TRANSMISSION FACTORS FOR MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE*

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ABSTRACT

Loma salmonae causes microsporidial gill disease (MGD) in farmed Pacific salmonids, *Oncorhynchus* spp., resulting in respiratory distress, secondary infections and often mortality. The infection occurs in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill. The overall research goal was to identify important host, parasite and environmental factors associated with the transmission of *L. salmonae* in rainbow trout (RBT). **Environmental Factors:** Water temperature is considered to be an important environmental variable in the transmission of many fish diseases. Using a cohabitation challenge model, RBT held at 19°C had the least number of days to the development of branchial xenomas compared to fish held at either 11° and 15°C and the latter two temperatures groups showed similar xenoma onset rate. A subsequent trial revealed that water temperature affects xenoma clearance and recovery time, so that as the water temperature increased, the time required for the dissolution of all branchial xenomas decreased. Additionally, a study comparing the effect of water temperature using per os and cohabitation challenge models revealed that the regulatory effects of water temperature on xenoma onset were dependent on experimental challenge model. The overall impact of water temperature was greater when RBT were per os exposed to *L. salmonae* compared to the cohabitation model, which exhibited a dampened temperature effect. A study manipulating flow rate revealed that RBT held in a low flow tank (0.83 L min^{-1}) developed xenomas the fastest with consistently higher numbers of xenomas per gill arch. **Host Factors:** Investigations under the domain of host factors centered on the effects of various feeding rates, the dependency of fish size at the time of exposure and further studies investigating the efficacy of monensin therapy. Although feeding rate did not alter the onset or resulting intensities of branchial xenomas, fish size was found to be a significant factor. Small RBT (17 to 23 g) had significantly faster rate of xenoma development and increased xenoma intensity with the median onset time approximately 1 week sooner compared to the 2 larger size groups. Another host factor considered was the potential use of monensin therapy to treat MGD by investigating the minimum dose and treatment time required for therapeutic success. RBT treated with monensin at 1000 ppm following a per os exposure to spores showed a reduction in xenoma intensity by 69% and 85% at weeks 7 and 8 post exposure (PE), respectively, compared to the similarly exposed non-treated fish. Prophylactic treatment beginning at the time of per os exposure to spores or one week before with monensin (at 1000 ppm) reduced xenoma formation compared to fish not treated. **Pathogen Factors:** The third domain of the disease triad contains factors that directly involve the pathogen. A novel challenge model using only effluent water revealed that *L. salmonae* was transmitted to naive RBT without the need for physical contact with infectious fish. Additionally, none of the RBT exposed to ultraviolet light-treated, *L. salmonae*-infected effluent water developed xenomas. The minimum infective dose investigated for disease transmission using a cohabitation challenge model was when 5 infectious fish were cohabited with 45 naive RBT for 1 hour. The contributions contained herein were partly novel observations identifying previously unexplored transmission factors and partly studies furthering knowledge involving known transmission factors. The importance of identifying specific transmission factors that alter the disease cycle is emphasized throughout the entire thesis.

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Thank you

DEDICATION

For Huub

♡

If A equals success, then the formula is:

$A=X+Y+Z$

X is work. Y is play. Z is keep your mouth shut.

Albert Einstein

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$\bar{X}$	mean
°C	degrees Celsius
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
B.C.	British Columbia
CCAC	Canadian Council on Animal Care
FCR	feed conversion ratio
FCT	fish contact time
G	instantaneous growth rate
GEE	generalized estimating equations
HIV	human immunodeficiency virus
HR	hazard ratio
IFAT	Indirect fluorescent antibody test
IP	intraperitoneal
IPN (V)	infectious pancreatic necrosis (virus)
ISA (V)	infectious salmon amenia (virus)
ISH	*in situ* hybridization
K-M	Kaplan-Meier
L	litre
ln	natural logarithm
MGD	microsporidial gill disease
min	minute
NSERC	Natural Sciences and Engineering Research Council
PCR	polymerase chain reaction
PE	post exposure
PH	proportional hazards
PIT	passive integrated transponder
ppm	parts per million
RBT	rainbow trout
SEM	standard error of the mean
SGR	specific growth rate
TEM	transmission electron microscopy
UV	ultraviolet
XCPGA	xenoma count per gill arch

CHAPTER 1 GENERAL INTRODUCTION

1.1 Salmon Aquaculture in British Columbia, Canada and the impact of *Loma salmonae*

Salmon farming in British Columbia (B.C.), Canada began in the early 1970s and the industry has grown to become the fourth largest producer of farmed salmon in the world after Norway, Chile and the United Kingdom (MAFF 2002). This prominent B.C. industry consists of twelve salmon producers and nine salmon hatcheries, which directly and indirectly support over 1800 and 2300 jobs, respectively. Many of these jobs represent full time employment in rural areas, allowing the salmon aquaculture industry to have large impacts on smaller rural communities. In 2002, the industry produced 73 119 tonnes of salmon (representing 40% of total aquaculture production in Canada) resulting in over $310 million for the B.C. economy (MAFF 2002, DFO 2002). The main fish species cultured in B.C. are Atlantic salmon (*Salmo salar*), chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) representing 82%, 15% and 3% of the production, respectively (British Columbia Salmon Farmers Association 2002). Although it appears that the chinook salmon production is minor compared to Atlantic salmon, the annual production for 2002 was over 11 000 tonnes of chinook salmon, which generated revenues of $90 million. Chinook salmon are the third most cultured aquaculture species in Canada (after Atlantic salmon and mussels) and the second most valuable cultured species (after Atlantic salmon).

Chinook salmon are reared in net-pens and this approach is economical because of the relatively low construction cost involved and there is no need to pump water (Kent 1998a). However, this form of aquaculture allows for the exacerbation of certain diseases and presents some unique fish health problems because of the shared ocean environment. Net-pen culturing allows for the potential to share pathogens amongst wild fish, pen-mates, neighbouring net-pens and other fish farms (Kent 1998a). Diseases are an integral part of the existence of all animal production systems including cultured fish populations (Hedrick 1998). Regarding Pacific salmon culture (chinook and coho salmon), microsporidial gill disease (MGD) caused by *Loma salmonae* was identified as one of the most problematic infectious diseases (Georgiadis et al. 2001). The first reported case of *L. salmonae* in B.C. was in coho salmon smolts from a hatchery on Vancouver Island in 1987 (Magor 1987). Since the initial description, *L. salmonae* has been identified as an important salmonid pathogen to the B.C. salmon industry causing severe gill infections, significantly reducing production and resulting in high mortality rates (Magor 1987, Kent et al. 1989, Kent et al. 1995, Speare et al. 1998a, Constantine 1999). *Loma salmonae* is transmitted horizontally using a resistant spore in both fresh- and seawater with rainbow trout (*O. mykiss*), chinook and coho salmon as usual host species. Additionally, as there are no efficacious drug therapies available to treat MGD, the control measures for this disease have focused on pathogen avoidance through changes in fish husbandry practices. A single infected salmon has the potential to release thousands of spores in the local environment, which leads to

difficulties in preventing the spread of the pathogen. Initial studies involving *L. salmonae* have described experimental challenge models, followed by investigations into specific transmission factors often identified as significant for other fish pathogens (e.g. water temperature and host species). However, several transmission factors identified as possibly affecting *L. salmonae* transmission have not been investigated and are the focus of this thesis.

1.2 Phylum Microspora

1.2.1 Introduction

Microsporidians have been reported to infect the members of nearly all of the invertebrate phyla, including ciliates, myxozoans, cnidarians, nematodes, rotifers, annelids, molluscs and arthropods, as well as all five classes of vertebrates (Wittner 1999). The first of these protozoan parasites reported to infect vertebrates was *Glugea anomala*, which causes subcutaneous cysts in fish (e.g. sticklebacks) (Wittner 1999). Subsequently other species such as *Pleistophora* spp., *Nucleospora* (*Enterocytozoon*) *salmonis* and *L. salmonae* were implicated in serious disease outbreaks amongst both wild and farmed fish populations (Hauck 1984, Magor 1987, Kent et al. 1989, Speare et al. 1989, Kent 1998b, Wittner 1999). Currently, more than 1200 species belonging to 143 genera are recognized with 156 species recorded in fish hosts separated into 14 genera (Sprague et al. 1992, Wittner 1999, Lom and Nilsen 2003).

Microsporidia are obligate, intracellular protozoan parasites of eukaryotes with the transmissible stage being a resistant spore, which is small, possesses a thick wall and contains a characteristic polar tube apparatus (Canning et al. 1986, Wittner 1999, Didier et al. 2000, Lom and Nilsen 2003). Microsporidians are unusual in that they lack mitochondria and presumably rely on their host cell for obtaining cellular energy (Canning et al. 1986). Additionally, members of this phylum have a Golgi apparatus possessing an accumulation of small, opaque vesicles enclosed by a single membrane forming a meshwork, instead of a series of stacked lamellar cisternae, more typically observed in eukaryotic cells (Vávra and Larsson 1999). The Golgi apparatus is the organelle that eventually forms the individual parts of the extrusion apparatus, which includes the polar tube (also known as the polar filament), the polaroplast and the posterior vacuole (Vávra and Larsson 1999). The polar tube is the defining characteristic for this phylum and is responsible for transferring the genetic material to the host cell (Vávra and Larsson 1999, Keeling and Fast 2002).

Initially, microsporidia were considered to be ancient organisms because they lack several features (specifically mitochondria) considered to be universal to eukaryotes and subsequently were placed on an early branch leading from prokaryotes to eukaryotes (Keeling and McFadden 1998, Franzen 2004). However, new evidence of mitochondria-derived genes (e.g. heat-shock protein 70) present in the genome places the phylum as part of the crown of eukaryotes, with animals, plants, fungi and other recently evolved protists (Keeling and McFadden 1998, Franzen 2004). Unique structural organization of the

microsporidian cell separates the phylum from other protists and indicates that microsporidians are a monophyletic group (Vávra and Larsson 1999). Taxonomic classifications for this phylum are based on life cycle and structural characteristics including the size and morphology of the spores and the number of coils in the polar tube. However, inadequate observation and judgement, in the absence of molecular analysis may result in lumping together into one genus several species that are not congeneric (Lom and Nilsen 2003). For example, the genus *Loma* contains ten species (Table 1.1) with considerable variation in ultrastructure features, whereby uninucleate meronts are observed only in *L. branchialis* and *L. fontinalis* and the other seven species have plasmodial meronts (Lom and Nilsen 2003). *Loma* is not considered a monophyletic grouping because *L. acerinae*, which infects the freshwater common ruff, *(Gymnocephalus cernuus*) and Balkhash lake perch (*Perka schrenki*) cannot be unambiguously placed on the microsporidian phyletic tree (Canning et al. 1986, Lom and Nilsen 2003). *Loma acerinae (*formerly *Pleistophora acerinae* and has similar spore description as *Glugea acerinae*) has been linked to both *Loma* and *Glugea* genera and possibly requires a new genus to be accurately placed on the microsporidian tree (Canning et al. 1986, Lom and Nilsen 2003). Phylogenetic analysis based on small sub-unit rDNA strongly supports the grouping of *L. salmonae*, *L. embiotocia* (infecting shiner perch, *Cymatogaster aggregata*) and *Loma* sp. (Shaw et al. 1997, Lom and Nilsen 2003).

1.2.2 Microsporidian Infection and Life Cycle

The life cycle for microsporidians is marked by three phases: the infective phase, the proliferative phase and the sporogonic phase (Fig. 1.1). The infective phase involves mature spores in the environment and their ingestion by the host species and finally culminates with spore germination and the transfer of sporoplasm into the host cell (Cali and Takvorian 1999). The focal point of the microsporidian infection strategy, life history and diagnosis is the spore, a single, highly organized cell (Fig. 1.2) (Keeling and Fast 2002). Generally, the life cycle for microsporidians begins and ends with a thick-walled spore with an exospore and endospore layers outside the plasma membrane (Vávra and Larsson 1999, Lom and Nilsen 2003). Microsporidian spores can range in size from 1 µm in *Enterocytozoon bieneusi* to 40 µm in *Bacillidium filiferum* and are generally uniform in size and shape for a particular species (Keeling and Fast 2002). Spores observed in *L. salmonae* infections are pyriform and range from 4.5 X 2.2 µm (fixed) and 7.5 X 2.4 µm (fresh) (Canning et al. 1986). In order for successful transmission to occur, spores must be liberated from the infected host, maintain their viability in the environment, encounter a new host and gain entry to host cells that can support multiplication of the parasite (Cali and Takvorian 1999). The three key features found in a mature microsporidan spore are: (1) exospore, (2) extrusion apparatus containing the polaroplast, posterior vacuole and the polar tube and (3) sporoplasm (Vávra and Larsson 1999, Lom and Nilsen 2003). The exospore is an outer electron dense layer that ranges from thin and unstratified (approximately 10 nm thick) to complex and multi-

layered (approximately 200 nm thick) (Vávra and Larsson 1999, Lom and Nilsen 2003). Conversely, the chitinous endospore is electron transparent and therefore appears to be a structureless layer below the exospore (Vávra and Larsson 1999). This layer is approximately 100 nm wide and is formed during late spore maturation (Vávra and Larsson 1999).

As part of the extrusion apparatus, the polaroplast is a system of complex smooth membranes arranged in layers or vesicles in the anterior part of the spore. The polaroplast occupies one third to one half of the spore volume, surrounds the straight section of the polar tube and ends at the level of the first polar tube coil. During spore germination, increased turgor within the spore, caused by swelling of the polaroplast is responsible for the initial stage of polar filament evagination (Vávra and Larsson 1999). Another element of the extrusion apparatus is the posterior vacuole, which is a large clear area in the posterior part of the spore and is enclosed with a unit membrane (Vávra and Larsson 1999). This vacuole expands during spore germination and pushes the spore contents into the evaginated polar tube (Vávra and Larsson 1999). Finally, the third member of the extrusion apparatus is the thread-like polar tube and it is considered the most conspicuous structure in a mature spore (Canning et al. 1986, Vávra and Nilsen 1999). The polar tube is inserted into the anchoring disc, which is a laminar structure at the anterior end of the spore (Dyková 1995). The tube extends obliquely from the anchoring disc to the posterior half of the spore, where it forms a coil beneath the spore wall (Dyková 1995). The number, arrangement and tilt of the polar tube coils is used to discriminate species of

microsporidians (Vávra and Larsson 1999). For *L. salmonae*, the polar tube forms a single layer of coils with 14-17 turns (Canning et al. 1986). The polar tube extrusion is triggered by an appropriate stimulus and everts from the spore turning itself inside out, creating a tube for the sporoplasm to travel through to the host cell. Spore germination begins with an environmental trigger that varies for different species depending on their habitat but is largely poorly understood (Keeling and Fast 2002). However, successful *in vitro* methods have used physical and chemical stimuli including, alterations in pH, presence of anions (e.g. chloride, iodide) and cations (e.g. potassium, sodium), exposure to ultraviolet light or hydrogen peroxide (Keohane and Weiss 1999, Keeling and Fast 2002). The spore contents are ejected into the host cell cytoplasm marking the end of the infective phase (Cali and Takvorian 1999). *Loma salmonae* spores infect fish through the gut and the organism migrates to the lamina propria, from there they are transported to other parts of the body inside infected cells to complete the life cycle (Sánchez et al. 2001). The discharged polar tube can range in length from 50 - 500 µm (up to 100 times the length of the spore) and the entire germination process takes place in less than 2 seconds (Keohane and Weiss 1999, Keeling and Fast 2002).

Microsporidians, especially those infecting fish hosts, are embedded directly in the cytoplasm of the host cell (Lom and Nilsen 2003). Typically, the parasite causes enormous hypertrophy of the cell to transform it into a special structure called the xenoma, in which the developing parasite and host cell represent a physiologically integrated unit (Lom and Nilsen 2003). Xenoma size is variable

amongst the genuses, for example xenomas during a *L. salmonae* infection are approximately 0.4 mm, whereas the xenomas produced during a *Glugea* infection can be up to 13 mm (Canning et al. 1986). Regardless of size, eventually the xenoma becomes too large and ruptures, releasing spore into the environment. The xenoma seems to secure advantage for both host and parasite (Canning et al. 1986). For the parasite, it provides a suitable environment for proliferation, while protecting it against host attack by masking it within a host component (Canning et al. 1986). The host benefits by confining the parasite and ensuring that free spread of the parasite does not take place (Canning et al. 1986). Although not restricted to the Phylum Microspora, xenoma formation seems to be an invention common to many intracellular parasites, such as the Apicomplexa (Lom and Nilsen 2003). There are two main features essential to the xenoma structure: (1) organization of the hypertrophied cell and (2) distribution of the parasite (Lom and Nilsen 2003). Regarding species in the genus *Loma*, there is a thick layer of finely granular, amorphous substance covering a plasmalemma, which has low irregular projections (Lom and Nilsen 2003). Additionally, the developmental stages of *L. salmonae* intermingle irregularly through out the host cell (Lom and Nilsen 2003). All species of *Loma* (with the exception of *L. camerounensis*) infect endothelial cells, causing the formation of xenomas throughout vascularized organs, including kidney, heart, spleen and liver but the majority of the infection occurs in the gills (Shaw and Kent 1999, Rodríguez-Tovar et al. 2002, Lom and Nilsen 2003).

Species belonging to the genera *Kabatana*, *Pleistophora*, *Nucleospora* and *Heterosporis* are considered non-xenoma forming and their development occurs in the host cell cytoplasm without any special boundary (Lom and Nilsen 2003).

The next stage of development for microsporidians is the proliferative phase, also known as merogony (Canning et al. 1986, Cali and Takvorian 1999, Lom and Nilsen 2003). The proliferative phase includes all cell growth and division from the sporoplasm through the parasite's commitment to spore formation (Cali and Takvorian 1999). The infective sporoplasm of *L. salmonae* seems to have initiated early merogonic development within five days following infection (Sánchez et al. 2001). During the proliferative phase, there is one, although it is repetitive, morphological generation of meronts (Lom and Nilsen 2003). Meronts are poorly endowed with organelles although they possess free ribosomes, a variety of small vesicles and cisternae of rough endoplasmic reticulum (Lom and Nilsen 2003). Generally, meronts grow into multi-nucleate plasmodia, which are mostly cylindrical and divide by plasmotomy or by multiple fission (Lom and Nilsen 2003). However, *L. salmonae* meronts are uninucleate structures, which develop into elongate plasmodia with at least five nuclei (Canning et al. 1986). Rodríguez-Tovar et al. (2002) reported that *L. salmonae* meronts were uninucleate or binucleate intracellular structures up to 3 µm in diameter. This was the earliest parasitic stage detected and it was recognizable by the third week of infection (Rodríguez-Tovar et al. 2002). The parasite cell membrane had a wavy appearance and was in close contact with the surrounding host cell

membrane (Rodríguez-Tovar et al. 2002). The proliferative phase during a *L. salmonae* infection is most frequently completed in the gills, where the sporogonic phase begins.

The sporogonic phase or sporogony culminates in the production of sporoblasts, which eventually undergo morphogenesis to develop into mature spores (Canning et al. 1986). Sporonts are the initial cells in this phase, giving rise to sporoblasts, which mature into spores (Cali and Takvorian 1999). The sporont stage is characterized by the formation of an uniformly thick, electron dense layer that becomes the exospore coat of the spore (Cali and Takvorian 1999, Lom and Nilsen 2003). The sporont cytoplasm contains a system of rough endoplasmic reticulum cisternae, which are generally more strongly developed than in meronts (Lom and Nilsen 2003). Additionally, the cytoplasm of the developing sporonts increases in density due to increases in the amount of rough endoplasmic reticulum and ribosomes (Dyková 1995, Cali and Takvorian 1999). The final division of sporonts gives rise to the sporoblasts, which undergo morphogenesis into spores (Cali and Takvorian 1999). Sporoblasts form the extrusion apparatus including the anchoring disc, polar tube, polaroplast membranes and the posterior vacuole (Cali and Takvorian 1999). The endospore forms the continuous deposition of chitin between the electron dense exospore and the plasmalemma (Cali and Takvorian 1999). At this stage, the sporoblasts become reduced in size and the density of the parasite cytoplasm increases (Bigliardi and Sacchi 2001). Finally, the sporoblast matures into a spore with the complete formation of all the internal structures.

1.2.3 Diagnosis and Treatment

An examination of the mature spore using light microscopy is a simple, robust and widely used approach for the morphological identification of microsporidia (Dyková 1995, Weber et al. 1999). Generally, a microsporidial infection is readily diagnosed by the presence of mature spores, which are differentiated from spores of other protozoa and fungi due to the uniform shape and size, large visible posterior vacuole and coiled polar tube (Vávra and Larsson 1999). However, transmission electron microscopy (TEM) is considered the gold standard for identifying microsporidians to the species level and is based on observing the number of coils of the polar tube (Dyková 1995, Didier 1998, Weber et al. 1999, Weiss 2001). TEM is a relatively costly and time consuming technique for routine diagnostic laboratories (Didier 1998). Histochemical methods developed for microsporidian detection rely on the use of chitin-staining fluorochromes, PAS (periodic acid Schiff) and Giemsa, however these techniques cannot differentiate between species (Dyková 1995, Weber et al. 1999). Microsporidians stain light blue with a dark nucleus using the commonly used Giemsa stain and they stain Gram positive with a purple appearance (Canning et al. 1986, Didier 1998, Vávra and Larsson 1999, Weber et al. 1999). Techniques such as indirect fluorescent antibody test (IFAT), in situ hybridization (ISH) and polymerase chain reaction (PCR) have been developed and assist diagnosis and identifying the parasite at the genus and species level (Didier 1998, Docker et al. 1997, Sánchez 2000).

Historically, it has proven difficult to develop effective control strategies and therapies against disease-causing microsporidia. This has mainly been attributed to their intracellular localization in host cells and due to the resistant infectious spore (Canning et al. 1986). The ongoing efforts to characterize the biology and epidemiology of microsporidians will be important for assessing risk factors and for identifying therapeutic and preventive strategies (Didier et al. 2000). Several drugs have been used to treat microsporidial infections in fish and humans, but mainly on an experimental basis. The major therapies described centre on the use of the antibiotic fumagillin and the antihelminthic albendazole (Chinabut et al. 1992, Didier 1998, Higgins et al. 1998, Speare et al. 1999, Conteas et al. 2000, Costa and Weiss 2000, Didier et al. 2000). Recently, a sodium ionophore, monensin was found to have potential for treating infections caused by *L. salmonae* (Speare et al. 2000, Becker et al. 2002).

Fumagillin is an antimicrobial agent used primarily for treating *Nosema apis* infections in honey bees. It is now the drug most widely used to treat microsporidiosis in fish (Kent 1998b). Fumagillin apparently acts by inhibiting RNA synthesis. It is not heat stable, thus the feed must be top-coated instead of being directly milled into the medicated feed (Kent 1998b, Shaw and Kent 1999). Various concentrations of the drug have been assessed, with 3 - 10 mg fumagillin kg^{-1} fish day^{-1} for approximately two weeks as the recommended dose for salmonids (Kent and Dawe 1994, Speare et al. 1999). Higher concentrations or prolonged treatment (e.g. 30 to 60 days) cause anorexia, poor growth, anemia, renal tubule degeneration and reduction in the hematopoietic tissue of

the kidney and spleen in salmonids (Hedrick et al. 1988, Laurén et al. 1989, Kent and Dawe 1994). Speare et al. (1999) found significant reductions in the numbers of xenomas in *L. salmonae*-infected rainbow trout treated with fumagillin (at high dose) or albendazole, although by week 8 post exposure, there was no effect for any of the therapies tested.

Albendazole is a benzimidazole derivative with broad-spectrum antihelminthic and antifungal activity. Albendazole interferes with tubulin polymerization and binds to the colchicine-binding site of beta tubulin and is effective against *Encephalitozoon* species that infect mammals (including humans) (Conteas et al. 2000, Didier et al. 2000). Benzimidazole sensitive microsporidians include *Enc. hellum*, *Enc. cuniculi* and *Enc. intestinalis*, however this drug is not effective against *Enterocytozoon bieneusi*, another important microsporidian in human medicine (Franssen et al. 1995, Kotler and Orenstein 1999, Conteas et al. 2000, Costa and Weiss 2000, Didier et al. 2000).

Recently, research involving the treatment of microsporidiosis caused by *L. salmonae* infections has involved the sodium ionophore monensin (Speare et al. 2000), which modifies intracellular ion channels and selectively acts on post-Golgi endosomes (Dinter and Berger 1998). In microsporidians, the Golgi apparatus is considered primitive, yet is a key cellular feature required for the development of the coiled polar tube through which the infective sporoplasm travels (Vávra and Larsson 1999). By inhibiting polar tube formation, transmission would be blocked because the sporoplasm would not be injected

from the spore into a host. Monensin treated rainbow trout exposed to an oral dose of *L. salmonae* spores showed a 93% reduction in xenoma production (Speare et al. 2000). During peak xenoma level at week 7 post exposure, the control fish had an average of 247 xenomas per gill arch, while the medicated fish had an average of 16 xenomas per gill arch (Speare et al. 2000). Additionally, the authors reported that during the experiment, all of the food offered to the fish was consumed and there was no evidence that monensin-treated feed had any deleterious effect on the fish behaviour or gross morphology (Speare et al. 2000).

1.2.4 Transmission

Microsporidians are unique globally distributed parasites infecting a diverse range of hosts from annelids to fish to humans, with many species having a wide host range (*Ent. bieneusi* infecting humans, dogs, cats, pigs), while others have a narrow range (*L. salmonae* infects only fish of the *Oncorhynchus* genus) (Franzen and Müller 1999, Didier et al. 2000). Only in the last 20 years has the importance of microsporidians in human medicine emerged, primarily due to secondary opportunistic infections diagnosed in immunocompromised patients (e.g. people with HIV/AIDS and following organ transplantation surgery). Within HIV-infected patients in North America, western Europe and Australia, the prevalence of microsporidiosis ranges from 2 to 50% with the main clinical manifestation being chronic diarrhea which also may be responsible for the

wasting disease observed in AIDS patients (Didier et al. 2000). However, serologic surveys for antibodies to *Encephalitozoon* spp. revealed seroprevalences of 8% amongst healthy Dutch blood donors, indicating that microsporidian infections are common in immunocompetent people (van Gool et al. 1997). Although direct zoonotic transmission has not been demonstrated, it is believed to be a source of infection because the main human-infecting microsporidians also infect pigs, dogs and cats (Franzen and Müller 1999, Didier et al. 2000). Additionally, all microsporidians share the common infection method with an environmentally resistant spore, leading to the possibility for waterborne transmission as a source of infection for humans and other mammals (Franzen and Müller 1999). Both *Ent. bieneusi* and *Enc. intestinalis*, the two most common human microsporidians have been detected using PCR in sewage, ground water and surface waters in the United States (Dowd et al. 1998). Other intestinal protozoa pathogenic to humans, such as *Giardia* and *Cryptosporidium* are commonly acquired through contaminated drinking water, leading to the suggestion that the same may be true for *Ent. bieneusi* and *Enc. intestinalis* (Bryan and Schwartz 1999).

Continued efforts to characterize the biology and epidemiology of microsporidian parasites will be important for assessing risk factors and for identifying therapeutic and preventive strategies (Didier et al. 2000). Microsporidian parasites infecting fish hosts have become of increasing importance and have the potential to severely impede the growth of select

sectors of the salmon aquaculture industry in Canada. Additionally, heavy infections with the microsporidian, *L. morhua* have been observed in the developing aquaculture species, Atlantic cod (*Gadus morhua*) causing reduced growth with infected fish susceptible to secondary infections (Barker and Davis 2004).

1.3 *Loma salmonae*

The emerging pathogen, *Loma salmonae* causes microsporidial gill disease (MGD) in farmed Pacific salmonids, *Oncorhynchus* spp., resulting in respiratory distress, secondary infections and often mortality (Kent et al. 1989, Speare et al. 1989, Weiss 2001; Ramsay et al. 2003). The infection occurs in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill (Speare et al. 1998a). In addition to causing severe disease on chinook salmon farms in B.C., *L. salmonae* has also been reported to cause MGD among rainbow trout on farms in Scotland and England and in hatcheries in Georgia, USA (Poynton 1986, Markey et al. 1994, Bruno et al. 1995, Gandhi et al. 1995, Bader et al. 1998).

The necessity to study the pathobiology of *L. salmonae* in a laboratory environment has led to the development and use of rainbow trout as an animal model for MGD (Speare et al. 1998a). Rainbow trout is a relatively hardy species of fish that are frequently used in laboratory studies (Gall and Crandell

1992). Speare et al. (1998a) reported that rainbow trout were an useful animal model for studying *L. salmonae* infections because the fish were readily prone to the parasite and within the fish, the parasite was able to complete a full life cycle including the production of spores. Unlike in chinook salmon, high mortality rates and secondary infections were not observed in rainbow trout infections, which enables researchers to study disease resistance with repeat exposures (Speare et al. 1998a). Although the focus for this thesis was investigating *L. salmonae* using rainbow trout as the animal model, several of the main themes can be applied to research involving other animal or human hosts because of the shared transmission characteristics (e.g. the spore) in all microsporidians. Disease research involving mammals and human hosts is often hindered for ethical reasons and the costs associated with housing animals and conducting the studies, many of these issues are minimized in studies using fish.

