomly sampling 3 fish from the tank and further processing of their heart and gills for DNA extraction, followed by PCR for detection of *L. salmonae*.

Group II consisted of naive fish which were injected with sera from "resistant" fish. The sera from the resistant fish was obtained as follows: six resistant rainbow trout were anaesthetized in 60 mg/L benzocaine and approximately 2 ml of blood was drawn from the caudal vein. The blood was let tc clot for 1 h at 4°C, and then centrifuged at 850 x g for 30

min at 4°C to separate the serum. The serum was transferred to a new tube. Sera were pooled and 100 µl were injected intra-peritoneally (IP) to each of the naive rainbow trout. The fish were left to recuperate for one day before infection with *L. salmonae*.

Group III consisted of naive rainbow trout used as controls.

Interspecies trial: Two circular 100-L fibreglass tanks with a habitable volume of 78 L were used. Sixty juvenile Atlantic salmon (~30 ± 1 g) were allocated to one tank (Group IV) and sixty juvenile brook trout (20 ± 1 g) were allocated to the second tank (Group V). The mean temperature in the tanks was 15°C. These fish were fed with minced gills from naive rainbow trout during the acclimation period to habituate them to eat gills before infection. Rainbow trout were used as controls (Group III; Resistance trial). This trial lasted 8 weeks.

4.3.3 Method of infection

Infected RT with visible xenomas were killed with an overdose of benzocaine (150 mg/L). The opercula from both sides were cut off to expose the gill arches. Each gill arch was excised and transversely cut in 1 mm sections, so that each section of cartilage had some gill filaments attached to it. The cut gill arches were weighed and distributed in 5 equal portions of gill material (3 g) per tank. Each portion was mixed with tank water just before infection. The water flow was stopped and the pieces of infected gill arch were evenly distributed in the tank. Feeding was visually monitored. All experimental fish were fasted for one day before infection, and inocula were used on the day of preparation.

4.3.4 Sampling and monitoring of disease development

Development of infection was monitored by weekly DNA extraction in the organs most commonly affected (heart, spleen, gill, small intestine), followed by PCR for the detection of *L. salmonae*. Five fish were randomly sampled at 3 and 7 days post infection, and every week after that for 8 weeks.

The fish were killed with an overdose of benzocaine before sampling. In all groups, organ samples were digested by incubation in separate 1.5 ml microcentrifuge tubes containing 750 µl of proteinase K buffer (10mM Tris pH 8, 10 mM EDTA pH 8, 1% SDS, 150 mM NaCl, 200 µg/ml proteinase K). The tissues were left to be digested for 20-24 h at 37°C. After digestion 20 µg of RNAse A was added to the tube and incubated for 1 h at room temperature. The homogenate was then extracted with phenol:chloroform:isoamyl alcohol (Sigma), and the aqueous phase was precipitated with a 1/10 volume of 3M sodium acetate pH 7 (Sigma) and 2 volumes of ice cold ethanol or 1 volume of isopropanol at room temperature. The precipitate was immediately centrifuged for 3 min at 16 000 x g (Eppendorf microcentrifuge model 5415C), and the pellet was let to air dry for 1 h, after which it was resuspended in 50 µl of sterile deionized water and kept at 5°C until use.

4.3.5 PCR for the detection of *L. salmonae*

The PCR protocol used the *L. salmonae*-specific primers (Docker et al. 1997) LS1 (5'- CTGGATCAGACCGATTTATAT-3') and LS2 (5'- ATGACATCTCACATAATTGTG-3') which produce an amplicon of 272-bp. The PCR reaction was carried out in 30 µl and consisted of the following: 5 µl of a stock of 10 pmol/µl of each primer (LS1 and LS2), 3 µl

of 10x PCR buffer (Pharmacia-Biotech), 0.3 µl of 2 mM dNTP (Pharmacia-Biotech), 0.25 µl of a stock of 5 units/µl of Taq DNA Polymerase (Pharmacia-Biotech), 3 µl of the extracted DNA and sterile deionized water up to a volume of 30 µl. Given the small quantities of reactants needed in each reaction, a master mix which included all of the reactants except the DNA was made by multiplying the number of reactions by the volume of reagent needed per reaction. Twenty seven µl of the mastermix were distributed in each PCR reaction tube and the 3 µl of the DNA were added afterwards. The reaction mixture was capped with 30µl of mineral oil.

The PCR reactions were performed in a PTC-200 Peltier Thermal Cycler (M.J. Research). The conditions of the PCR were 3 min at 95°C of initial denaturation, followed by 30 cycles consisting of 3 steps of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 3 min extension at 72°C, followed by a 10 min final extension at 72°C. A soak cycle at 4°C was added at the end.

The PCR reactions were screened for correct amplicon size by electrophoresis on a 1 % agarose gel, in a Wide Mini Sub Cell GT electrophoresis chamber (BIORAD) in 1x TBE electrophoresis buffer for 1 h at 100 V.

4.4 RESULTS

Naive rainbow trout (Control fish)

Loma salmonae exhibits a consistent pattern of migration in naive fish (Table 4.1) consisting of the rapid appearance of the parasite in the heart (3 days post exposure [PE]), where it remains for two weeks before it reaches the gills (2 weeks PE). Parasite DNA was

detected in the heart and the gills until the end of the trial. Detection of *L. salmonae* in the intestine was inconsistent, and no pattern was discerned.

Table 4.1. Presence of *L. salmonae* DNA in selected control rainbow trout organs at different times PE at 15°C (# positive / # examined).

	Number of rainbow trout positive for *L. salmonae* by PCR								
Organ	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Heart	4/5	3/5	4/5	4/5	5/5	5/5	5/5	5/5	4/5
Gills	0/5	0/5	2/5	5/5	5/5	5/5	5/5	4/5	4/5
Spleen	0/5	0/5	0/5	1/5	5/5	1/5	1/5	1/5	0/5
Intestine	0/5	0/5	1/5	3/5	5/5	0/5	1/5	2/5	0/5
Total	4/5	3/5	4/5	5/5	5/5	5/5	5/5	5/5	4/5

Resistant rainbow trout (previously exposed to L. salmonae).

The distribution of the parasite in fish that had previously been exposed to *L. salmonae* differed to that observed in control fish. Parasite DNA was not detected in any of the organs sampled, including the heart of the infected fish until 3 weeks PE, where it was detected for only two weeks. Parasite DNA was never detected in the gills (Table 4.2).

Naive rainbow trout passively immunized with sera from resistant fish.

In naive fish that were passively immunized with sera from resistant fish (Table 4.3), the parasite DNA was not detected in the heart until week 2 PE, when it was also detected in the gills. From week 3 onwards, the parasite DNA could be detected in the heart and gills until the end of the trial. Detection was erratic in the intestine and spleen.

Table 4.2. Presence of *L. salmonae* DNA in selected organs of "resistant" rainbow trout at different times PE at 15°C (# positive / # examined).

	Number of fish positive by PCR.								
Organ	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Heart	0/5	0/5	0/5	4/5	4/5	0/5	0/5	0/5	0/5
Gills	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5
Intestine	0/5	0/5	0/5	4/5	3/5	0/5	0/5	0/5	0/5
Total	0/5	0/5	0/5	4/5	4/5	0/5	0/5	0/5	0/5

Table 4.3. Presence of *L. salmonae* DNA in selected organs of passive immunized rainbow trout at different times PE at 15°C (# positive / # examined).

	Number of fish positive by PCR								
Organ	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Heart	0/5	0/5	2/5	4/5	5/5	1/5	4/5	3/5	4/5
Gills	0/5	0/5	1/5	3/5	4/5	5/5	5/5	4/5	3/5
Spleen	0/5	0/5	0/5	2/5	2/5	0/5	0/5	2/5	0/5
Intestine	0/5	0/5	0/5	5/5	3/5	0/5	0/5	2/5	0/5
Total	0/5	0/5	2/5	5/5	5/5	5/5	5/5	4/5	4/5

Non-susceptible species (Atlantic salmon and brook trout).

In Atlantic salmon and brook trout, *L. salmonae* showed a very different pattern of migration from that observed in the susceptible control species. Parasite DNA was detected in the heart, gills, spleen and intestine of infected Atlantic salmon 3 days PE (Table 4.4). The parasite was thereafter detected in the heart and gills only until week 2 PE. In brook trout (Table 4.5) *L. salmonae* was detected in the heart and gills 3 day PE, where it stayed

only until 2 weeks PE.

Table 4.4. Presence of *L. salmonae* DNA in selected organs of Atlantic salmon at different time PE at 15°C (# positive / # examined).

	Number of fish positive by PCR								
Organ	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Heart	5/5	4/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5
Gills	3/5	3/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Intestine	5/5	1/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5
Total	5/5	4/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5

Table 4.5. Presence of *L. salmonae* DNA in selected organs of brook trout at different times PE at 15°C (# positive / # examined).

	Number of fish positive by PCR								
Organ	Day 3	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Heart	3/5	3/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5
Gills	3/5	2/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	0/5	1/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5
Intestine	3/5	0/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5
Total	3/5	3/5	4/5	0/5	0/5	0/5	0/5	0/5	0/5

4.5 DISCUSSION

Following the progression of microsporidian pathogens from their port of entry to the site of sporogenesis is difficult because of a lack of suitable labels as compared to

extracellular parasites (Albright et al. 1999). Studies have addressed these questions by means of histology and immunohistochemistry, which may fail to detect all the developmental stages (Cox et al. 1979; Kondova et al. 1998; Shaw et al. 1998). The PCR has been extensively used as a diagnostic tool for the detection of a wide variety of pathogens in different hosts (Olsen et al. 1996; Docker et al. 1997; Brand et al. 1999; Esteban-Redondo et al. 1999; Gray et al. 1999; Zhang and Tarleton 1999), in the study of the epidemiology of human diseases (Baron et al. 1996), in the identification of cell subpopulations (Von Laer et al. 1996), and to determine the impact of vaccination (Griffiths et al. 1998). The current study has capitalized on the combination of a highly reproducible infection model (Speare et al. 1998a), and the availability of *L. salmonae*-specific PCR primers (Docker et al. 1997) which previously have been shown to identify most of the stages of the parasite life cycle (Chapter 2). In chapter 2, this combination of methods allowed us to determine the fate of *L. salmonae* when water temperatures were not favourable to its development. The potential utility of this approach, and in particular, the use of DNA primers in a PCR, has been previously hypothesized (Bell et al. 1999) to be a valuable way of tracking the migration routes of microsporidia, and our results confirm this.

Based on the current results, PCR has also allowed us to partially understand the progression of *L. salmonae* infection when host conditions are not favourable to its life cycle. Particularly, when the parasite was fed to rainbow trout with acquired resistance to reinfection, naive rainbow trout passively immunized with sera from resistant rainbow trout, and naturally resistant, non-susceptible hosts like Atlantic salmon and brook trout, as compared to naive rainbow trout.

The results in the control group confirm many of the observations previously described for the time-course tissue distribution of *L. salmonae* in the naive rainbow trout (Chapter 2). The appearance of the parasite in the heart 3 days post exposure (PE), and in the gills 2 weeks after, appear to be a characteristic developmental feature of *L. salmonae* in naive rainbow trout at 15°C. In contrast, the differences in the sequential tissue distribution of *L. salmonae* in resistant and passively immunized rainbow trout, as well as in non-susceptible salmonid species, suggest a range of interesting possibilities relating to the role of innate and acquired resistance factors.

In resistant rainbow trout, the results suggest that there are at least two stages in the progression of the infection with *L. salmonae* where the fish immune system can inhibit parasite development. The first stage occurs at the beginning of the infection, when the parasite traverses the intestinal mucosa before arriving in the heart, as can be seen by the delays of 3 and 1 weeks observed in the resistant and the passively immunized rainbow trout, respectively. This also points out that passive immunization has a role in delaying the parasite arrival in the heart. The second stage appears to be in the cardiac phase, where the passage of *L. salmonae* into the gills in resistant fish was completely blocked, suggesting that the heart, and the phagocytic cells that line it, may play a crucial role in the progression of the pathogenesis of *L. salmonae*. Furthermore, *L. salmonae* seems to have been cleared from the body of resistant fish from week 5 PE onwards, suggesting that the fish probably do not become carriers. Other studies have reported the presence of free spores in the kidney of resistant Chinook salmon (Kent et al. 1999), where they might act as immunological stimulators to reinforce resistance to new *L. salmonae* infections. However, it is not known

if these renal spores are empty and these have not been detected in rainbow trout kidney.

Studies on the progression of infection with microsporidia in fish are few (Dyková and Lom 1978; Cox et al. 1979; Kim et al. 1996; Kondova et al. 1998) and have not focused on contrasting the timing and initial fate of the parasite in naive and resistant hosts. However, in examples taken from other parasite and host species, delays in the timing of intestinal invasion, have been observed between naive and immune chickens infected with *Eimeria maxima* (Riley and Fernando 1988; Vervelde et al. 1995; Jeurissen et al. 1996). Fish intestinal enterocytes are able to take up antigens and present them to macrophages and lymphocytes in the lamina propria (Ellis 1998), and possibly "wandering cells" (intraepithelial lymphocytes ?) may be used as transport by *L. salmonae* to move from the intestine to other parts of the body (Canning et al. 1986; Shaw et al. 1998). If that is the case, it could be hypothesized that the passage of *L. salmonae* is delayed at the lamina propria level as with *Eimeria*, although it ultimately is able to pass and reach the heart

Little is known about the stages in the life cycle of *L. salmonae* that can induce immunity and those that are most susceptible to the effects of the immune system of resistant fish. Previous studies suggest that *L. salmonae* may still be in the merogonic stage when it is rejected from fish at 21°C two weeks PE (chapter 2), and that this stage is capable of inducing an immune response and resistance in the fish (Beaman et al. 1999a). This suggests that, as in other animal models (Rose and Hesketh 1976), the merogonic stages of *L. salmonae* may be highly immunogenic, and that completion of the life cycle may not be necessary to induce immunity in fish. Furthermore, the present study suggests that the merogonic stages of *L. salmonae* are very susceptible to the effects of the immune system

in resistant fish, as the parasite wasn't able to reach the gills nor (possibly) the sporogonic stage.

The immune system of fish is capable of mounting cellular and humoral responses (Woo 1992; Woo 1997) with the characteristics of specificity and memory found in the immune response of mammals and birds (van Muiswinkel 1995), and at an early stage of development (Tatner 1986). Acquired resistance in fish against a wide range of parasites, including *Amyloodinium ocellatum* (Woo 1997; Cobb et al. 1998), *Cryptobia salmositica* (Woo 1997; Woo 1998), and *Ichthyophthirius multifiliis* (Clark and Dickerson 1997; Woo 1997; Dickerson and Clark 1998) has been reported, and in some cases, this protection can be passively transferred to other fish (Lin et al. 1996). However, the type of response behind the acquired resistance to *L. salmonae* remains unknown. The induction of humoral immune responses by *L. salmonae* has not been detected and the humoral responses reported in fish against other microsporidians are often weak or non-existent (Leiro et al. 1993; Leiro et al. 1996). It is unknown to which stage (spore, sporoplasm, meront, or sporont) humoral responses are directed. Fish immunoglobulins have been detected in the intestine and mucus (Woo 1992), suggesting the possibility of some early protection at the level of the gut epithelium as one of the reasons behind the delay observed in resistant rainbow trout, by favouring macrophage uptake of the spores (Leiro et al. 1996), blocking the germination of the spores or delaying the passage of meronts to the lamina propria (Chapter 3).

The objective of passive immunization of naive rainbow trout was to examine whether humoral factors play a role in protection against *L. salmonae*. The 11-day delay of *L. salmonae* in reaching the heart in the passively immunized fish (similar but shorter than

that observed in resistant fish) suggests that humoral factors may play a limited role in the defence of the fish against the initial invasion of the parasite at the intestinal level, possibly reducing its capabilities of cell invasion, and exposing it to the phagocytic action of macrophages, as has been reported for other pathogens (Ellis 1999). However, the injected antiserum did not seem to exert any other effects in the progression of the infection after the arrival of the parasite to the heart. Studies of passive immunization in fish against a number of pathogens like enteric red mouth disease (Olesen 1991), infectious haematopoietic necrosis virus (LaPatra et al. 1994), channel catfish virus (Hedrick and McDowell 1987), and *Aeromonas salmonicida* (Ellis et al. 1988; Marquis and Lallier 1989) with fish antisera report that the protection against pathogen challenge is weak or partial (Akhlaghi et al. 1996; Akhlaghi 1999), while the use of antisera of mammalian origin achieves high levels of protection, although this protection is not lasting (Akhlaghi et al. 1996; Akhlaghi 1999). The positive (though partial) results on passive immunization against other pathogens, especially bacteria and viruses, may be due to the occurrence of extracellular stages of the pathogen, which can be bound by the antibodies while it is spreading (during a viraemia, for example), after replication at the site of infection. The lack of evidence for the existence of such extracellular stages in *L. salmonae* may provide an explanation as to why passive immunization against *L. salmonae* was not as effective in protecting the naive fish, suggesting that the mechanism of resistance in immune fish against this microsporidian may be mainly cellular. The importance of the cellular immunity in fish microsporidiosis with *L. salmonae* is no doubt important, because the parasite is able to survive inside infected cells (Chapter 3). There are, however, limitations to the interpretation of results from the

passive immunization experiment, as a control group injected with normal serum was not included.

The fate of *L. salmonae* in species traditionally considered non-susceptible and naturally immune, such as Atlantic salmon and brook trout is not known. Innate immunity in fish against parasites has been observed (Forward et al. 1995; Forward and Woo 1996), and differences among fish in the response to different antigens have been reported between (Eide et al. 1994; Hedrick et al. 1999), and within, species due to genetic variation (Stromsheim et al. 1994a; Stromsheim et al. 1994b). Similar observations have been made in mammalian models with *Encephalitozoon intestinalis* (El Fakhry et al. 1998). This study suggests that, in apparently non-susceptible species, the parasite is able to invade the fish. However, once inside the host, the typical parasite tropism shows a marked deviation from the normal pattern of pathogenesis observed in susceptible species like rainbow trout. This colonization is very rapid and short lived, and parasite DNA could not be detected in the sampled tissues after week 2 PE, which may indicate control and clearance of the parasite by the immune system, thus preventing its progression to the xenoma stage. It is difficult to surmise where and what factors affect the development of the infection in the foreign hosts (Atlantic salmon and brook trout), but the absence of positive signals by PCR after 2 weeks PE, may indicate that the fish did not become reservoirs after infection. The inability of *L. salmonae* to infect closely related species, is an indication of the high specificity of this parasite, a specificity that has been reported for other fish microsporidia (Pomport-Castillon et al. 1999). Host specificity is judged in other parasites by the completion of the life cycle, (Long and Millard 1979), thus, in *L. salmonae* this would mean the completion of the

sporogonic stage with the corresponding formation of xenomas. However, transient infection of non-susceptible hosts by other pathogens has also been reported, although in the end, infections in non-natural hosts are rejected faster than in natural hosts that have acquired the immunity (Long and Millard 1979), a pattern observed with *L. salmonae* DNA which was detected longer in immune fish (up to 5 weeks PE) than in the non-susceptible host (up to 2 weeks PE). These results, regarding the infection of foreign hosts, pinpoint the importance that host genetic factors may have in controlling disease susceptibility, although it is not known how this genetic control is exerted, but differences in cellular immunity may account for differences in resistance (Bumstead et al. 1995). A study contrasting the cell-mediated immune response between non-susceptible and susceptible species when challenged to *L. salmonae* should be undertaken.

In summary, fish exposed to *L. salmonae* become resistant to further infection with this parasite, which is delayed at the intestinal level and blocked in the heart. The immune response behind the resistance in the fish seems to be cellular, as passive immunization with sera from resistant fish did not confer protection against infection. *Loma salmonae* exhibits a high host specificity and is unable to infect non target species such as AS and BT. This is important for the aquaculture industry in British Columbia, because the presence of *L. salmonae* susceptible fish such as Chinook salmon will not likely affect the culture of Atlantic salmon. Further studies on the mechanisms of resistance both in "resistant" fish and in non-susceptible species are warranted, specially for the development of methods to induce resistance in susceptible fish such as Chinook salmon before they are stocked in net pens.

5 HISTORY, BIOLOGICAL CHARACTERISTICS AND HOST RANGE OF A LOMA SALMONAE VARIANT ISOLATED FROM BROOK TROUT.

5.1 ABSTRACT

This study describes the isolation of a new variant of *Loma salmonae*, here termed *L. salmonae* SV (*Salvelinus* variant). A trial was conducted to study the development of *L. salmonae* in brook trout (*Salvelinus fontinalis*), a species normally considered refractory to infection with this parasite. However, in this study, 20% of a group of 40 brook trout developed xenomas following infection with *Loma salmonae* spores pooled from naturally infected Chinook salmon (*Oncorhynchus tschawytscha*) and experimentally infected rainbow trout *(O. mykiss)*. The spores collected from these fish were able to infect a second group of naive brook trout, and a 50% infection rate was observed in the infected fish. After this second passage in brook trout, the resulting *L. salmonae* SV spores exhibited a high preference for development in brook trout, resulting in 100% point prevalence and high xenoma intensity in the gill filaments. In contrast, the virulence towards rainbow trout was very low. A study of the host range of the *L. salmonae* SV showed that high numbers of xenomas developed in Arctic char (*Salvelinus alpinus*), whereas Atlantic salmon (*Salmo salar*), Chinook salmon, rainbow trout, and coho salmon (*O. kisutch*) only showed sporadic xenomas in a small number of fish. Spores collected from the infected Arctic char were used to infect another group of naive Arctic char, and the fish that recovered from the infection developed resistance to reinfection with the *L. salmonae* SV. Based on its ultrastructural, molecular, and antigenic characteristics, *L. salmonae* SV retains sufficient similarity to

typical *L. salmonae* such that it is not considered herein to be a new species. However, its range of biological differences suggests it to be a variant.

5.2 INTRODUCTION

Loma salmonae is a microsporidian parasite of salmonid fish, whose impact to the Chinook salmon *(Oncorhynchus tshawytscha)* aquaculture industry of British Columbia has been recently recognized (Hauck 1984; Kent et al. 1995; Constantine 1999; Scholz 1999). This parasite primarily infects species of the genus *Oncorhynchus* (Shaw and Kent 1999), and produces branchial xenomas containing infective spores (Morrison and Sprague 1983; Hauck 1984; Magor 1987; Kent et al. 1989; Bruno et al. 1995; Kent et al. 1998). However, *L. salmonae* has also been reported in other genera of salmonids, including brown trout *Salmo trutta* (Poynton 1986) and brook trout *Salvelinus fontinalis* (Bader et al. 1998). A growing list of species within the genus *Loma*, which include *L. dimorpha* (Arias et al. 1999), *L. salmonae* (Morrison and Sprague 1983), *L. camerounensis* (Fomena et al. 1992), *L. morhua* (Morrison and Sprague 1981a), *L. branchialis* (Morrison and Sprague 1981b), *L. fontinalis* (Morrison and Sprague 1983), *L. diplodae* (Canning et al. 1986), and *L. embiotocia* (Shaw et al. 1997), and the reports of *L. salmonae* infections in species from genus other than *Oncorhynchus*, questions our current understanding of the host range of *L. salmonae*.

It has been proposed that microsporidia evolved with a particular host and are adapted to a specific host or host group (Solter et al. 1997; Solter and Maddox 1998a), and thus have a narrow range of species that they can infect. Assessment of the range of species that can be infected with *L. salmonae* has yielded somewhat conflicting results under laboratory

conditions. Spores that were originally derived from Chinook salmon and that have been repeatedly passaged through rainbow trout were unable to complete their life cycle in Atlantic salmon *Salmo salar*, Arctic char *Salvelinus alpinus*, and brook trout (Speare et al. 1998a). Previous studies (Chapter 4), using spores derived from Chinook salmon and passed repeatedly through rainbow trout, support Speare et al. (1998a), and show that *L. salmonae* can infect Atlantic salmon and brook trout, although the life cycle is truncated early, with no xenoma development. In contrast, parasites derived from naturally infected farm-reared Chinook salmon (Shaw et al. 2000), were able to complete the life cycle in at least a small proportion of brook trout. This suggests that farm-reared Chinook salmon may carry more than one variant of *L. salmonae*, i.e., it may carry both the variant which readily infects species within the genus *Oncorhynchus*, and that we have exploited in experimental models, and an atypical variant or species of *L. salmonae* which is also capable of infecting salmonids of the genus *Salvelinus*, e.g. brook trout. The purpose of this study was to test this hypothesis by firstly isolating an atypical *L. salmonae* whose host range includes *Salvelinus*, and secondly to assess selected biological characteristics, in particular its virulence within a range of salmonid species.

5.3 MATERIALS AND METHODS

To achieve the purpose of this study, several trials were designed. Trial 1 was conducted to test the ability of a mixed inoculum of *L. salmonae* spores (from laboratory infected rainbow trout and naturally infected Chinook salmon) to infect naive brook trout. Trial 2 was conducted with the objective to isolate and amplify the *Salvelinus*-infecting

variant (SV) of *L. salmonae* obtained in Trial 1. Trials 3 and 3a were designed to compare the infectivity of *L. salmonae* SV spores in brook and rainbow trout. Trial 4 was designed to study the intial stages of infection of the *L. salmonae* SV in brook trout. Trial 5 and its sub-trials 5a, 5b, 5c, and 5d were conducted to study the host range of this *L. salmonae* variant.

5.3.1 Experimental fish

Juvenile brook trout, rainbow trout, Atlantic salmon, and Arctic char were acquired from a certified disease-free (notifiable pathogens) commercial hatchery on Prince Edward Island, with no history of *L. salmonae*. Chinook and coho salmon (*O. kisutch*) were acquired from the Puntledge River Hatchery, in Courtenay, B. C., a certified disease free federal hatchery, (Department of Fisheries and Oceans). All procedures were conducted according to the guidelines of the Canadian Council on Animal Care (Anonymous 1993). All fish were acclimated in their experimental fibreglass tanks for one week before the start of the trials. All tanks were circular, and had a habitable volume of 78 L. The tanks used in Trial 5a in British Columbia were oval and made of fibreglass, with a habitable volume of 80 L. The fish stayed in the experimental tanks throughout the duration of the trials. Water temperature was 15±0.5°C. Details on the species used, number of fish, weight of fish and duration of trial are found in Table 5.A.

5.3.2 Experimental design

Trial 1: Designed to test the ability of a mixed inoculum of *L. salmonae* spores

(harvested from laboratory-infected rainbow trout and naturally-infected Chinook salmon gills [from British Columbia]) to infect and produce xenomas in naive brook trout juveniles under experimental conditions.

Trial 2: Designed to test the ability of *L. salmonae* SV spores harvested from brook trout in trial 1, to infect and produce xenomas in naive brook trout.

Trial 3: Designed to compare and contrast the ability of the *L. salmonae* SV spores harvested from the infected fish in trial 2 to invade the fish and to form xenomas in naive brook and rainbow trout. A second group of naive brook and rainbow trout were infected with *L. salmonae* spores derived from experimentally infected rainbow trout as a control. Twenty brook trout, and twenty rainbow trout, were randomly assigned and housed in tank A, while the remaining twenty brook trout, and twenty rainbow trout, were housed in tank B. The experimental populations were distributed as follows:

Tank A: Brook and rainbow trout were intubated and dosed with an inoculum of *L. salmonae* SV spores. The two species of fish were cohabitated in the same tank throughout the duration of the trial.

Tank B: Brook and rainbow trout were intubated and dosed with an inoculum of *L. salmonae* spores derived from rainbow trout gills. The two species were cohabitated in the same tank throughout the duration of the trial.

Trial 3a: The purpose of this trial was to repeat the experiment of infecting brook and rainbow trout with *L. salmonae* SV spores and validate the observations made in trial 3.

Trial 4: This trial was conducted to study the initial stages of infection of the *L.*

salmonae SV in brook trout.

Trial 5: The purpose of this trial was to study the host range of the *L. salmonae* SV, and it consisted of a series of sub-trials.

Trial 5a tested the infectivity and ability of the *L. salmonae* SV spores to develop xenomas in species of the genus *Salvelinus* (naive brook trout, brook trout previously exposed to the *L. salmonae* SV, Arctic char), *Oncorhynchus* (Chinook and coho salmon, rainbow trout), and *Salmo* (Atlantic salmon). Seven experimental fish populations were used in this experiment, each housed in a separate tank. The infections of Atlantic salmon, Arctic char, 'resistant' brook trout and naive brook trout were carried out at the Atlantic Veterinary College in PEI. The infections of Chinook salmon, coho salmon, and rainbow trout were carried out simultaneously at the Pacific Biological Station in Nanaimo, BC. All fish (in BC and PEI) were infected by intubation with the same *L. salmonae* SV inoculum.

Trial 5b: This trial tested the ability of *L. salmonae* SV spores obtained from infected Arctic char (Trial 5a) to infect naive Arctic char. At the end of the trial the fish were left to clear the infection for 8 weeks.

Trial 5c: The purpose of this trial was to assess if exposure of Arctic char to the *L. salmonae* SV resulted in the development of resistance to reinfection with *L. salmonae* SV spores harvested from brook trout. The recovered Arctic char from trial 5b were used, after clearance of infection, assessed by the absence of branchial xenomas prior to reinfection (15 weeks after first challenge).

Trial 5d: The objective of this trial was to assess the effects that stress may have on the infection of Atlantic salmon with the *L. salmonae* SV. The fish used in this trial were

stressed daily by chasing them in the tank with a net, capture and subsequent emersion for 30 sec (Sanchez et al. 1997).

Table 5.A Details of the fish and the duration of the different trials.

Trial	Species	No. fish	Mean Weight	Duration
1	Brook trout	40	15 g	7 weeks
2	Brook trout	20	15 g	7 weeks
3	Brook trout	40	15 g	7 weeks
	Rainbow trout	40	20 g	7 weeks
3a	Brook trout	40	20 g	7 weeks
	Rainbow trout	60*	30 g	7 weeks
4	Brook trout	80	15 g	7 weeks
5a	Atlantic salmon	35	75 g	7 weeks
	Arctic char	20	200 g	7 weeks
	rBrook trout[1]	15	25 g	7 weeks
	Brook trout[2]	40	15 g	7 weeks
	Chinook salmon	35	10 g	7 weeks
	Coho salmon	35	10 g	7 weeks
	Rainbow trout	35	15 g	7 weeks
5b	Arctic char	10	200 g	7 weeks
5c	rArctic char[3]	10	200 g	7 weeks
5d	Atlantic salmon	30	75 g	7 weeks

[1]Brook trout previously exposed to *L. salmonae* SV spores; [2]naive brook trout; [3]"resistant" Arctic char previously exposed to *L. salmonae* SV spores; *The excess (40) rainbow trout were left to recover from infection for eight weeks after the end of this trial, to be used in another experiment at a later date.

5.3.3 Method of infection

Fish in every trial were infected either by feeding or by gastric intubation (Table 5.B).