Several experimental challenge models have been developed for *L. salmonae* with the most popular being per os and cohabitation (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998a, Ramsay et al. 2001, Becker et al. 2003, Becker and Speare 2004). Compared with the high dose per os model, the cohabitation model exposes fish to a low dose of spores over a longer time and is considered more representative of the actual challenges occurring in the sea cage (Becker et al. 2003). The xenoma intensity and infection prevalence were significantly higher for per os exposed fish compared to those with a cohabitation exposure (Ramsay et al. 2001). The usual pathogenesis of MGD, with a cohabitation challenge model, is the development of branchial xenomas approximately 3 to 8

weeks post exposure (PE), with xenoma dissolution occurring 1 to 5 weeks after development (Ramsay et al. 2001, Becker et al. 2003, Ramsay et al. 2003, Becker et al. 2004). However, this time-line is influenced by water temperature and challenge model (Ramsay et al. 2001, Becker et al. 2003). The cohabitation challenge model is used extensively throughout this thesis to investigate transmission factors because it more accurately represents disease events occurring in the sea cage compared to the per os model. Nevertheless, the per os model is relevant considering the potential disease transmission amongst caged chinook salmon, juvenile wild chum salmon (*O. keta*) that often reside in a net-pen for several months and the consumption of *L. salmonae*-infected fish carcasses, with any of these groups acting as a source of spores (Kent et al. 1995).

Studies using both the per os and intraperitoneal (IP) experimental challenge models demonstrated the ability of rainbow trout and chinook salmon to resist the formation of xenomas subsequent to an earlier challenge with *L. salmonae* spores (Speare et al. 1998b, Beaman et al. 1999b, Kent et al. 1999). Furthermore, rainbow trout initially exposed to *L. salmonae* spores at non-permissive temperatures (e.g. 10°C) demonstrated resistance to reinfection when they were shifted to permissive temperatures (e.g. 15°C) and rechallenged with spores (Speare et al. 1998b). Although exposure to *L. salmonae* at 10°C was not able to elicit the development of xenomas, it was able to induce a protective host response to future challenges with this pathogen (Speare et al. 1998b, Beaman et al. 1999b). Unfortunately, the mechanisms used by the host

immune system against *L. salmonae* have not been determined (Rodríguez-Tovar 2002). Recently, it was reported that rainbow trout receiving live spores orally for the initial challenge began to develop resistance at week 2 post exposure (PE) and complete resistance was observed at week 8 PE (Rodríguez-Tovar 2002). The rapid onset of functional resistance that develops in fish after exposure to this microsporidian makes *L. salmonae* an ideal candidate for vaccine development (Speare et al. 1998b, Kent et al. 1999, Rodríguez-Tovar 2002).

1.4 Host, Pathogen and Environment Relationship

Diseases are an integral part of the existence of all animals including both cultured and wild fish populations (Hedrick 1998). The artificial rearing of fish has led to the exacerbation of certain diseases that previously existed in wild populations (Reno 1998). *Loma salmonae* is a common parasite found in wild salmon populations caught in the coastal waters off British Columbia, Canada (Kent et al. 1998c). Generally, this parasite does not cause severe disease in wild fish, although it is considered to be a major pathogen to the chinook salmon aquaculture industry (Kent 2000). Typically, diseases among cultured fish can cause death, poor growth and food conversion, increased production costs and interrupted production schedules (Hedrick 1998). During a *L. salmonae* outbreak, fish mortality is considered a direct cost, while reduced growth rates and increased feed conversion ratios represent the indirect costs to the

aquaculture farmer (Constantine 1999). However, the impact of a disease outbreak is dependent on the interactions of variables defined for the host, the pathogen and the environment, which is often depicted as three interlocking circles with disease occurring at the intersection (Hedrick 1998). Making sense of the complex interactions, within and amongst the domains of host, pathogen and environment requires an understanding of many variables, some of which may not be known. Moreover, an understanding of the epidemiology and pathogenesis of infectious agents through investigating disease events may result in improved management strategies.

Scientific research investigating fish disease, in particular for those pathogens infecting cultured fish species, often begins with the development of efficient experimental challenge models. Generally, this is followed by successions of infection trials aimed at identifying basic disease parameters, including water temperature, which is paired with parasite development and host immune response, susceptible host species, resistence to reinfection and dose effects. The overall goal of this thesis was to identify important host, parasite and environmental transmission factors associated with microsporidial gill disease caused by *L. salmonae* using rainbow trout as the animal model.

Generally, transmission factors that fall under the umbrella of host factors are considered constantly present, such as host species, fish size, population size or nutritional status (Hedrick 1998). For MGD caused by *L. salmonae*, the identified host risk factors are (1) host species with chinook salmon being more susceptible compared to coho salmon and rainbow trout (Ramsay et al. 2002)

and (2) host strain differences seen in chinook salmon from British Columbia (Shaw et al. 2000). The research described in this thesis will investigate the relative importance of two related host nutritional factors in the disease paradigm responsible for MGD in rainbow trout. Additionally, further studies into the therapeutic effects of dietary treatment with monensin in rainbow trout infected with *L. salmonae* will assist in developing a strategic management plan for this disease.

Transmission factors associated with the pathogen generally include the infective dose or the number of pathogens available, how they are delivered to the host and duration of exposure, which directly influences the severity of the resulting infection (Hedrick 1998, Lapatra 1998). Ramsay et al. (2001) reported a difference in the transmission potential when using either high dose per os or presumed low dose cohabitation challenge models. Fish that were exposed using the per os model developed xenomas faster and with greater intensity than those exposed using the cohabitation model (Ramsay et al. 2001). Although *L. salmonae* is readily transmissible through a variety of methods, the minimum infective dose for this parasite had not been investigated. Additionally, the described transmission models all use direct fish-to-fish contact through cohabitation or by feeding infective material. A non-contact transmission model, using only effluent water to infect fish could identify a potential source of disease between groups of fish that share the same ocean environment but are not in direct contact (e.g. cultured salmon and migrating wild salmon).

The environment is perhaps the least defined element of the host, pathogen and environment triad, especially when considering wild fish populations (Hedrick 1998). In laboratory models, the critical environmental parameters, including temperature and flow rate, can be more closely controlled and monitored (Hedrick 1998). Water temperature affects the development rate of fish and their immune system and the development of parasites. Generally, as water temperature increases, the rate of parasite development increases. This is readily demonstrated for *L. salmonae* and another microsporidian infecting salmon, *Nucleospora* (*Enterocytozoon*) *salmonis* (Beaman et al. 1999a, Antonio and Hedrick 1995). Research conducted on the regulatory effects of water temperature on the pathogenesis of *L. salmonae* concluded that temperature has a defining role in the life cycle of this pathogen (Beaman et al. 1999a). The temperature range in which this parasite can proceed to sporogony and xenoma formation is between 9° to 20°C (Beaman et al. 1999a). Fish exposed to *L. salmonae* outside of this temperature range failed to develop xenomas. Further investigations involving water temperature are warranted to describe the regulatory effects using different challenge models and the impact of water temperature on the later stages of the parasite life cycle during xenoma dissolution.

Another environmental variable considered important to parasite transmission in fish is flow rate or the rate of water exchange (Hedrick 1998). Theoretically, the faster infective spores are cleared from the water column, the less likely a susceptible host will ingest them and develop disease. Currently, there have not

been any investigations into the role of flow rate on *L. salmonae* transmission. Understanding the role environmental factors play in disease management would be helpful in choosing appropriate sites for salmon farms or possibly predicting disease events (e.g. *L. salmonae* outbreaks occur in late summer when water temperatures peak).

The use of epidemiological methods is still in its infancy, with regard to fish health management (Georgiadis et al. 2001). Survival analysis is used extensively throughout this thesis to assess the significance of the transmission factor being investigated. Survival analysis is a collection of statistical procedures for the analysis of data in which the outcome variable of interest is time until an event occurs (Kleinbaum 1996). An event is any designated experience of research interest, for example death, disease event, recovery or sero-conversion. Generally when using survival analysis, the time variable is referred to as survival time and the event is referred to as failure (Kleinbaum 1996). Typically, the data generated herein defined the survival time as the number of days post exposure until the first xenoma was observed on the gill of an exposed fish. Survival analysis has been used to model the effect of water temperature on *Vibrio anguillarum* transmission, a significant bacterial pathogen to salmon aquaculture in juvenile Atlantic halibut (*Hippoglossus hippoglossus*) and a viral pathogen in largemouth bass (*Micropterus salmoides*) (Hoare et al. 2002, Grant et al. 2003). These techniques have also been used to model factors affecting challenge pressures during infectious pancreatic necrosis epidemics in rainbow trout, the variation in virulence of *Renibacterium*

salmoninarum observed in rainbow trout and horizontal transmission of *Piscirickettsia salmonis* in Atlantic salmon (Almendras et al. 1997, Dale et al. 1997, Nordmo and Ramstad 1999, Bebak-Williams et al. 2002).

1.5 Research Objective and Specific Aims

The overall research goal was to identify important host, parasite and environmental factors associated with the transmission of microsporidial gill disease caused by *Loma salmonae* in rainbow trout.

1.5.1 Specific Aims

1. Demonstrate successful cohabitation transmission of *L. salmonae* in a rainbow trout population. Using the cohabitation model, determine the transmission potential of *L. salmonae* to naive fish under different temperature and flow rate regimes (Chapter 2).

2. Critically examine the hypothesis (derived from observations generated in chapter 2) that a change in water temperature (particularly a decline in water temperature) may cause an accelerated dissolution of pre-formed xenomas. Also, provide a repeat observation of the rate of xenoma formation and dissolution at three constant permissible temperatures to determine whether the laboratory strain of *L. salmonae* presently in use acts similarly to strains which had generated the historical data (Chapter 3).

3. Evaluate the regulatory effects of water temperature on the development of branchial xenomas caused by *L. salmonae* using a high dose per os challenge model and a low dose cohabitation challenge model (Chapter 4).

4. Further investigate the therapeutic effect of dietary treatment with monensin on rainbow trout infected with *L. salmonae*. Specifically to determine an acceptable dose of monensin and to determine the shortest duration of treatment with the most desired effect on reducing clinical disease (Chapter 5).

5. Determine the influence of feeding rate and fish size on onset time of branchial xenomas in naive rainbow trout exposed to *L. salmonae* (Chapter 6).

6. Investigate the possibility of a non-contact horizontal transmission of *L. salmonae* and to provide proof of principle evidence that UV light sterilization of water can render *L. salmonae* spores functionally non-infective (Chapter 7).

7. Investigate the minimum exposure time required between naive rainbow trout and *L. salmonae*-infected cohorts by determining the effect of adding low (1), medium (5) or high (10) numbers of infectious cohorts into a tank containing naive RBT and by varying the available contact time between five infectious cohorts and a population of naive rainbow trout (Chapter 8).

Table 1.1. Description of the species assigned to the genus *Loma* (family Glugeidae).

Species	Host	Geographic Distribution	Site of Infection	Reference
branchialis (morhua) (type species)	*Gadus morhua* (Atlantic cod)	Boreo-arctic	gills	Canning et al. 1986
dimorpha	*Gobius niger* (black goby)	France	digestive tract (connective tissue)	Canning et al. 1986
diplodae	*Diplodus sargus* (sea bream)	France	efferent blood vessels of gill filaments	Canning et al. 1986
fontinalis	*Salvelinus fontinalis* (brook trout)	Halifax, Nova Scotia	gill lamellae	Canning et al. 1986
salmonae	*Oncorhynchus* spp. (Pacific salmonids)	North America, salmonid hatcheries	secondary lamellae of gills	Canning et al. 1986
acerinae	*Gymnocephalus cernuus* (common ruff) *Perka schrenki* (Balkhash lake perch)	France Lake Balkash, Kazakhstan	mesentery and intestinal walls	Canning et al. 1986
embiotocia	*Cymatogaster aggregata* (shiner perch)	British Columbia	secondary lamellae of gills	Shaw et al. 1997
camerounensis	*Oreochromis niloticus* (Nile tilapia)	Cameroon	gut, duodenum	Fomena et al. 1992
myrophis	*Myrophis platyrhynchus* (broadnose worm eel)	Amazon River, Brazil	connective tissue of the intestine	Azevedo & Matos 2002
sp.	*Tilapia melanopleura* (tilapia)	Bénin, Africa	adductor muscle of gills filaments	Canning et al. 1986

Generalized Life Cycle of the Microsporidia

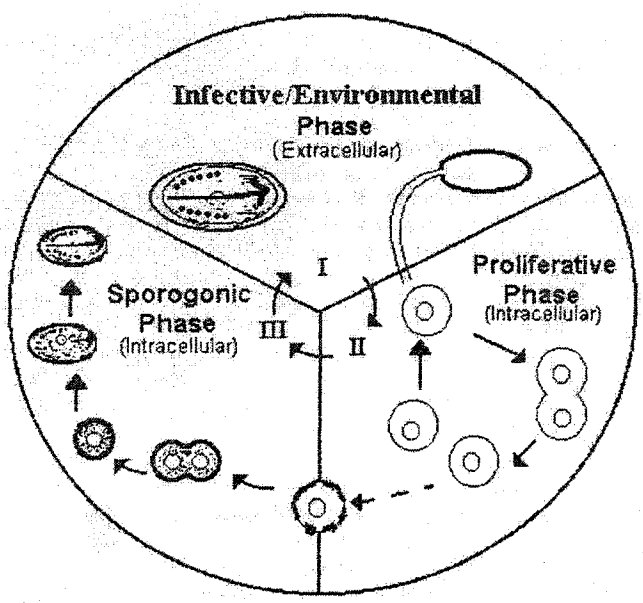

Figure 1.1. General life cycle of the microsporidia indicating the three phases of development. Phase I is the extracellular infective/environmental stage, followed by the intracellular proliferative phase II and the sporogonic phase III (Adapted from Cali and Takvorian 1999).

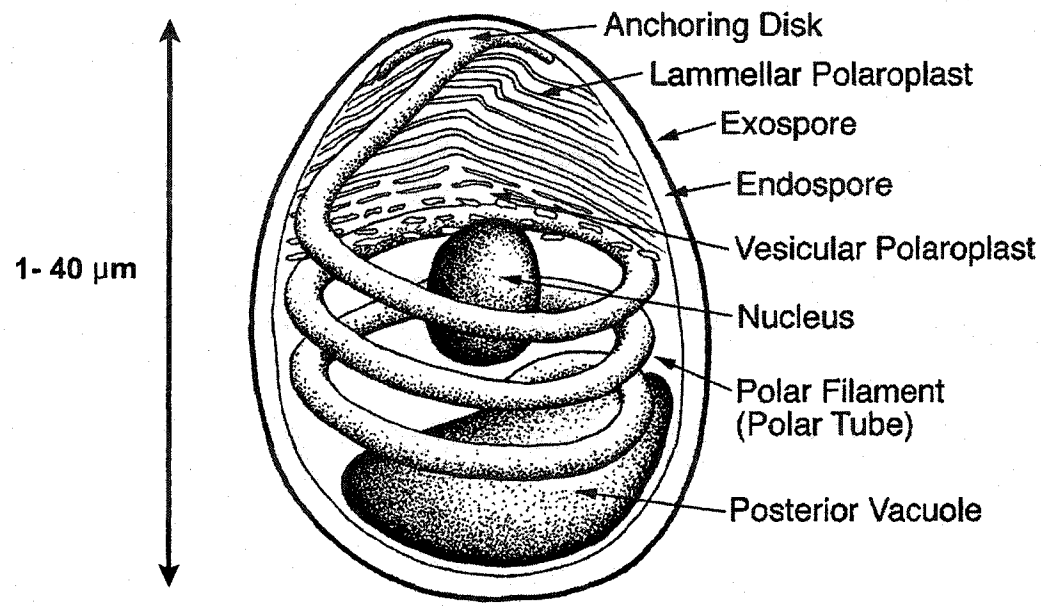

Figure 1.2. Diagram of the internal structure of a typical microsporidian spore (adapted from Keeling and Fast 2002).

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CHAPTER 2

EFFECT OF WATER TEMPERATURE AND FLOW RATE ON THE TRANSMISSION OF MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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2 EFFECT OF WATER TEMPERATURE AND FLOW RATE ON THE TRANSMISSION OF MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

2.1 ABSTRACT

Two studies were designed to quantify the effect of water temperature and flow rate on the transmission potential of the important salmonid gill pathogen, *Loma salmonae*. Using survival analysis, increased water temperature and low flow rates were determined as risk factors for the transmission of microsporidial gill disease caused by *L. salmonae* in rainbow trout. Fish were experimentally infected with *L. salmonae* via a cohabitation exposure model and monitored for the development of branchial xenomas. On any given day, fish held at 11°C and 15°C had a hazard ratio equal to 0.80 and 0.68, respectively, for the development of branchial xenomas compared with fish held at 19°C. In the flow rate trial, fish housed in a low flow tank (0.83 L min^{-1}) had an increased hazard of developing branchial xenomas when compared to fish in tanks at normal (1.67 L min^{-1}) and high (2.5 L min^{-1}) flow rates.

2.2 INTRODUCTION

Diseases are an integral part of the existence of all animals including cultured fish populations with the disease response dependent on the interactions of variables defined for the host, the pathogen and the environment (Hedrick 1998). This interaction is often diagrammed as three interlocking circles with disease occurring at the intersection of the circles. A recent examination of the health management problems for the principal fish species (salmon, rainbow trout and channel catfish) produced by North American aquaculture revealed infectious diseases as a major obstacle to future growth (Georgiadis et al. 2001). The diseases that were identified as the most problematic in North American salmon aquaculture share common characteristics in that they are almost all infectious agents that respond poorly to drug therapy and often have transmission patterns that are unknown or poorly understood (Georgiadis et al. 2001). Regarding Pacific salmon culture, microsporidiosis caused by *Loma salmonae* was identified as one of the most problematic infectious diseases.

Infections caused by the protozoan parasite, *L. salmonae* are becoming more important in salmon aquaculture because of their high prevalence and severe mortality rates (Magor 1987, Kent et al. 1995, Ramsay et al. 2001). All species of *Oncorhynchus* are susceptible to *L. salmonae* infection, although both cultured and wild chinook salmon (*Oncorhynchus tshawytscha*) are the most vulnerable (Kent et al. 1995, Shaw et al. 2000). The infection occurs in the gills and in other vascularized tissues with final developmental stages consisting of a

spore-filled xenoma within the endothelial and pillar cells of the gill (Speare et al. 1998b). Experimental infection models have been developed for *L. salmonae* using a high dose oral exposure (per os) and a low dose cohabitation model (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998a, Ramsay et al. 2001). Rainbow trout per os exposed developed significantly higher numbers of branchial xenomas compared with cohabitation exposed RBT (Ramsay et al. 2001). Additionally, the per os exposed fish were able to infect naive RBT (also with per os exposure) until week 20 PE compared to cohabitation exposed RBT being able to infect naive fish until week 11 PE (also with cohabitation exposure) (Ramsay et al. 2001). The observed differences in the ability of an infected fish to transmit *L. salmonae* to a naive fish between these two experimental models indicated the transmission potential of this pathogen appeared to extend beyond the visible signs of disease (Ramsay et al. 2001). This indicated that the cohabitation model may be a more reasonable demonstration of the actual disease events occurring in the sea cage. The cohabitation model allowed for chronic low dose exposure to spores as compared to a single point exposure to a large number of spores. Although microsporidian research has become increasingly important in veterinary and human medicine, there is still little knowledge regarding mechanisms of pathogenesis (Becker et al. 2002).

The environment is perhaps the least defined element of the host, pathogen and environment paradigm mentioned above (Hedrick 1998). In an aquaculture situation, specific components of the environment (e.g. dissolved gases, pH, temperature, flows and turbidity) are evident and are monitored either

continuously or after specific times (e.g. after disease outbreaks) (Hedrick 1998). Temperature has been identified as having a defining role in the life cycle of *L. salmonae* (Beaman et al. 1999). The permissible temperature range for this parasite to proceed to sporogony and xenoma formation is between 9° and 20°C (Beaman et al. 1999). Additionally, a thermal unit model was developed for predicting disease onset based on temperature (Beaman et al. 1999, Speare et al. 1999). However, these studies were completed using a high dose oral point exposure infection model. Recently, the oral infection model has been shown to produce significantly higher disease prevalence and xenoma intensity than fish exposed via a cohabitation infection model (Ramsay et al. 2001).

Flow rate is another environmental factor that was identified as a possible disease transmission modifier in an aquaculture environment (Hedrick 1998, Reno 1998). Flow rates are quite easily explored in the laboratory setting and currently there are no reports investigating the transmission potential of *L. salmonae* under flow rate manipulation. Under the cohabitation infection model, presumably the faster the infective spores are cleared from the water column, the less likely a susceptible host will ingest a spore and develop disease.

Initially, a pilot study was designed to describe the transmission of *L. salmonae* using a cohabitation exposure model to determine the feasability of the experimental design and the usage of survival analysis to evaluate the time to onset of branchial xenomas. Subsequently, a study was completed with the objective of using the cohabitation model with a chronic low dose exposure to

infective spores to determine the transmission potential of *L. salmonae* to naive fish under different temperature and flow rate regimes.

2.3 MATERIALS AND METHODS

2.3.1 Pilot Study

Juvenile rainbow trout (RBT) (*Oncorhynchus mykiss*) (approximately 23 g each) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no previous record of *L. salmonae.* Two weeks prior to the start of the trial, RBT were distributed to five separate tanks so that three tanks (46 L habitable volume) each held 45 fish (of which one served as the control tank and did not receive any infected fish) and two tanks (70 L habitable volume) each held 75 naive fish to achieve an equivalent stocking density of 24 g L^{-1}. All of the tanks were held at a water temperature of 15°C (± 0.3°C) from a well source as described below in Trial I with a minimum flow rate of 2 L min^{-1}. Tanks with 45 fish were labeled Tank A and B and tanks with 75 fish were labeled Tank C and D.

2.3.2 Trial I

The purpose of this trial was to evaluate the effect of water temperature on *L. salmonae* transmission in a rainbow trout population. Juvenile rainbow trout *(O. mykiss*) (RBT) (15-25 g) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no history of *L. salmonae.*

Approximately 75 RBT were randomly distributed to each circular fibreglass tank (for a total of six tanks) with a flow through system of 70 L of habitable volume. The tanks were supplied from a well water source with constant aeration and the oxygen levels were monitored using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, Utah). The RBT were acclimatized to these conditions for 8 days prior to initiating the experiments. The flow rate was maintained at 2.0 L min^{-1} because water turnover rate may affect the transmission of *L. salmonae* (Speare et al. 1998c). All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993).

The water temperature treatments were 11° (cold), 15° (moderate) and 19° (warm) (± 0.3) C, which were completed in duplicate with the second 15°C tank being the control tank (not exposed to *L. salmonae*). This was chosen as the control tank because 15°C is the optimum temperature for *L. salmonae* proliferation (Beaman et al. 1999, Speare et al. 1998a). A two header tank system was used to ensure precision and consistency in water temperatures. One tank contained ambient well water at approximately 11°C, the other contained heated well water at 27°C. Heated water passed through an aeration/degassing column to prevent gas supersaturation. The two tanks of water mixed just before entering the experimental tanks to provide the desired temperatures. Water temperatures were monitored daily using a Fluke® armoured thermocouple (Fluke Corp., Everett, WA, USA) and were recorded every 10 minutes using a Campbell Scientific Data Logger.

2.3.3 Trial II

The purpose of this trial was to evaluate the effect of flow rate on *L. salmonae* dissemination in a rainbow trout population. Juvenile RBT (30-35 g) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no history of *L. salmonae*. Fish (approximately 40 per tank) were housed in a flow through system of 50 L (habitable volume) circular fibreglass tanks with a diameter and water depth of 50 cm and 32 cm, respectively. The tanks were supplied from the same water source as described above with the same monitoring systems for temperature and water quality. Fish were acclimatized to these conditions for 5 days prior to initiating the experiments. The flow rate treatments were set at 0.83 L min^{-1} (low), 1.67 L min^{-1} (normal) or 2.5 L min^{-1} (high). Each flow rate was carried out with 2 replicates with one control tank not exposed to *L. salmonae* with a flow rate adjusted for 1.67 L min^{-1} (normal rate).

2.3.4 Cohabitation Exposure Model

Separately for each trial, a large pool of rainbow trout was per os exposed (high dose) to *L. salmonae* spores via infected gill macerated material as previously described and subsequently held at 15°C (Kent et al. 1995; Speare et al., 1998a). These RBT were routinely non-lethally evaluated for the intensity of visible xenomas (Table 2.1). Once a sufficient number of highly infected RBT scoring a 3 or higher on the intensity index (Table 2.1) were observed (20, 25

and 30 RBT for the pilot study, trial I and II, respectively), these fish were fin-clipped for identification and randomly allocated to the treatment tanks so that five highly infected RBT were added to each tank. This was considered the initiation of each trial and subsequently referred to as day 0 post exposure (PE). The infected fish remained in the tanks for the entire duration of the pilot study. However, for the temperature and flow rate trials, the infected fish were allowed to cohabitate with the naive RBT in the treatment tanks until day 21 PE.

2.3.5 Screening Methods and Data Collection

Beginning at day 21 PE and continuing on a biweekly basis until day 98 PE for the pilot study, day 91 PE for trial I and day 80 PE for trial II, the first left gill arch for all fish in the treatment and control tanks were non-lethally examined under a stereoscope to monitor development of branchial xenomas. In the case that *L. salmonae* was detected, the adipose fin was clipped and the fish was declared disease positive for the remainder of the trial. Furthermore, xenoma intensity was measured by an index value based on the number of visible xenomas on the first left gill arch (Table 2.1). Fish were anaesthetized using benzocaine at a concentration of 60 mg L^{-1} in water for all screening procedures.

2.3.6 Data Analysis

Overall, survival analysis is a collection of statistical procedures for the analysis of data in which the outcome variable of interest is time until an event

occurs (Kleinbaum 1996). An event is any designated experience of interest, for example death, disease event, recovery or sero-conversion. Generally when using survival analysis, the time variable is referred to as survival time and the event is referred to as failure (Kleinbaum 1996). Survival analysis was used to interpret the data collected from each study with the survival time defined as the number of days PE until first visible branchial xenoma and the failure described as the first visible branchial xenoma. All of the survival analyses were completed using the software package STATA™ (Stata™ Corporation, College Station, Texas) using -st- (survival time) procedures as outlined by Cleves et al. (2002).

The first step in the data analysis was obtaining survival probabilities for the different treatment groups, which is in the form of the survivor function. The survivor function S(t), is the probability that an individual will survive longer than some specified time, t (Kleinbaum 1996). Survival functions start at one and drop to zero if all subjects experience the outcome of interest (Kleinbaum 1996). For both the temperature and flow rate studies, the Kaplan-Meier (K-M) estimator (as described in Cleves et al. 2002) was used to calculate the survivor curve for each tank. The Wilcoxon's Test was used to compare the survivor functions. This test was specifically used to identify possible differences within replicates of a given treatment group. For each trial, fish that did not experience the disease event were considered censored observations for the survival analysis.

The next step was to fit a proportional hazards model to each of the data sets. The hazard function is the probability of a fish failing at a specific time (i.e. showing a visible branchial xenomas) given that it did not show xenomas before

that time (Kleinbaum 1996). In other words, the hazard function represents the instantaneous failure rate over time. A parametric proportional hazards model based on a Weibull distribution was chosen over the commonly used semi-parametric Cox proportional hazard regression model. Semi-parametric methods proceed by making comparisons between individuals at the times when failures happen to occur whereas parametric methods use probabilities that depict what occurs over the whole time interval (Cleves et al. 2002). Consequently, parametric models are statistically more efficient, provided that the distribution assumptions are fully met. In this study, a Weibull hazard function was assumed and this was evaluated by graphing the empirical hazard function for each group. A Weibull function is either monotonically increasing or decreasing with the shape of the curve depending on the shape and scale parameters (Cleves et al. 2002).

The final step in the survival analysis process was to assess the validity of the proportional hazards model by ensuring that no assumptions were violated and completing residual diagnostic procedures. A proportional hazard regression model assumes that the hazard ratio is constant over time. This assumption can be evaluated by looking for parallel lines on a plot of the log of the cumulative hazard against log time or by testing for a zero slope in a linear regression of the scaled Schoenfeld residuals over time (Kleinbaum 1996, Cleves et al. 2002). The assumption of a Weibull distribution was investigated as described above. Additionally, the possibility of tank effect was investigated by building a shared frailty model. A frailty model is a generalization of a survival regression model

and accounts for heterogeneity and random effects (Gutierrez 2002). Finally, Cox-Snell residuals were calculated and graphed to identify any influential observations and to investigate outliers.