Table 5.B. Details of infection for each trial

Trial	Inoculum source	Species infected	Method of infection	Spore count
1	Mixed gills from rainbow trout and Chinook salmon (BC)	Brook trout	Feeding	N.A.
2	Brook trout[1] from Trial 1	Brook trout	Feeding	N.A.
3	Brook trout[1] from Trial 2	Brook trout (Tank A)	Gastric Intubation	N.A.
	Brook trout[1] from Trial 2	Rainbow trout (Tank A)	Gastric Intubation	N.A.
	Rainbow trout[3]	Brook trout	Gastric Intubation	N.A.
	Rainbow trout[3]	Rainbow trout	Gastric Intubation	N.A.
3a	Brook trout[1]	Brook trout	Gastric Intubation	N.A.
	Brook trout[1]	Rainbow trout	Gastric Intubation	N.A.
4	Brook trout[1]	Brook trout	Gastric Intubation	1 x 10^6/fish
5a	Brook trout[1]	Arctic char	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	Atlantic salmon	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	rBrook trout[2]	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	Chinook salmon	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	Coho salmon	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	Rainbow trout	Gastric intubation	1 x 10^6/fish
	Brook trout[1]	Brook trout	Gastric intubation	1 x 10^6/fish
5b	Arctic char[1]	Arctic char	Gastric intubation	1 x 10^6/fish
5c	Brook trout[1]	rArctic char[2]	Gastric intubation	1 x 10^6/fish
5d	Brook trout[1]	Atlantic salmon	Gastric intubation	1 x 10^6/fish

[1] *L. salmonae* Sv derived from naturally infected Chinook salmon and passaged in brook trout; N.A. = not assessed; [2] r = resistant (fish previously exposed to the same parasite variant); [3] *L. salmonae* derived from naturally infected Chinook salmon and passaged 3 times in rainbow trout.

Infection by feeding: infected gill arches were repeatedly cut transversely so that the inoculum consisted of approximately 2 gill filaments connected by the cartilaginous arch. Water flow in the tanks was stopped just before the feeding-infection process. The gill inoculum was mixed with 5 ml of tank water and distributed evenly in the tank. Fish were observed while they fed to ensure that all the gill inoculum was consumed.

Infection by gastric intubation: gill filaments alone were finely macerated and diluted with tank water in a 1:1 ratio. Spores were counted with a haemocytometer and dilutions made with deionized distilled water to obtain a final concentration of 10×10^6 spores/ml. Fish were intubated with 0.1 ml of prepared tissue through a 1 ml syringe with attached tubing. The fish were lightly anaesthetized with benzocaine to facilitate intubation.

5.3.4 Sampling and assessment of infection

All fish were anaesthetized with benzocaine (60 mg/L) for non-lethal examination, unless the fish were euthanised; then the benzocaine concentration was increased to 150 mg/L. Details for infection assessment are shown in Table 5.C.

Trial 1. All fish were examined for the presence of xenomas in the gills in the dissecting microscope 7 weeks PE.

Trial 2. All fish were examined for the presence of xenomas by using a dissecting microscope 7 weeks PE. From the gills sampled for histology, the number of xenomas per gill filament was assessed by counting the number of filaments present in the gill arch and the number of xenomas detected. A xenoma index was obtained by dividing the number of xenomas observed by the number of filaments counted.

Trial 3. All fish were euthanised 7 weeks PE. A second xenoma index (Number of xenomas per gill arch) was assessed by counting the total number of xenomas observed in a wet mount of the entire gill arch.

Trial 3a. Twenty brook trout and twenty rainbow trout were euthanised 7 weeks PE. The xenoma index of trial 3 was used to assess the intensity of infection.

Trial 4. Five fish were sampled and euthanised at the specified times PE. From each sampling day, 14 serial sections 5 μm thick were cut from the sampled organs. Each third section was processed for ISH. Number of xenomas per gill filament for each fish sampled at week 6 and 7 PE were assessed as described for trial 2.

Trial 5a: Five fish from each group were sampled at the specified times PE, except for Arctic char where only 3 fish were sampled at a time, and rBrook trout (2 fish/sampling time). For the Arctic char, number of xenomas per gill filament was assessed from the fish sampled at 7 weeks PE.

Trial 5b: All fish were observed for the presence of xenomas in the gills by using the dissecting microscope at 7 weeks PE.

Trial 5c: All fish were euthanised 7 weeks PE and the first left gill arch processed for histology.

Trial 5d: All fish were euthanised 7 weeks PE and the first left gill arch processed for histology.

Table 5.C Sampling and assessment of infection

Trial	Sampling Time	Assessment of infection	Method
1	7 weeks PE[1]	Non-lethal	Xenoma detection by dissecting microscope. Positive fish euthanised. First left gill arch sampled for PCR.
2	7 weeks PE	Non-lethal	Xenoma detection by dissecting microscope. Fish with xenomas euthanised. First left gill arch sampled for histology.
3	7 weeks PE	Lethal	Xenoma counts in wet mount of first left gill arch by microscope. Second left gill arch sampled for histology.
3a	7 weeks PE	Lethal	Xenoma counts in wet mount of first left gill arch by microscope. Second left gill arch sampled for histology.
4	Daily 1-7 days PE	Lethal	Heart and first left gill arch sampled for histology.
	Weekly 1-7 weeks PE	Lethal	Heart and first left gill arch sampled for histology.
5a	Weekly 1-7 weeks PE	Lethal	First left gill arch (heart and first left gill arch for Chinook and coho salmon) sampled for histology.
5b	7 weeks PE	Non-lethal	Xenoma detection by dissecting microscope.
5c	7 weeks PE	Lethal	First left gill arch sampled for histology.
5d	7 weeks PE	Lethal	First left gill arch sampled for histology.

[1]PE - weeks elapsing since exposure to infective inoculum

5.3.5 Histology of gills

All samples fixed for histology in phosphate buffered formalin were further processed

in an automatic tissue processor. Five μm thick sections were cut and processed for routine histological staining with H&E, immunohistochemistry with the anti-*L. salmonae* MAb 4H8 (Speare et al. 1998c), or for *in situ* hybridization (ISH) with a *L. salmonae* ssDNA probe (Sanchez et al. 1999). Specific details for each trial are presented in Table 5.D

Table 5.D Analytical method used for histological section in the different trials

Trial	Method
2	*In situ* hybridization and immunohistochemistry (anti-*L. salmonae* MAb 4H8)
3	Haematoxylin and Eosin
3a	Haematoxylin and Eosin
4	*In situ* hybridization
5a	*In situ* hybridization and immunohistochemistry (anti-*L. salmonae* MAb 4H8)
5c	*In situ* hybridization
5d	*In situ* hybridization

5.3.5.1 In situ hybridization

Sections were deparaffinized in xylene (7 min), rehydrated in a descending ethanol series (100, 95, 70, 50%), washed in distilled water, and equilibrated in 50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, pH 7.4 (TES) for 10 minutes. After equilibration the sections were permeabilized with proteinase K (15μg/ml in TES) for 15 min at 37°C. Proteolysis was stopped with two 5 min washes in PBS with 2 mg/ml glycine (Fisher Scientific). Tissue sections were immersed in aqueous 20% acetic acid at 4°C for 15 sec, and washed in two changes of distilled water for 10 min each. The tissues were briefly postfixed in 0.4% paraformaldehyde for 5 min, rinsed in distilled water and air dried.

For the hybridization 30 ng of digoxigenin-labelled probe mixed with 100 μl of

hybridization buffer (5µl 100x Denhardt's solution, 50µl dextran sulfate 50%, 10 µl sperm DNA 100 mg/ml, 100 µl 20X SSC, DW to 250 µl, and 250 µl formamide) were applied onto the permeabilized tissue sections, the sections were covered with hybri-slips [Sigma], and tissue DNA was denatured at 90-95°C for 3 min on a heating block. The slides were then transferred to a humidified box and incubated at 40°C overnight.

After incubation, excess probe was washed off the sections by immersion in 4x SCC for 5 minutes at room temperature. The slides were equilibrated in 50 mM Tris-HCl, 100 mM NaCl, pH 7.2 (TBS) for 5 min, and blocked with Tris-buffered saline, 3% BSA, 0.5% Triton X-100 (TBT) at room temperature for 10 min. After equilibration, the slides were transferred to a slide incubation tray and incubated with alkaline phosphatase conjugated anti-digoxigenin sheep Fab fragments [Boehringer Mannheim] diluted 1:600 in TBT for 1 h. The slides were then washed in TBS for 5 min. The signal was developed by applying 300 µl of a mix containing 45 µl nitroblue tetrazolium (NBT) [Boehringer Mannheim] and 35 µl bromo-chloro-indolyl-phosphate (BCIP) [Boehringer Mannheim] in 10 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [Sigma], pH 9.5 for 5 h in the dark. Colour development was stopped by washing in distilled water for 5 min. The slides were counterstained with 10% methyl green, rinsed in distilled water, air dried and mounted with Permount (Fisher Scientific).

5.3.5.2 Immunohistochemistry

Sections were deparaffinized in xylene for 5 minutes, rehydrated in descending ethanol series (100%, 95%, and 70% for 5 minutes each), and washed in distilled water. All

incubations were done in a moist chamber. Tissue sections were blocked with 5% hydrogen peroxide in methanol for 20 minutes, to remove endogenous peroxidase activity. Then the tissues were rinsed and soaked in PBS for 10 minutes before blocking with normal goat serum diluted 1/20 in PBS for 10 minutes. After blocking the sections were incubated with primary antibody (anti-*L. salmonae* undiluted monoclonal antibody supernatant) for 1 hour. The sections were then rinsed in PBS for 10 minutes before incubation with peroxidase labelled goat antimouse IgG diluted 1:100 in PBS for 30 minutes. The incubation was followed by rinsing in PBS for 10 minutes. Two hundred microlitres of substrate (20 ml of PBS, 200 µl of H_2O_2, and one 10 mg tablet of diaminobenzidine) were then applied to the slides and incubated for 3-5 minutes, before the reaction was stopped with distilled water. The slides were briefly counterstained with methyl green, before being mounted with Permount (Fisher Scientific). Sections were viewed and photographed with an Olympus BX50 photomicroscope.

5.3.6 Data Analysis

For trial 3 and 3a, statistical comparisons were made between data recorded for each group of fish, depending on the species infected and the origin of the infection inoculum. The unit of study was the individual fish. After ANOVA, a t-test was used to compare the number of xenomas present in the sampled gill arch between the two groups in tanks A and B. For the xenoma indices in trials 2, 4 and 5a, mean and standard deviation were calculated. All statistical analyses were carried out using commercial software (MINITAB™ Inc.). Differences were considered significant at the $\alpha = 0.05$ level of probability.

5.4 RESULTS

Trial 1. This trial assessed the infectivity of a *L. salmonae* mixed inoculum derived from spores harvested from laboratory-infected rainbow trout and naturally infected Chinook salmon, and its ability to produce xenomas in brook trout. Xenoma formation was observed in twenty per cent of the exposed brook trout (Table 5.E). Few xenomas (2-3 per gill arch) were observed in the fish that developed xenomas. The PCR done in the gills of positive fish with the *L. salmonae*-specific primers (Docker et al. 1997) confirmed the identity of the parasite as *L. salmonae*.

Trial 2 tested the ability of the spores collected from the xenomas found in the brook trout from Trial 1 to infect and complete the *L. salmonae* life cycle in brook trout, and further, to isolate the brook trout variant. The spores were able to infect and complete the *L. salmonae* life cycle, as fifty percent of the exposed fish developed xenomas (Table 5.E). Histological examination of the gills by ISH revealed the presence of xenomas in the gill filaments of infected fish (Fig. 5.1). This trial also resulted in the amplification and possible purification of the *L. salmonae* SV, as more xenomas were present (0.325 xenomas/filament; ~8 xenomas/gill arch) compared with exposed fish in trial 1.

Trial 3 was conducted to contrast the ability of the *L. salmonae* SV spores harvested from the infected fish in trial 2 to infect and complete the parasite's life cycle in naive brook and rainbow trout. Results of H&E stained samples from this trial suggest that the *L. salmonae* SV show a marked preference for xenoma development in brook trout over rainbow trout. Brook trout infected with the *L. salmonae* SV exhibited 100% prevalence (number of fish infected/total number of fish). In addition, the intensity (number of

xenomas/fish host) of xenomas in the gills was very high (Table 5.E), and accompanied with extensive epithelial hyperplasia and lamellar fusion, completely obliterating the normal structure of some gill filaments (Fig. 5.2). In contrast, rainbow trout infected with the *L. salmonae* SV exhibited very low prevalence and xenoma intensity in the gill arch (Table 5.E). Microscopic examination revealed that rainbow trout gill was not affected by infection with this variant of *L. salmonae* (Fig. 5.3). The number of xenomas was significantly higher in brook trout compared to rainbow trout exposed to *L. salmonae* SV ($P \leq 0.0001$).

In contrast, Chinook salmon derived *L. salmonae* spores passaged 2 times in rainbow trout developed to xenomas better in rainbow trout compared with brook trout. The control group of brook trout infected with laboratory rainbow trout-derived *L. salmonae* spores were lightly infected (8 xenomas/gill arch) without noticeable alteration of the normal gill structure (Fig. 5.4), despite showing a prevalence of 100%. Rainbow trout infected with the same inoculum showed significantly higher xenomas per gill arch than brook trout ($P \leq 0.009$; Table 5.E), although the gill structure was not extensively affected (Fig. 5.5).

Trial 3a repeated the results obtained in Trial 3 for brook and rainbow trout exposed to *L. salmonae* SV spores (Table 5.E), confirming the preference that this variant has for infection and development in brook trout. A high intensity of xenomas in the gills of the brook trout infected with the *L. salmonae* SV spores was observed (Fig. 5.6), with extensive epithelial hyperplasia in the gill lamellae (Fig . 5.7). This contrasted with the infected rainbow trout, which had a very low or no incidence of xenomas in the gills (Fig. 5.8) and very low prevalence (Table 5.E). The number of xenomas per gill arch in brook trout were significantly higher than those observed in rainbow trout ($P \leq 0.0001$).

Trial 4 studied the initial stages of infection of the *L. salmonae* SV in brook trout. Although intestine was not sampled in this trial, this parasite moved through the host within host cells, in a similar way to that described for the rainbow trout-derived *L. salmonae* (Chapter 3). Cells harbouring early stages of *L. salmonae* SV could be identified by ISH in the heart as early as 2 days PE associated with the ventricular endocardial lining (Fig. 5.9). These early stages were better visualized at 5 days PE (Figs. 5.10 & 5.11). Dividing stages were also observed in infected blood cells (Fig. 5.12), not only associated with the ventricular endocardium. Xenomas were observed in the heart of some of the fish at weeks 5, 6, and 7 PE (Figs. 5.13, 5.14, 5.15), and usually only one xenoma was detected in each fish heart. Microscopic assessment of gills at the end of the trial revealed an intensity of 2.76 xenomas per gill filament (Table 5.E).

Trial 5 was conducted to study the host range and biological characteristics of *L. salmonae* SV.

Subtrial 5a tested the ability of *L. salmonae* SV spores to form xenomas in a number of different salmonid species. The results from this trial confirmed the preference that the *L. salmonae* SV has for brook trout, and expanded the range of susceptible species to Arctic char, while retaining the capability of infection and developing (although with low incidence of disease) in Chinook and coho salmon, and rainbow trout.

Specifically, the results show that the life cycle of *L. salmonae* SV was completed in Arctic char; prevalence was 100% and xenoma intensity was 2.166 per gill filament (Table 5.E). Histological examination by ISH revealed numerous xenomas of varying sizes in the gills of exposed Arctic char (Fig. 5.16). Xenoma development was not observed in

the gills of brook trout that had been previously exposed to *L. salmonae* SV (Table 5.E). Gill structure was preserved and no signs of disease development were observed (Fig. 5.17). *Loma salmonae* SV spores had limited development in Atlantic salmon, as only one mature xenoma was found at the base of a filament of one fish 7 weeks PE (Fig. 5.18). In Chinook salmon, low prevalence was observed (Table 5.E) as only one fish developed xenomas in the gills (Fig. 5.19) and in the heart (Fig. 5.20). Xenoma intensity in the gills of the one Chinook salmon with xenomas was low (8 xenomas/gill arch). Inoculation of *L. salmonae* SV spores in coho salmon resulted in a single fish becoming infected with development of a single xenoma (Table 5.E; Fig. 5.21). In rainbow trout three fish developed one xenoma each (Table 5.E; Fig. 5.22); by contrast, the naive brook trout showed high numbers of xenomas and 100% prevalence (Table 5.E; Fig. 5.23).

Subtrial 5b tested the ability of the *L. salmonae* SV spores harvested from infected Arctic char from Subtrial 5a to infect other fish from the same species. The results showed that the spores were capable of infecting and developing xenomas in all the naive Arctic char (Table 5.E), but their number and intensity in the gills was not assessed because the fish were left to clear the disease.

Trial 5c assessed the development of resistance in Arctic char after infection with the *L. salmonae* SV. Xenoma development was not observed using ISH in any of the previously infected fish 7 weeks after the second exposure to *L. salmonae* SV (Table 5.E). The gill structure was normal and had no signs of disease with *L. salmonae* SV (Fig. 5.24).

Trial 5d assessed the effects of stress on the infection of Atlantic salmon with the *L. salmonae* SV. No xenomas were observed by ISH in the Atlantic salmon infected with *L.*

salmonae SV spores from brook trout (Table 5.E; Fig. 5.25).

Table 5.E Summary of the results from trials 1 - 5.

Trial	Species	Inoculum Source (Trial)	Xenoma formation	Point Prevalence	Xenoma index
1	Brook trout	Rainbow trout + Chinook salmon	Yes	8/40	N.A.*
2	Brook trout	Brook trout (1)	Yes	10/20	0.325[1]
3	Brook trout	Brook trout (2)	Yes	12/12	437[a]
	Rainbow trout	Brook trout (2)	Yes	1/20	0.05[a]
	Brook trout	Rainbow trout*	Yes	20/20	8[b]
	Rainbow trout	Rainbow trout *	Yes	20/20	80[b]
3a	Brook trout	Brook trout (3)	Yes	20/20	206[c]
	Rainbow trout	Brook trout (3)	Yes	3/20	0.15[c]
4	Brook trout	Brook trout (3a)	Yes	80/80	2.763[2]
5a	Arctic char	Brook trout (4)	Yes	20/20	2.166[3]
	rBrook trout[r]	Brook trout (4)	No	0/15	N.A.
	Atlantic salmon	Brook trout (4)	Yes	1/30	N.A.
	Chinook salmon	Brook trout (4)	Yes	1/30	N.A.
	Coho salmon	Brook trout (4)	Yes	1/30	N.A.
	Rainbow trout	Brook trout (4)	Yes	3/30	N.A.
	Brook trout	Brook trout (4)	Yes	40/40	N.A.
5b	Arctic char	Arctic char (5a)	Yes	10/10	N.A.
5c	rArctic char[r]	Brook trout	No	0/10	N.A.
5d	Atlantic salmon	Brook trout	No	0/30	N.A.

[a]xenomas/gill arch (P = 0.0001); [b]xenomas/gill arch (P = 0.009); [c]xenomas/gill arch (P = 0.0001); [1] xenomas/filament (n = 10); [2] xenomas/filament (n = 10); [3]xenomas/filament (n = 5); [r] = resistant fish; *N.A. = not assessed; * inoculum derived from naturally infected Chinook salmon passaged 3 times in rainbow trout; values sharing the same superscript letter are significantly different.

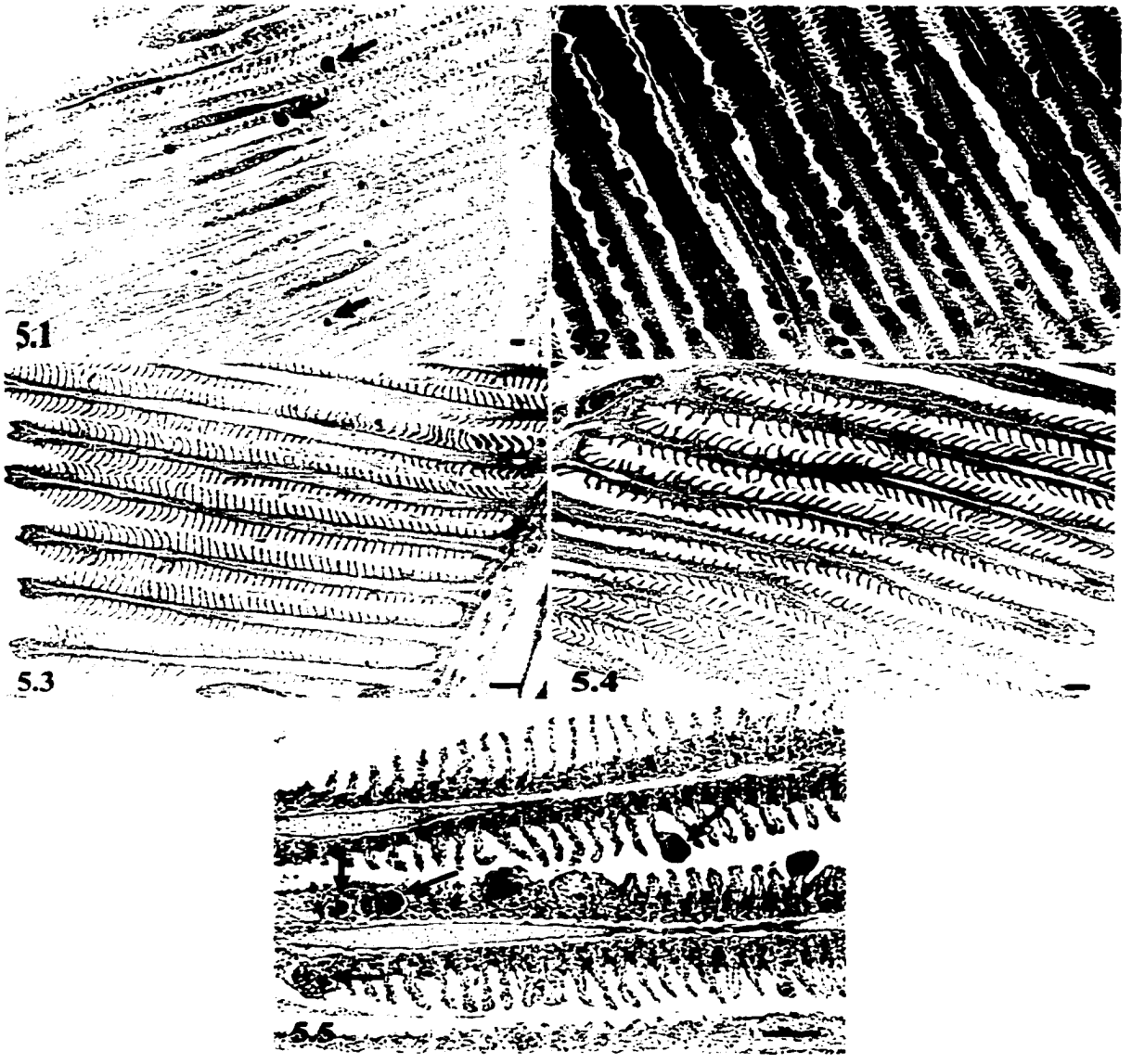

Fig. 5.1 Brook trout (*Salvelinus fontinalis*) gills 7 weeks PE to *Loma salmonae* SV spores. Several xenomas are visible in the gills (arrows) . *In situ* hybridization (ISH) with *L. salmonae* ssDNA digoxigenin (DIG)-labelled probe and alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments; methyl green counterstain. Bar = 100 μm. Fig. 5.2 Brook trout gills 7 weeks PE to *L. salmonae* SV spores. Numerous xenomas are visible (arrows); evident epithelial hyperplasia and the filament structure is lost. H&E. Bar = 100 μm. Fig. 5.3 Rainbow trout (*O. mykiss*) gills 7 weeks PE to *L. salmonae* SV spores. No xenomas are observed and the filament structure is preserved. H&E. Bar = 160 μm. Fig. 5.4 Brook trout 7 weeks PE to wild-type *L. salmonae* spores. No xenomas are observed, fish had very light infections. H&E. Bar = 100 μm. Fig. 5.5 Rainbow trout 7 days PE to wild-type *L. salmonae* spores. Several xenomas are visible along the gill filament (arrows). H&E. Bar = 100 μm.

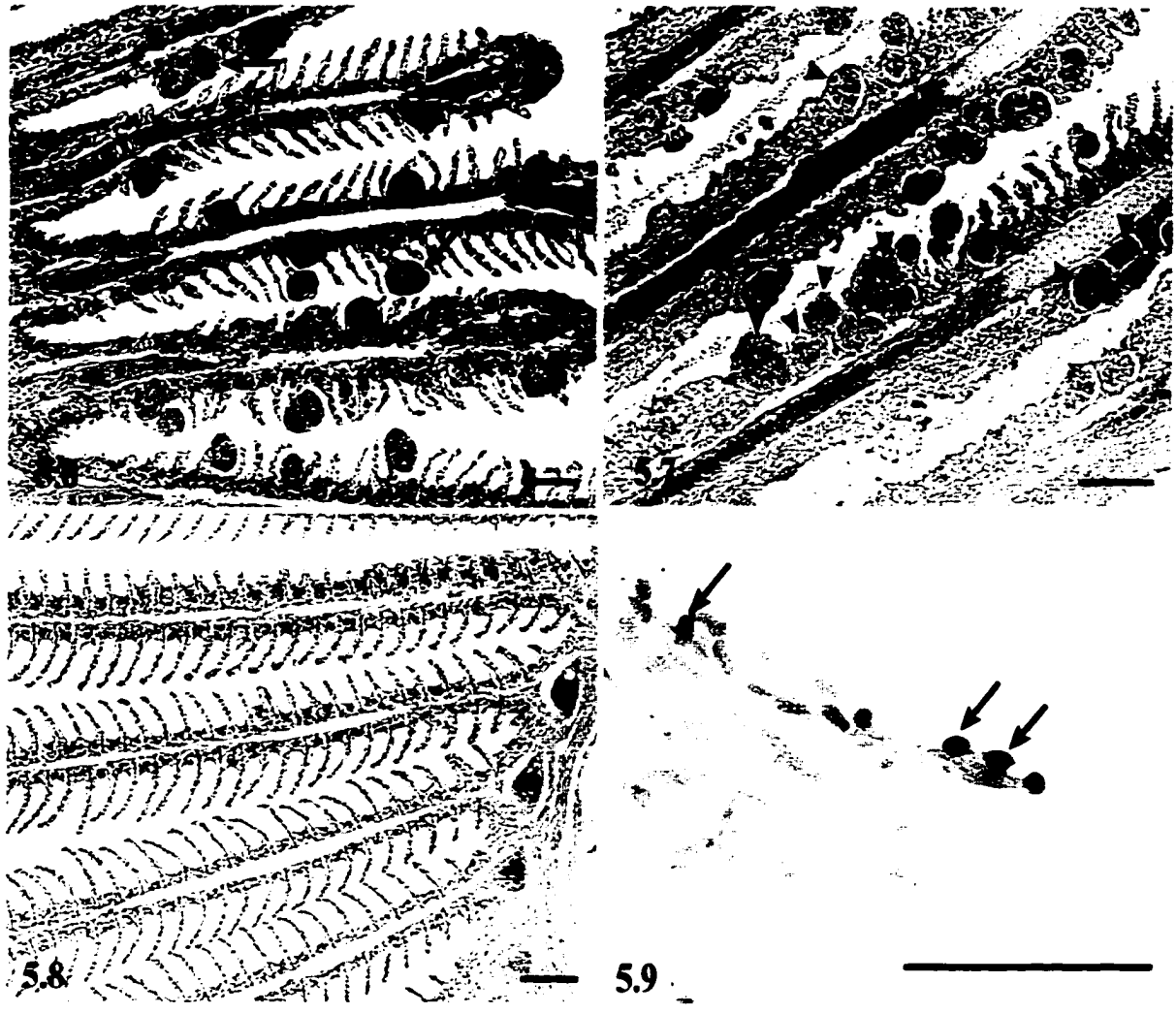

Fig. 5.6 & 5.7 Brook trout gill filaments 7 weeks PE to *L. salmonae* SV spores. Many xenomas are visible (arrows and arrowheads). Extensive epithelial hyperplasia is observed and high density of xenomas (Fig. 5.7, arrowheads). H&E. Bar = 100 μm. Fig. 5.8 Rainbow trout gill filaments 7 weeks PE to *L. salmonae* SV spores. No xenomas are observed, the gill filament structure is conserved. H&E. Bar = 100 μm. Fig. 5.9. Brook trout heart (ventricle) 2 days PE to *L. salmonae* SV spores. Three infected cells are seen associated with the endocardium; very early stages of the parasite, possibly sporoplasm or early meronts are observed (arrows). ISH with *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments; methyl green counterstain. Bar = 100 μm.

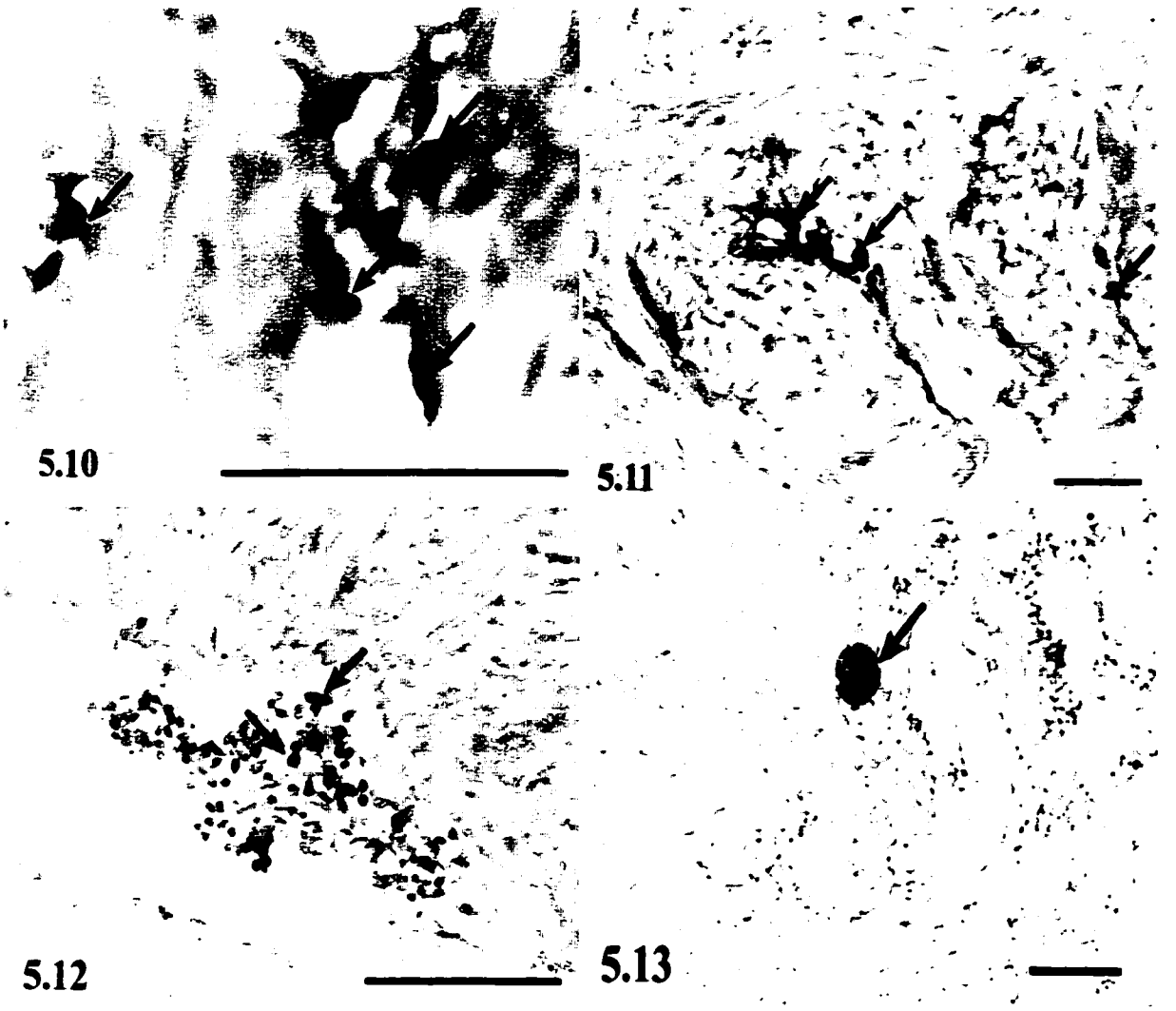

Fig. 5.10 , 5.11 and 5.12 Brook trout heart (ventricle and bulbus arteriosus) 5 days PE to *L. salmonae* SV spores. Infected cells (arrows) can be seen in association to the endocardial lining (Fig. 5.10) or mixed with other blood cells (Fig. 5.12). Fig. 5.13 Brook trout heart (ventricle) 5 weeks PE to *L. sal-salmonae* SV spores. One xenoma was visible (arrow). ISH with *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments; methyl green counterstain. Bars = 100 μm.