For each trial, the mean xenoma intensity score was calculated for each treatment separately on all sample days using SAS 8 (SAS Institute Inc., Cary, NC, USA version 8). The means were graphed and analyzed using repeated measures analysis of variance (ANOVA). Because of the low replication, it was assumed that there was equal correlation across time for the ANOVA calculations in both the temperature and flow rate trials. Bonferroni corrections were made for all within time period comparisons.

2.4 RESULTS

2.4.1 Pilot Study

During the pilot study, 97.9% of the naive cohorts developed branchial xenomas and none of the non-exposed control fish developed xenomas and were subsequently not included in the data analysis. Figure 2.1 displays the Kaplan-Meier survivor curves from each tank separately, with tanks A and B having a sample size of 45 and tanks C and D having a sample size of 75. The median survival times (whereby 50% of the fish in the tank showed branchial xenomas) were 49, 39, 39 and 35 days PE for tanks A, B, C and D, respectively. Using the Wilcoxon's Test, there was at least one significant difference amongst the survivor curves, which was further elucidated using proportional hazard

modeling. From the proportional hazard model, on any given day, a fish in tank B, C or D had a hazard ratio for developing branchial xenomas equal to 1.73, 1.60 or 1.76, respectively, compared with a fish from tank A. The experimental design was satisfactory and trials were designed to evaluate environmental risk (water temperature and flow rate) factors for microsporidial gill disease (MGD) caused by *L. salmonae*.

The mean xenoma intensity score for each tank was calculated on all sample days and plotted in Figure 2.2. The curves follow an arched pathway ranging from mild to moderate to mild levels of disease. The curves for tanks B, C and D are not significantly different from each other. Although, the curve for tank A was much shallower, it follows a similar pattern. Using a repeated measures ANOVA, the mean intensity values for tank A are significantly lower than those of the other three tanks ($p < 0.0001$). Tanks B, C and D showed no significant differences ($p > 0.05$).

2.4.2 Trial I and Trial II

During the temperature and flow rate transmission trials, 97.4 and 92.3 % of the naive RBT developed branchial xenomas and were subsequently identified as disease positive for *L. salmonae*, respectively. None of the non-exposed control fish developed branchial xenomas in either trial and therefore were not included in the data analysis. On day 21 PE, the original highly infected fish were removed from the treatment tanks and their gills were examined for branchial xenomas. No xenomas were detected on any of these fish for both the

temperature and flow rate trial, suggesting that all xenomas ruptured and released spores during the three week cohabitation period.

2.4.2.1 Survival Curves

There were no significant differences (all $p > 0.15$) between replicate cold or warm tanks in the temperature trial so data were combined to generate warm, moderate and cold treatment groups. The Kaplan-Meier survival curves were plotted for each temperature group (Fig. 2.3). Fish held at 19°C had significantly reduced survival throughout the entire trial ($p = 0.004$). These fish were the more likely to develop xenomas when compared to the moderate and cold water groups. The median survival time was 33.5 days for the warm water fish, whereas it was 39 days for the moderate and cold water fish.

In the flow rate trial, the within group replicates were not significantly different from each other (all $p > 0.15$) and were combined to generate low, normal and high flow rate treatment groups. The Kaplan-Meier survival curves revealed that the low flow tanks had significantly lower probability of surviving compared to the normal and high flow tanks ($p = 0.006$) (Fig. 2.4). The median survival time for the fish in a low flow tank was 31 days, whereas it was 35 days for both the normal and high flow tanks. The survival probabilities for the normal and high flow tanks were not significantly different from each other.

2.4.2.2 Proportional Hazards Model

For both the temperature and flow rate trials, the hazard curves in all the treatment groups had a monotonically increasing Weibull distribution. Subsequent to this, a parametric proportional hazards model was fit to each data set to determine the relationship amongst the hazard functions. The estimated hazard function for each temperature group was plotted in Fig. 2.5 with the warm water fish always having the highest hazard function. From the proportional hazards model, on any given day, a fish in cold water has a hazard ratio for developing branchial xenomas of 0.80 compared with a fish in warm water. Also, on any given day, a fish in moderate water has a hazard ratio for developing xenomas equal to 0.68 of that of a fish in warm water. Similar procedures were completed on the data generated from the flow rate trial and the hazard curves for these groups are plotted in Fig. 2.6 with the low flow rate group having the highest hazard. The hazard curve for the normal and high flow rates are indistinguishable from each other. From the proportional hazards model, on any given day a fish in either a normal or high flow tank had a hazard ratio for developing xenomas equal to 0.69 compared to a fish in a low flow tank.

Once a proportional hazards model was developed for each trial, the model assumptions were tested, the possibility of tank effect was evaluated and the residuals were examined for possible outliers or influential observations. The assumption that the hazard ratio was constant over time was examined and there was no evidence of a violation for either temperature or the flow rate trials ($p > 0.10$). Shared frailty models were developed and produced similar

estimates for the proportional hazards for both models with the tank effect estimators near zero. Consequently, it was not necessary to add tank effects to the model. Finally, examination of the Cox-Snell residuals did not reveal any outliers or influential observations. The proportional hazards model as described above for each trial was considered appropriate.

2.4.2.3 Xenoma Intensity Index

For both trials, the mean xenoma intensity score for each tank was calculated on all sample days (Fig. 2.7). For the temperature trial, the level of disease peaked twice at day 35 and day 46 with little variation among the replicate tanks at all temperatures. The xenoma scores were consistently higher in the warm water tanks with the combined overall warm water mean score equal to 0.772. The overall mean score for the moderate and cold water tanks was 0.634 and 0.661, respectively. The repeated measures ANOVA was completed with only mean xenoma scores up to day 50 PE because after this time the curves overlap and are not different from each other. Up to day 50 PE, there was a marginally significant difference in the mean xenoma intensity scores across temperatures ($p = 0.061$).

For the flow rate trial, the xenoma intensity curves gradually increased to a peak at approximately day 45 PE and then began to decline. The repeated measures ANOVA revealed there was little within group variation amongst the replicates. The ANOVA data set only used mean score values up to day 45 PE because after this time there was no differences detected amongst the curves. Up to day 45 PE, there were significant differences in the mean xenoma scores amongst the different flow rates ($p = 0.015$). The low flow tanks had consistently higher mean xenoma scores with a combined overall mean score of 0.824 whereas the moderate and high flow overall means were 0.693 and 0.706, respectively.

Table 2.1. Xenoma intensity index for measurement of the number of visible branchial xenomas during a *Loma salmonae* infection in rainbow trout.

Xenoma Intensity Score	**Description**
0	No visible xenomas
1	1 visible xenoma per 6 gill filaments
2	1 visible xenoma per 3 gill filaments
3	1 visible xenoma per gill filament
4	>1 visible xenoma per gill filament

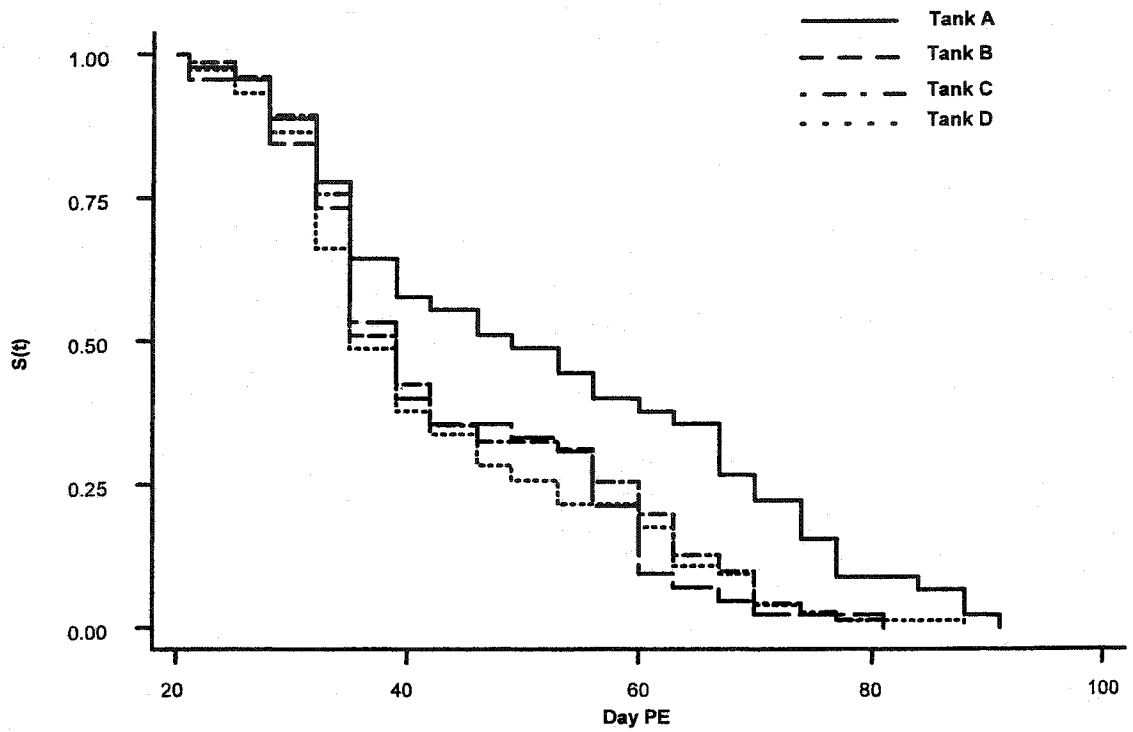

Figure 2.1. Kaplan-Meier survivor curves for the transmission of *Loma salmonae* in a rainbow trout population. Tank A and B had 45 naive fish on day 0 post exposure (PE) and tank C and D had 75 naive fish.

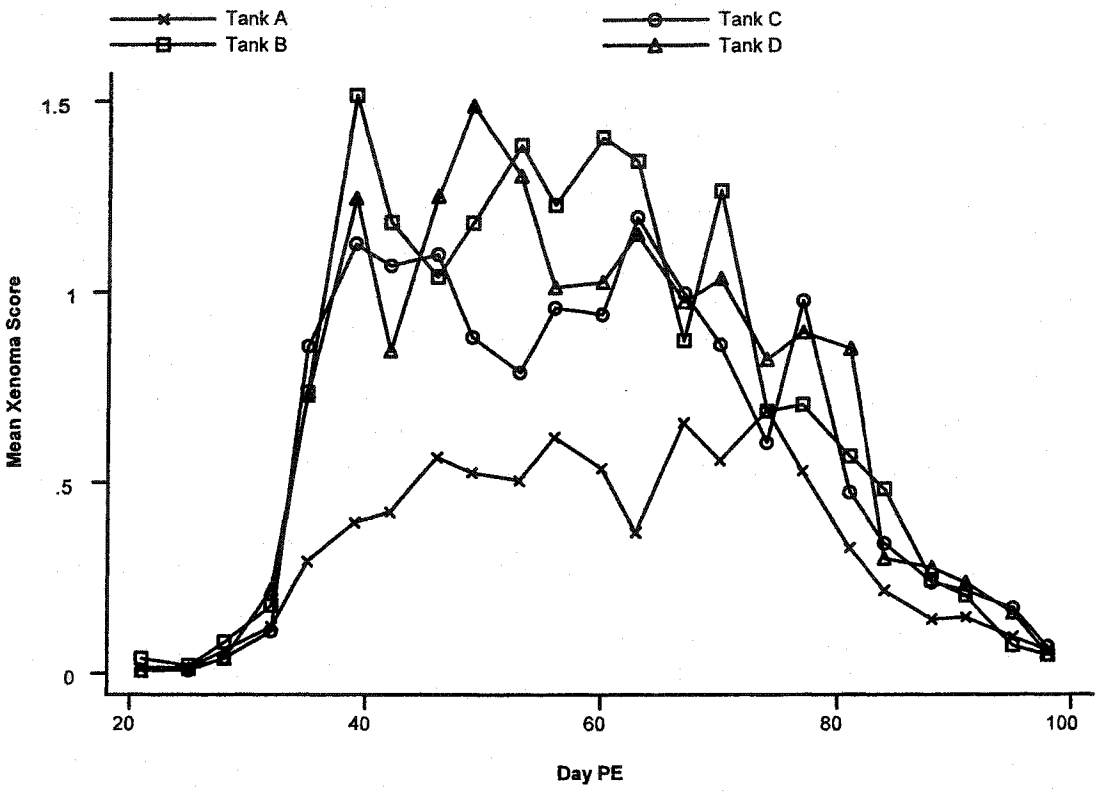

Figure 2.2. Mean xenoma intensity in rainbow trout infected with *Loma salmonae* through cohabitation with infectious cohorts for two different population sizes of 45 (tank A and B) and 75 (tank C and D) naive rainbow trout.

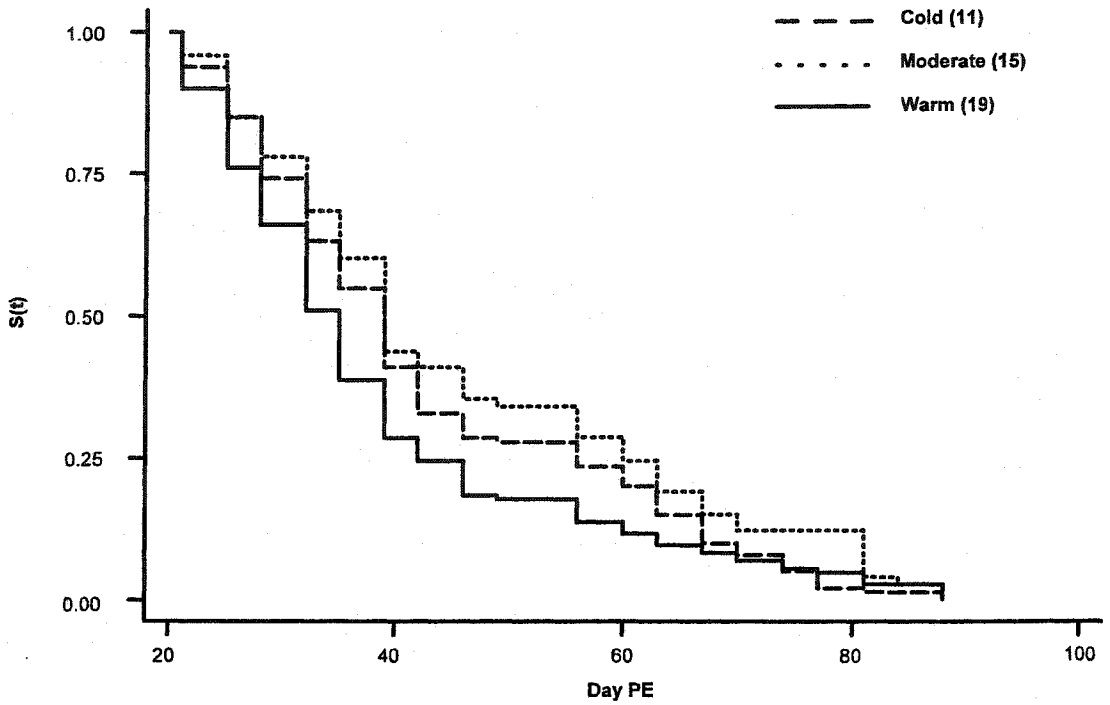

Figure 2.3. Kaplan-Meier survivor curves for the three temperature groups (°C) post exposure (PE) to a chronic low dose of *Loma salmonae* spores in a rainbow trout population.

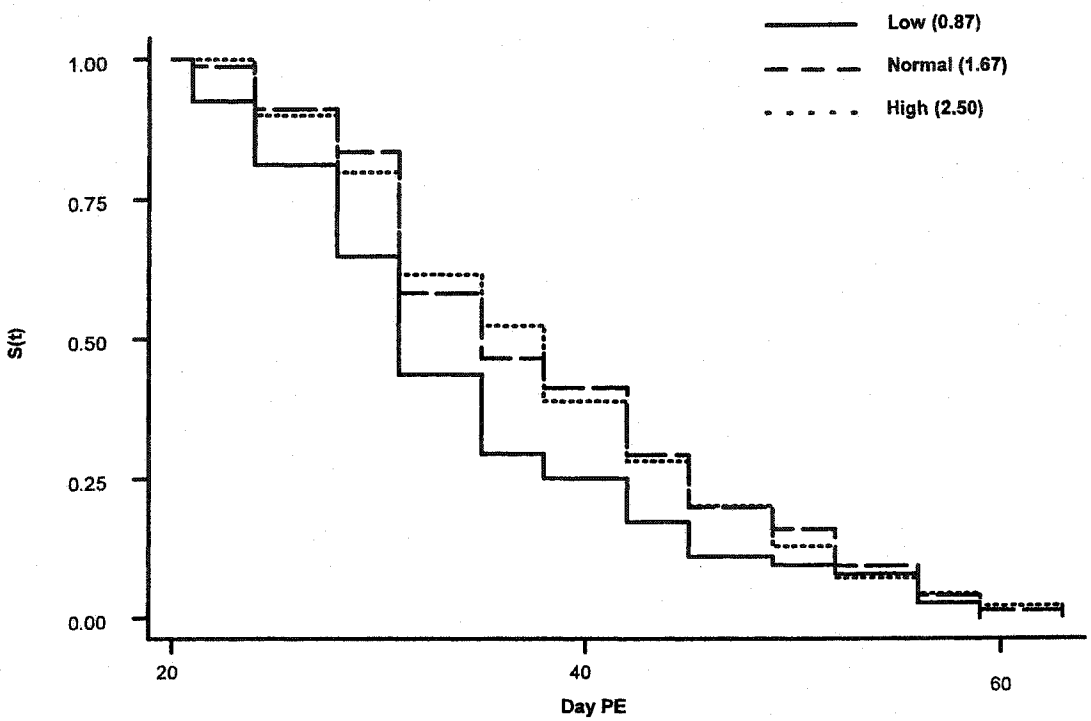

Figure 2.4. Kaplan-Meier survivor curves for the three flow rate groups (L min^{-1}) post exposure (PE) to a chronic low dose of *Loma salmonae* spores in a rainbow trout population.

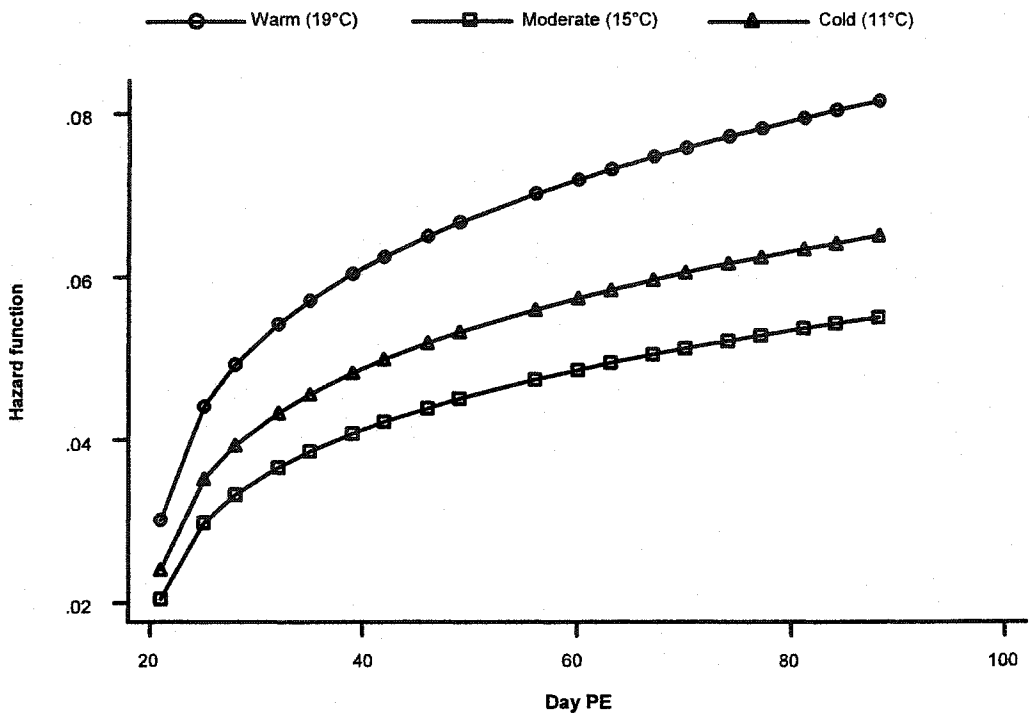

Figure 2.5. Hazard function for each temperature group (°C) post exposure (PE) to a chronic low dose of *Loma salmonae* spores in rainbow trout population.

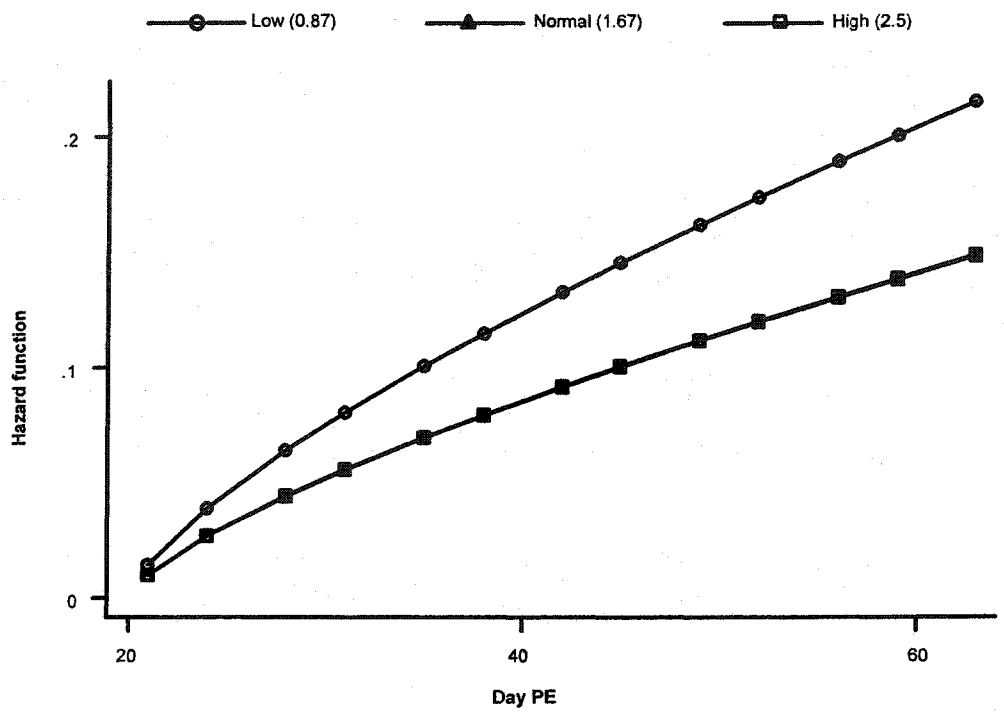

Figure 2.6. Hazard function for each flow rate group (L min^{-1}) post exposure (PE) to a chronic low dose of *Loma salmonae* spores in rainbow trout population.

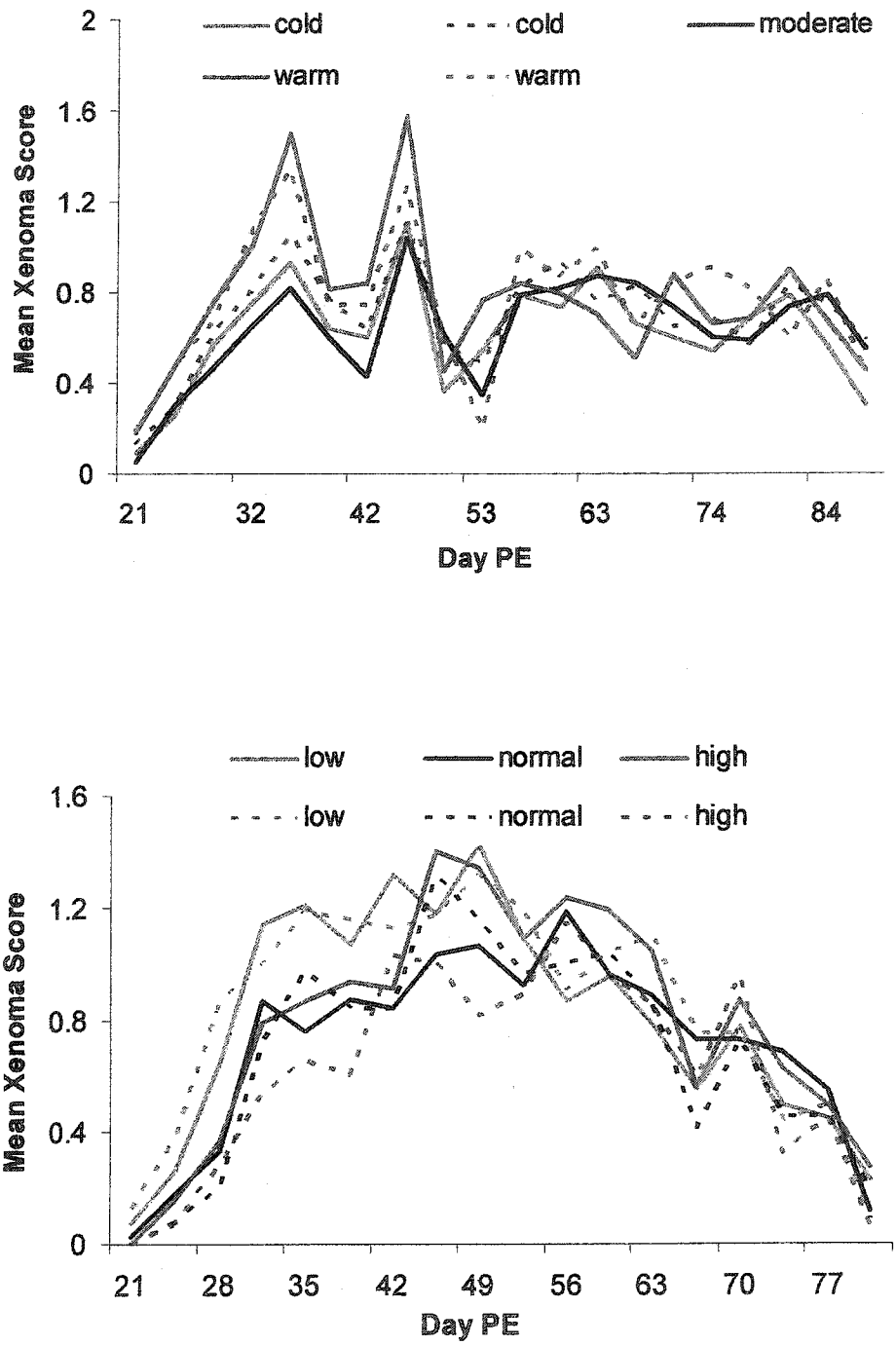

Figure 2.7.

Figure 2.7. The mean xenoma intensity for a *Loma salmonae* infection with a low dose cohabitation infection model beginning at day 21 post exposure (PE). The top graph shows the xenoma scores for the three different temperature groups, cold (blue), moderate (black) and warm (red). The bottom graph shows the xenoma scores for the three different flow rate groups, low (blue), normal (black) and high (red). For both graphs, the solid and dotted lines show the replicates in each group.

2.5 DISCUSSION

Survival analysis was used to identify differences in the transmission potential of *L. salmonae* under different temperature and flow rate regimes. Additionally, from the pilot study a simple experimental design was used to gather the required data for the survival analyses. This statistical technique is relatively new in the analysis of fish health studies. A recent study used survival analysis to effectively quantify the effect of fish density and number of infected fish during an laboratory based challenge to infectious pancreatic necrosis (Bebak-Williams et al. 2002). For our study, the proportional hazard models developed for each trial revealed that warm water and low flow rates are risk factors which enhance the development of branchial xenomas in RBT exposed to *L. salmonae* spores.

The pilot study was used to identify experimental design shortcomings and to reveal any unforeseen sampling and data analysis complications. Three of the four treatment tanks were indistinguishable from each other in both the time to onset of xenomas and the intensity of the infections. This provided evidence that there was no difference in the transmission of this pathogen between the two different sample sizes (n = 45 or n = 75) used in this trial. Therefore future trials were completed using approximately 45 naive fish.

There was a small anomaly in that the survivor curve and the intensity of secondary infections in tank A, which were both slower and less severe, respectively compared with the other three tanks. The RBT in tank A required about 10 days longer to show 50% of the population with positive signs of *L.*

salmonae. When interpreting this result, it is plausible that tank B is the anomaly and the tank A results are accurate. However, the combination of both the transmission and the intensity results, it is more likely that tank A is the anomaly. Presumptively, the initial five infected RBT that were added to tank A on day 0 PE were less able to transmit the pathogen to the naive fish. To reduce this bias in future trials, the treatment groups will be randomly allocated to the tanks as well as the assignment of the infected fish. Another design feature improved for further studies was that the cohabitation period was reduced so that the infected fish are removed on day 21 PE.