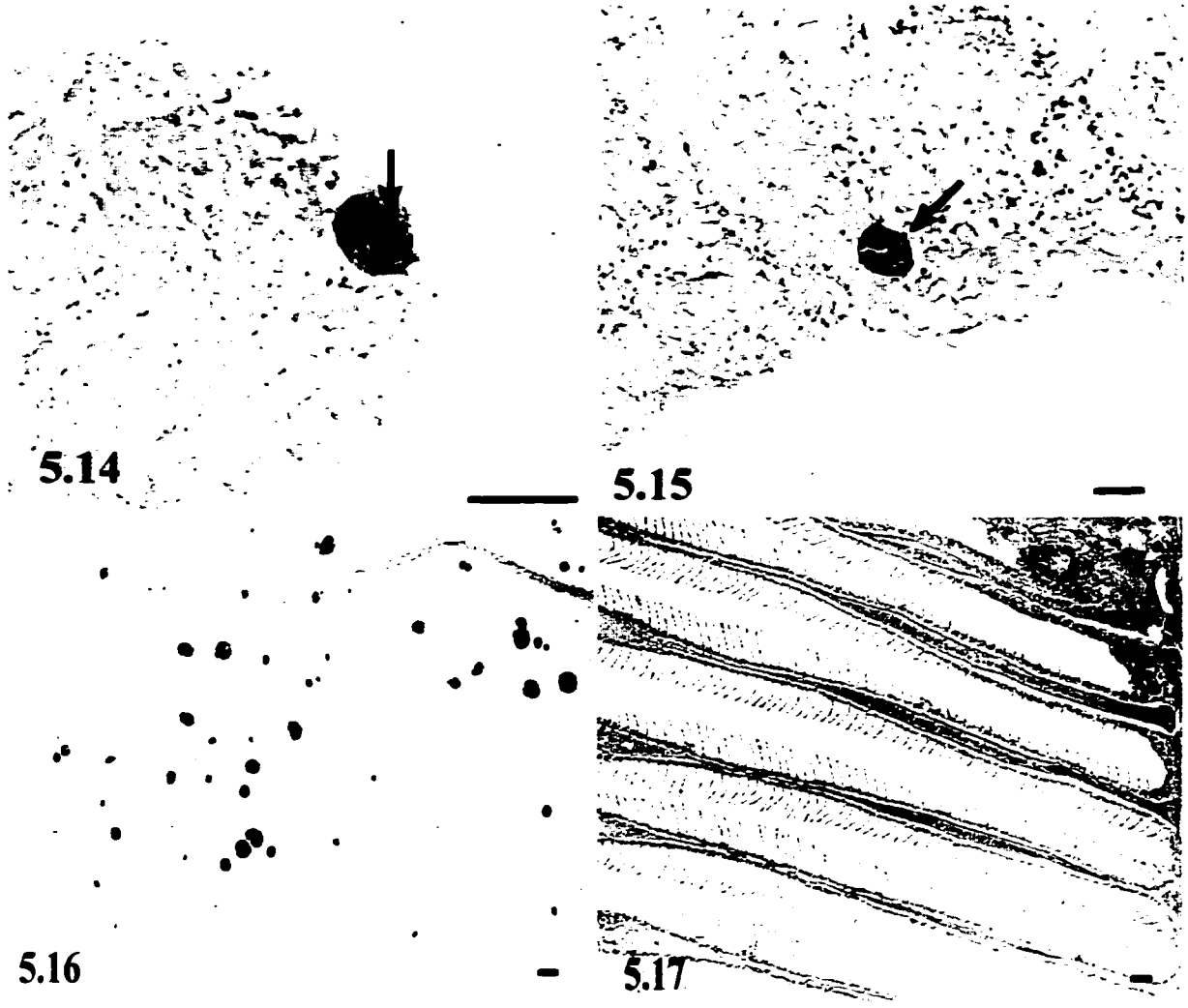

Fig. 5.14 Brook trout heart (atrium) 6 weeks PE to *L. salmonae* SV spores. One mature xenoma is visible (arrow). Fig. 5.15 Brook trout (ventricle) 7 weeks PE to *L. salmonae* SV spores. A single xenoma is visible (arrow). Fig. 5.16 Arctic char (*Salvelinus alpinus*) 7 weeks PE to *L. salmonae* SV spores. Numerous xenomas of various sizes are visible along the gill filaments. Fig. 5.17 Resistant brook trout 7 weeks PE to *L. salmonae* SV spores. This fish had been previously exposed to spores from the same parasite variant and had cleared the disease before rechallenge. No xenoma formation was observed. H&E. Figs. 5.14, 5.15, and 5.16 ISH with *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments; methyl green counterstain. Bars = 100 μm.

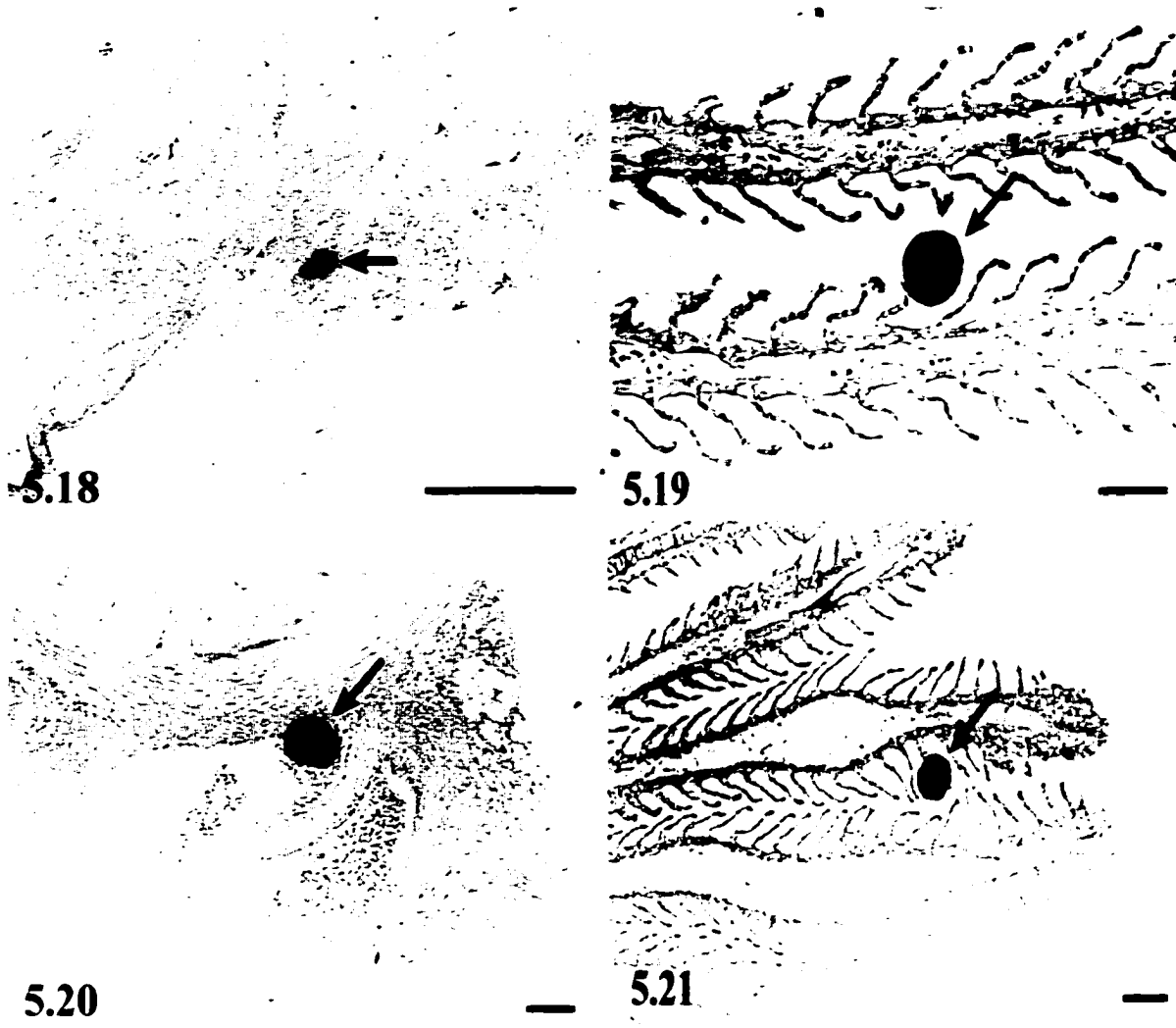

Fig. 5.18 Atlantic salmon (*Salmo salar*) 7 weeks PE to *L. salmonae* SV spores. A single dissolving xenoma (arrow) is visible at the base of the gill filaments. Fig. 5.19 Chinook salmon (*O. tschawytscha*) gill filaments 7 weeks PE to *L. salmonae* SV spores. A single xenoma (arrow) is visible at the tip of a lamella. Fig. 5.20 Chinook salmon heart (ventricle) 7 weeks PE to *L. salmonae* SV spores. A single mature xenoma is visible (arrow). Fig. 5.21 Coho salmon (*O. kisutch*) 7 weeks PE to *L. salmonae* SV spores. A single xenoma (arrow) is visible a the tip of a lamella. ISH with a *L. salmonae* ssDNA DIG-labelled probe and AP-conjugated anti-DIG Fab fragments; methyl green counterstain. Bars = 100 µm.

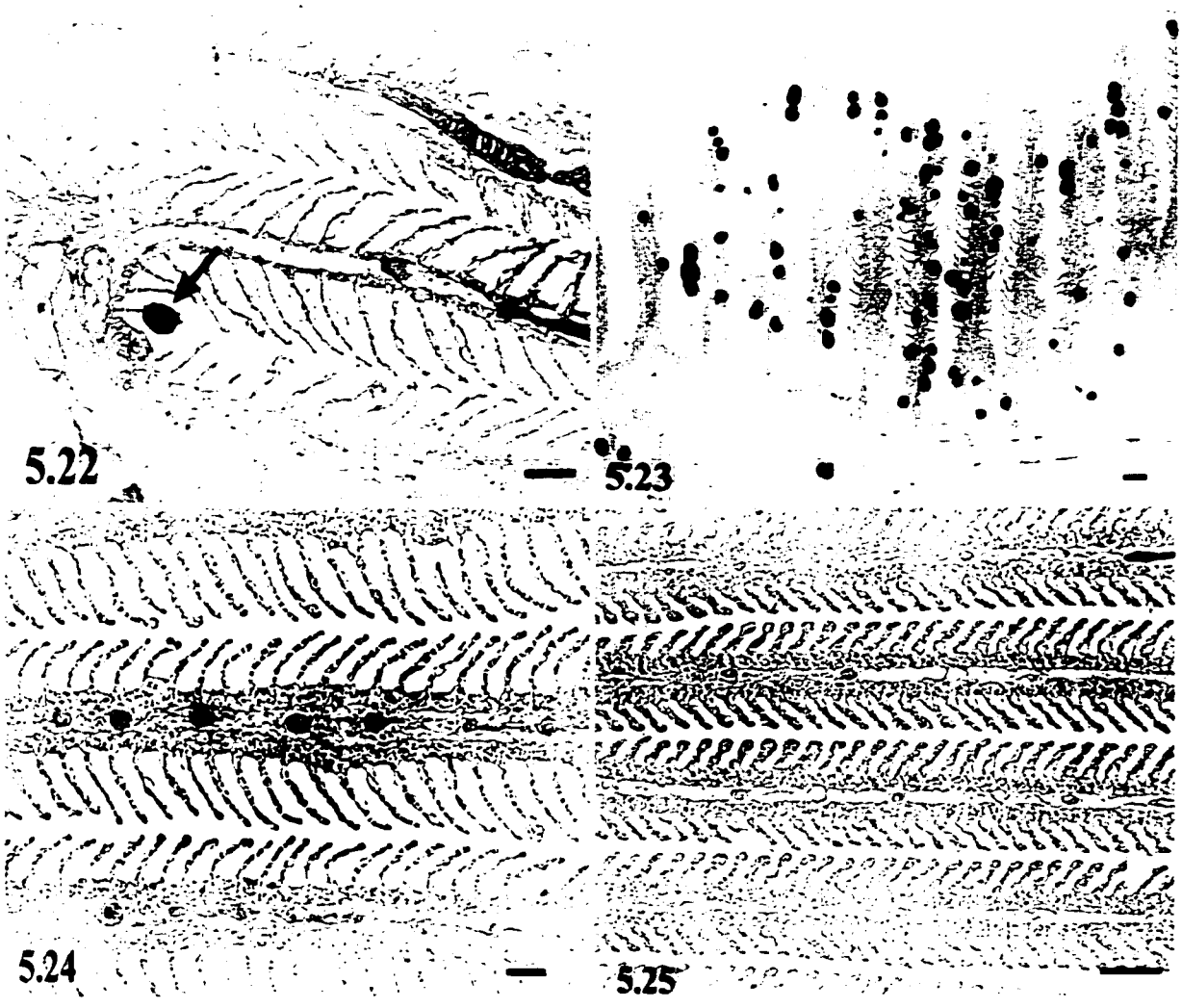

Fig. 5.22 Rainbow trout gill 7 weeks PE to *L. salmonae* SV spores. A single xenoma (arrow) is visible at the tip of a lamella. Bar = 100 µm. Fig. 5.23 Brook trout gill filaments 7 weeks PE to *L. salmonae* SV spores. Numerous xenomas are visible along the gill filaments. Bar = 100 µm. Fig. 5.24 Recovered Arctic char 7 weeks PE to *L. salmonae* SV spores. No xenomas developed in these fish. Bar = 100 µm. Fig. 5.25 Atlantic salmon gill filaments 7 weeks PE to *L. salmonae* SV spores. No xenomas are observed. Bar = 100 µm. ISH with *L. salmonae* ssDNA digoxigenin-labelled probe and AP-conjugated anti-digoxigenin Fab fragments. Methyl green counterstain.

5.5 DISCUSSION

This study describes the isolation, biological characterization, and preference for fish of the genus *Salvelinus* by a variant of *Loma salmonae* which is herein called *L. salmonae* SV (*Salvelinus* variant). Previous attempts to infect brook trout with inocula derived from Chinook salmon and extensively passaged in experimentally-infected rainbow trout had failed to produce xenomas (Chapter 4) (Speare et al. 1998a), therefore, because the parasite failed to develop and reproduce (Wakelin 1988), brook trout had previously been considered naturally resistant to disease with *L. salmonae*. However, the natural infection of brook trout with *L. salmonae* has since been reported (Bader et al. 1998), as well as experimental infection of brook trout with *L. salmonae* using spores harvested from naturally-infected Chinook salmon (Shaw et al. 2000). The results of this study confirmed the latter two reports and showed that after infection with a mixed inoculum of *L. salmonae* spores derived from infected gills from naturally infected Chinook salmon and laboratory-infected rainbow trout, xenomas formed in the infected brook trout.

An explanation for this result is that the Chinook salmon sample of the inoculum (originating in British Columbia) used in the initial infection may have carried spores from two variants of *L. salmonae*. The majority of the spores present in the Chinook salmon inoculum possibly belonged to the typical *Oncorhynchus*-infecting *L. salmonae*, whereas a small percentage of the spores probably belonged to the *Salvelinus*-infecting variant. Evidence for this lies in the small number of brook trout with xenomas at the end of Trial 1, and in the low numbers of xenomas observed. More evidence suggesting the presence of two variants of *L. salmonae* in the Chinook salmon inoculum is that, when *L. salmonae*-infected

Chinook salmon gills have been used to infect rainbow trout, serial passage in this species apparently leads to the selection of a variant that is infective to salmonids of the genus *Oncorhynchus*, but that is not able to complete its life cycle in brook trout and Arctic char (Speare et al. 1998a). Further evidence is found in the observation that Chinook salmon could be successfully infected with the *L. salmonae* SV, hence they could act as carriers.

Upon exposure to a new host, parasites may be able to invade it, before being rejected, as has been documented in parasites such as *Eimeria* sp. (Long and Millard 1979) and *L. salmonae* (Chapter 4). Results show that the *L. salmonae* SV was able to survive longer in brook trout than the rainbow trout laboratory-derived *L. salmonae* used in chapters 2, 3, and 4, leading to the completion of the life cycle (xenoma production) in a low percentage of the infected fish. This may suggest a selection for a *L. salmonae* variant capable of evading the defence system of brook trout, and to adapt to the new host, thus enhancing its survival. The low percentage (20%) of brook trout that developed xenomas may be an indication that the numbers of *L. salmonae* SV spores present in the original mixed inoculum was low, while the 80% of the fish in which *L. salmonae* did not complete its life cycle and that did not exhibit xenomas, were probably infected with the typical variety of the parasite, which fails to complete its life cycle in brook trout (Chapter 4).

Susceptibility of a host to disease caused by parasites may depend on factors such as diet, stress and genetics (Wakelin 1988; Forward et al. 1995), as well as the presence and virulence of the pathogen, and environmental conditions (Arkoosh et al. 1998). Parasite differences which cause variable levels of disease in the same host genotype, and host individual variation within the same population may explain the differences observed in a

group of individuals when infected with the same parasite (Hanson 1988; Ebert and Hamilton 1996). It has been postulated that within a susceptible host species, individuals resistant to infection to that same parasite may exist (Forward et al. 1995); this intra-species variation in the susceptibility to a pathogen has been reported in brook trout (Bakke et al. 1990; Woo 1992; Forward et al. 1995). Another possibility for disease susceptibility is stress-induced immunosuppression, since corticosteroids enhance the development of parasites in normal and abnormal hosts (Kent and Hedrick 1987), and increase the susceptibility of fish to disease (Woo et al. 1987). In this study, stress could have been a result of the gastric intubation, since cortisol levels in brook trout after application of an acute stressor can remain elevated for at least 3 days after the stressor is applied (Sanchez et al. 1997), and previous studies show that by this time *L. salmonae* has already invaded the fish (Chapter 4). However, this does not explain satisfactorily the results observed, because all the fish were subjected to the same stressor, suggesting again that only those fish infected with the *L. salmonae* SV developed xenomas. Therefore, the existence of variants within *L. salmonae* may provide a better explanation of the results observed in the experimental population of brook trout used in trial 1, suggesting that the *L. salmonae* SV was able to infect the fish while the rainbow trout-derived spores were not.

The exact conditions that allowed the *L. salmonae* SV to surmount the immunological barriers of the brook trout and Arctic char are not known. However, the adaptation and amplification of the parasite in the new host subsequently led to an increase in the level of parasitism and prevalence, as was observed when the xenomas from the infected brook trout from Trial 1 were harvested and used as inoculum to infect a naive

group of brook trout in Trial 2. This resulted in the amplification of this novel variant, as the results show that 50% of the infected fish developed xenomas, likely due to an increase in the percentage of spores belonging to the *L. salmonae* SV (which is apparently more virulent to brook trout) in the inoculum used to infect the second group of brook trout.

Further *in vivo* passage of this *L. salmonae* SV through brook trout suggests a separation, purification and amplification of the number of spores from the *L. salmonae* SV present in the inoculum, as the levels of parasitism and prevalence increased. Gastric intubation (which provides a better control in the infection of fish, because the amount of inoculum per fish is constant) of brook and rainbow trout with *L. salmonae* SV spores illustrated the marked preference and virulence of this parasite variant for brook trout. Virulence is considered as the net effect of parasites on their hosts, and it can be estimated by parasite reproduction, infectivity or by damage caused to the host (Toft and Karter 1990; Ebert and Mangin 1997). In brook trout the point prevalence of infection was 100%, and parasitism (number of xenomas observed in the gill arches) was very high, resulting in gill arches that were completely obliterated with xenomas, possibly impairing respiratory function (Morrison and Sprague 1981a). This may have lead to death of some of the brook trout used in this trial, since only 12 fish survived until week 7 PE (Table 5.E). *Loma salmonae* SV, while being highly infective to brook trout, exhibited minimal virulence towards rainbow trout infected and cohabitated with the brook trout. In contrast, *L. salmonae* spores derived from Chinook salmon and passaged 3 times in rainbow trout showed a marked preference for rainbow trout over brook trout infected with the same inoculum and cohabitated in the same tank. Brook trout probably developed xenomas in this

trial because of the low number of passages in rainbow trout of the Chinook salmon derived spores. This suggests that the purification of spores of the typical *Oncorhynchus*-infecting spores was not thoroughly complete and some spores belonging to the SV were still present, further supporting the existence of two variants of the parasite.

The adaptation of a parasite to a new host genotype that was formerly rare or absent often results in the loss or a decline of virulence in the host of origin (Ebert and Hamilton 1996), and as virulence in the new host increases, attenuation in the former host increases as well, suggesting a trade off between parasite fitness on different hosts (Ebert 1998). The results from the present study cannot confirm or deny an increase or loss of virulence in *L. salmonae* SV; however, it could indicate that the high virulence of this variant towards brook trout was already present, and that the isolation and amplification of the *L. salmonae* SV only magnified this already existing virulence. On the other hand, it has also been hypothesized that severe disease represents a lack of co-adaptation between a host and a parasite (Bakke et al. 1990). Accordingly, the high numbers of xenomas observed in brook trout infected with the *L. salmonae* SV might suggest that this species is not the natural host for *L. salmonae* SV, but is nonetheless susceptible to disease, since the parasite was able to establish successfully in the host and form xenomas (Ebert 1994). The low virulence that this variant exhibits towards rainbow trout, suggests that it may become a possible candidate for use as a live vaccine, especially since other methods of disease control, like culling and/or chemotherapy (Reno 1998) may not be sufficient to control the disease.

It could be speculated that the *L. salmonae* SV is *L. fontinalis*, a microsporidian reported to affect brook trout (Morrison and Sprague 1983), but ultrastructural studies (L.

Rodríguez, personal communication) suggest that this may not be the case (Table 5.F). Ultrastructurally, the number of polar filament coils observed in the spores, and the morphology of the parasite appear closer to that of *L. salmonae*. Furthermore, this variant was positive for *L. salmonae* by PCR, ISH and immunohistochemistry. Based upon the current observations, this organism is referred to as *Loma salmonae* SV, pending definitive identification.

Table 5.F Comparison of *L. salmonae* spore ultrastructure.

Parasite	Spore width (µm)	Spore length (µm)	Coils in filament	Reference
L. salmonae	2.84 - 6.16	1.42 - 2.84	13 - 17	(Bruno et al. 1995)
L. salmonae	4 - 5.6	2 - 2.4	14 - 17	(Kent et al. 1989)
L. salmonae	4.5	2.2	14 - 17	(Morrison and Sprague 1983)
L. fontinalis	3.7	2.2	14 - 15	(Morrison and Sprague 1983)
L. salmonae[1]	3.8±0.25	1.64±0.11	16 - 17	L. Rodriguez (P.C.)
L. salmonae[2]	3.57±0.12	1.92±0.22	16 - 17	L. Rodriguez (P.C.)
L. salmonae[3]	3.36±0.01	1.71±0.03	N.A.	L. Rodriguez (P.C.)
L. salmonae[4]	3.68±0.24	1.91±0.09	17	L. Rodriguez (P.C.

[1]Host rainbow trout; [2]Host brook trout; [3]Chinook salmon; [4]Arctic char; P.C. personal communication. [1,3] Typical *Loma salmonae*. [2,4] *Loma salmonae* SV.

The study of the initial stages after infection, suggest that the *L. salmonae* SV moves through the body of the fish in the same way as the typical *L. salmonae*. Although the highest prevalence of the parasite is in the gills, merogony may start before the dividing parasite arrives in the gills, as seen by the replicating stages observed in the heart of the infected fish, indicating that the parasite reaches the heart and the gills inside an as yet unidentified infected cell via the blood (Matthews and Matthews 1980; Tzipori et al. 1996; Didier et al. 1998). Some infected cells were seen in close association with the phagocytic

cells (Hauck 1984) of the endocardium, but others were seen in cells mixed with uninfected blood cells. Interestingly, xenoma formation was observed in the hearts of some fish, although only 1 xenoma could be observed in most cases. Gastric intubation of these fish with high numbers of spores, did not translate into higher numbers of infective stages seen in the heart. While infected cells with dividing stages were observed within days post infection, the level of parasitism was not as high as expected, suggesting that a large number of spores may just pass through the gut and be lost in the faeces of the fish before they can germinate. The passage of intact spores through the GI tract was observed by immunoflourescent antibody test (IFAT) with a rabbit anti-*L. salmonae* polyclonal antibody on faecal smears obtained from a fish intubated with a large number of spores 12 and 24 h PE.

Variability in the susceptibility of different salmonid species to a common pathogen exists (Bower and Margolis 1984). Studies on the host range for *L. salmonae* SV, showed that the parasite variant exhibited a marked preference for two of the six species (brook trout, rainbow trout, Arctic char, Atlantic salmon, Chinook salmon, and coho salmon) experimentally infected. Both species belong to the genus *Salvelinus* (brook trout and Arctic char), suggesting that the virulence observed in the *L. salmonae* SV might be directed not only to one particular species (brook trout), but also to other species within the same genus. Furthermore, the low virulence observed in rainbow trout was also observed in other species within the genus *Oncorhynchus*, as Chinook and coho salmon had low parasitism and even lower morbidity. Atlantic salmon (genus *Salmo*) showed very little susceptibility to disease with this variant of the parasite.

Arctic char developed xenoparasitic complexes typical for *L. salmonae* after infection with the *L. salmonae* SV. This is the first successful attempt to experimentally infect Arctic char with *L. salmonae*, as previous attempts with rainbow trout-derived *L. salmonae* failed to produce xenomas in fish when they were experimentally infected (Speare et al. 1998a). In addition, the *L. salmonae* SV spores collected from the xenomas that developed in the gills of infected Arctic char were capable of infecting and producing xenomas in naive Arctic char, providing further evidence for the preference of the *L. salmonae* SV to fish from the genus *Salvelinus*. Xenoma development in the Arctic char and brook trout after exposure to spores of the *L. salmonae* SV, also resulted in the development of resistance to *L. salmonae* SV. Fish that had been previously exposed to *L. salmonae* SV failed to become infected and develop xenomas when they were experimentally challenged with *L. salmonae* SV spores harvested from brook trout. These results increase the number of species that can develop resistance to *L. salmonae* and its variants, since acquired resistance to *L. salmonae* has been previously reported in rainbow trout (Speare et al. 1998b) and Chinook salmon (Kent et al. 1999). Furthermore, the results suggest that the acquired protection to reinfection is complete, as neither the brook trout nor the Arctic char presented any signs of the parasite when their gills were histologically examined by ISH.

Xenoma development in Atlantic salmon has been difficult to achieve after infection with *L. salmonae* (Kent et al. 1989; Speare et al. 1998), and thus Atlantic salmon have been considered naturally immune to disease with this parasite. PCR monitoring of the progression of infection with *L. salmonae* in Atlantic salmon (Chapter 4) suggests that after entry in the host, *L. salmonae* is controlled and cleared from the fish system approximately

2 weeks PE. Interestingly in this study, one mature xenoma was observed in Atlantic salmon in the middle of a gill filament after exposure to *L. salmonae* SV. This is the first time that a *L. salmonae* xenoma has been visualized in an Atlantic salmon, thus increasing the host range known for this parasite. The factors involved behind the ability of *L. salmonae* SV to form a xenoma in one Atlantic salmon are not known. It was hypothesized that stress may have been behind the parasite's capability of establishing itself in this species. However, attempts at infecting Atlantic salmon with the *L. salmonae* SV, followed by a regime of acute stressors (chasing in housing tank and emersion) were not successful, and xenomas were not detected by ISH, which is a very sensitive method of detection, and is able to detect low-level infections that are missed by other methods. This suggests that stress may not be a factor responsible for permitting *L. salmonae* SV to complete its life cycle and form xenomas.

Loma salmonae is the cause of extensive economic losses in the aquaculture industry in British Columbia (Constantine 1999), affecting Chinook (Hauck 1984; Kent et al. 1995) and coho salmon (Magor 1987; Kent et al. 1989). Exposure of Chinook and coho salmon to *L. salmonae* SV resulted in the development of xenomas in one fish from each species. *Loma salmonae* SV exhibited low-virulence towards Chinook and coho salmon, similar to that observed in Trial 3 against rainbow trout. The low prevalence and the low numbers of xenomas observed in the one infected Chinook salmon (7 xenomas/gill arch), may give an idea of the numbers of xenomas present in the original Chinook salmon inoculum used in Trial 1 belonging to *L. salmonae* SV. This suggests that in the Chinook salmon inoculum carrying the two variants, the number of xenomas derived from *L. salmonae* SV spores likely

constituted a small percentage of the total number of xenomas present in the inoculum, explaining the low prevalence initially observed in brook trout. Furthermore, the low prevalence of xenomas in the Chinook salmon infected with the *L. salmonae* SV spores after isolation and amplification of this variant, reinforces the observations made before about the strong preference that the *L. salmonae* SV has for species of the genus *Salvelinus*. Especially because even after the isolation and amplification of the *L. salmonae* SV, the number of xenomas that developed in the *Oncorhynchus* species from which it was originally isolated was small.

The low-virulence exhibited by *L. salmonae* SV towards rainbow trout, Chinook and coho salmon, should be explored further to study the possible use of this low-virulence variant as a live vaccine to induce protection against typical *Oncorhynchus*-infecting *L. salmonae* in susceptible species. This is particularly important since *L. salmonae* is the cause of high morbidity and mortality in Chinook salmon (Scholz 1999) leading to high economic losses (Constantine 1999) in British Columbia.

In conclusion, this study describes the isolation and amplification of a *Salvelinus*-infecting variant of *L. salmonae*, as it was passed through brook trout. *Loma salmonae* SV exhibited high virulence and preference for species within the genus *Salvelinus* (brook trout and Arctic char), and low virulence and preference for species of the genus *Onchorhynchus* (rainbow trout, chinook salmon, coho salmon) or *Salmo* (Atlantic salmon). This *L. salmonae* SV has sufficient biological differences from the regular *L. salmonae* to be considered a strain, but it retains sufficient features of *L. salmonae* and it should not be considered a new species, although definitive determination is pending.