Temperature has a well-defined role in the development and transmission of *L. salmonae* within the permissible temperature range (Beaman 1998). Using the high dose oral exposure model, Beaman et al. (1999) determined that as water temperature increased, the mean number of days until disease onset decreased. Similar results were found in this cohabitation infection model with the warm water having the least number of days to disease onset. However, unlike the oral exposure model, there was no difference in the time to onset and subsequent survival curves for the moderate and cold water fish in the cohabitation model. The fish held at 11°C were expected to have a lagged onset time because of the delayed parasite development reported by Beaman et al. (1999). A simple interpretation for this observation was a difference in the xenoma resolution time and subsequent spore release in the infected fish at different temperatures. The large pool of infected RBT were held at 15°C during their entire course of infection including the exposure point, incubation period

and disease onset. Once these fish were inducted into the temperature trial, they were placed into either 11°, 15° or 19°C. The immediate temperature change may have caused the xenomas to rupture more quickly for the 11°C group and conversely may have delayed the rupture for the 19°C group. Therefore, the differential xenoma rupture times would have obscured the lag period. A further trial to specifically investigate the possibility of differential xenoma rupture times with immediate temperature changes is required to support this proposed theory.

To our knowledge, this is the first report measuring the effect of flow rate on the transmission potential of *L. salmonae*. From this study, fish in a low flow tank had a greater risk of developing microsporidial gill disease when compared to fish at normal and high flow rates. Under normal RBT rearing conditions in a circular tank (body weight >15g), the flow rate usually will not exceed two exchanges per hour (Sedgwick 1990, Ross et al. 1995, Pennel and Barton 1996). Therefore, the flow rates for this study were set at one (low), two (normal) and three (high) exchanges per hour, assuming constant mixing. Anecdotally, increasing flow rates has been generally recommended for both disease and stress reduction in salmonid culture for many years. Presumably, fewer water exchanges per hour will increase the contact time the fish have with a pathogen thus increase the probability of disease, as was the situation in this study.

For both a monogenean and copepod gill parasite infecting wild eels (*Anguilla rostrata*) in Atlantic Canada, Barker and Cone (2000) found that increasing flow rate over 5 cm per second significantly reduced the abundance of the parasites.

The reduced level of disease was attributed to the faster clearance of the free-swimming stages of the pathogens (Barker and Cone 2000). Although microsporidians do not have a free-swimming stage, the infective spore themselves are free-floating in the water column. Therefore, the use of high flow regimes presumably removes the infective spores from the water more quickly and thus the fish have a reduced probability of contacting a spore and developing microsporidial gill disease. Additionally, increased flowing water was found to be an effective treatment for ichthyophthiriasis (ich) caused by *Ichthyophthirius multifiliis* in channel catfish, *Ictalurus punctatus* (Bodensteiner et al. 2000). Compared to over 99% mortality at the base flow rate of 0.5 exchanges per hour, it was determined that 2.5 exchanges per hour greatly reduced ich-related deaths to less than 10% and 4.5 exchanges per hour, deaths were reduced to 7% and the pathogen was removed from the raceway (Bodensteiner et al. 2000). The use of a flowing water treatment instead of a chemical treatment (e.g. formalin) was advantageous because it is non-toxic, safe for all species, easily applied and does not require any government approvals (Bodensteiner et al. 2000). Similar to the situation for ich, the treatments available for *L. salmonae* are limited (Becker et al. 2002). The inclusion of increasing flow rates when possible in a health management plan for microsporidial gill disease could reduce the transmission of the pathogen in a high density sea cage. Additionally, the potential reduction in *L. salmonae* transmission through flow rate manipulation would be relatively practical if the aquaculture industry moves towards land-based rearing facilities.

Fish health management in aquaculture is mainly population-based medicine; therefore epidemiologic methods and tools are a natural choice for addressing fish-disease problems (Georgiadis et al. 2001). It is well known that occurrence of infectious disease in a population depends on interactions among a pathogen, the host and the environment. Realistic experimental challenge models can provide valuable data for identifying potential risk factors for disease and these results can be used to specifically design an epidemiological study to further investigate those factors. The development of a comprehensive health management plan for controlling microsporidial gill disease caused by *L. salmonae* requires a collaborative effort amongst culturists, veterinarians and epidemiologists in the fish health community.

2.6 ACKNOWLEDGMENTS

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CHAPTER 3

IMPACT OF A WATER TEMPERATURE SHIFT ON XENOMA CLEARANCE AND RECOVERY TIME DURING A *LOMA SALMONAE* (MICROSPORIDIA) INFECTION IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

Adapted from:

BECKER JA, SPEARE DJ. Impact of a water temperature shift on xenoma clearance and recovery time during a *Loma salmonae* (Microsporidia) infection in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2004; 58: 185-191.

3 IMPACT OF A WATER TEMPERATURE SHIFT ON XENOMA CLEARANCE AND RECOVERY TIME DURING A *LOMA SALMONAE* (MICROSPORIDIA) INFECTION IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

3.1 ABSTRACT

Previous studies have modeled the relationship between water temperature and the rate of sporulation as defined by xenoma formation during Microsporidial Gill Disease (MGD) in salmon caused by *Loma salmonae*. Although offering insight into the epidemiology of MGD, a key unexplored area is the role of temperature in the rate of xenoma dissolution including spore release into the environment as this is crucial to our ability to model horizontal transmission of MGD within confined net pen populations of farmed salmon. Results from a previous trial have led to the hypothesis that xenoma dissolution may be dramatically hastened as water temperature declines, thus introducing a critical anomaly into any predictive exercise. Data generated herein were evaluated using the statistics of survival analysis to re-establish the baseline relationship of xenoma formation and dissolution relative to water temperature and to compare these results with those of previous studies. Thirty fish were fed infected macerated xenoma-laden gill material (per os exposure) and afterwards allocated to tanks with water temperatures of 11°, 15°, or 19°C and followed through a disease cycle. Xenoma onset and clearance times were similar to previous findings with both events being accelerated at higher water

temperatures, thereby suggesting a similar temperature response in the current strain and ones used in previous studies. Another group of 45 fish was infected with *L. salmonae* and held at 15°C until xenomas formed and subsequently shifted to 11°, 15°, or 19°C. The median xenoma dissolution time in these tanks was 49, 35 and 28 days post exposure, respectively which was similar to rates observed when water temperature remained constant. Thus we rejected the hypothesis that a sudden change in water temperature triggers rapid or anomalous xenoma dissolution.

3.2 INTRODUCTION

Within intensive fish rearing systems, there has been an observed exacerbation of infections by parasites that are not considered severe pathogens in salmonids from other habitats (Kent 2000). One example of this observation is Microsporidial Gill Disease (MGD) caused by *Loma salmonae* in net-pen reared chinook and coho salmon (*Oncorhynchus tshawytscha* and *O. kisutch*) in coastal British Columbia, Canada. Although common, *L. salmonae* is not usually considered a severe pathogen in wild salmon, however it is an important cause of disease in pen reared chinook and coho salmon (Kent 2000, Georgiadis et al. 2001).

To provide a basis for the epidemiology of MGD, the rate of sporulation (xenoma formation) and spore release (xenoma dissolution) has been the subject of intensive investigation and in large part the rate of these processes is

determined by water temperature (Beaman et al. 1999, Becker et al. 2003), although the effect of host species is not negligible (Ramsay et al. 2002). The pathogenesis of MGD has recently been elucidated and the key events include oral uptake of an infectious spore, intragastric spore germination, a brief residence period in the gut lamina propria, a two-week merogony-like phase in the cardiac subendothelium, followed by a macrophage-mediated transport of the parasite to the gill where final development (further merogony and sporogony) occurs within gill pillar cells with the results of sporogony leading to the formation of a distended spore-filled xenoma (Sánchez et al. 2000, Sánchez et al. 2001, Rodríguez-Tovar et al. 2002). The development to the xenoma stage has been effectively modeled relative to the effects of temperature on the rate of xenoma formation. The permissible temperature range for this parasite to proceed to sporogony and xenoma formation is between 9°C and 20°C with optimum parasite development at 15°C (Beaman et al. 1999). Using the direct exposure model with gastric intubation, Beaman et al. (1999) reported the mean xenoma onset time as 70, 37 and 30 days post exposure (PE) for rainbow trout (RBT) reared at 11°, 15° and 19°C, respectively.

Similar modeling of the rate of xenoma clearance has been more elusive and yet is the key event regarding transmission of infection, recovery of infected fish and involves the induction of protective immunity. However, a recent report stated that the median clearance time for branchial xenomas was 10 and 12 weeks PE using the direct per os exposure model and indirect cohabitation model, respectively (Ramsay et al. 2003). The xenoma clearance time was

reportedly more variable than xenoma onset and could possibly be dependent upon the initial dose of spores received (Ramsay et al. 2003). This was the first report to identify the importance of accurately quantifying the recovery period of MGD caused by *L. salmonae* by measuring xenoma clearance and the duration of infectivity.

A critical unknown, hampering the direct application of laboratory studies (using constant water temperature) to the aquaculture setting (with fluctuating water temperatures as dictated by the ocean location), is the effect of water temperature fluctuation on disease transmission. For example, with constant water temperature, the rate of xenoma formation is highly predictable (Beaman et al. 1999). However, Speare et al. (1999) reported that when water temperature changed during a trial, the rate of xenoma formation was best predicted by the water temperature during early merogony and was not predicted by an accumulation of thermal units throughout the trial duration. Additionally, similar vagaries may frustrate our ability to predict the clearance of xenomas in conditions of water temperature change. Specifically, results from a previous trial led to the hypothesis that a sudden drop in water temperature may cause pre-formed xenomas to undergo a rapid rate of dissolution (Becker et al. 2003). A biological explanation could be that the temperature decline signals to the parasite the need to transmit to another naive fish. In support of this, Speare et al. (1999) reported that the early stages of the parasite can remain dormant within an infected fish when at 5°C (which is below the permissive water temperature) and these parasites were able to re-enter their typical life cycle

once temperatures rose above permissible levels to 15°C. However, an equally plausible alternate explanation for the previously observed anomaly was that the life cycle thermal dependance of the laboratory strain of *L. salmonae* used in the trial may have differences from strains used in the previous trials. The ciliate, *Ichthyophthirius multifiliis*, the causative agent for ich in freshwater, showed a reduction in infectivity after 25 serial passages in channel catfish (*Ictalurus punctatus*) (Xu and Klesius 2004).

The objectives of the current study were two-fold and interconnected. The main objective was to critically examine the hypothesis that a change in water temperature (particularly a decline in water temperature) may cause an accelerated dissolution of pre-formed xenomas. A secondary objective was to provide a repeat observation of the rate of xenoma formation and dissolution at three constant permissible temperatures to determine whether the laboratory strain of *L. salmonae* presently in use acts similarly to strains which had generated the historical data.

3.3 MATERIALS AND METHODS

3.3.1 Fish Husbandry and Maintenance

Juvenile RBT (15-25 g) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no history of *L. salmonae*. Fish were housed in a flow through system of 70 L (habitable volume) circular fibreglass tanks (n = 4) with a well water source. The tanks had constant

aeration and the oxygen levels were monitored using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, UT, USA). The flow rate was maintained at 2.0 L min^{-1} because water turnover rate affects the transmission of *L. salmonae* (Becker et al. 2003). The RBT were acclimatized to these conditions for 5 days prior to initiating the experiments. In all cases, RBT were anaesthetized and euthanized using benzocaine at a concentration of 60 and 100 mg L^{-1}, respectively. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.2 Temperature Control and Monitoring

The water temperatures were 11°, 15° (two tanks) and 19° (± 0.3) C. A two header-tank system was used to ensure precise and consistent water temperatures. One header-tank contained ambient well water at approximately 11°C, the other contained heated well water at 27°C. Heated water passed through an aeration/degassing column to prevent gas supersaturation. The water from the two header-tanks mixed just before entering the experimental tanks to provide the desired temperatures. Water temperatures were monitored daily using a Fluke® armoured thermocouple (Fluke Corp., Everett, WA, USA) and were recorded every 10 minutes using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, Utah).

3.3.3 Experimental Design and Method of Infection

For the per os disease exposure, a pool of 80 naive RBT maintained at 15°C were fed infectious macerated gill material obtained from laboratory maintained *L. salmonae* infections and this was subsequently considered day 0 post exposure (PE) (Kent et al. 1995, Shaw and Kent 1998; Speare et al. 1998, Ramsay et al. 2001). Twenty-four hours PE, 30 of these per os exposed RBT were randomly allocated to the other experimental tanks (10 fish per tank) held at 11°, 15° and 19°C, respectively. Therefore, this group of fish (referred to as satellite fish) were exposed to *L. salmonae* spores at 15°C and subsequently transferred to 11°, 15° or 19°C for the incubation and development of xenomas.

Beginning at week 4 PE and continuing twice weekly, the satellite fish were examined under a stereoscope to determine if there were visible xenomas on the gill filaments. Xenoma intensity was measured by an index value based on the number of visible xenomas on the first left gill arch (Table 3.1). The intensity score data generated from the satellite was recorded and tallied. The pool of 50 remaining RBT (referred to as the first cohort fish) held at 15°C were also scored twice weekly for the appearance of branchial xenomas. Once a fish was identified as having a score of 3, a dorsal fin tag was sewn into the fish and it was randomly allocated to one of the temperature tanks (already containing the satellite fish) held at 11°, 15° or 19°C. Xenoma monitoring continued until no branchial xenomas were visible.

3.3.4 Data Analyses

Survival analysis is a relatively new and useful tool used in the analysis of disease transmission models in fish research (Bebak-Williams et al. 2002, Becker et al. 2002, Becker et al. 2003, Ramsay et al. 2003). These statistical procedures are used for the analysis of data in which the outcome variable of interest is time until an event occurs with the event defined as any designated experience (e.g. death, sero-conversion or xenoma clearance). Survival analysis was used to analyze the data collected from the first cohort of RBT to evaluate the effect of a temperature shift on branchial xenoma clearance during a *L. salmonae* infection. For this study, the survival time was calculated as the number of days after the shift in temperature until no branchial xenomas were visible. Using this data, survival probabilities were calculated and plotted for each temperature group in the form of a survivor function S(t) as defined as the probability that an individual will survive longer than some specified time, t (Kleinbaum 1996). Survival functions are non-increasing and start at one and drop to zero. Specifically, the Kaplan-Meier (K-M) estimator (as described in Cleves et al. 2002) was used to calculate the survivor function for each group of fish held at 11°, 15° or 19°C, respectively. The Wilcoxon's Test was used to compare the survivor curves to determine statistical differences.

A proportional hazards (PH) model was fit to the survival data generated from the first cohort of RBT. This model calculates the hazard function for each temperature group, which is the probability of a fish failing at a specific time given that it did not fail before. In other words, the hazard function represents

the instantaneous failure rate over time (Becker et al. 2003). For this study, on any given day the hazard function represents the probability of a fish no longer showing visible xenomas. The PH model compares the hazard of one temperature group to the hazard of another in the form of a ratio (Kleinbaum 1996). The PH model assumes that the hazard ratio is constant over time and is evaluated by looking for parallel lines on a plot of the log of the cumulative hazard against log time (Kleinbaum 1996, Cleves et al. 2002). Cox-Snell residuals were calculated to identify outliers. All of the survival analysis was completed using the software package STATA™ (Stata™ Corporation, College Station, Texas) using -st- procedures as outlined by Cleves et al. (2002).

For the satellite fish, the mean xenoma intensity score was calculated for each temperature group separately on all sample days using STATA. The means were graphed using lowess smoothed graphs with a band width equal to 0.15.

3.4 RESULTS

The Kaplan-Meier survival curves measuring the time to branchial xenoma dissolution after the temperature shift for each group for the first cohort are plotted in Figure 3.1. As water temperature decreased, the amount of time required to clear all branchial xenomas increased. Fish transferred to the 19°C tank after developing a severe level of branchial xenomas had a significantly reduced xenoma survival ($p < 0.0001$). In other words, fish shifted to 19°C cleared their xenomas the fastest when compared to fish shifted to 15° and

11°C. The median xenoma clearance time was 49, 35 and 28 days after transfer to treatment tanks held at 11°, 15° or 19°C, respectively. The RBT held at 11°C experienced a lagged or retarded branchial xenoma recovery period and did not show a rapid rate of dissolution with a sudden drop in temperature.

A parametric PH model using a Weibull distribution was fit to the xenoma clearance data set to determine the relationship amongst the hazard functions for each temperature. The estimated hazard functions defined as the probability of a fish clearing all visible branchial xenomas for each temperature were plotted in Figure 3.2. At any given time, a fish that was shifted to 15°C had a hazard that was 3.2 times greater than the hazard experienced by a fish transferred to the 11°C tank. Similarly, at any given time, a fish shifted to 19°C had a hazard that was 11.5 times greater than the hazard experienced by a fish transferred to 11°C. In other words, on any given day fish shifted to either 15° or 19°C were 3.2 and 11.5 times more likely to clear all branchial xenomas compared to fish shifted to 11°C, respectively. The PH model assumption that the hazard ratio is constant over time was investigated and there was no evidence of a violation ($p = 0.60$). Examination of the Cox-Snell residuals did not reveal any outliers.

The mean xenoma intensity scores calculated from the satellite RBT were plotted in Figure 3.3. Each temperature group experienced a double peak in the mean xenoma scores followed by rapid decline and absence of xenomas. Satellite fish that were shifted to 19° and 15°C one day after *L. salmonae* exposure had xenoma scores that quickly peaked at day 32 and 42 PE and day 35 and 42 PE, respectively, whereas the fish shifted to 11°C were much slower

Table 3.1. Xenoma intensity index for measurement of the number of visible branchial xenomas during a *Loma salmonae* infection in rainbow trout.

Xenoma Intensity Score	Description
0	No visible xenomas
1	1 visible xenoma per 6 gill filaments
2	1 visible xenoma per gill filament
3	More than 1 visible xenoma per filament

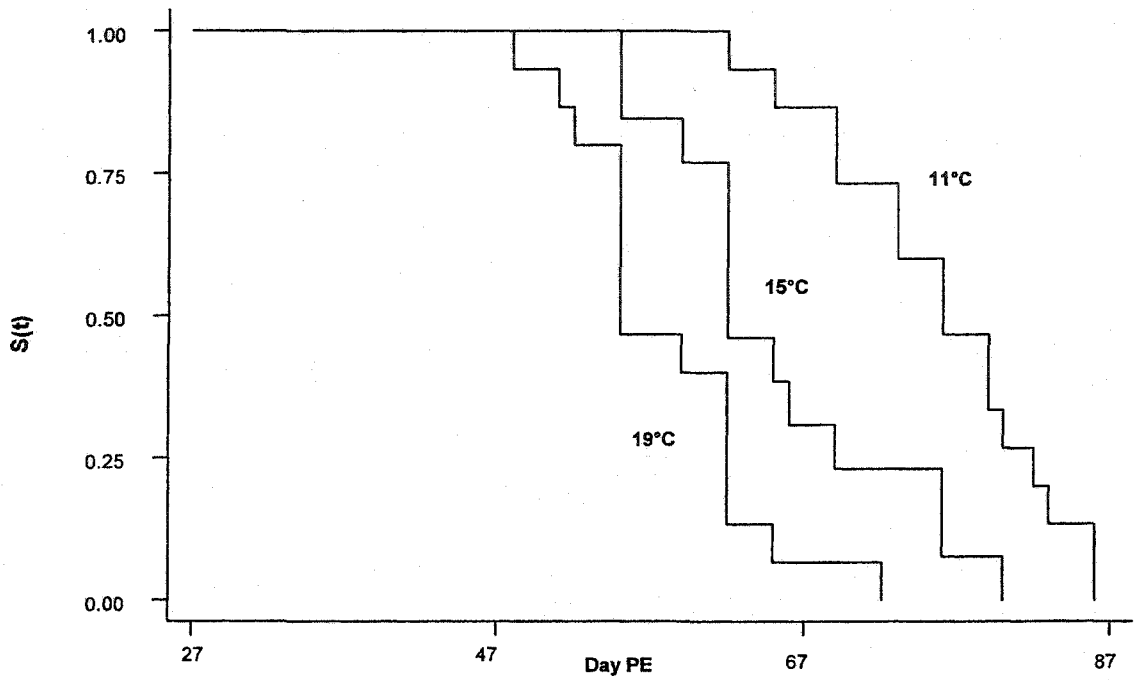

Figure 3.1. Kaplan-Meier survivor curves measuring the branchial xenoma clearance time post exposure (PE) for rainbow trout (*Oncorhynchus mykiss*) (held at 15°C) per os exposed to *Loma salmonae* spores and shifted to one of three temperatures (11°, 15° or 19°C) after experiencing peak xenoma development.

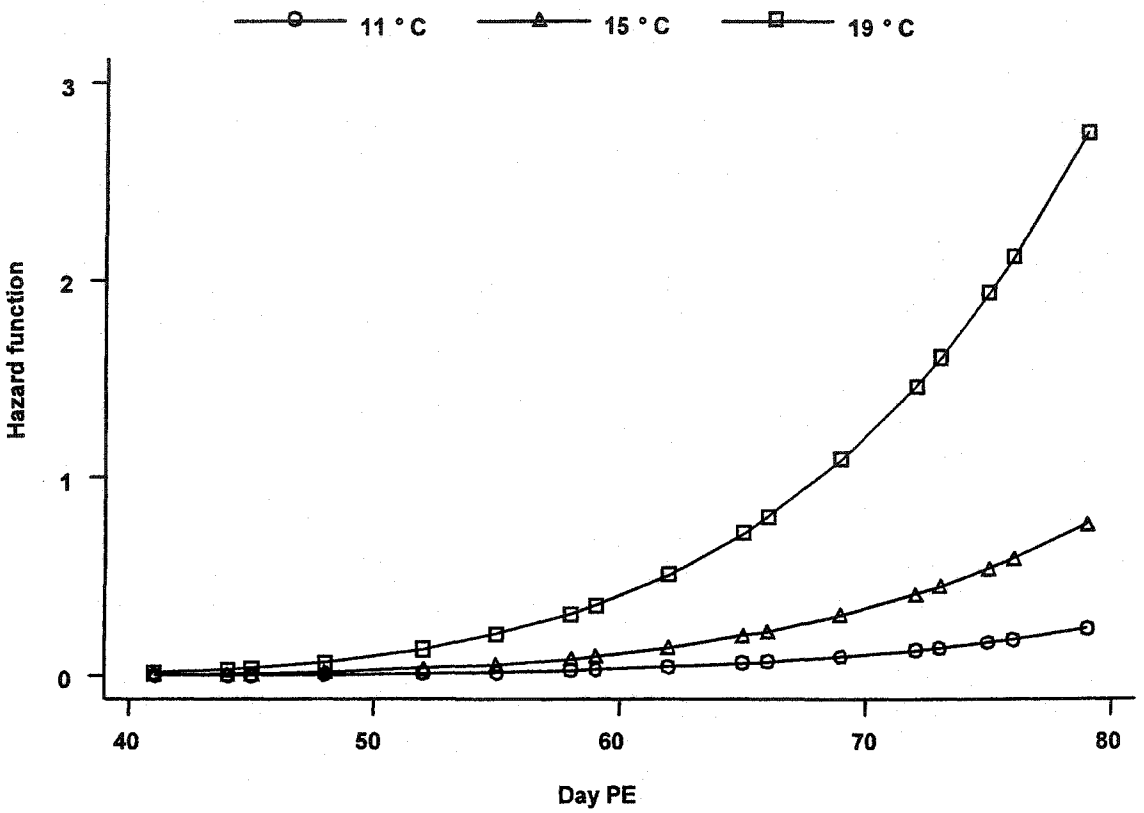

Figure 3.2. Hazard function measuring branchial xenoma clearance in the first cohort of fish at each temperature post exposure (PE) to an oral high dose of *Loma salmonae* spores.

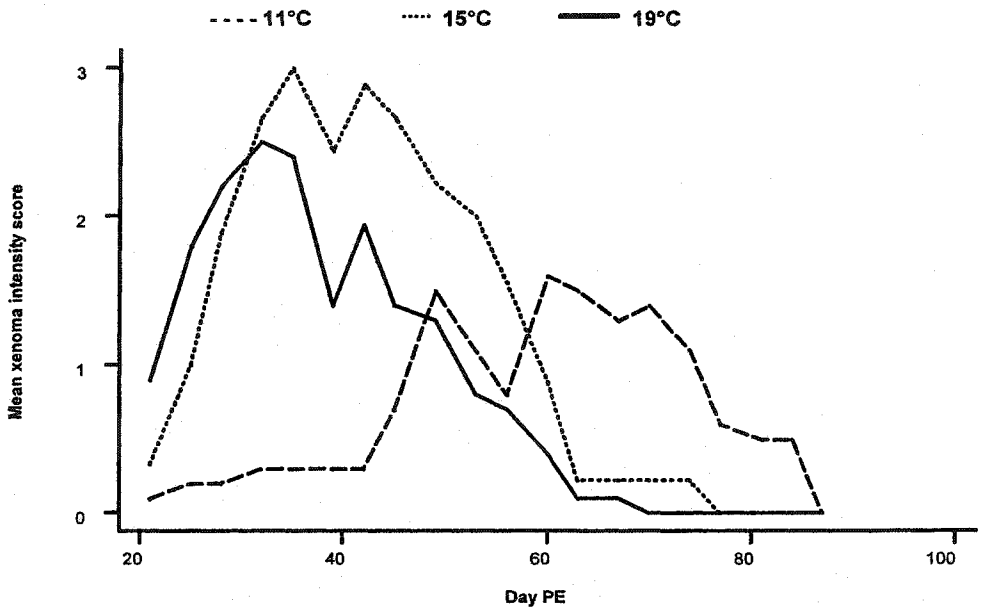

Figure 3.3. The mean xenoma intensity for satellite rainbow trout *(Oncorhynchus mykiss)* held at 11° (short dash), 15° (dotted) or 19°C (long dash) during an *Loma salmonae* infection using the high dose infection exposure model on day 0 post exposure (PE).

to peak at approximately day 49 and 60 PE. The fish held at 11°C displayed delayed xenoma formation with a peak xenoma score equal to 1.6.

3.5 DISCUSSION

The defining role of temperature in the life cycle of *L. salmonae* now includes the disease recovery period during which branchial xenomas rupture and are no longer visible. This study is the first to accurately measure the impact of a temperature shift on the xenoma dissolution using the per os exposure model. As the water temperature of the transfer tank increased, the amount of time required for the dissolution of all branchial xenomas decreased. Therefore, the temperature during the exposure period or during the early developmental stages of merogony did not fix the development rate of this parasite. Additionally the acute temperature shift to colder water did not cause an accelerated dissolution of xenomas. The RBT shifted to 11°C during the disease recovery period experienced a temperature associated lag period in the rate of xenoma dissolution. This result does not support the theory proposed regarding differential xenoma rupture with sudden temperature change (Becker et al. 2003). Additionally, there was no evidence to suggest that the associated temperature dependency of the life cycle of *L. salmonae* is a result of strain differences. However, the observed double peak at all three temperatures may account for the absence of a lag period reported by Becker et al. (2003) in the RBT held at 11°C using the cohabitation exposure model. The double peak in the mean xenoma scores suggests the possibility of early and late developing

xenomas within an individual host. This information would be critical for efficiently managing a current outbreak of MGD caused by *L. salmonae* and for prevention of future disease episodes.

Water temperature is often recognized by fish farmers and scientists to be the dominant environmental factor associated with several severe infectious diseases including *L. salmonae*, *Gyrodactylus* spp. and *Nucleospora* (*Enterocytozoon*) *salmonis*. There are a large number of gyrodactylids that parasitize cold water fish including salmonids (Anderson and Buchmann 1998). *Gyrodactylus derjavini* is widely distributed on Danish RBT farms and is clearly influenced by temperature. Anderson and Buchmann (1998) reported 100% infection was possible at 5.5°, 11.6° and 18.7°C however RBT held at the coldest temperature were significantly slower to reach maximum incidence. A rapid decrease in the parasite numbers was evident after the peak abundances occurred (Anderson and Buchmann 1998). The quick recovery from the parasite invasion was also seen in the present study with the dramatic reduction in branchial xenomas in the satellite fish after the peak was reached at all three temperatures investigated. Anderson and Buchmann (1998) suggested that a host response was involved in the elimination of *G. derjavini* following the peak abundance. Additionally, temperature affects the ability to mount an immune response as the phagocytic activity of macrophages is dependent on temperature (Shaw and Kent 1999).

The non-xenoma forming microsporidian, *N. salmonis* is associated with severe anemic conditions in chinook salmon with packed blood cell volume as low as 5% (Shaw and Kent 1999). The optimum temperature for the progression of infections occurred between 15° and 18°C with the permissible range demonstrated between 9 and 21°C (Antonio & Hedrick 1995). It was reported at the low temperatures of 9°C, mortality due to *N. salmonis* was delayed and the resultant infections were considered mild (Antonio and Hedrick 1995). A similar pattern was found with *L. salmonae* in that the satellite fish at 11°C displayed a delayed rate of xenoma development of several weeks when compared to fish held at 15° and 19°C. Although 100% of the satellite fish at 11°C developed MGD, they showed considerably lower mean numbers of xenomas and were classified as having mild to moderate levels of disease. Interestingly, fish infected with *N. salmonis* at 9°C and subsequently shifted to 15°C at week 12 PE developed severe infections with significantly higher levels of mortalities than the fish that remained at 9°C throughout the course of the disease (Antonio and Hedrick 1995). Therefore, even at relatively non-permissive temperatures of 9°C, *N. salmonis* infection occurs and can rapidly progress to severe disease with an upward shift in temperature to 15°C (Antonio and Hedrick 1995). In the present study, *L. salmonae*-exposed fish initially held at 15°C and subsequently shifted down to 11°C or up to 19°C showed an extended or reduced disease recovery period, respectively. As with *N. salmonis*, temperature was able to influence the final progression of MGD irrespective of the investigated water

temperatures during the early stages of the parasite life cycle. This observation emphasizes the importance of perpetual disease surveillance as transmission potential of MGD is quite substantive at near non-permissible water temperatures with mild levels of disease.