6 *LOMA SALMONAE* SV AS A VACCINE TO INDUCE PROTECTION IN SALMONID SPECIES AGAINST *LOMA SALMONAE* INFECTION

6.1 ABSTRACT

Recovery from infection with *Loma salmonae* affords protection of rainbow trout against reinfection with this parasite. It is possible that exposure to a variant with lower virulence may provide similar protection. Rainbow trout juveniles, orally exposed to viable spores from *L. salmonae* SV, derived from multiple passages in brook trout, were subsequently re-challenged 16 weeks later with rainbow trout-derived *L. salmonae* spores. The subsequent infection was compared to that in a group of naive control rainbow trout challenged only with the rainbow trout-derived *L. salmonae* spores. Fish with prior exposure to the low-virulence variant had 14 times fewer xenomas per filament (0.044 versus 0.641, $P = 0.0001$) than the naive controls, as determined morphometrically by *in situ* hybridization at the peak of the disease (between weeks four and six post exposure). The marked degree of reduction in numbers of xenomas that formed after challenge suggests that use of the low-virulence variant should be further considered as a means to protect fish in regions where the parasite is endemic. Exposure of susceptible salmonids to the low virulence *L. salmonae* SV during hatchery rearing and before exposure to virulent *L. salmonae*, may protect them during grow out periods.

6.2 INTRODUCTION

The microsporidian *Loma salmonae*, an intracellular gill parasite, is a cause of serious seasonal morbidity and mortality in farm-reared Chinook salmon in coastal British Columbia (Hauck 1984; Kent et al. 1989; Speare et al. 1989; Kent et al. 1995; Scholz 1999). It continues to be of increasing concern because of a current lack of approved therapeutic agents (Mullins et al. 1994) and vaccines. Moreover, *L. salmonae* has been identified in feral stocks of Pacific salmon and these likely serve as natural reservoirs (Kent et al. 1998). The importance of the latter finding is supported by the ease with which horizontal infection with parasites can occur in fish farming situations (van Muiswinkel 1995; Woo 1997), including infections with *L. salmonae* (Kent et al. 1989; Kent et al. 1995; Shaw et al. 1998; Speare et al. 1998a). *Loma salmonae* infections result in the formation of xenoparasitic complexes (xenomas) that cause significant branchitis when they rupture, leading to death (Speare et al. 1989; Kent et al. 1995). Mortality rates have been used to assess the influence of *L. salmonae* on profitability in affected farms (Constantine 1999); however, observations on the subclinical effects in survivors, including significant reductions in growth rates need to be more completely considered (Speare et al. 1998c).

Fish can develop immunity after exposure to parasitic pathogens (Woo 1987; Woo 1992; Lin et al. 1996; Woo 1997), and the vaccination with attenuated strains of parasites as demonstrated with *Cryptobia salmositica* has been successful in the induction of immunity in fish (Woo 1998). Several recent studies have shown that species within the genus *Oncorhynchus* can develop protection against *L. salmonae* upon recovery from non-lethal infections (Speare et al. 1998; Beaman et al. 1999b; Kent et al. 1999). A reasonable control

strategy to consider is the development of a vaccine against *L. salmonae* that could be applied during the freshwater rearing phase to induce a protective immune response in Chinook salmon before their release into marine net-pens where the pathogen is endemic. However, to date, the development and use of an attenuated strain of *L. salmonae*, as a vaccine, has not been attempted.

Recently, the low-virulence *L. salmonae* SV was isolated following serial passage of infective gill inoculum derived from Chinook salmon and rainbow trout through brook trout, *Salvelinus fontinalis* (Chapter 5). It has been suggested that this isolate represents a variant of *L. salmonae* based on assessment with PCR, *in situ* hybridization, immunohistochemistry with anti-*L. salmonae* monoclonal antibodies and transmission electron microscopy. A biological characteristic of this variant is that the strain has a marked preference for species within the genus *Salvelinus*, where far more xenomas develop, as compared with *Oncorhynchus*.

The intent of this study was to determine whether exposure of rainbow trout to *L. salmonae* SV could induce protection against xenoma development when the fish were challenged with the homologous virulent typical strain.

6.3 MATERIALS AND METHODS

6.3.1 Experimental population

One hundred juvenile rainbow trout (~30 ± 2 g) and 40 brook trout (~20 ± 1 g) were purchased from a certified disease-free (notifiable pathogens) commercial hatchery on Prince Edward Island, with no history of *L. salmonae*. The fish were held at 10°C in a common

tank, before allocation to experimental tanks. All procedures were conducted according to the guidelines of the Canadian Council on Animal Care (Anonymous 1993).

6.3.2 Experimental design

This trial was divided in two phases:

1st phase: Exposure of naive rainbow trout and brook trout to L. salmonae SV. Sixty juvenile rainbow trout and forty brook trout were cohabitated in one circular fibre glass tank with a habitable volume of 70 L and acclimated for one week before the beginning of this phase of the trial. The water temperature was kept at 15±0.5°C. The fish were infected with an inoculum of *L. salmonae* SV. This phase lasted 16 weeks.

2nd phase: Exposure of "vaccinated" rainbow trout (previously exposed to a low-virulence strain of L. salmonae), and naive rainbow trout to the typical Oncorhynchus-infecting L. salmonae. Two circular fibre glass tanks with a habitable volume of 70 L were used. The water temperature was kept at 15±0.5°C. Forty juvenile rainbow trout that had previously been infected with the low-virulence *L. salmonae* SV, and that were negative for infection (as judged by the absence of xenomas in the gills) were kept in one tank. Forty naive size-graded juvenile rainbow trout with an average weight of 30 g were used as controls and kept in a separate tank. The two groups of fish were acclimated for one week before the beginning of the trial. These fish were infected with wild-type *L. salmonae* spores. This trial lasted for seven weeks.

6.3.3 Method of infection

All fish were infected by gastric intubation with a homogenate prepared from finely cut gills from *L. salmonae*-infected fish. The gill material used as inoculum was harvested and processed just before the infection. Following removal of the cartilaginous arches, gill filaments were finely macerated into a pulp and then diluted with tank water in a 1:1 ratio. The fish were intubated with 0.1 ml of prepared tissue through a 1 ml syringe fitted with a 5 cm attached rubber tubing (diameter 0.2 mm). The fish were lightly anaesthetized with benzocaine during intubation.

Fish in the first phase were infected with an inoculum harvested from infected brook trout that carried the low-virulence (for rainbow trout) *L. salmonae* SV. Fish in the second phase of the trial were infected with the typical strain of *L. salmonae*, harvested from rainbow trout.

6.3.4 Sampling, histology and assessment of infection

1st Phase: Eight weeks post exposure (PE), twenty rainbow trout and twenty brook trout were euthanised with a benzocaine overdose. The first left gill arch of each fish was cut off. The presence of xenomas in their gills was assessed by wet mounts. Remaining rainbow trout were left undisturbed for another 8 weeks, while remaining brook trout were euthanised and their gills used as inocula in other trials.

2nd Phase: Five fish from each group were randomly sampled and euthanised with a benzocaine overdose each week PE. The first left gill arch was removed from the sampled fish and was fixed in 10 % neutral buffered formalin. The fixed gill arches from infected fish

were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Five μm thick sagittal sections were cut and mounted on glass slides (1 section of 5 gills per slide). The slides were further processed for *in situ* hybridization (ISH) and for an immunohistochemical assay (sections 6.3.4.1 and 6.3.4.2). Total number of filaments, and total number of pre-xenomas or xenomas visible by ISH and immunohistochemistry, were counted for each fish gill arch sampled. A ratio (mean number of xenomas per gill filament) was calculated. The ratio between vaccinated and naive fish was compared weekly through t-test using MINITAB™ statistical software. At the peak time of disease, between weeks 4 and 6 PE, the ratios (xenoma/filament) observed by ISH were pooled for each group of fish (vaccinated versus naive) to get a general indication of the level of protection induced during this time.

6.3.4.1 *In situ* hybridization

A more detailed protocol is found in Chapter 3. Briefly, the slides were deparaffinized in xylene for seven minutes, rehydrated in descending ethanol series (100, 95, 70, 50%), and washed in distilled water. The tissues were then equilibrated in TES (50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, pH 7.4) for 10 minutes and then treated with proteinase K (15μg/ml in TES) for 15 min at 37°C. Proteolysis was halted with two washes in PBS with 2 mg/ml glycine (Fisher Scientific) for 5 min per wash. The tissue sections were then immersed in aqueous 20% acetic acid at 4°C for 15 sec, and washed in two changes of distilled water for 10 min each. The tissues were briefly postfixed in 0.4% paraformaldehyde for 5 min, rinsed in distilled water and air dried.

Hybridization was done by applying 100 μl of hybridization buffer (5μl 100x

Denhardt's solution, 50µl dextran sulfate 50%, 10 µl sperm DNA 100 mg/ml, 100 µl 20X SSC, DW to 250 µl, and 250 µl formamide) with 30 ng of digoxigenin-labelled probe onto the permeabilized tissue sections. The slides were covered with hybri-slips [Sigma], placed onto a preheated baking tray and incubated at 90-95°C for 3 min to denature the DNA. The slides where then transferred to a humidified box and incubated at 40°C overnight.

After the overnight incubation, the sections were washed twice with 4x SCC for 5 minutes at room temperature. The sections where then equilibrated in tris buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.2) for 5 min and then blocked with TBT (Tris-buffered saline, 3% BSA, 0.5% Triton X-100) at room temperature for 10 min. After equilibration, the slides were then transferred to a slide incubation tray and the sections incubated with alkaline phosphatase conjugated anti-digoxigenin Fab fragments [Boehringer Mannheim] diluted 1:600 in TBT for 1 h. The sections were then washed in tris-buffered saline for 5 min. The signal was developed by using 45 µl nitroblue tetrazolium (NBT) [Boehringer Mannheim] and 35 µl bromo-chloro-indolyl-phosphate (BCIP) [Boehringer Mannheim] in 10 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ [Sigma], pH 9.5 for 5 h in the dark. Colour development was ended by washing in distilled water for 5 min.

6.3.4.2 Immunohistochemical assay

The slides were deparaffinized in xylene for 5 minutes, rehydrated in descending ethanol series (100%, 95%, and 70% for 5 minutes each), and washed in distilled water. All incubations were done in a moist chamber. The sections were treated with 5% hydrogen peroxide in methanol for 20 minutes, to remove endogenous peroxidase activity. Then the

sections were rinsed and soaked in PBS for 10 minutes before blocking with normal goat serum diluted 1/20 in PBS for 10 minutes. After blocking, the sections were incubated with the primary antibody (anti-*L. salmonae* undiluted monoclonal antibody supernatant [MAb]) for 1 hour. Sections were then rinsed in PBS for 10 minutes, before incubation with peroxidase labeled goat anti-mouse IgG diluted 1:100 in PBS for 30 minutes. The incubation was stopped by rinsing in PBS for 10 minutes. Two hundred microlitres of substrate (20 ml of PBS, 200 µl of H_2O_2, and one 10 mg tablet of diaminobenzidine) were then applied to the slides and incubated for 3-5 minutes, before stopping the reaction by soaking the sections in distilled water for 5 minutes. The tissues were counterstained for 10 seconds with 10% aqueous methyl green, before being mounted with Permount (Fisher Scientific).

6.4 RESULTS

Eight weeks PE, rainbow trout infected with the low-virulence *L. salmonae* SV, showed no signs of disease; only one xenoma was observed in one of the 20 fish sampled. Brook trout showed high xenoma intensity (437 xenomas/gill arch) and 100% prevalence. In phase 2, naive fish showed 100 % prevalence, while "exposed" fish had lower levels of prevalence (Table 6.1). Presence of *L. salmonae* was detected as early as 2 weeks PE when ISH was used, but not by using the anti-*L. salmonae* MAb. Both naive and previously exposed fish showed some pre-xenomas at this time, but because the numbers were low, no ratios were calculated. For all the other weeks, both recovered and naive fish showed xenomas in the gills by ISH and by anti-*L. salmonae* MAb. From week 3 to 7, it was

observed that the fish that had been previously exposed to *L. salmonae* SV spores showed lower numbers of xenomas per filament when compared with the naive fish (Table 6.2). The differences were significant ($P \leq 0.05$) except for week 7 PE.

At the peak of disease, between weeks 4 and 6 PE, vaccinated fish had 14 times fewer xenomas than the naive fish (0.044 xenomas/filament in vaccinated fish versus 0.641 xenomas/filament observed in the naive fish; $P = 0.0001$).

Table 6.1 Prevalence of fish with *L. salmonae* xenomas

	Week 3	Week 4	Week 5	Week 6	Week 7
Naive RT	5/5	5/5	5/5	5/5	5/5
Resist. RT	2/5	2/5	4/5	2/5	3/5

Table 6.2. Mean ratio ± standard deviation of number of xenoma per filament in naive rainbow trout and rainbow trout previously infected with a low-virulence *L. salmonae* from brook trout, at selected times after infection with the wild-type *L. salmonae* detected by ISH.

	Mean ± S.D. number of xenomas/filament observed by ISH (n = 5)				
	Week 3	Week 4	Week 5	Week 6	Week 7
Naive RT	0.152 ± 0.09*	0.888 ± 0.48*	0.400 ± 0.26*	0.582 ± 0.32*	0.164 ± 0.13
Resist. RT	0.032 ± 0.05*	0.030 ± 0.03*	0.068 ± 0.05*	0.049 ± 0.09*	0.029 ± 0.03

* Denotes significant differences between the two groups assessed for a given week.

Comparing the two methods of detection (Table 6.3) shows that ISH was more sensitive in the early stages of infection, especially at weeks 2 and 3 PE, when it detected parasite stages missed by the MAb. From week 5 to week 7 PE the number of xenomas detected by both methods was similar.

Table 6.3. Mean number of total xenomas observed per gill arch in naive rainbow trout and rainbow trout previously infected with a low-virulence *L. salmonae* from brook trout, at different times after infection with the wild-type *L. salmonae*.

	Mean number of xenomas observed (n = 5)						
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
ISH							
Naive RT	0	1	5.8	33.6	15	21.6	7
Exposed RT	0	0.6	1.4	1.2	2.8	1.6	1.2
MAb							
Naive RT	0	0	0.8	23.4	13.4	25.8	7.4
Exposed RT	0	0	0.6	1.2	1.6	2	1.2

6.5 DISCUSSION

In the present study an attempt was made to use a strain of *L. salmonae* with low virulence for rainbow trout (Chapter 5) as a live parasite vaccine in this species. Pre-exposure of rainbow trout to the low-virulence *L. salmonae* SV spores resulted in protection, as seen in the significantly reduced numbers of xenomas/filament in the exposed fish when compared to those in the naive fish, when both groups were challenged with a normal wild-type strain of the parasite. A reduced infection manifested as lower numbers of xenomas/filament, is beneficial to the fish because of the detrimental effects of xenoma formation on gill pathology and pathophysiology (Speare et al. 1998c).

The results of this study suggest that a single exposure to the low-virulence *L. salmonae* SV induced protection from disease in juvenile rainbow trout when challenged 16 weeks later with typical, virulent spores. However, the duration of this immune state is unknown, as is the time required to develop resistance. Studies with other fish parasites

show variability in the length of time the fish are protected against reinfection. Vaccination with attenuated *C. salmositica* last for 24 months in rainbow trout (Woo 1998), while protection against *Ichthyophthirius multifiliis* lasts at least 12 months in channel catfish *Ictalurus punctatus* (Dickerson and Clark 1998).