Understanding how a pathogen such as *L. salmonae* moves from fish to fish is crucial to effectively controlling its dissemination (Ramsay et al. 2001). During the course of outbreaks of MGD caused by *L. salmonae*, there are very few options available to fish farmers or animal health professionals to combat disease. Ramsay et al. (2001) reported that the transmission potential of *L. salmonae* appears to extend beyond the signs of disease with RBT being able to infect naive fish up to 8 weeks after xenoma clearance. This observation in combination with the influence of temperature on the clearance of xenomas from the gill filaments may have serious consequences on the possible control and reduction of this disease. Typical control methods for many infectious salmon pathogens include fallowing to break the disease cycle and separation of year classes to prevent possible spread of disease. These methods have also been suggested as possible measures for MGD control (Ramsay et al. 2001). However with the new knowledge regarding the effect of temperature on the recovery period, water temperature must be considered in the attempt to control this pathogen. Fallowing periods should be extended or reduced to reflect the water temperature at the outbreak site. The transmission potential of fish infected with *L. salmonae* in cold water is much greater than fish in warm water because the length of time in which they are able to release infective spores is

significantly longer. Outbreaks of *L. salmonae* with high mortality levels in chinook salmon tend to occur in late summer when water temperatures peak. Many fish health professionals have focused on high water temperatures as a key risk factor for disease (Beaman et al. 1999, Speare et al. 1999, Becker et al. 2003). However, this study reflects that the transmission potential in cold water is also a key factor in disease control. For example, chinook salmon that survive the MGD outbreak in late summer possibly have the ability to release infective spores for many weeks or months as the water temperature begins to drop as winter approaches. Additionally, chinook salmon have a much longer natural course of disease with the majority of fish clearing branchial xenomas by week 20 PE (Kent et al. 1999). This observation in combination with the cold water, greatly extends the infectious stage of MGD caused by *L. salmonae* in chinook salmon. Theoretically, it may be possible that a recovered chinook salmon is able to harbour a minimal number of infectious spores over the winter to be released when water temperatures are permissive in the spring and a naive population in the form of newly transferred smolts is available for infection. Therefore, recovered salmon from the previous year class would provide a functional perpetual pathogen reservoir for naive salmon.

3.6 ACKNOWLEDGMENTS

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CHAPTER 4

REGULATORY EFFECTS OF WATER TEMPERATURE ON XENOMA DEVELOPMENT WAS DEPENDENT ON CHALLENGE MODEL FOR *LOMA SALMONAE* (MICROSPORA) IN RAINBOW TROUT

4 REGULATORY EFFECTS OF WATER TEMPERATURE ON XENOMA DEVELOPMENT WAS DEPENDENT ON CHALLENGE MODEL FOR *LOMA SALMONAE* (MICROSPORA) IN RAINBOW TROUT

4.1 ABSTRACT

The objective of this study was to evaluate the regulatory effects of water temperature on the development of branchial xenomas caused by *Loma salmonae* using a high dose per os challenge model and a low dose cohabitation challenge model. Approximately 275 juvenile rainbow trout, *Oncorhynchus mykiss* (RBT) were randomly distributed to six tanks with two tanks each maintained at 11°, 15° and 19°C. Fish in one tank from each temperature setting were per os exposed to macerated *L. salmonae*-infected gill material and fish in the other tank from each temperature setting were exposed to *L. salmonae* using the cohabitation challenge model. Fish were monitored for the development of branchial xenomas beginning at day 21 post exposure (PE). Survival analyses were used to evaluate the effect of water temperature and challenge model on the number of days until the first visible branchial xenoma. The survivor curves for the per os challenge model revealed that there was at least one significant difference, whereas the cohabitation challenge did not reveal any significant differences amongst the temperature settings. The proportional hazards model revealed a significant interaction between the challenge model used and water temperature. This indicated that the effect of

water temperature was different depending on challenge model. From the mean xenoma intensities, on average the per os challenged fish showed higher xenoma intensity compared to the cohabitation challenged fish. Overall, the impact of water temperature on disease pathogenesis was greater when the RBT were per os challenged compared to using the cohabitation model.

4.2 INTRODUCTION

Loma salmonae is a relatively newly described member of the phylum Microspora, which are obligate intracellular protozoan parasites characterized by the production of spores (Canning et al. 1986, Rodriguez-Tovar et al. 2002). Infections caused by the emerging pathogen, *L. salmonae* are gaining in importance in salmon aquaculture because of their high incidence and severe mortality rates (Magor 1987, Bruno et al 1995, Georgiadis et al. 2001). This xenoma-forming microsporidian infects net-pen reared chinook salmon, *Oncorhynchus tshawtscha* and coho salmon, *O. kisutch* in coastal British Columbia (Morrison and Sprague 1983, Kent et al. 1989, Speare et al. 1989, Ramsay et al. 2001). Xenomas occur in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill (Speare et al. 1998b). Although microsporidian research has become increasingly important in veterinary and human medicine, there is much to be learned regarding the pathogenesis of microsporidian diseases (Becker et al. 2002).

Several experimental challenge models have been developed for *L. salmonae* with the most popular being per os and cohabitation (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998a, Ramsay et al. 2001). Compared with the high dose per os model, the cohabitation model exposes naive fish to a chronic low dose of spores over a longer time period and is considered to be more representative of the actual challenges occurring in the sea cage (Becker et al. 2003). Differences between per os and cohabitation exposure have been demonstrated in terms of the ability of an infected fish to transmit *L. salmonae* to a naive fish (Ramsay et al. 2001). The xenoma intensity and percent infected were significantly higher for per os exposed fish compared to those with a cohabitation exposure (Ramsay et al. 2001).

The development to the xenoma stage has been effectively modeled relative to the effects of temperature on the rate of xenoma formation using both the per os and the cohabitation challenge models (Beaman et al. 1999, Speare et al. 1999, Becker et al. 2003). The permissible temperature range for this parasite to proceed to sporogony and xenoma formation is between 9°C and 20°C with optimum parasite development at 15°C (Beaman et al. 1999). Using the per os challenge model with gastric intubation, Beaman et al. (1999) reported the mean xenoma onset time as 70, 37 and 30 days post exposure (PE) for rainbow trout (RBT) reared at 11°, 15° and 19°C, respectively. However, Becker et al. (2003) reported the median xenoma onset time as 39, 39 and 33.5 days for RBT challenged with *L. salmonae* using the low dose cohabitation model at 11°, 15° and 19°C. Similar results were found between the per os and cohabitation

infection models with fish held in warm water having the least number of days to disease onset. However, unlike the per os challenge model, there was no difference in the time to onset and subsequent survival curves for fish held at 15°C and 11°C using the cohabitation model. Therefore, it appears that the delay observed in xenoma development for per os exposed fish held at 11°C was absent with cohabitation exposed fish at the same temperature.

The objective of this study was to evaluate the regulatory effects of water temperature on the development of branchial xenomas caused by *L. salmonae* using a high dose per os challenge model and a low dose cohabitation challenge model.

4.3 MATERIALS AND METHODS

4.3.1 Fish, Animal Husbandry and Maintenance

From a large pool of over 1000 naive juvenile rainbow trout (*O. mykiss*), approximately 275 healthy fish were selected (based on a visual inspection) to participate in the study. The RBT with a mean (± standard deviation) weight of 34.2 g (± 9.40) were randomly distributed to six 70 L (habitable volume) round fibreglass tanks. As described in section 2.3.2, all tanks were supplied with a well water source with constant aeration with two tanks maintained at each of 11°, 15° or 19°C. Water temperature and oxygen levels were monitored using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, Utah). The flow rate was maintained at 2.0 L min^{-1} because it

affects the transmission of *L. salmonae* (Becker et al. 2003). Approximately, three weeks after the fish were placed in the tanks, all RBT were implanted with a passive integrated transponder (PIT) tag (AVID Canada, Calgary, Alberta) for individual fish identification during the sampling procedures. In all cases, RBT were anaesthetized and euthanized using benzocaine at a concentration of 60 and 100 mg L^{-1} in water, respectively. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993).

4.3.2 Experimental Design, Challenge Models and Sampling

One week following the PIT tag implantation, fish in one tank from each temperature setting (11°, 15° and 19°C) were fed *L. salmonae* infected macerated gill material, obtained from laboratory maintained infections as previously described (Kent et al. 1995, Speare et al. 1998a, Ramsay et al. 2001). Gills of the donor fish were examined to ensure the presence and intensity of xenomas on the gills arches. This represented day 0 PE for the high dose per os exposed tanks held at either 11°, 15° or 19°C. Beginning on day 21 PE and continuing biweekly until day 137 PE, the PIT tag number was recorded for each fish along with xenoma intensity score (see Table 4.1) based on a non-lethal inspection of the first left gill arch under a stereoscope.

On day 25 PE, five per os exposed fish from each temperature setting showing a xenoma intensity score equal to 3 were removed and their PIT number recorded. These infected fish were placed into the corresponding

temperature tank containing the naive RBT transferred several weeks earlier. This marked the initiation of the three week cohabitation challenge period at each temperature setting (11°, 15° and 19°C) and was considered day 0 PE for the low dose cohabitation exposed fish. As with the per os exposed fish, the first left gill arch of the cohabitation exposed fish was non-lethally examined under a stereoscope beginning on day 21 PE and continuing biweekly until day 113 PE. Additionally, the five per os exposed cohorts were removed on day 21 PE to mark the end of the cohabitation period. Individually for all fish, the PIT tag identification number and xenoma intensity score were recorded.

4.3.3 Data Analysis

Individually for all fish, the xenoma onset time was identified as the first day an individual showed a xenoma intensity score greater than zero (Table 4.1). Survival analysis techniques were applied to the onset time data to calculate survival probabilities and subsequently plot the Kaplan-Meier survivor curves for both per os and cohabitation challenge models at each of the three temperatures. Within each challenge model, the survivor curves for each temperature were compared using the Wilcoxon's Test. Next, a proportional hazards (PH) model was fit to the onset time data. This model calculates the hazard function for each challenge model at each temperature setting, which is the probability of a fish "failing" (i.e. having its first visible xenoma) at a specific time given that it did not fail before. The PH model compares the hazard of one group (i.e. 11°C tank with per os exposure) to the hazard of another (i.e. 19°C

tank with cohabitation exposure) in the form of a ratio (Kleinbaum 1996). The PH model assumes that the hazard ratio is constant over time and this assumption is evaluated by looking for parallel lines on a plot of the log of the cumulative hazard against log time (Kleinbaum 1996, Cleves et al. 2002). Cox-Snell and deviance residuals were calculated to validate the model and to identify outliers, respectively. All survival analyses were completed using the software package STATA™ (Stata™ Corporation, College Station, Texas) using -st- (survival time) procedures as outlined by Cleves et al. (2002).

Separately for each challenge model at each of the three temperatures, the mean xenoma intensity score was calculated on all sample days. The means were graphed using lowess smoothed graphs with a bandwidth equal to 0.40. To determine significant differences ($\alpha = 0.05$) amongst the infection curves, generalized estimating equations (GEE) were used for the linear regression model to account for the repeated measures data (i.e. mean xenoma scores within a tank were recorded on many sample days). This type of model adds fixed effects to the model and generates population average estimates of the coefficients. Additionally, the peak mean xenoma score was calculated for each treatment group.

4.4. RESULTS

The Kaplan-Meier survival curves were plotted separated by challenge model for each temperature setting (Fig. 4.1). For the per os challenge model, there

was at least one significant difference amongst the curves for fish maintained at 11°, 15° and 19°C (Fig. 4.1a) ($p = 0.0007$). Per os challenged fish held at 19°C developed xenomas faster than similarly challenged fish held at either 11° or 15°C. On the first sampling period at day 21 PE, 91.8 % of the fish at 19°C were positive for branchial xenomas compared to 69.2% and 53.7% infected fish held at 15° and 11°C, respectively. Additionally, 100% infection was observed in fish held at 19° and 15°C on day 28 PE and fish held at 11°C showed maximum infection at day 32. Conversely to the high dose oral challenge, the survival curves for the cohabitation challenged fish did not reveal any significant differences amongst the three temperatures (Fig. 4.1b) ($p = 0.408$). On day 21 PE, 55.8% of the fish held at 19°C were positive for branchial xenomas compared to 35.7% and 51.2% positive fish held at 15° and 11°C, respectively. Also, 100% infection was observed at day 66, 73 and 77 PE for fish held at 19°, 15° and 11°C, respectively.

A proportional hazard model was fit to the xenoma onset data consisting of the per os and cohabitation challenged tanks at each temperature setting. The PH model revealed a significant interaction between water temperature and challenge model, indicating that the effect of water temperature was different depending on the challenge model. From the PH model, on any given day, a per os challenged fish held at 11° or 15°C had a hazard ratio equal to 0.724 or 0.829 compared with a per os challenged fish held at 19°C (Table 4.2). Regarding the cohabitation challenged RBT, a fish held at 11° or 15°C had a hazard ratio equal to 0.793 or 0.876 compared to a fish held at 19°C (Table 4.2). Although the

interaction between water temperature and challenge model was significant, the numerical differences in the hazard ratios were small, so the interaction effect was minimal. The PH model assumption that the hazards ratio was constant over time was investigated and there was no evidence of a violation ($p > 0.970$). Additionally, an examination of the deviance residuals did not reveal any outliers.

The mean xenoma intensity score was calculated and plotted on each sample day separately for each tank (Fig. 4.2). On average, per os challenged RBT showed higher xenomas scores, with peak scores greater than 2.3 compared to cohabitation challenged fish, with peak scores less than 1.5 (Table 4.2). For the per os challenged tanks, the highest mean xenoma scores were observed in the fish held at 15°C with a mean ± standard deviation score equal to 1.46 ± 1.10, which was followed closely by the fish at 11°C and then lowest scores were observed in the RBT held at 19°C (Fig. 4.2a, Table 4.2). From the GEE model, the predicted mean xenoma score for the per os challenged RBT held at 15°C was significantly higher than the fish held at 19°C but not significantly different from the 11°C tank. For the cohabitation challenged tanks, RBT held at 19°C had the highest observed mean xenoma scores, followed by the 15° and 11°C tanks (Fig. 4.2b, Table 4.2). Fish held at 11°C had significantly lower predicted mean xenoma scores as determined from the GEE model compared to RBT held at both 15° and 19°C, which were not significantly different from each other (Table 4.2).

Table 4.1. Xenoma intensity index for measurement of the number of visible branchial xenomas during a *Loma salmonae* infection in rainbow trout.

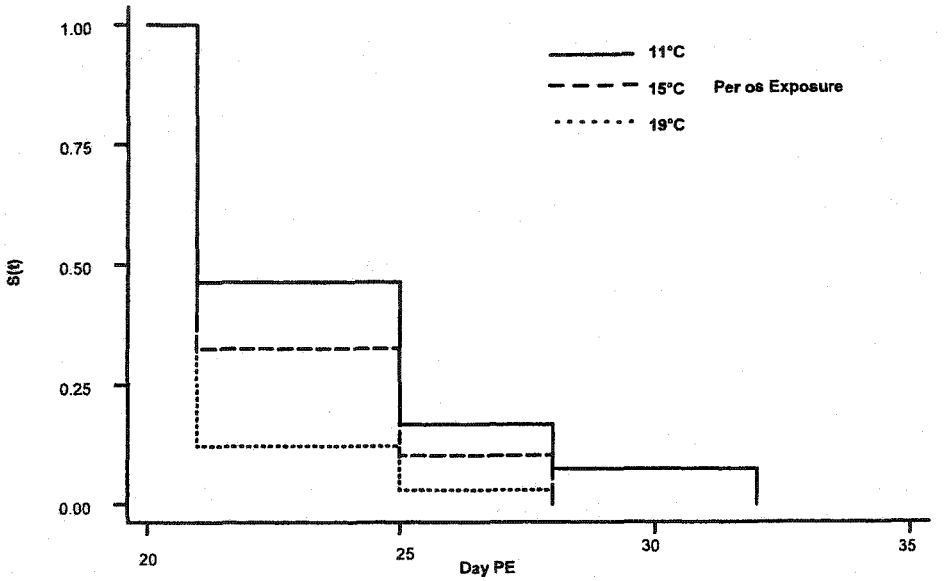
Xenoma Intensity Score	Description
0	No visible xenomas
1	<1 visible xenoma per gill filament
2	1 visible xenoma per gill filament
3	More than 1 visible xenoma per filament

Table 4.2. Hazard ratio (HR), overall mean ($\bar{x}$) ± standard deviation (sd) and peak mean xenoma score (Xn) for each challenge model at each temperature (T).

Challenge Model	T (°C)	HR	$\bar{x}$ ± sd Xn	Peak $\bar{x}$ Xn
i) Per os	11	0.724	1.41 ± 1.04	2.56
	15	0.829	1.46 ± 1.10	2.56
	19	1.00*	1.19 ± 1.04	2.37
ii) Cohabitation	11	0.793	.464 ± .549	.957
	15	0.876	.683 ± .738	1.27
	19	1.00*	.747 ± .688	1.31

* set as the baseline level in proportional hazards model

(a)



(b)

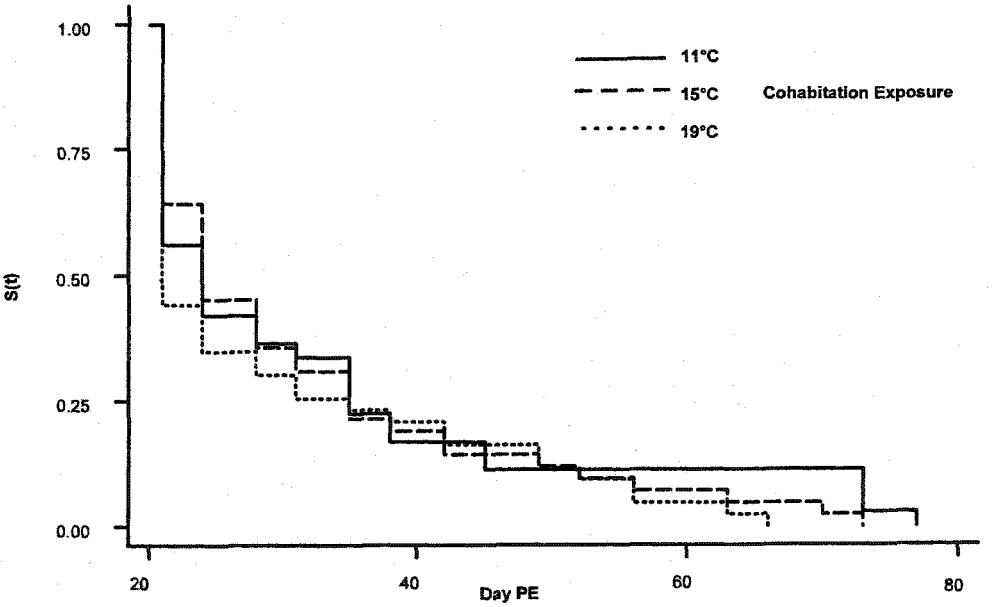


Figure 4.1.

Figure 4.1. Kaplan-Meier survivor curves for rainbow trout, *Oncorhynchus mykiss* exposed to *Loma salmonae* using the (a) per os and (b) cohabitation experimental challenge models at three temperatures.

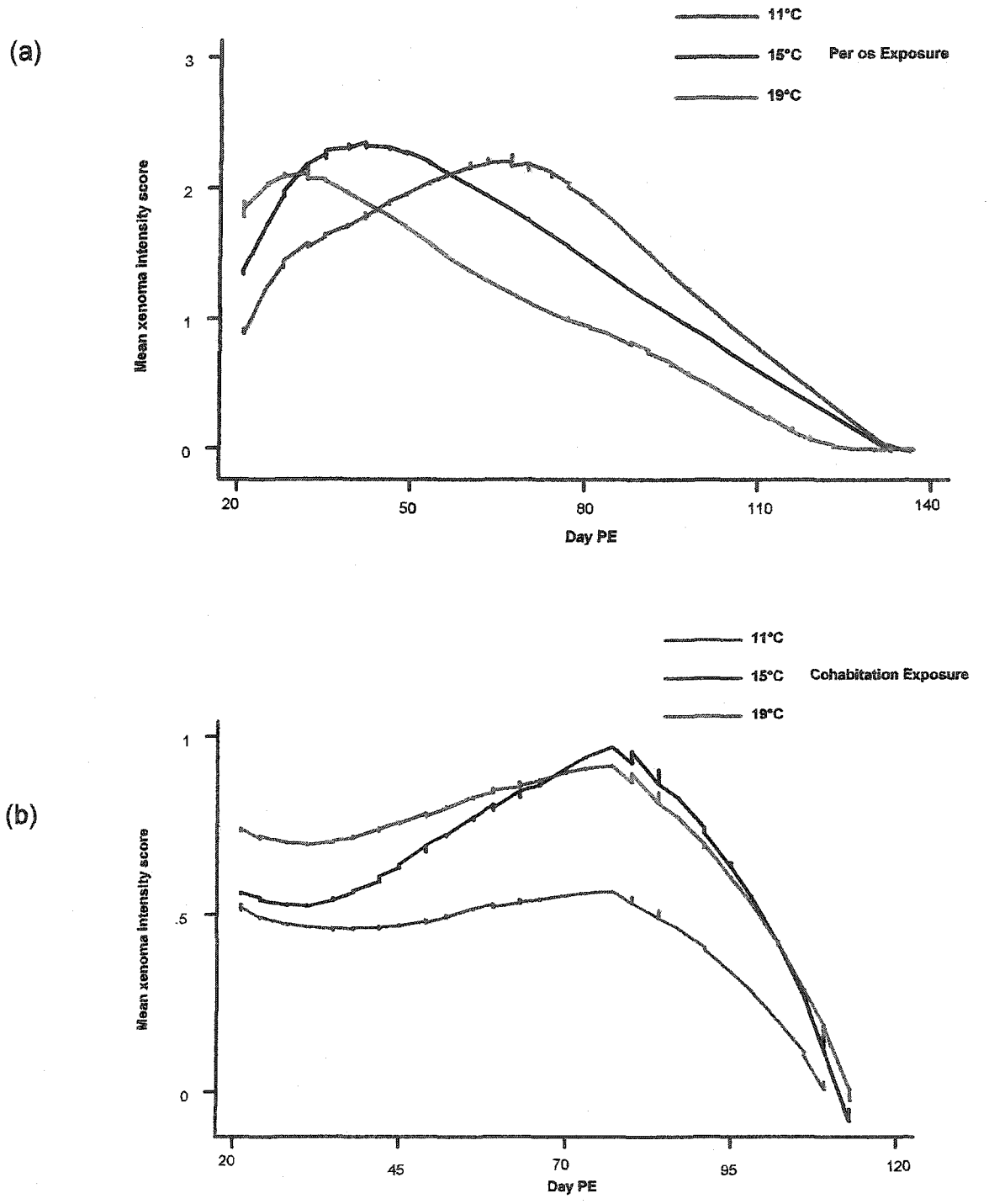


Figure 4.2.

Figure 4.2. Mean xenoma intensity for rainbow trout, *Oncorhynchus mykiss* exposed to *Loma salmonae* using the (a) per os and (b) cohabitation experimental challenge models at three temperatures.

4.5 DISCUSSION

The overall aim of this study was to investigate the impact of water temperature and experimental challenge model, two substantive transmission factors, on xenoma development during a *L. salmonae* infection and to determine the interaction of these two factors. The regulatory effects of water temperature on xenoma onset during a *L. salmonae* infection were dependent on experimental challenge model. The overall impact of water temperature on disease pathogenesis was greater when the RBT were fed *L. salmonae*-infected macerated gill material for the per os challenge model compared to the cohabitation model. Recognizing that due to the lack of repetition, the reported interaction between water temperature and challenge model cannot be differentiated from a tank effect. Notwithstanding, the results were consistent with the apparent differences in xenoma onset and intensity as reported by Beaman et al. (1999) and Becker et al. (2003) investigating the regulatory effects of temperature using the per os and cohabitation model, respectively. However, the effect of water temperature appears to be dampened when using the cohabitation exposure model, which is considered to give a low dose of spores compared to the per os challenge. The advantage of cohabitation exposure models is that they result in a subtle challenge that is not likely to overwhelm the host and they more closely simulate the natural routes of infection (Murray et al. 1992, Becker et al. 2003).

Overall, the intensity of branchial xenomas resulting from the cohabitation

challenge were considerably less than those developed from the per os challenge, which may account for the reduced impact of water temperature. Additionally, the mean xenoma intensity increased with increasing water temperature for either challenge model. Both of these observations were in agreement with other studies investigating the effect of water temperature on *L. salmonae* transmission, separately for each exposure model (Speare et al. 1998a, Ramsay et al. 2001, Becker et al. 2003). Previous reports showed that the per os challenge model produced significantly higher disease incidence and xenoma intensity compared to the cohabitation model (Speare et al. 1998a, Ramsay et al. 2001).

Several experimental challenge models have been developed for many fish pathogens, notably Bacterial Kidney Disease (BKD) caused by *Renibacterium salmoninarum.* Similar to *L. salmonae*, BKD can be horizontally transmitted and can cause high mortality rates amongst intensively reared chinook salmon in British Columbia (Murray et al. 1992, Dale et al. 1997, Wiens and Kaattari 1999). Murray et al. (1992) developed cohabitation and immersion challenge models for BKD, whilst noting the benefits of low dose challenge models by accurately simulating naturalistic disease events occurring in the sea cage aiding in vaccine and disease resistence research. Regarding *L. salmonae*, differences observed between the per os and cohabitation models have been demonstrated in terms of the ability of an infected fish to transmit *L. salmonae* to a naive fish (Ramsay et al. 2001). Therefore, investigations regarding the regulatory effects of water

temperature on *L. salmonae* transmission should be specific for the chosen challenge model.

Water temperature has been often noted as a key component of disease pathogenesis by affecting both incidence and intensity, whilst playing a major role in transmission of many notable salmonid diseases including *L. salmonae* (as described above), *Gyrodactylus derjavini*, *Aeromonas salmonicida* (Mo 1997, Anderson and Buchmann 1998, Nordmo and Ramstad 1998, Beaman et al. 1999, Becker et al. 2003). There are a large number of gyrodactylids that parasitize cold water fish including salmonids (Anderson and Buchmann 1998, Soleng et al. 1999). The common monogenean parasite, *G. derjavini* infecting Atlantic salmon (*Salmo salar*) and Brown trout (*S. trutta*) in Norway was reported to increase in prevalence and intensity concurrently with increasing water temperatures in a river near Oslo, Norway (Mo 1997). Furthermore, *G. derjavini* is widely distributed on Danish RBT farms and is clearly influenced by temperature. Anderson and Buchmann (1998) reported 100% infection was possible at 5.5°, 11.6° and 18.7°C however RBT held at the coldest temperature were significantly slower to reach maximum percent infected. Similar results were reported in this study for both exposure models for *L. salmonae*, the colder temperatures were slower to reach maximum prevalence although all three temperatures investigated were able to evoke 100% infection.

Scientific research investigating fish disease, in particular for those pathogens infecting cultured fish species often begins with the development of efficient

experimental challenge models. Generally, this is followed by successions of infection trials aimed at identifying basic disease parameters, in particular water temperature which is paired with parasite development and host immune response, susceptible host species, resistence to reinfection, fish size and dose effects. Specifically for *L. salmonae*, many experimental models were developed including per os, intra-peritoneal, cohabitation and non-contact horizontal transmission using effluent water (Speare et al. 1998a, Shaw et al. 1998, Ramsay et al. 2001, Becker and Speare 2004). However until this study, regulatory role of water temperature has only been investigated using the per os and cohabitation models, whereby divergences were noted in the disease onset time under various temperature regimes (Beaman et al. 1999, Becker et al. 2003). The combination of observations from this study and previous work by Beaman et al. (1999) and Becker et al. (2003), lead to the novel conclusions that the regulatory role of water temperature in the pathogenesis of *L. salmonae* is dependent on the chosen experimental challenge model.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

EFFECTS OF MONENSIN DOSE AND TREATMENT TIME ON XENOMA REDUCTION IN MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

Adapted from:

BECKER JA, SPEARE DJ, DALEY J. DICK P. Effects of monensin dose and treatment time on xenoma reduction in microsporidial gill disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2002; 25: 673-680.

5 EFFECTS OF MONENSIN DOSE AND TREATMENT TIME ON XENOMA REDUCTION IN MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

5.1 ABSTRACT

The objectives of the study were (1) to determine an acceptable dose of dietary monensin effective against microsporidial gill disease (MGD) of salmon caused by *Loma salmonae* and (2) to determine the life stage of *L. salmonae* that the action of monensin was most effective at reducing clinical disease. Rainbow trout (RBT) were fed monensin diets at the concentrations of 0, 10, 100, 1000 and 5000 ppm starting at 1 week before the per os exposure to *L. salmonae* infected gill material. Lethal sampling occurred at week 6, 7, 8 and 9 post exposure (PE). The acceptable dose of dietary monensin was determined to be 1000 ppm because it was the lowest concentration that produced a significant and constant reduction in xenoma formation. To determine the effect of treatment duration on *L. salmonae* development, rainbow trout were fed monensin at 1000 ppm beginning at 0, 1, 2 and 3 weeks before exposure and at 1, 2 and 3 weeks after exposure, continuing until week 9 PE. Treatment initiated at the time of exposure or 1 week before, generated the greatest reduction in xenoma production when compared to the untreated RBT. In conclusion, monensin could be included as part of an integrated fish farm management strategy; monensin use could significantly reduce xenoma

production and thus reduce production of infective spores for transmission. Also, monensin accelerates xenoma rupture and clearance and this could further reduce respiratory stress.

5.2 INTRODUCTION

Microsporidians are common obligate, intracellular parasites in the aquatic environment and many of them cause disease in fish and invertebrates (Canning and Lom 1986). These poorly understood pathogens have a potentially complex life cycle with an unique way of infecting host cells, which culminates in the production of spores with a coiled polar tube. Microsporidian research has recently become of increasing importance in veterinary and human medicine, however there is still little knowledge regarding basic mechanisms of pathogenesis.

One important microsporidian parasite is *Loma salmonae*. This is a xenoma-forming microsporidian which infects net-pen reared chinook salmon, *Oncorhynchus tshawtscha* and coho salmon *O. kisutch*, in coastal British Columbia (Morrison and Sprague 1983, Kent et al. 1989, Speare et al. 1989, Ramsay et al. 2001). The infection occurs in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill (Speare et al. 1998a). *Loma salmonae* infections can cause a large number of mortalities in net pens of chinook salmon, especially when fish are closest to harvest and have the largest

economic value. The usual pathogenesis of the disease at 15°C, with an experimental per os exposure, is the development of xenomas approximately 5 weeks post exposure (PE), which rupture between week 8 and 10 PE, causing the potentially lethal branchitis (Kent et al. 1995). Although several causes of disease in pen-reared salmonids have been identified, generally there are few drugs available for their treatment (Kent 1998). Currently, there are no pharmacological agents licensed for use in controlling *L. salmonae* or other microsporidian parasites affecting farmed fish (Speare et al. 1998a). Therefore, most fish diseases, including those caused by microsporidians are controlled by changing the husbandry practices that avoid infections or improve the overall health status of the fish (Kent 1998).

One of the major management goals for *L. salmonae* involves blocking sporogony, as this would have the combined effect of limiting transmission to naive cohorts, as well as reducing the intensity of branchitis in those already infected (Speare et al. 2000). Various compounds with the shared mechanism of inhibiting DNA/RNA synthesis have shown some effectiveness (Speare et al. 1999). However to date, the most effective drug has been monensin, a sodium ionophore. This drug is selectively active on post-Golgi endosomes and Golgi subcompartments (Dinter and Berger 1998). In microsporidians, the Golgi apparatus is considered primitive but is a key cellular feature required for the development of the coiled polar tube through which the infective sporoplasm travels (Vávra and Larsson 1999). Theoretically by inhibiting sporogony, transmission would be blocked because the development of the pathogen is

arrested and could not produce viable spores.

A pilot study completed in 1999 indicated that monensin was more effective than all previously tested drugs by reducing the average number of xenomas per gill arch by 94% over the control fish (Speare et al. 2000). This study used top-coated feed at a concentration of 10 000 ppm of monensin, with a feeding rate of 2% of fish biomass per day. Therefore, on average, the dose rate of monensin consumed per day was 200 mg of monensin kg^{-1} of fish. The objective of this study was to further investigate the therapeutic effect of dietary treatment with monensin on rainbow trout (RBT) infected with *L. salmonae*. The specific aims were: 1) to determine an acceptable dose of monensin and 2) to determine the shortest duration of treatment with the most desired effect on reducing clinical disease. The definition of an acceptable dose of monensin was defined as fish continually eating the medicated feed, normal weight gain during the treatment and a minimum of a 50% reduction of xenomas during the peak formation period.

5.3 MATERIALS AND METHODS

5.3.1 Fish, Husbandry and Maintenance

Juvenile RBT (15-30 g) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no history of *L. salmonae*. Fish were housed in a flow through system of 70 L (habitable volume) circular fibreglass tanks. The tanks were supplied from a well water source and

maintained at 15°C (± 0.3) as measured with a Fluke7 armoured thermocouple (Fluke Corp., Everett, WA, USA). The tanks had constant aeration and the oxygen levels were monitored using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, UT, USA). The flow rate was maintained at 2.0 L min^{-1} because water turnover rate may affect the transmission of *L. salmonae* (Speare et al. 1998b). The RBT were acclimatized to these conditions for 8 days prior to initiating the experiments. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993).

5.3.2 Trial I

The purpose of this trial was to the determine an acceptable dose of monensin during a *L. salmonae* infection. Approximately 1 week before the trial began, 300 naive RBT were randomly allocated to 6 tanks (50 per tank) and were fin clipped for identification purposes. Fish in all tanks were fed once daily at 2% of the biomass, which was determined on a weekly basis. The commercial feed used was Corey 7 High-Pro 3 GR parr and smolt feed (Corey Feed Mills Ltd, Fredericton, New Brunswick, Canada). Fish in the treatment tanks received feed top-coated with gelatin containing the specified concentration of monensin. (Elanco, London, Ontario). The lowest dosage group was 10 ppm (10 mg active ingredient kg^{-1} feed) and then 100 ppm (100 mg active ingredient kg^{-1} feed), 1000 ppm (1 g active ingredient kg^{-1} feed) and finally the largest dosage group was 5000 ppm (5 g active ingredient kg^{-1} feed). Feed

given to the positive (non-medicated and exposed) and negative (non-medicated and non-exposed) control groups was only coated with gelatin. The formulated diets were administered for one week prior to exposure to *L. salmonae* and continued until the end of the trial. If the drug acted on the very early life stages, this ensured that the RBT would have a level of monensin in their body.

5.3.3 Trial II

The purpose of this experiment was to use the acceptable dose of monensin (from Trial I) and to further determine the life stage of *L. salmonae* in which the action of monensin is most effective at reducing the xenoma formation. This was accomplished by initiating the therapy with monensin at different times before and after exposure to *L. salmonae*. Approximately one week before the trial began, 405 RBT were randomly allocated to 9 tanks (45 per tank). The treatment groups were defined by initiation of the medicated diets as being either so many weeks before or after the exposure. The treatment groups began at 0, 1, 2, and 3 weeks before exposure and 1, 2, and 3 weeks post exposure (PE). The most beneficial dose of monensin, as determined from Trial I was fed to all tanks, except the non-medicated control group, as described above. Fish were maintained on the medicated diet until the end of the trial at week 9 PE. The ninth tank contained non-medicated and non-exposed fish to serve as a negative control group.

5.3.4 Exposure Method

In preparation for exposure, feed was withheld from the experimental animals for 1 day and they were pooled into 2 tanks. Pooling fish during the crucial exposure period minimized differential exposure levels amongst fish. During exposure to *L. salmonae*, fish were fed infectious macerated gill material, obtained from laboratory maintained *L. salmonae* infections as previously described (Kent et al. 1995; Speare et al. 1998a; Ramsay et al. 2001). Gills of the donor fish were examined to ensure the presence and intensity of xenomas on the gill arches. In all cases, RBT were anaesthetized and euthanized using benzocaine at a concentration of 60 mg L^{-1} and 100 mg L^{-1} in water, respectively. After three hours, fish were identified based on fin clipping and returned to their original tanks for the remainder of the experiments and the feeding schedule resumed. In both trials, exposure was considered time 0.

5.3.5 Data Collection and Analyses

In both trials, at weekly time intervals beginning with week 6 PE until week 9 PE, 10 fish were randomly collected from each tank for lethal sampling. From each fish, the first left gill arch was removed for a whole mount and the number of xenomas were counted using a stereoscope.

For both trials, statistical comparisons were performed using the mean xenoma count per gill arch (XCPGA) as the outcome variable. All comparisons were made against the positive control group (untreated and exposed) within a

specified time interval. Furthermore due to the natural pathogenesis of disease, time PE was an interaction term so the comparisons were only done within each time interval. Also because of the disease pathogenesis, the data was skewed to the right so a natural logarithm transformation was required, creating a robust analysis with valid residual diagnostics. One-way analysis of variance (ANOVA) was used to compare the treatment means and this was followed by Dunnett's post-hoc multiple comparisons test to identify where the differences occurred. The Dunnett's comparisons expresses the difference between each treatment mean and the control mean as a two-sided confidence interval. For all comparisons in trial I, the family error rate (α) was 0.05 and individual error rate was 0.0148. For all comparisons in trial II, the family error rate (α) was 0.05 and individual error rate was 0.0092. The statistical analyses were performed using MINITAB™ (MINITAB™ Inc., State College, PA, USA, version 12) and SAS (SAS Institute Inc., Cary, NC, USA. version 6.11).

From trial I, weekly tanks weights were used to calculate growth parameters. Specific growth rate (SGR) and the feed conversion ratio (FCR) was calculated for each tank during each elapsed time period (1 week PE). The formulas used for SGR and FCR were :

$$\text{SGR (\%)} = \frac{\ln(\text{final weight}) - \ln(\text{initial weight}) \times 100}{\text{period of time elapsed}}$$

$$\text{FCR} = \frac{\text{weight of feed consumed (as fed)}}{\text{wet weight of fish gained}}$$

The growth parameters were separately analyzed using one-way ANOVA to compare the treatment groups, followed by Tukey's Honestly Significant Difference test to specifically identify any differences. These comparisons were performed using SAS 6.11 and analyzed with a family error rate of 0.05.

5.4 RESULTS

5.4.1 Trial I

During the trial, all of the food offered to the fish was eaten except in the 5000 ppm treatment tank. The majority of feed in this tank was not eaten by the fish and therefore these animals presumably did not receive their target dose (Table 5.1.). Although, the 5000 ppm group had the largest FCR, an ANOVA revealed no significant difference in the FCR measurements amongst the monensin treatment groups ($p = 0.173$). However, an ANOVA revealed a significant difference in the SGR measurements amongst the treatment groups ($p = 0.0037$). Multiple comparison testing identified the 5000 ppm group as having a significantly lower SGR when separately compared to all of the other groups. The other dose groups (not including the 5000 ppm) were not different from each other according to the multiple comparison analysis. Excluding the 5000 ppm group, fish showed predicted growth parameters when fed daily at a rate of 2% biomass, indicating the fish received the target dose of monensin.

The proportion of fish exposed to *L. salmonae* which then developed the disease was 100%. None of the fish in the unexposed control group developed

signs of *L. salmonae* and subsequently were not included in the statistical analysis of xenoma counts. From trial I, the acceptable level of monensin was 1000 ppm and this dose level was subsequently used in trial II.

At week 6 post exposure (PE), rainbow trout treated with monensin at 1000 ppm and 5000 ppm had significantly ($p = 0.0148$) lower mean XCPGA values compared with the control group from that time interval (Figure 5.1a.). As expected, the intensity of xenomas was highest at week 6 PE for all groups of fish and then gradually decreased with time. During week 7 and 8 PE, the two highest doses of treatment continued the reducing trend with significantly lower mean XCPGA when compared to the control group (Figure 5.2a.). Moreover, at week 8 PE, all of the monensin treated fish showed significantly lower XCPGA than the control group. There was no change in mean xenoma count in fish from the control group between week 7 PE and week 8 PE. Meanwhile, the monensin treated tanks continued to decrease in mean XCPGA from the previous sample period. Finally at week 9 PE, there were no significant differences in the mean XCPGA amongst all the groups of rainbow trout. Standard residual diagnostics were completed and found to validate the model.

In addition to statistical analysis, treatment associated (mean) XCPGA-reduction was expressed in proportion to the (mean) XCPGA of the control fish. This simple mathematical calculation gives another measure of the main effect and was calculated as:

$$\% \text{ reduction} = 1 - [(\bar{x}XCPGA_{treatment}) / (\bar{x}XCPGA_{control})] \times 100.$$

At week 6 PE when the peak xenoma formation occurred, the mean XCPGA was reduced by 68% and 93% for the 1000 and 5000 ppm treatment groups, respectively (Figure 5.1a.). This same trend for percent reduction was seen across week 7 and 8 PE sample periods for these two treatment groups. At week 8 PE when all of the treatment groups showed significantly lower mean XCPGA, the smallest percent reduction, compared to control fish was seen in the 10 ppm group (the lowest dose) and it was 60% and this value increased linearly to 87% in the largest dosage group.

5.4.2 Trial II

During the trial, all of the food offered to the fish was eaten and therefore fish ingested (on average) the target dose of 1000 ppm. As with the first trial, the proportion of fish exposed to *L. salmonae* that developed the disease was 100%. None of the fish in the unexposed control group developed signs of *L. salmonae* and subsequently were not included in the statistical analysis.

At week 6 PE, the lowest mean XCPGA values were seen in the tanks when the treatment was initiated at exposure and one week earlier (Figure 5.1b). Fish that were treated with monensin beginning at one week before exposure to *L. salmonae* and at the time of exposure had significantly fewer xenomas than the fish that were not treated with monensin ($p = 0.0092$). In each of these significantly lower tanks, the percent reduction in mean XCPGA was 73% compared to the non-treated fish. Furthermore, fish that were treated beginning

at three and two weeks before exposure showed 57% fewer xenomas than the control fish. Finally, as the treatment initiation moved further ahead after the exposure date, the xenoma production increased.

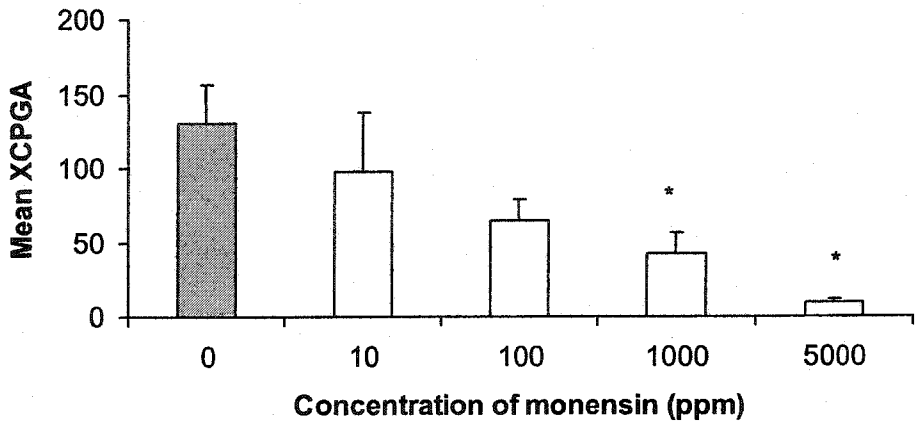
The above stated trends continued at week 7 and 8 PE, where fish treated at time of exposure or before were producing less xenomas than fish not treated with monensin (Figure 5.2b). At week 7 PE, fish treated at exposure and three weeks before exposure showed a 75% and 72% reduction in xenoma formation, respectively, although not statistically significant. Whereas, fish treated one week and two weeks before exposure only showed a 19% and 25% reduction, respectively. These same trends were carried into the week 8 PE sample period, with the largest percent reductions favouring prophylactic treatment of monensin on microsporidial gill disease. Fish treated a week before exposure had significantly fewer xenomas than the control fish at week 9 PE. This represented a 67% decrease in the mean number of xenomas found on a gill arch.

Table 5.1. The mean (± SEM) feed conversion ratio (FCR) and the mean specific growth rate (SGR) for each monensin dose group during the course of a *Loma salmonae* infection in rainbow trout. Within each growth measurement column, FCR and SGR, different letters (a, b) indicate a significant difference ($p<0.05$).

Monesin Dose, ppm	FCR ± SEM	SGR ± SEM
5000	3.30 ± 0.582^{a}	3.83 ± 0.853^{a}
1000	1.26 ± 0.227^{a}	9.81 ± 1.57^{b}
100	1.12 ± 0.0864^{a}	10.9 ± 0.972^{b}
10	1.15 ± 0.148^{a}	12.1 ± 1.93^{b}
0	2.61 ± 1.53^{a}	9.79 ± 1.38^{b}
Control*	1.38 ± 0.149^{a}	9.90 ± 1.26^{b}

* fish in this tank were not exposed to *L. salmonae*

(a)



(b)

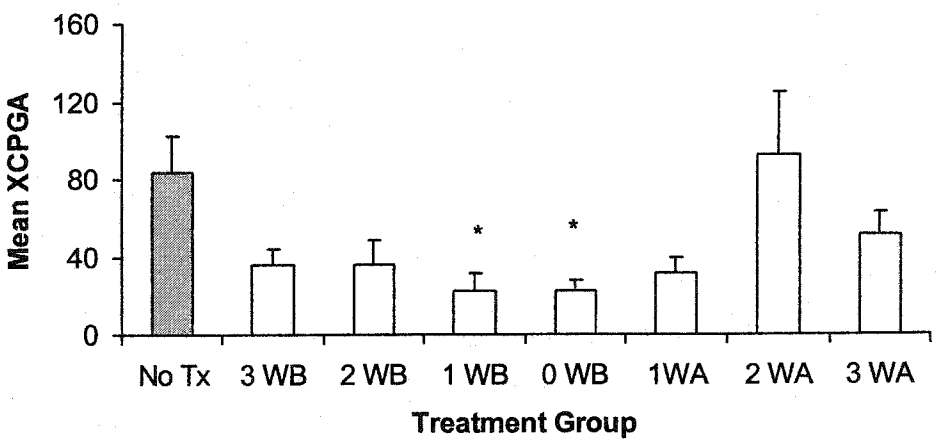
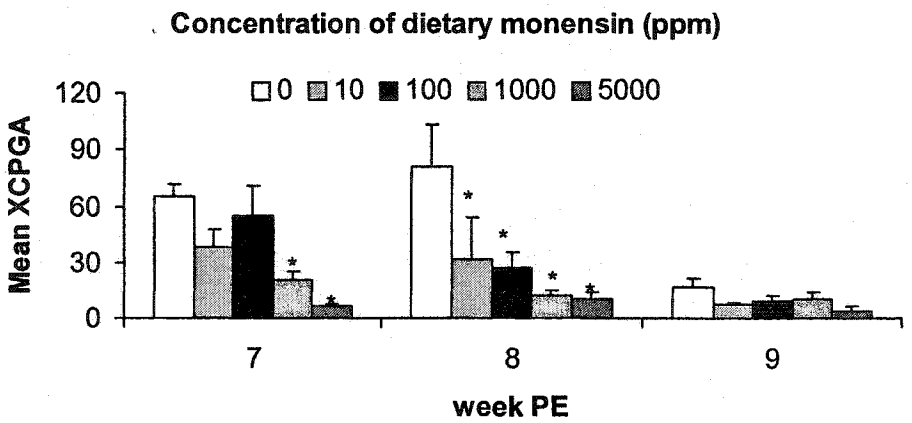


Figure 5.1.

Figure 5.1. The effect of dietary monensin treatment on microsporidial gill disease in rainbow trout measured at week 6 after *Loma salmonae* exposure. Graph (a) shows the dose response of monensin on the mean xenoma count per gill arch (XCPGA) (± SEM). Graph (b) shows the response of prophylactic treatment beginning at a specific week (W) before (B) or after (A) exposure to the pathogen on the mean XCPGA (±SEM). Statistical comparisons were done against the control group [0 ppm and No Tx (no treatment), respectively] within each time interval and significance is marked by '*'.

(a)



(b)

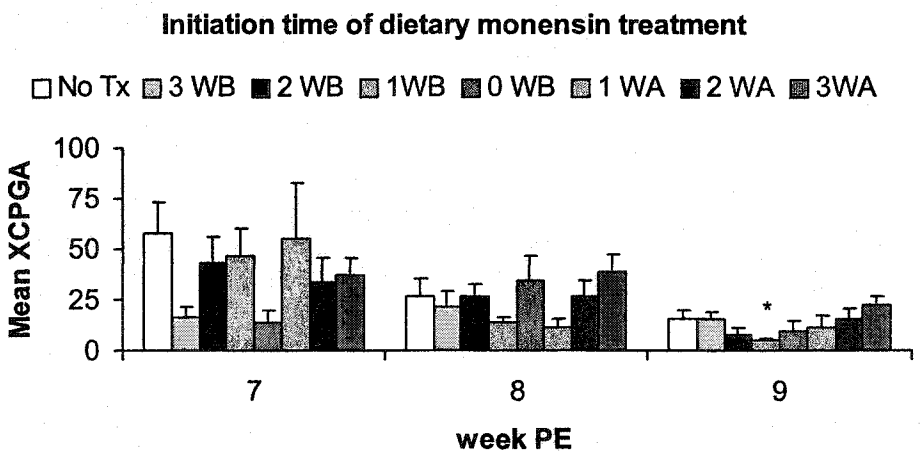


Figure 5.2.

Figure 5.2. The effect of dietary monensin treatment on microsporidial gill disease in rainbow trout measured at week 7, 8 and 9 post exposure (PE) to *Loma salmonae*. Graph (a) shows the dose response of monensin on the mean xenoma count per gill arch (XCPGA) (± SEM). Graph (b) shows the response of prophylactic treatment beginning at a specific week (W) before (B) or after (A) exposure to the pathogen on the mean XCPGA. Statistical comparisons were done against the control group [0 ppm and No Tx (no treatment), respectively] within each time interval and significance is marked by an '*'.

5.5 DISCUSSION

Dietary monensin treatment was effective at reducing the xenoma intensity on gill arches of RBT infected with *L. salmonae*. The acceptable dose was 1000 ppm (1 g active ingredient kg $^{-1}$ feed) because this was the lowest concentration that produced significantly fewer xenomas at each sample period from week 6 PE to week 8 PE. The 1000 ppm treatment group satisfied the criteria set for the acceptable dose, in that fish consumed all the feed offered, the weight gain was normal and xenomas reduction at peak formation was well over 50%. The 5000 ppm dose resulted in a significant reduction in XCPGA at each sample period but the fish in this tank did not consume all the feed offered so their target dose was unknown, but it was presumably between 1000 and 5000 ppm. Consequently, the SGR for the 5000 ppm dose was significantly reduced compared to all other tanks and these tanks were not significantly different from each other. Unfortunately there was no method to accurately quantify the amount of uneaten food in 5000 ppm tank. However, since the goal of the trial was to identify the lowest acceptable dose and there was a lower dose (1000 ppm) that did meet the pre-set requirements, the 5000 ppm tank was not interpreted further.

It has been suggested (Speare et al. 1999) that a drug which delays the formation of xenomas may be beneficial in regions where *L. salmonae* is endemic. The rationale behind this is that many outbreaks occur in the early autumn, just before the water cools to temperatures likely to retard parasite development. The delay in parasite development as a result of therapy could

delay the xenoma rupture and the potentially fatal respiratory effects to the late autumn, when the colder water temperatures would favour the increased oxygen demand from diseased fish (Speare et al. 1999). However, the possibility of blocking xenoma formation from occurring is a better strategic plan for developing a disease management program against *L. salmonae*. Results from Trial I are in agreement with the results from the previous study (Speare et al. 2000) in that monensin appears to reduce the intensity of xenomas on the gills of rainbow trout exposed to *L. salmonae*. Other drugs (fumagillin and albendazole) that were determined to be efficacious for treating *L. salmonae* infections reduced the mean XCPGA by 56% and 67% respectively at week 7 PE (Speare et al. 1999, Speare et al. 2000). In comparison, the monensin treated fish (1000 ppm) reduced the xenoma formation by 69% and 85% at weeks 7 and 8 PE, respectively. Furthermore, the above reductions were accomplished with fumagillin delivered at 750 ppm and albendazole delivered at 75 ppm (fed at 2% body weight) for three weeks, starting at 5 days PE. The most efficacious monensin dose tested was higher at 1000 ppm than the therapeutic doses for fumagillin and albendazole. However, monensin produced the more desired effect of reducing xenoma intensities compared with the observed delay in the onset of xenomas as seen with fumagillin and albendazole. The combination of these results and those from a previous study (Speare et al. 2000), provides strong evidence that monensin is valuable in the development of a strategic control program for *L. salmonae*.

The acceptable monensin dose at 1000 ppm was carried over to the second trial, which emphasized the impact of prophylactic treatment on xenoma formation. Prophylactic treatment with monensin reduced the xenoma formation on the gills of rainbow trout exposed to *L. salmonae*. When treatment was started at the time of exposure or one week before, significantly lower mean number of xenomas were produced than if the fish were not treated. It has been shown that the early stages of *L. salmonae* were localized in rainbow trout with the parasite detected in the gut at 24 hours PE and in the heart at 2 days PE (Sánchez et al. 2001b). A further study showed the initial detection of *L. salmonae* in the gills was at 2 weeks PE with visible xenomas forming by week 4 PE (Sánchez, et al. 2001a). The combination of these results with this study suggests that the window of opportunity for executing a treatment program with monensin is approximately two or three weeks. The effect of monensin on reducing xenoma production was minimal once the parasite became localized in the gill tissue (at approximately 2 weeks PE). The ideal treatment time would focus on the earlier stages when *L. salmonae* is in the heart and gut and this would be approximately 1 week before exposure and up to and including 1 week PE.

Although the significant reduction in xenomas did not carry over to the longer durations of treatment (2 and 3 weeks before exposure), their respective mean counts were in line with the others and they did produce a considerable percent reduction (57%). Moreover, as the duration of treatment was reduced (e.g. initiated after pathogen exposure), the xenoma formation increased and became

similar to the untreated group. The tank of RBT which were given monensin beginning at 1 week before exposure, showed significantly fewer xenomas at week 9 PE than the untreated fish. This may provide evidence that monensin aids in accelerating the rate of xenoma rupture and clearance in the gill tissue.

Understanding the natural disease pathogenesis of a parasite, such as *L. salmonae*, is crucial to effectively controlling its dissemination. Historically, microsporidians have been very elusive for developing effective control strategies and therapies. This has mainly been attributed to their intracellular localization in host cells and due to the resistant infectious spore (Canning and Lom 1986). Microsporidia are now recognized as emerging agents of infectious diseases in mammals, insects and fish (Weiss 2001). For mammalian microsporidia research, continual efforts to characterize the biology and epidemiology of microsporidiosis have been identified as important for assessing risk factors and for identifying therapeutic and preventive strategies (Didier et al. 2000, Weiss 2001).

Currently, the best form of control of *L. salmonae* in aquaculture is through avoidance with good farm management practices, which is unrealistic in net-pen culture environments. However, it has been suggested (Ramsay et al. 2001) that with the apparent longevity of *L. salmonae* within fish recovering from disease and the existence of wild fish and environmental reservoirs (Kent et al. 1989), alternative strategies may be needed. These alternative strategies may require the use of chemotherapeutics to reduce the pathogen burdens within cultured fish. Therefore using monensin as part of an integrated farm

management strategy may have a two fold effect on microsporidial gill disease. The first being that it significantly reduced the xenoma intensity during the course of infection and thus the number of infective spores produced for disease transmission and secondly, monensin may accelerate the dissolution of the xenomas and thus possibly reduced the lethal respiratory stress. Results from these two trials are in agreement with each other and with the results from previous treatment investigations using dietary monensin therapy. Although this does provide a basis for developing an integrated control program, it would be useful to further examine the effect of monensin on growth parameters, the possibility to reduce the treatment duration and to complete drug residue studies. Additionally, a clinical trial should be performed to evaluate the efficacy of monensin in a field situation.

5.6 ACKNOWLEDGMENTS

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CHAPTER 6

INFLUENCE OF FEEDING AND SIZE ON SUSCEPTIBILITY TO MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

6 INFLUENCE OF FEEDING AND SIZE ON SUSCEPTIBILITY TO MICROSPORIDIAL GILL DISEASE CAUSED BY *LOMA SALMONAE* IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

6.1 ABSTRACT

Two trials were designed to quantify the effect of feeding rate and fish size on the cohabitation transmission of *Loma salmonae*, the causative agent for microsporidial gill disease (MGD), in Pacific salmonids (*Oncorhynchus* spp.). To evaluate the effect of feeding rate on disease onset, groups of 45 rainbow trout (RBT) were fed daily at 1% (low), 2% (medium) or 4% (high) of the fish biomass in the tank. There were three tanks at each feeding level: two tanks were exposed to the pathogen and one was a control tank. For the second objective, 300 rainbow trout were separated into seven tanks so that the weight classifications were small (17-23 g), medium (32-38 g) and large (57-63 g). Each size class was done in duplicate with one control tank containing medium-sized fish. Separately for each trial, on day 0 post exposure (PE) five highly infected RBT were added to each tank (not including the control tanks) to begin the cohabitation exposure period. Beginning on day 21 PE and continuing bi-weekly until day 70 PE and 77 PE for the feeding rate and fish size trial, respectively, each fish was evaluated for visible branchial xenomas to determine disease onset time. Using survival analysis, the survival curves for the low, medium and high feeding rates were not significantly different from each other. The median

number of days until disease onset was 45, 42 and 42 PE, respectively. In the fish size trial, there were significant differences amongst the small, medium and large weight classes. The median numbers of days to the development of branchial xenomas was 31, 38 and 42 for small, medium and large size fish, respectively. On any given day, a medium or large sized fish had a hazard ratio for developing branchial xenomas of 0.66 and 0.63 compared to a small fish, respectively. In addition to host species and host strain differences, fish size is now considered a host risk factor for the development of MGD.