Previous work (Chapter 5) described the *in vivo* isolation of the low-virulence *L. salmonae* SV after several serial passages in brook trout. *Loma salmonae* SV exhibited a high virulence in brook trout, while simultaneously showing low virulence in rainbow trout, as evidenced by the absence of xenomas in the infected fish. Studies on the effect of vaccination with the low-virulence *L. salmonae* SV in Chinook and coho salmon, two species for which this strain also shows low virulence (Chapter 5), should be undertaken. Parasites such as *Cryptobia salmositica* can be attenuated by serial passage through cell culture (Woo 1998). Experimental infection of rainbow trout with the avirulent strain does not cause disease in rainbow trout and these fish are protected against subsequent virulent challenge (Ardelli et al. 1994). The results of this study concur with those observed for other attenuated pathogens (Woo 1997; Roberti and Rohovec 1998), in that the *L. salmonae* SV used as a vaccine had low-virulence (as determined by xenoma absence) and conferred protection against subsequent virulent challenge as determined by the low parasite load observed. Rainbow trout exposed to the low-virulence strain became resistant to the branchial disease, but were not protected from being infected and invaded by the parasite, i.e., they were not resistant to infection, as the parasite could enter the body of the fish and reach the gills where it developed into xenomas, although at much lower numbers than those seen in the naive rainbow trout. Therefore, it is important to recognize the distinction

between infection and disease.

Cell-mediated immunity may be an important mechanism of protection against this parasite, as has been reported for other microsporidia (Khan et al. 1999). Humoral immunity does not seem to play a major role in the protection against infection (Chapter 4), although passively transferred immune serum delayed the progression of infection from the intestine to the heart of *L. salmonae* in rainbow trout. Furthermore, it is not known how infection with this low-virulence strain affects the growth of juvenile rainbow trout or, whether there is a bioenergetic cost to the fish associated with *L. salmonae* SV infection.

Cross-protection occurs when fish that become infected with one pathogen become resistant to infection by other related but different pathogens, e.g., fish immunized with *Tetrahymena* become resistant to *Ichtyophthirius multifiliis* (Dickerson et al. 1984), and fish immunized against *Vibrio salmonicida* become significantly protected against *Aeromonas salmonicida* (Hoel et al. 1998). Mammalian examples show cross-protection in mice against *Plasmodium berghei* when they are infected with *P. falciparum* (Sina et al. 1993), and monkeys immunized with *Leishmania donovani* become protected against infections with *L. major* (Gicheru et al. 1997). The factor or factors recognized in the attenuated strain by rainbow trout that induces increased protection against infection with typical *L. salmonae*, are not known. The exospore layers of the two strains may share similar antigens, in the same way as the exospores of *Encephalitozoon cuniculi, Glugea hertwigi, G. stephani* and *Nosema algerae* do (Niederkorn et al. 1980). The sharing of similar antigens has also been reported in other pathogens. An exopolysaccharide layer similar to that produced by *R. salmoninarum* is produced by another non-virulent bacterium and can be used to induce

protection against BKD (Griffiths et al. 1998).

This study also demonstrated that ISH for *L. salmonae* (Sanchez et al. 1999) allowed the detection of the presence of the parasite in the gills at stages when they could not be detected by immunohistochemistry using the MAb, especially at weeks three and 4 PE, but that the MAb works as the spores develop in the xenoma and enter sporogony. Differences in the number of xenomas detected by ISH and the MAb, suggest that not all the parasites contained in xenomas enter sporogony simultaneously, resulting in some xenomas with sporogonic stages that can be detected with the MAb while others not yet at the sporogonic stage are only detectable by ISH. The use of ISH proved useful for the assessment of the prevalence of infection.

In conclusion, control of fish diseases by vaccination has advantages over chemotherapeutic methods, in that it is preventive rather than curative and that concerns about development of drug resistance, toxicity and withdrawal times are overcome by vaccination (Ellis 1989; van Muiswinkel 1995). Vaccination of rainbow trout with an attenuated strain of *L. salmonae* seems an option in the control of microsporidiosis with this parasite. Vaccination resulted in the development of protective immunity against the typical *L. salmonae,* observed as lower xenoma numbers than naive fish. This attenuated strain of *L. salmonae* shows promise, but further studies on its effects on vaccinated salmonids, especially Chinook salmon, need to be done before it can be considered for use.

7 GENERAL DISCUSSION

Considerable economic losses are attributed to the microsporidian parasite *Loma salmonae* in the seawater netpen culture of Chinook salmon in British Columbia (Constantine 1999). Despite this, no methods of controlling the disease attributed to *L. salmonae* are available because of the paucity in the understanding of the basic biology of this parasite, and the absence of licenced effective pharmacological agents (Speare et al. 1999a) or vaccines. One way to identify processes that may help control the outcome of disease with infectious agents, is to study host-parasite relationships, and this can be achieved in different ways. It is important to understand what a parasite does once it has invaded its host, what the routes of infection are, if the disease occurs in a site different from the site of infection, what (if any) effects the immune status of the fish has in the development of the disease, and if external factors have any influence on parasite development. A better understanding of the occurrence of these events can suggest what strategies may be taken to control disease.

The purpose of this thesis was to use an *in vivo* model of infection in a susceptible species to define the progression of infection with *L. salmonae* (Speare et al. 1998a), and to determine the factors that modulate the progression of infection. This thesis contains several studies that probed the progression of *L. salmonae* infection in rainbow trout under optimal rearing conditions, when the fish were housed at high (21°C) and low (5°C) temperatures, in fish that had been previously exposed to *L. salmonae* and were resistant to reinfection, in fish that were passively immunized with sera from resistant fish, and in fish vaccinated with

a low-virulence (for rainbow trout) variant of *L. salmonae*. In addition, parasite development in species that do not usually develop disease after infection (Atlantic salmon and brook trout) was also examined.

Different approaches are available to detect infections with a pathogen, and some have been used to monitor the progression of infection with different pathogens. In this study, the monitoring of *L. salmonae* infection by the polymerase chain reaction (PCR) offered a particularly useful means for tracking this parasite, because of its intracellular nature. This is the first time that PCR has been used to study the progression of infection of a microsporidian parasite of salmonid fish, although PCR has been previously used in time course distribution studies of other pathogens in experimentally infected fish, such as infectious salmon anaemia virus in Atlantic salmon (Rimstad et al. 1999). ISH has been used in studies that followed the tissue distribution of foreign DNA ingested by mice (Schubbert et al. 1997). ISH has also helped in the study of the life cycle of myxosporidian parasites of fish (Antonio et al. 1998), especially the initial developmental stages in fish and in intermediate hosts (Antonio et. al. 1999). For the study of the initial stages of *L. salmonae* infection, an *in situ* hybridization (ISH) protocol especially designed for this study was developed. This is the first time that *in situ* hybridization has been used in the localization of a fish-infecting microsporidian, and is one of the few reports where ISH has been used for the study of microsporidian infections, which focus mainly in human-infecting microsporidia (Velasquez et al. 1999).

Infection of rainbow trout with *L. salmonae* at 15°C had been well studied, and the timing of xenoma formation in the gills of infected fish was well established (Beaman et al.

1999a). However, little was known about the route the parasite takes after entering the gut of the host (Shaw and Kent 1999) to the first appearance of xenomas in the gills. This was addressed in chapters 2 and 3, where the progression of *L. salmonae* infection was monitored at a temperature considered to be optimal for parasite development, thus providing a better understanding of the sequence of events, the main organs affected, and approximate times when this occurs. Although the parasite could be detected by PCR in all the organs sampled (except the kidney), the main pattern of tissue distribution observed was from the intestine to heart, and then to the gills. In a manner similar to the majority of microsporidial infections of insects and mammals (Cali and Takvorian 1999), spores germinate in the GI tract, where the proper environmental stimuli needed for polar tube extrusion and injection of the sporoplasm are found. Dissemination of microsporidia of both mammals and insects from their site of infection to their site of development has been noted. In both the bee-infecting microsporidian *Nosema apis* and the human-infecting *Septata intestinalis* the transport of the parasite takes place inside macrophages and fibroblastic cells (Cali 1993; Cali et al. 1993). In *L. salmonae* infections the infective sporoplasm moves to the lamina propria of the intestine (as early as 12 h post exposure [PE]) after germination, and from the lamina propria *L. salmonae* (and *L. salmonae* SV [*Salvelinus* variant]) are transported inside infected cells (which had never been visualized) via the blood to the heart (as early as 3 days PE). The heart is an important development site for *L. salmonae*, and results suggest that a developmental step occurs here before migration to the gill at 2 weeks PE at 15°C. ISH allowed the first observation of *L. salmonae*-infected cells during their passage through the heart and showed that merogony starts before putative infected transport cells reach the gills.

This is the first time that ISH has been used in the study of the progression of infection of a microsporidian parasite, although the technique has been used succesfully in the diagnosis of human infections with *E. bieneusi* (Mansfield et al. 1996; Tzipori et al. 1996).

After the route by which *L. salmonae* migrates in a susceptible species was established at a permissible temperature, subsequent studies examined factors within and outside the fish for their role in modifying this developmental process. Temperature is a very important extrinsic factor for poikilothermic animals such as fish, but also for the pathogens that affect them. Chapter 2 showed that extreme temperatures can have profound effects in the progression of infection, and can be used effectively in the control of infections with *L. salmonae*. At both 5° and 21°C, the heart phase observed at 15°C before arrival in the gill is absent, and *L. salmonae* was cleared from the fish system within 3 weeks PE, showing that the absence of xenomas in fish held at high or low temperatures is due to death and subsequent clearing of the parasite from the host. The influence of temperature in the development of microsporidian species in homeothermic and poikilothermic hosts has been studied. *Glugea stephani* development is arrested when water temperatures are below 15°C (Olson 1981). In insects, microsporidia usually develop at the ambient temperature for their developmental range (Cali and Takvorian 1999), and so temperature can act as a barrier for infection in non-targer hosts, e.g. *Nosema algerae* cannot develop when it is experimentally infected in mice (Trammer et al. 1997). Temperature has been demonstrated to affect infectivity in *Encephalitozoon hellem*, a microsporidian that infects birds and mammals, and also affects the rate of replication (Didier and Bessinger 1999).

Previous studies (Speare et al. 1998; Kent et al. 1999), and the present study have shown that exposure to *L. salmonae* or a variant thereof, results in development of protective immunity to reinfection, observed as the absence of xenomas in challenged rainbow and brook trout, Chinook salmon, and Arctic char that have been previously exposed to the parasite. For Chinook salmon and rainbow trout, the stage at which parasite development was inhibited was not known, nor whether this inhibition was permanent or the parasite stayed latent. These questions were addressed in chapter 4, in which the progression of infection with *L. salmonae* in recovered and resistant rainbow trout showed that parasite development is inhibited in at least two stages. The first inhibition stage is at the beginning of the infection, and delays arrival of the parasite in the heart; the second stage occurs subsequently, and appears to block the passage of *L. salmonae* from the heart into the gills. Furthermore, the parasite is also cleared from the heart of the resistant fish. Thus, the acquired immunity developed by the fish against reinfection with *L. salmonae* is complete. Resistance to lethal microsporidioses in mammals has been found to be dependent on functional T cell responses (Didier and Bessinger 1999), and athymic and severe combined immunodeficient (SCID) mice die after inoculation with *E. cuniculi* or *E. hellem* (Didier et al. 1994; Schmidt and Shadduck 1983). Fish do posses T cells (Partula 1999), so it is possible that the resistance observed by the exposed and recovered fish is due to cell mediated responses. However, immunity was not transferable to naive fish when they were passively immunized with sera from resistant fish, although a short delay in the arrival of the parasite in the heart of passively immunized fish was observed. Mammalian hosts infected with microsporidia express antibodies against the parasite usually for the life of the host, but

although antibodies appear to contribute to resistance, they are insufficient to prevent lethal microsporidioses (Didier and Bessinger 1999), and passive immunization of hyperimmune serum from mice does not protect athymic mice inoculated with *E. cuniculi* (Schmidt and Shadduck 1983).

The results obtained in the present study suggest a possible management strategy for the control of *L. salmonae* infections. Studies on the infection of rainbow trout with *L. salmonae* at temperatures non-permissive for xenoma formation (Beaman et al. 1999b) show that immunity develops in the absence of xenoma formation. The results from the present study show that blocking of the progression of infection at 5° and 21°C occurs early during disease development, at a stage when the parasite has not yet entered sporogony. This provides further evidence that completion of the life cycle is not necessary to induce immunity in exposed fish, and suggests that early parasite stages, such as meronts, are immunogenic. Therefore fish could be exposed to *L. salmonae* at low temperatures, and immunity could be induced, thereby avoiding a reservoir state. This is certainly an attractive alternative for control.

The results of chapter 4 show that the parasite is able to invade non-target species considered naturally resistant to this parasite, such as Atlantic salmon and brook trout. However, this invasion is transient, and the parasite is cleared from the fish as early as 2 weeks PE. Innate resistance to infection with microsporidia in mammals results from a combination of host specificity, physical an chemical barriers, and defense responses observed shortly after infection with a microsporidian (Didier and Bessinger 1999). The host specificity exhibited by *L. salmonae* (Shaw and Kent 1999) is an important factor to consider

for the aquaculture industry in British Columbia, because the presence of *L. salmonae* in susceptible fish like Chinook salmon will not likely affect the culture of Atlantic salmon, the other species that forms the backbone of the BC salmon industry. Studies in mammalian species, correlate host specificity of microsporidia to the genetic background (Niederkorn et al. 1981) or the body temperature (Undeen and Maddox 1973) of the species infected. In insects, microsporidian species that exhibit host specificity such as *Nosema locustae* have been exploited as microbial insecticides, because it affects grasshopers while being non-infections to honey bees (Brooks 1986), however, other insect microsporidia are able to infect and develop in non-target species (Becnel and Andreadis 1999).

An attractive alternative for control of disease with *L. salmonae* which could exploit the development of immunity in exposed fish is vaccination. Fish may be vaccinated with killed spores, with attenuated variants of the parasite, or with closely related species. The isolation from Chinook salmon of *L. salmonae* SV (*Salvelinus* variant) provided an opportunity to test if vaccination with a low-virulence variant of *L. salmonae* would confer protection against xenoma formation. *Loma salmonae* SV exhibits a strong preference for species of the genus *Salvelinus* (brook trout and Arctic char), and a low preference for species of the genus *Oncorhynchus* (rainbow trout, Chinook salmon, coho salmon) or *Salmo* (Atlantic salmon), which could be exploited for the control of typical *Oncorhynchus*-infecting *L. salmonae* when used as a live vaccine. The potential value of this variant as a vaccine strain was addressed in chapter 6. The results of this study show that a single exposure of naive rainbow trout to the low-virulence *L. salmonae* SV induced protection from disease in juvenile rainbow trout. The logistics of mass production of spores from the

low-virulence variant to use as a vaccine in susceptible fish may be difficult to accomplish; however, this study demonstrated that vaccination is a viable option for control of fish microsporidian diseases.

This study looked at some aspects of the biology of the infection of the microsporidian parasite *L. salmonae*. It involved the study of the progression of infection with this parasite in a susceptible species under different conditions, to observe how these influenced the outcome of disease development, while at the same time elucidating the time frame and the location (in the host's body) where this occurs. The conclusion of this study is that temperature provides an excellent way to modulate the progression of disease with *L. salmonae*, as the parasite development is completely blocked in the early stages of infection. Furthermore, fish exposed to a temperature modulated infection are all protected against homologous challenge under optimal conditions. Blocking of parasite development occurs in the heart, an organ in which the interaction of the parasite with its host should be studied in more detail. A strategy for disease control with *L. salmonae*, could include exposure to the pathogen followed by brief holding at non-permissive temperatures. Another significant conclusion from this work is that vaccination of fish against *L. salmonae* is a feasible option which works by diminishing the effects of disease (number of xenomas in the gill filaments) and is an effective way to control disease.

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