6.2 INTRODUCTION

The emerging pathogen, *Loma salmonae* causes microsporidial gill disease (MGD) in farmed Pacific salmonids, *Oncorhynchus* spp., resulting in respiratory distress, secondary infections and often mortality (Kent et al. 1989, Speare et al. 1989, Weiss 2001; Ramsay et al. 2003). The infection occurs in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill (Speare et al. 1998a). Several experimental infection models have been developed for *L. salmonae* with the most popular being per os and cohabitation (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998a, Ramsay et al. 2001). Compared with the per os model, the cohabitation model exposes naive fish to a chronic low dose of spores over a longer time period and is considered to be a more representative of the actual challenges occurring in the sea cage (Becker et al. 2003).

Exposure to infectious disease agents can be a continual process during the life span of any organism including farmed fish (Lapatra 1998). However, exposure to a pathogen does not necessarily result in infection or the development of clinical disease. This depends on the interaction of several factors under the comprehensive categories of host, pathogen and environmental factors. The focus of this study was the interaction of two host factors and MGD caused by *L. salmonae*. Specifically, the factors that have been suggested to affect fish host susceptibility to disease include nutritional status and size (Lapatra et al. 1990, Hedrick 1998). The inter-connection of fish nutrition and growth impacts on the management practices and profitability of any fish rearing system. The addition of a stressor, such as an infectious disease agent, will impact on the health status (e.g. additional energy demands) of the fish, which in turn could have a detrimental effect on other production elements including a reduction in water quality.

The overall objective of this study was to determine the relative importance of two related nutritional factors in the disease paradigm responsible for MGD in rainbow trout (*Oncorhynchus mykiss*). The specific aims were to determine the influence of feeding rate and fish size on onset time of branchial xenomas in naive rainbow trout exposed to *L. salmonae* using an experimental cohabitation exposure model.

6.3 MATERIALS AND METHODS

6.3.1 Trial I

The purpose of this trial was to evaluate the effect of feeding rate on *L. salmonae* transmission in a naive rainbow trout (RBT) population. Juvenile RBT (approximately 15 g each) were obtained from a certified specific pathogen-free commercial hatchery on Prince Edward Island with no history of *L. salmonae*. Approximately one week before the initiation of the trial, 45 RBT were randomly distributed to each of nine circular fibreglass tanks with a flow through system of 70 L of habitable volume. The tanks were held at 15°C ± 0.3 supplied from a well water source with constant aeration and the oxygen levels were monitored using a Campbell Scientific Data Logger (model CR-7, Campbell Scientific Data Logging Inc., Logan, Utah). The flow rate was maintained at 2.0 L min^{-1} because water turnover rate affects the transmission of *L. salmonae* (Becker et al. 2003). All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993). Following the transfer to the experimental tanks, feeding at three rates was initiated and continued until the final sampling on day 70 post exposure (PE). The feeding rates were set at 1% (low), 2% (medium) and 4% (high) of the total fish biomass in the tank (referred to as tank weight (tw)) with three tanks in each feeding group. Tank weights were determined twice weekly and adjustments were made to the daily ration. The daily ration for the tanks fed at 4% was divided into two equal portions to facilitate total consumption. The cohabitation exposure model as described by

Becker et al. (2003) and previously described in section 2.3.4 was used. To initiate the trial on day 0 PE, five RBT with severe MGD caused by *L. salmonae* were added to each of two tanks in each of the three feeding rates. One tank from each of the feeding groups did not receive infectious fish to serve as a control.

6.3.2 Trial II

The purpose of this trial was to determine if fish size at the time of exposure to infectious *L. salmonae* cohorts affects the onset time of branchial xenomas in a naive rainbow trout population. Approximately 300 juvenile RBT originating from the same stock were obtained from a hatchery in Nova Scotia, Canada with no reported history of *L. salmonae.* Seven weeks prior to day 0 PE, fish were distributed to each of seven tanks (approximately 40 fish per tank) based on body weight and through manipulation of feeding regimes, the weight ranges for the trial were 17-23 g (small), 32-38 g (medium) and 57-63 g (large). Each size group was completed in duplicate with one control tank containing medium sized naive RBT which did not receive infectious fish. A similar tank design as described above was used with water temperature held at 15°C ± 0.3 and flow rate equal to 2 L min^{-1}. To maintain the distinct size groupings, fish were fed to satiation twice weekly. Once again a cohabitation exposure model was used by placing five infectious RBT in each tank on day 0 PE (the control tank did not receive infectious fish) as described in section 2.3.4.

6.3.3 Screening Methods and Data Collection

Beginning at day 21 PE and continuing on a biweekly basis until day 70 PE for trial I (feeding rate) and day 77 PE for trial II (fish size), the first left gill arch for all fish in the treatment and control tanks was non-lethally examined under a stereoscope to determine if the RBT showed branchial xenomas. In the case that *L. salmonae* was detected, the adipose fin was clipped and the fish was declared disease positive for the remainder of the trial. Furthermore, xenoma intensity was measured by an index value based on the number of visible xenomas on the first left gill arch (Table 6.1). Fish were anaesthetized using benzocaine at a concentration of 60 mg L^{-1} in water for all screening procedures.

6.3.4 Data Analysis

Survival analysis is a relatively new and useful tool used in the analysis of disease transmission models in fish research (Bebak-Williams et al. 2002, Becker et al. 2002, Becker et al. 2003, Ramsay et al. 2003). These statistical procedures are used for the analysis of data in which the outcome variable of interest is time until an event occurs with the event defined as any designated experience (e.g. death, sero-conversion or xenoma appearance). Survival analysis was used to analyze the data collected from both trials to evaluate the effect of feeding rate and fish size on the development of branchial xenomas. For this study, the survival time was calculated as the number of days from the start of cohabitation exposure until the first appearance of visible branchial

xenomas. Using these data, survival probabilities were calculated and plotted for each temperature group in the form of a survivor function S(t) as defined as the probability that an individual will survive longer than some specified time, t (Kleinbaum 1996). For each trial, the Kaplan-Meier estimator (as described in Cleves et al. 2002) was used to calculate the survivor function for each feeding rate and fish size group. The Wilcoxon's Test was used to compare the survivor curves to determine if statistically significant differences existed between study groups.

A proportional hazards (PH) model was also fit to the data for each trial. This model calculates the hazard function for each treatment group, which is the probability of a fish "failing" (i.e. having its first visible xenoma) at a specific time given that it did not fail before. In other words, the hazard function represents the instantaneous failure rate over time (Bebak-Williams et al. 2002; Becker et al. 2003). The PH model compares the hazard of one study group to the hazard of another in the form of a ratio (Kleinbaum 1996). The PH model assumes that the hazard ratio is constant over time and this assumption is evaluated by looking for parallel lines on a plot of the log of the cumulative hazard against log time (Kleinbaum 1996; Cleves et al. 2002). Cox-Snell residuals and deviance residuals were calculated to assess the overall fit of the model and to identify outliers, respectively. Tank effect was evaluated using shared frailty models, which add random effects to the regression model to account for the fact that fish were held in different tanks. All survival analyses were completed using the

software package STATA™ (Stata™ Corporation, version 7.0 College Station, Texas) using -st- (survival time) procedures as outlined by Cleves et al. (2002).

Separately for each trial, the mean xenoma intensity score was calculated for each treatment group on all sample days. The means were graphed using lowess smoothed graphs with a bandwidth equal to 0.40. To determine if significant differences existed amongst the intensity score curves, generalized estimating equations (GEE) were used for the linear regression model to account for the repeated measures data (i.e. mean xenoma scores within a tank were recorded on many sample days). For the fish size trial, day PE was centered and then entered in the model as both linear and quadratic terms.

Additionally, the growth data generated from trial I were used to calculate instantaneous growth rate (G) (formula below) for each tank, which was used for linear regression analysis to confirm that the fish were actually consuming the feed offered (Busacker et al. 1990; Hopkins 1992). A plot of the residuals against the predicted values was examined for heteroscedasticity. All analyses were carried out using STATA (version 7.0).

$$G = \frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{period of time elapsed (days)}}$$

6.4 RESULTS

During the feeding rate and fish size trials, 98.9 and 99.6 % of the naive RBT developed branchial xenomas and were consequently identified as disease positive for *L. salmonae*, respectively. None of the non-exposed control fish developed branchial xenomas in either trial and therefore were not included in the data analysis. On day 21 PE, the original highly infectious fish were removed from the treatment tanks and their gills were examined for branchial xenomas. Very few xenomas were detected (xenoma index score equal to 1 or 2; Table 6.1) on these fish for both trials, suggesting that the vast majority of xenomas ruptured and released spores during the three week cohabitation period.

6.4.1 Trial I

The Kaplan-Meier survival curves were plotted for each feeding group with combined replicate tanks (Fig. 6.1). There was no significant difference amongst the survivor curves for the combined feeding groups ($p = 0.356$). The median survival time for the low, medium and high feeding rates was 45, 42 and 42 days PE, respectively. From the proportional hazards model, the hazard ratio for the medium and high feeding rates was equal to 1.08 and 1.13 compare to the low feeding rate, respectively. The 95% confidence intervals for both of these hazard ratio estimates contained the value 1, suggesting there is no evidence that the hazard of developing branchial xenomas was different amongst the feeding groups. However, the shared frailty model revealed a significant tank

effect ($p = 0.007$). Because no significant effects were reported, this clustering effect, which would tend to inflate statistical significance was of little concern. Additionally, there were no significant differences in the mean xenoma intensity index for the three different feeding rates ($p = 0.806$) (Figure 6.2).

To confirm that feed consumption and assignment of feeding rates were as designed, G for all tanks at each feeding rate, low, medium, high including the non-exposed control tanks was calculated on each sample day. Since there was a control group for each feeding rate, direct comparisons were made between exposed and non-exposed tanks. From the linear regression, all tanks performed as expected regarding G and there were no significant differences amongst the three tanks within a feeding rate ($p > 0.832$). The regression equation was:

$$\text{Predicted } G = 0.00970 + 0.0106\ (2\%\ tw) + 0.0155\ (4\%\ tw)$$

From this equation, the predicted G (ln g day^{-1}) values for the 1%, 2% and 4% tanks were 0.00970, 0.0203 and 0.0252, respectively.

6.4.2 Trial II

There were no significant differences (all $p > 0.15$) between the replicate small, medium and large tanks, therefore data were combined for the remainder of the analysis. The Kaplan-Meier survival curves were plotted for each size group (Fig. 6.3) with the median survival time for the small, medium and large

groups was 31, 38 and 42 days PE, respectively. Using the Wilcoxon's test, there was at least one significant difference amongst the three survival curves ($p = 0.024$). From the proportional hazards model, on any given day, a medium or large sized fish had a hazard ratio for developing branchial xenomas of 0.66 or 0.63 compared with a small fish, respectively. The assumption for the proportional hazards model (that the hazard ratio was constant over time) was examined and there was no evidence of a violation ($p > 0.44$). An examination of the Cox-Snell residuals did not reveal any outliers and the proportional hazards model developed was considered appropriate. Tank effect was not significant as determined by the shared frailty model ($p = 0.499$).

From the experimental design for trial II, all tanks were fed a maintenance diet (satiation twice weekly) to maintain separation in the weight classes during the entire trial. The small, medium and large sized fish challenged with *L. salmonae* maintained their separated weight classes with minimal growth during the 11 week study period (Table 6.2). However, the non-exposed medium sized fish experienced the largest growth rate, which could indicate a growth suppression in exposed fish.

The mean xenoma intensity score for each tank was calculated on all sample days (Fig. 6.4). From the GEE model, the mean xenoma intensity observed in small sized fish was significantly greater than the other two size groups over the entire duration of the study ($p < 0.001$). The predicted values calculated from the regression model for the mean xenoma scores for each size group are plotted in Figure 6.5.

Table 6.1. Xenoma intensity index for measurement of the number of visible branchial xenomas during a *Loma salmonae* infection in rainbow trout.

Xenoma Intensity Score	Description
0	No visible xenomas
1	<1 visible xenoma per gill filament
2	1 visible xenoma per gill filament
3	More than 1 visible xenoma per filament

Table 6.2. From trial II, the average weight (g) per fish and instantaneous growth rate (G) for each tank during *Loma salmonae* infection in rainbow trout.

Weight Class	Initial Weight	Final Weight	G†
Small	21.55	24.98	0.00192
Small	20.97	26.58	0.00204
Medium	36.15	43.85	0.00251
Medium	38.13	43.41	0.00168
Medium*	34.08	42.85	0.00297
Large	66.94	79.71	0.00227
Large	57.37	68.29	0.00226

* Fish were not exposed to *L. salmonae* to serve as the control group

† Units for G are ln g day^{-1}

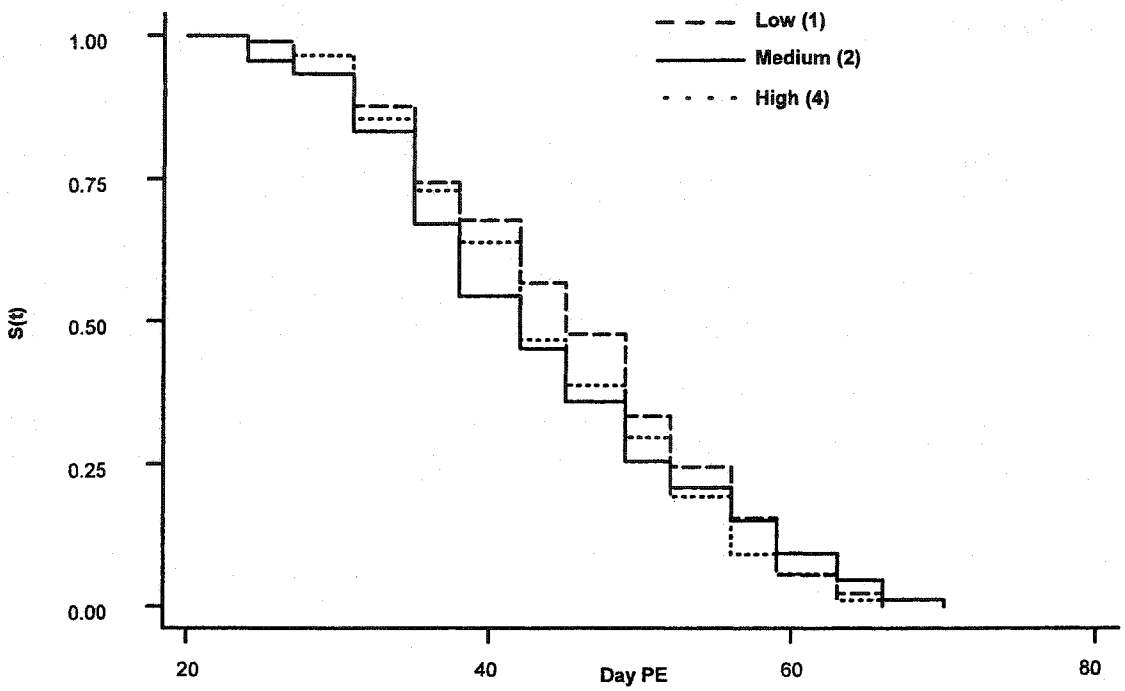

Figure 6.1. Kaplan-Meier survivor curves for the three feeding rates (% tank weight) post exposure (PE) to a chronic low dose of *Loma salmonae* spores in a rainbow trout population.

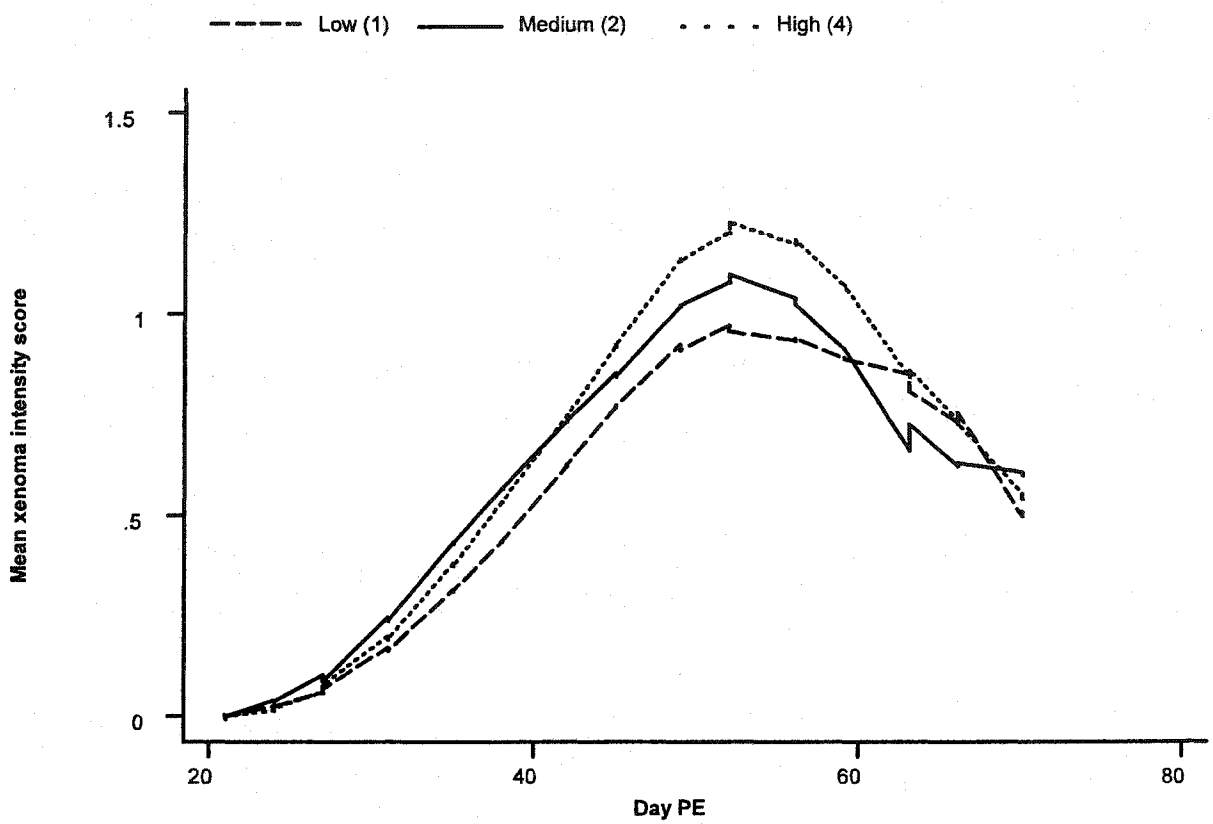

Figure 6.2. The mean xenoma intensity score for each feeding group (% tank weight) during a *Loma salmonae* infection with a cohabitation exposure model beginning on day 21 post exposure (PE). The curves are lowess smoothed with a bandwidth equal to 0.40.

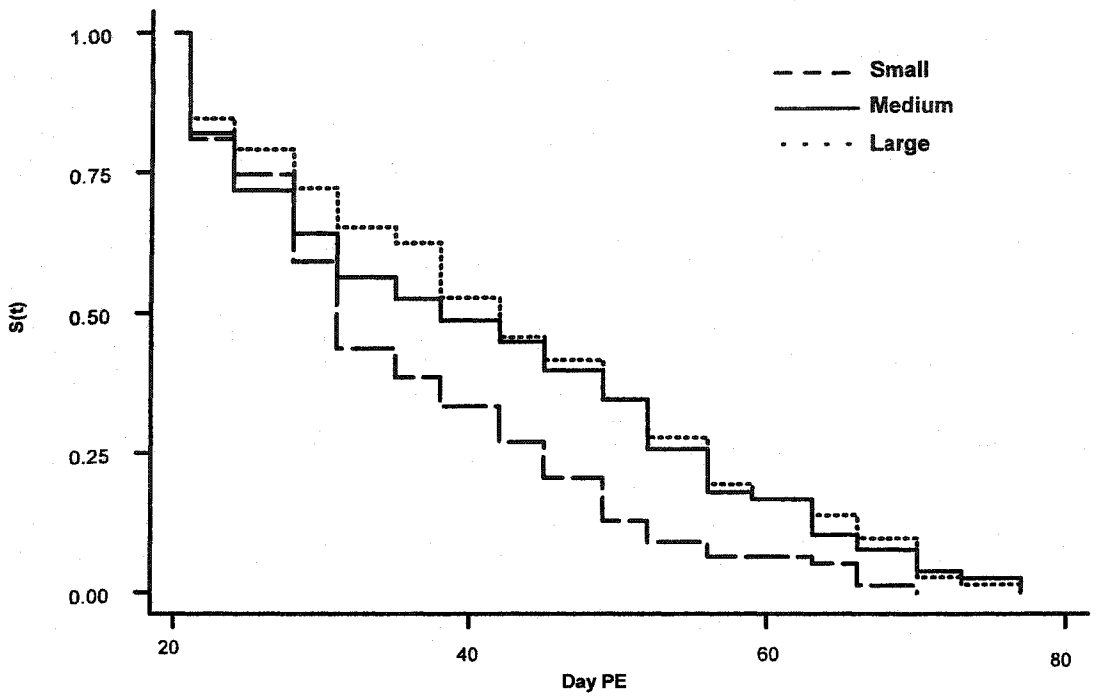

Figure 6.3. Kaplan-Meier survivor curves for the three fish size groups post exposure (PE) to a chronic low dose of *Loma salmonae* spores in a rainbow trout population. The weight ranges for the small, medium and large groups were 17 - 23, 32 - 38 and 57 - 63 g, respectively.

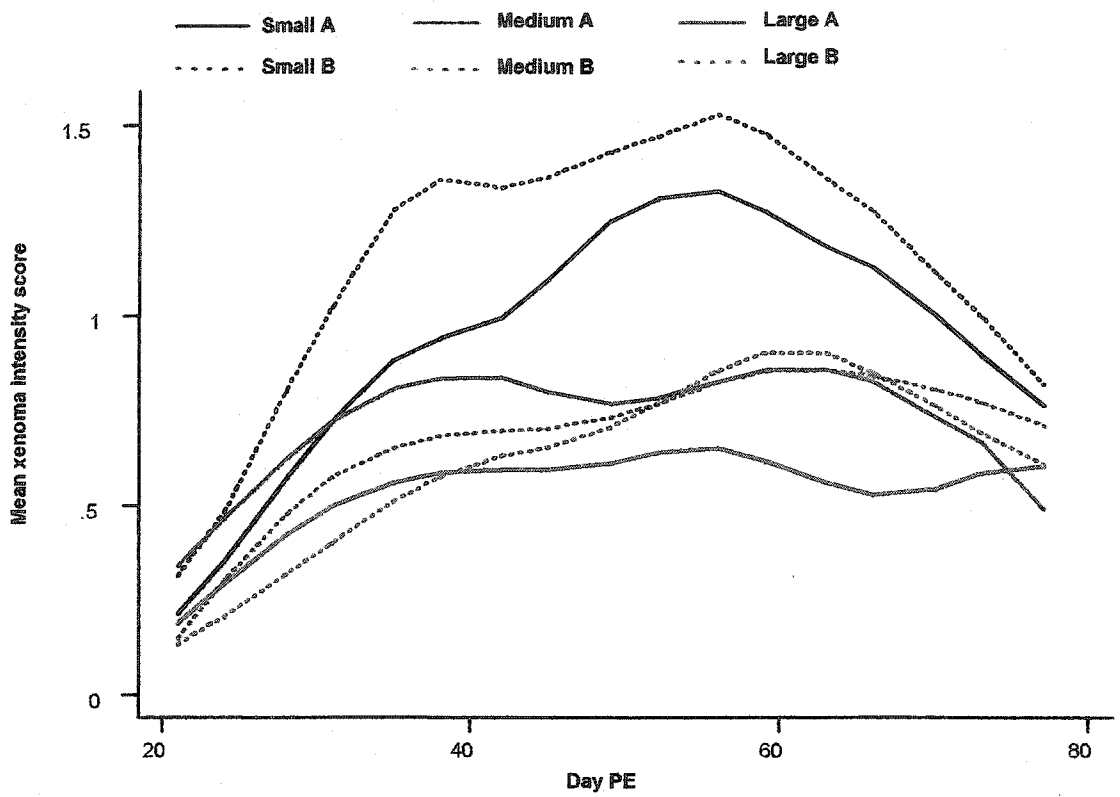

Figure 6.4. The mean xenoma intensity for each tank separated by size grouping during a *Loma salmonae* infection with a cohabitation exposure model beginning on day 21 post exposure (PE). The curves are lowess smoothed with a bandwidth equal to 0.40.

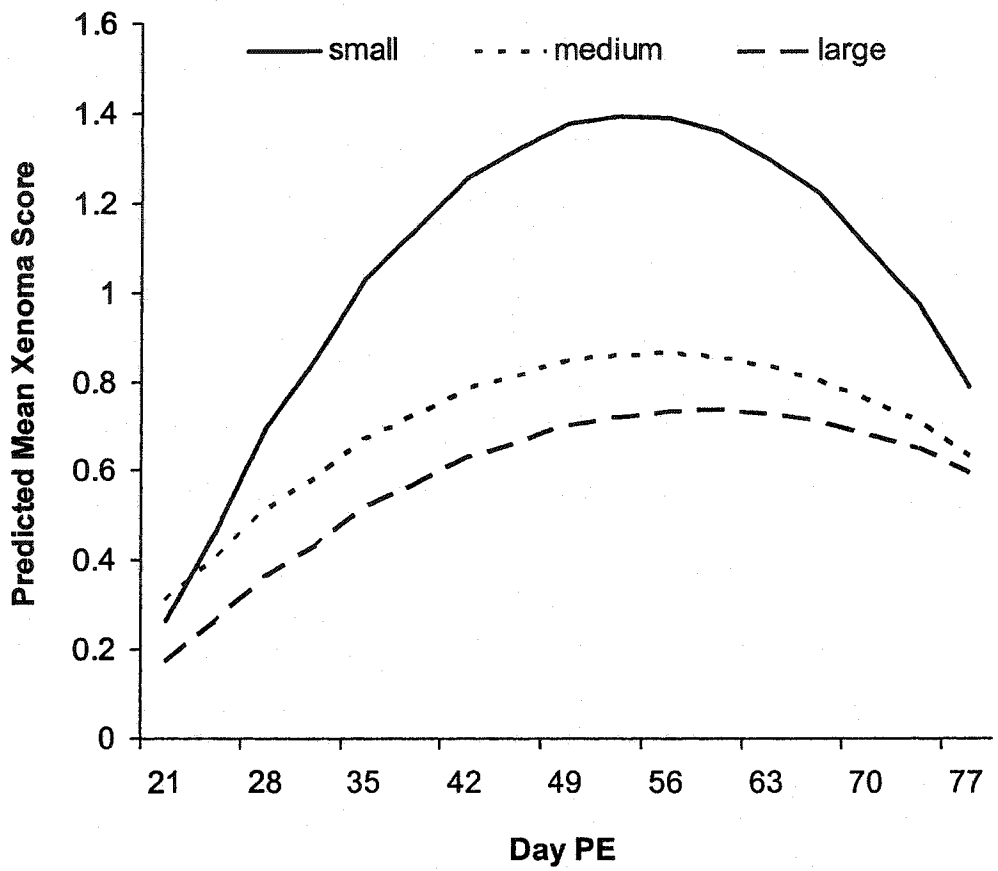

Figure 6.5. The predicted values generated from a GEE model for the mean xenoma intensity score for each fish size group during a *Loma salmonae* infection in rainbow trout, *Oncorhynchus mykiss* with a cohabitation exposure model beginning on day 21 post exposure (PE).

6.5 DISCUSSION

Under the described feeding groups, there was no evidence that host feeding rate affected the onset time or the numbers of branchial xenomas during a *L. salmonae* infection in RBT with a cohabitation exposure model. Although tank effect did not alter the significance of the feeding groups, it was found to be a significant factor. This clustering of observations may result in an erroneous declaration of non-significant results as being significant and would be minimized by increasing the number of tanks in study.

Direct comparisons between the control and infectious tanks showed that the G was not significantly different within each of three feeding groups. Therefore, fish in the control tanks grew at the same rate as their infected counterparts, which may indicate that the development of branchial xenomas did not create a negative energy balance within the host. In contrast, previous work evaluating the effect of feeding rates on disease onset, Speare et al. (1998b) studied the effect of disease onset on growth by calculating specific growth rate (SGR). Rainbow trout fed to satiation once daily were exposed using a high dose oral intubation to *L. salmonae* spores and compared these fish to naive cohorts placed in the tank two days after intubation. It was reported that a reduction in SGR corresponded to weeks 4 to 10 PE, whereby the highest proportion of fish showed xenomas (Speare et al. 1998b). However, the growth rates of the infected fish recovered and exhibited a compensatory growth to match those of the control fish by the conclusion of the trial at week 12 PE. In the current study,

none of the three feeding groups revealed growth suppression in relation with the onset of disease suggesting that the lower parasite burdens observed in fish exposed using the cohabitation method may not influence growth rate.

Although not significant in this study, nutrition status has been identified as an important host defense tool for the salmon hemoflagellate, *Cryptobia salmositica* (Li and Woo 1991, Beamish et al. 1996). Li and Woo (1991) reported that anorexia is a clinical sign for cryptobiosis and onset of disease was correlated with increased parasitemia. As the numbers of parasites declined, food consumption increased to levels similar to control fish (Li and Woo 1991). Parasite replication was related to plasma protein levels and thus the reduction in these levels via an anorexic state may have aided in reducing the cryptobiosis (Beamish et al. 1996).

Fish size was a significant factor affecting the onset time of branchial xenomas caused by *L. salmonae* in a rainbow trout population. During the course of the study period, fish ranging from 17 to 23 g had significantly faster rate of development of branchial xenomas compared to the other two size groups. Additionally, on average, the smallest weight group had markedly higher number of xenomas observed on the gill filaments. It was reported by Speare et al. (1998a) that susceptibility to *L. salmonae* did not differ between 35 g and 114 g fish intubated with high dose of infective spores. The current study is consistent with these prior results and goes further to identify a size-related susceptibility in fish ranging between 17 and 23 g, which were not investigated

by Speare et al. (1998a). Size-related susceptibility has been reported in other significant pathogens from many different fish species from both farmed and wild populations (Lapatra et al. 1990, Bowser et al. 1997, Aranguren et al. 2002, Perelberg et al. 2003). Notably, it has been demonstrated that small (1.7 g and 0.2 g, respectively) RBT and kokanee (*O. nerka*) are more susceptible to infectious hematopoietic necrosis virus compared with larger sizes (7.4 g and 7.2 g, respectively) (Lapatra et al. 1990, Lapatra 1998). Similarly, research on a devastating unidentified viral pathogen of carp in Israel has demonstrated significantly higher mortality rates in smaller carp (2.5 and 6 g) compared with adult carp (230 g) (Perelberg et al. 2003).

The practice of fish farming has often provided the conditions for manifestation of disease in populations of fish and the subsequent identification of pathogens that have evolved with their hosts in natural environments (Lapatra 1998). For MGD caused by *L. salmonae*, the identified host risk factors are (1) host species with chinook salmon (*O. tshawytscha*) being more susceptible compared to coho salmon (*O. kisutch*) and RBT (Ramsay et al. 2002); (2) host strain differences seen in chinook salmon from British Columbia (Shaw et al. 2000), and with the conclusions of this study (3) fish size at the time of exposure to *L. salmonae* is included as a host risk factor for MGD. An understanding of the epidemiology and pathogenesis of infectious agents through investigating disease events may result in improved management strategies. Regarding MGD

caused by *L. salmonae*, the challenge remains for fish health scientists to continue identifying host, environment and pathogen risk factors and their respective interactions for a more complete understanding of this disease pathogenesis.

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CHAPTER 7

ULTRAVIOLET LIGHT CONTROL OF HORIZONTAL TRANSMISSION OF *LOMA SALMONAE*

Adapted from:

BECKER JA, SPEARE DJ. Ultraviolet light control of horizontal transmission of *Loma salmonae*. J Fish Dis 2004; 27: 177-180.

7 ULTRAVIOLET LIGHT CONTROL OF HORIZONTAL TRANSMISSION OF *LOMA SALMONAE*

7.1 ABSTRACT

Microsporidial gill disease (MGD) caused by the xenoma-forming protozoan parasite, *Loma salmonae* is gaining in importance in salmon aquaculture because of the high infection prevalence and severe mortalities. The objectives of study were firstly to determine if physical fish-to-fish contact is necessary for the horizontal transmission of *L. salmonae* and secondly to determine if UV light sterilization of water can render *L. salmonae* spores non-infective. Three pairs of tanks each consisting of an upper and lower tank with the effluent of the upper tank serving as the exclusive water source of the lower tank. In two of the paired systems, juvenile rainbow trout (RBT) with severe MGD infections were placed in the upper tanks with naive fish in the lower tanks. The naive RBT were monitored beginning at day 21 post exposure (PE) for visible xenomas on the gill arches. The third pair of tanks contained only naive RBT to serve as the control. Subsequently, a similar experiment was prepared except the effluent from the upper tanks containing the infectious fish traveled through an UV light sterilizer before entering the lower tanks containing naive RBT. Horizontal transmission of *L. salmonae* without physical contact was readily demonstrated. For the two lower tanks the median survival time, whereby 50% of the fish were showing branchial xenomas was at day 45 PE. For the second objective, naive RBT were

exposed for 80 days to UV treated *L. salmonae*-infectious water without any fish developing xenomas. This is the first report to document a non-contact transmission model for *L. salmonae* and the effects of UV to control the transmission of MGD.

7.2 INTRODUCTION

Loma salmonae is a xenoma-forming microsporial gill pathogen responsible for Microsporidial Gill Disease (MGD) of both wild and cultured Pacific salmon. Although common, this obligate intracellular protozoan is not usually considered a severe pathogen in wild salmon, however it is an important cause of disease in net pen reared chinook salmon (*Oncorhynchus tshawytscha*) on the west coast of Canada (Kent 2000). The pathogenesis of MGD has recently been elucidated and the key events include oral uptake of an infectious spore, intragastric spore germination, a brief residence period in the gut lamina propria, a two-week merogony-like phase in the cardiac subendothelium, followed by a macrophage-mediated transport of the parasite to the gill where final development (further merogony and sporogony) occurs within gill pillar cells with the results of sporogony leading to the formation of a distended spore-filled xenoma (Sánchez et al. 2000, Sánchez et al. 2001, Rodríguez-Tovar et al. 2002). The key to success for all microsporidia including those infecting fish, lies in the diversity and flexibility of their transmission strategies and life cycles (Dunn and Smith 2001). Several transmission exposure models have been reported for

L. salmonae including the commonly used per os and cohabitation methods (Shaw et al. 1998, Ramsay et al. 2001). However, a non-contact transmission model to assess the requirements for physical fish-to-fish contact remains elusive.

Biocontrol of the incoming water to a hatchery or the water in a recirculation system is essential in minimizing the spread of diseases amongst fish due to infectious agents such as *L. salmonae*. The most widely used and effective method for water disinfection is short wave ultraviolet (UV) irradiation, with a wavelength at or near 253.7 nm because there are no harmful by-products and UV does not interfere with nitrifying bacteria within the biofilter and culture system (Gratzek et al. 1983, Losordo et al. 1999). The control and prevention of MGD is hampered by the ability of the resistant spore to maintain infectivity in the environment for extended periods of time even with extreme environmental changes (Canning and Lom 1986, Becker et al. 2002).

The objectives of this study were two-fold in that the first aim was to determine if physical fish-to-fish contact is necessary for horizontal transmission of *L. salmonae* and secondly to provide proof of principle evidence that a UV light sterilizer of water can render *L. salmonae* spores functionally non-infective.

7.3 MATERIALS AND METHODS

To accomplish these objectives three pairs of 70 L flow through circular tanks were established with each pair consisting of an upper and lower tank with

effluent of the upper tank functioning as the exclusive water source of the coupled lower tank. The upper tanks received fresh water at 2.0 L min^{-1} and all tanks were maintained at 15°C because both flow rate and temperature alter the transmission of this parasite (Becker et al. 2003). Juvenile rainbow trout, *Oncorhynchus mykiss* (RBT) (17 ± 3 g) were obtained from a certified specific pathogen-free hatchery on Prince Edward Island with no prior history of *L. salmonae*. For the first trial, two of the upper tanks each contained 12 highly infected per os exposed RBT, while approximately 42 naive fish were placed into each of the corresponding lower tanks. To obtain the highly infected fish required for the two trials, naive rainbow trout were fed infectious mascerated gill material with laboratory maintained *L. salmonae* infections as described by Kent et al. 1995 and Ramsay et al. 2001. Additionally similar numbers of naive RBT were placed into the upper and lower tanks of the third pair to serve as a control. A similar system design was used for the second trial, except the effluent from the upper tanks containing the *L. salmonae* infected RBT traveled through an UV sterilizer (Model # 02318, 18 W UV Sterilizer, Emperor Aquatics Inc, Pottstown, Pennsylvania USA) before entering the lower tanks. The pair of control tanks with only naive RBT did not have a UV sterilizer. Using an 18 W UV sterilizer with a flow rate at 2.0 L min^{-1}, the predicted UV dose was 283 500 μ W-s cm^{-2}.

Beginning on day 21 post exposure (PE) and continuing twice weekly until all *L. salmonae* exposed fish were infected in the first trial and until day 80 PE for the second trial, the first left gill arch of each fish was non-lethally examined

under a stereoscope for the presence of visible branchial xenomas to determine disease onset time. In the case that *L. salmonae* was detected, the adipose fin was clipped and the fish was declared disease positive for the remainder of the trial. The non-exposed control fish were examined in an identical fashion. The first day that branchial xenomas were observed was recorded for each fish in order to calculate the median xenoma onset time using survival analysis techniques (as described by Cleves et al. 2002). For all procedures, fish were anaesthetized using benzocaine at a concentration of 60 mg L^{-1} in water.

7.4 RESULTS AND DISCUSSION

We found that *Loma salmonae* was horizontally transmitted to naive RBT without physical contact with the infectious fish. The median survival time, as defined as the day whereby fifty percent of the naive fish within the lower tank exhibited branchial xenomas, occurred at day 45 PE. Additionally, the Kaplan-Meier survival curves revealed no significant differences between the two replicate tanks and therefore indicated there was no tank effect ($p = 0.70$) (Figure 7.1). All of the non-exposed control fish in both the upper and lower tanks remained negative for xenomas, thus indicating the absence of *L. salmonae* spores in the water supplied to the facility. The infectious fish in the two upper tanks cleared their branchial infections during the course of trial, thus demonstrating that they were presumably releasing spores in to the water. A similar study using the cohabitation exposure model for *L. salmonae* with

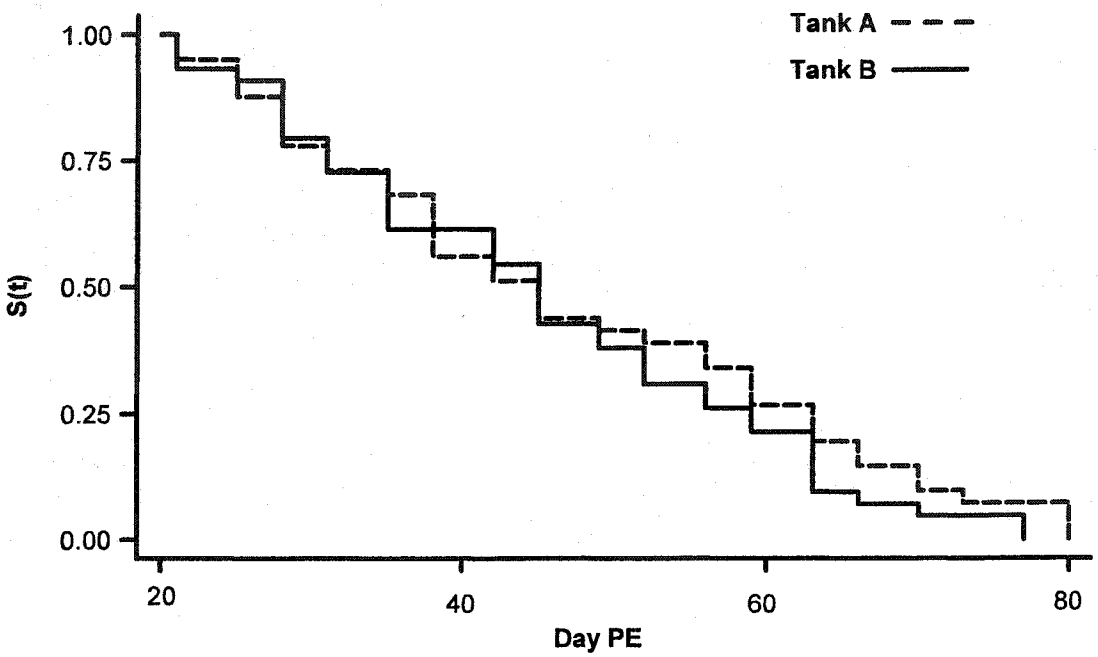

Figure 7.1. Kaplan-Meier survivor curves for naive rainbow trout exposed to *Loma salmonae* through water only contact beginning on day 0 post exposure (PE). Replicate Tanks A and B do not differ in their survivorship (p = 0.70).

physical contact and similar experimental conditions (e.g. fish were maintained at 15°C and 2 L min^{-1}) reported day 39 PE as the median survival time (Becker et al. 2003). Thus, there is evidence that the non-contact model for MGD caused by *L. salmonae* results in a slightly delayed xenoma development rate when compared with the contact model. This temporal difference between infection models may be an important consideration for selecting research models through which to more fully probe the disease transmission risk amongst net-pen chinook salmon and wild fish sharing the nearby waters. Similarly to the current study, horizontal transmission of the salmon pathogen *Piscirickettsia salmonis* without physical contact between fish was possible, but onset was delayed, as in our study, when compared to a physical contact transmission model (Almendras et al. 1997) Additionally, a non-contact horizontal transmission model has been developed for infectious salmon anemia virus (ISAV), a severe pathogen affecting Atlantic salmon aquaculture industry in Canada (Jones and Groman 2001). The feasibility of transmitting ISAV without physical contact, Jones and Groman (2001) indicated that to properly assess the risk of disease transmission, it is important to establish the duration of infectivity of virus-contaminated water. Similarly this is required for non-contact horizontal transmission model for *L. salmonae* and will be a subject of future study. Due to the nature of microsporidians and their resistant spores and the combination of little or no effective chemotherapies available, the transmission potential and disease impact of MGD is considered to be high.

The second aim of the study was to demonstrate the possibility of using UV light to control the non-contact horizontal transmission of *L. salmonae*. Approximately 44 naive RBT in each of two tanks were exposed for 80 consecutive days to UV treated *L. salmonae*-infectious water without any fish developing branchial xenomas. Additionally, none of the non-exposed control fish developed xenomas and the infectious fish in the upper tanks recovered from MGD with the rupture of branchial xenomas and presumed release of spores. This is the first report to document the effects of UV to control the transmission of *L. salmonae*. The use of UV irradiation is common in aquaculture and is a key component in maintaining high water quality for rearing fish and for the treatment of waste water from hatcheries and abattoirs (Øye & Rimstad 2001). The UV dose used in this study was purposely high at an estimated 283 500 μ W-s cm^{-2}, however the manufacturer recommends 336 000 μ W-s cm^{-2} and 200 000 μ W-s cm^{-2} for the protozoan *Ichthyophthirius* spp. tomite and tomont stages respectively. A higher UV dose is generally recommended for larger organisms such as the protozoans, however, Gratzek et al. (1983) controlled the spread of *I. multifiliis* using 91 900 μ W-s cm^{-2} in a recirculation system stocked with channel catfish, *Ictalurus punctatus*. The next research step in examining UV control of *L. salmonae* transmission should be to increase the flow rates through the UV sterilizers to a typical rate used in commercial scale hatchery or recirculating systems and determine the minimum dose of UV light required to prevent the spread of disease.

Loma salmonae was initially identified as a freshwater pathogen and considered a hatchery associated disease issue, although infections persisted after chinook salmon were transferred to sea water (Kent et al. 1995). The first report of *L. salmonae* in British Columbia was in coho salmon (*O. kisutch*) which occurred in a hatchery on Vancouver Island with the reported incidence equal to 28% (Magor 1986). Similarly, at a river water supplied trout hatchery in Buford, Georgia, USA, infections caused by *Loma* cf. *salmonae* negatively impacted the potential production of RBT, brown (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) with as many as 27% of moribund trout sampled were affected by this parasite (Bader et al. 1998). Both Magor (1986) and Bader et al. (1998) recognized the importance of identifying and controlling disease caused by *Loma* spp. to improve salmonid productivity in the hatchery. Certainly for hatcheries, the water-borne transmission of severe pathogens such as *L. salmonae* is very important and the practicality of using UV is of utmost importance. The most practical application of these results will be in those situations, such as hatcheries supplied with surface water, where UV treatment of water can mitigate against the influx of viable spores from water in which feral *L. salmonae* infected fish are present. Furthermore, the results are applicable to situations of serial re-use or recirculation of water within a facility which would otherwise promote prolonged and repeated contact of fish with infective spores.

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CHAPTER 8

EFFECT OF THE NUMBER OF INFECTED FISH AND ACUTE EXPOSURE PERIOD ON THE HORIZONTAL TRANSMISSION OF *LOMA SALMONAE* (MICROSPORIDIA) IN RAINBOW TROUT

BECKER JA, SPEARE DJ, DOHOO IR. Effect of the number of infected fish and acute exposure period on the horizontal transmission of *Loma salmonae* (Microsporidia) in rainbow trout, *Oncorhynchus mykiss* Submitted to Aquaculture June 2004.

8 EFFECT OF THE NUMBER OF INFECTED FISH AND ACUTE EXPOSURE PERIOD ON THE HORIZONTAL TRANSMISSION OF *LOMA SALMONAE* (MICROSPORIDIA) IN RAINBOW TROUT

8.1 ABSTRACT

Loma salmonae is an intracellular obligate parasite of farmed chinook salmon (*Oncorhynchus tshawtscha*) and is the causative agent for microsporidial gill disease. The infection occurs mainly in the secondary lamellae of the gills, with the formation of large white spore-laden xenomas within the endothelial and pillar cells. The overall objective of the study was to investigate the minimum exposure time required for disease transmission between naive rainbow trout (*O. mykiss*) (RBT) and *L. salmonae*-infected fish using the cohabitation challenge model. In trial I, low (1), medium (5) or high (10) numbers of infected RBT were added to tanks containing 45 naive fish for a cohabitation period of 21 days. Trial II limited the cohabitation period between five *L. salmonae*-infected fish in a tank of 45 naive RBT to 1 hr, 12 hr, 24 hr or 96 hr. For both trials, each treatment level was completed in duplicate and an additional tank of 45 naive RBT served as the control and did not receive infected fish. Beginning on day 21 post exposure (PE) and continuing bi-weekly until day 83 PE and 105 PE for each trial, respectively, fish were evaluated for branchial xenomas to determine disease onset time. Using survival analysis, there were no significant differences amongst the survivor curves for low, medium or high numbers of infected fish

added to a tank of naive fish (trial I). Additionally, there were no differences observed in the mean xenoma intensity amongst the groups. For trial II, there were significant differences observed amongst the survivor curves for the fish exposed to infected RBT for 1hr, 12 hr, 24 hr and 96 hr. However, 12 hr exposure group violated the proportional hazards (PH) model assumption because the hazard ratio for this group (relative to the 1 hr baseline group) was not constant over time. A time-dependent covariate was added to the PH model for this group and the increased hazard observed in the 12 hr group was negated by day 68 PE. Additionally, the mean xenoma intensities for the 12 hr group were significantly higher over the duration of the study. This study demonstrated that *L. salmonae* transmission was possible with a minimum of one hour exposure time between cohabiting infected and naive RBT. Adding five *L. salmonae*-infected fish (at week 7 PE) to a group of naive RBT for a period of one hour resulted in over 94% disease incidence in cohabiting fish.

8.2 INTRODUCTION

The emerging pathogen, *Loma salmonae* causes microsporidial gill disease (MGD) in farmed Pacific salmonids, *Oncorhynchus* spp., resulting in respiratory distress, secondary infections and often mortality (Kent et al. 1989, Speare et al. 1989, Weiss 2001, Ramsay et al. 2003). The infection occurs in the gills and to a lesser extent in other vascularized tissues with a final development stage of a spore-laden xenoma within the endothelial and pillar cells of the gill (Speare et

al. 1998). The usual pathogenesis of MGD, with a cohabitation challenge model, is the development of branchial xenomas approximately 3 to 8 weeks post exposure (PE), with xenoma dissolution occurring 1 to 5 weeks after development (Ramsay et al. 2001, Becker et al. 2003, Ramsay et al. 2003, Becker et al. 2004). However, this time line is influenced by water temperature and challenge model (Ramsay et al. 2001, Becker et al. 2003). An examination of the health management problems for the principal fish species (salmon, rainbow trout and channel catfish) present within North American aquaculture revealed infectious diseases as a major obstacle to future growth (Georgiadis et al. 2001). Regarding Pacific salmon culture, microsporidiosis caused by *L. salmonae* was identified as one of the most problematic infectious diseases. Due to the nature of microsporidians, their resistant spore and the combination of little or no effective chemotherapies available, the transmission potential and disease impact of MGD is considered to be high (Becker and Speare 2004).

Over the last decade, many experimental challenge models (per os, cohabitation, intraperitoneal) have been developed for *L. salmonae* with the most recent being a non-contact horizontal transmission model using only effluent water from a tank containing *L. salmonae*-infected fish (Kent et al. 1995, Shaw et al. 1998, Speare et al. 1998, Ramsay et al. 2001, Becker and Speare 2004). The cohabitation challenge model was chosen for this study because it allowed for chronic low dose exposure to spores, which we believe more closely simulates spore transmission between pen-mates in a salmonid net-pen. Ramsay et al. (2001) showed that *L. salmonae* transmission was possible with

cohabitation periods as brief as 96 hours (hr) between infected fish (n = 37) and naive fish (n = 25). Additionally, Speare et al. (1998) showed a high prevalence of branchial xenomas using a longer cohabiting period of 12 weeks with sixty of each naive and infected RBT.

The overall objective of this study was to investigate the minimum exposure time required between naive rainbow trout and *L. salmonae*-infected fish. The specific aims were (1) to determine the effect of adding low (1), medium (5) or high (10) numbers of infected fish into a tank containing naive RBT and (2) to determine if contact time as low as 1 hr with five infected fish was sufficient to initiate a disease cycle.

8.3 MATERIALS AND METHODS

8.3.1 Trial I

The objective of this trial was to determine the effect of low, medium and high numbers of cohabiting infected fish on the transmission of *L. salmonae* to naive tank-mates. Juvenile rainbow trout (approximately 20 g each) were obtained from a certified specific pathogen-free hatchery on Prince Edward Island with no history of *L. salmonae*. At little more than two weeks before the initiation of the trial, 45 naive RBT were randomly allocated to each of seven circular fibreglass tanks with a flow through system of 70 L of habitable volume. Tanks were held at 15 ± 0.3°C supplied from a well water source with constant aeration and the oxygen levels were monitored using a Campbell Scientific Data Logger (model

CR-7, Campbell Scientific Data Logging Inc., Logan, Utah). The flow rate was maintained at 2.0 L min^{-1} in each tank because it has been shown to influence *L. salmonae* transmission (Becker et al. 2003). Fish were fed to satiation twice weekly and all procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 1993).

For the experimental challenge, a large pool (n = 100) of per os *L. salmonae*-infected RBT were examined for branchial xenomas at week 7 PE. The first 32 fish with high numbers of branchial xenomas (score = 3, see Table 6.1) were adipose fin clipped and used in the experiment as the source of spores for the cohabitation challenge. To initiate the trial on day 0 PE, 1, 5 or 10 infected fish were added to a tank of naive RBT, beginning the cohabitation exposure period. Each treatment was completed in duplicate and the 7^{th} tank served as a control and did not receive infected fish. To minimize the possibility of tank effect, the infected RBT were removed from the tanks on day 7 and day 14 PE, pooled together in a large container (with similar water parameters and constant aeration) and were subsequently randomly re-allocated to the treatment tanks. Ultimately, the infected fish were removed on day 21 PE to end the cohabitation period.

8.3.2 Trial II

The objective of this trial was to investigate *L. salmonae* transmission during short periods of cohabitation between infected and naive RBT. Three weeks,

prior to the initiation of the trial, 45 naive RBT were transferred to each of nine tanks (as described above) maintained at 15 ± 0.3°C with a flow rate of 2.0 L min^{-1}. Separately, a large pool of per os infected RBT were examined at week 7 PE for branchial xenomas. The first 40 fish showing high numbers of xenomas (score = 3, see Table 6.1) were adipose fin clipped (for identification) and used in the trial as the source of spores for the cohabitation challenge. Five infected fish were randomly allocated to each of the eight tanks containing naive RBT. The length of the cohabitation periods were 1, 12, 24 and 96 hrs. Each treatment group was completed in duplicate with the 9^{th} tank serving as the control, which did not receive infectious fish.

8.3.3 Screening Methods and Data Collection

Beginning on day 21 PE and continuing on a biweekly basis until day 83 PE for trial I and day 105 PE for trial II, the first left gill arch for all fish in the treatment and control tanks was non-lethally examined under a stereoscope to determine if xenomas had formed. Fish with branchial xenomas had their adipose fin clipped and the fish was declared disease positive for the remainder of the trial. Furthermore, the xenoma intensity for all fish was measured by an index value based on the number of visible xenomas on the first left gill arch (see Table 6.1). Data collection was not completed on day 25 PE during trial II due to adverse weather (Hurricane Juan). Fish were anaesthetized using benzocaine at a concentration of 60 mg L^{-1} in water for all screening procedures.

8.3.4 Data Analyses

Survival analysis was used to evaluate the effect of the number of infected fish and acute exposure on the development of branchial xenomas during a *L. salmonae* infection. Survival time was calculated as the number of days from the start of the cohabitation period until the first appearance of branchial xenomas. As previously described (section 6.3.4), the Kaplan-Meier estimator was used to calculate and plot the survivor function separately for each trial. The Wilcoxon's test was used to compare the survivor curves to determine the existence of statistical differences. A Cox proportional hazards (PH) model was fit to the data for each trial as described in section 6.3.4. Similar to previously described analyses, the PH model assumptions were evaluated, shared frailty models were used to identify tank effects and Cox-Snell and deviance residuals were examined to assess the overall fit of the model and to identify outliers.

Data generated from trial II violated the PH model assumption in that the hazard ratio was not constant over time. To account for this violation, an interaction term between the treatment group (number of hours of contact time with infectious fish) and survival time (number of days until first visible xenoma) was added to the PH model. The value of this time-dependent covariate changes with time, which indicates that the hazard ratio changed over time. An example of a situation where the hazard ratio may change over time is an experiment with a group of fish receiving a new vaccine and another group receiving a placebo, which is followed by a chronic disease challenge. The mortality records reveal that vaccinated fish begin to show high levels of mortality

beginning at day 85 PE, while all of the non-vaccinated fish were dead by day 10 PE. The hazard for a vaccinated fish is very low during the first 85 days however at this time, the protection of the vaccine is minimal and the hazard for the vaccinated fish increases. A time-dependent covariate term added to the PH model accounts for the change in vaccine efficacy over time.

Fish that did not develop xenomas, as well as fish that died were considered censored observations for the survival analysis and the assumption of censoring being independent from the outcome was evaluated. The data were examined under specific hypothetical situations of complete positive or negative correlation in a sensitivity analysis to assess the independence of the censoring. For complete positive correlation, the model was re-fit with all the censored observations re-assigned to experience the outcome event (e.g. developing xenomas) instead of being censored at the point in time at which they were originally censored. For complete negative correlation, the model was re-fit with all of the censored observations experiencing the outcome event (e.g. develop xenomas) on the very last sample day (e.g. day 110 PE). All survival analyses were completed using the software package STATA™ (Stata™ Corporation, version 7.0, College Station, Texas) using survival time (-st-) procedures as outlined by Cleves et al. (2002).

Separately for each trial, the mean xenoma intensity score was calculated for each treatment group on all sample days. Generalized estimating equations (GEE) were used for the linear regression as described in section 6.3.4. All analyses were carried out using STATA (version 8). The mean xenoma score

was calculated on each sample day in each treatment groups and graphed using lowess smoothing (bandwidth = 0.40). Additionally, the fish contact time (FCT) was calculated as the predicted infection dose available to naive fish in both trials, as summarized in Table 8.1. The FCT was calculated as a combination of the number of infected fish added to each tank and the length of cohabitation period so that for trial I, the FCT was for 21 days with either 1, 5 or 10 infected fish and for trial II, the FCT was for 1, 12, 24 or 96 hrs with 5 infected fish.

8.4 RESULTS

In each trial, over 94% of the naive RBT developed branchial xenomas and were consequently identified as positive for *L. salmonae*. None of the non-exposed control fish developed xenomas in both trials and therefore were not included in the data analysis.

8.4.1 Trial I

The Kaplan-Meier survivor curves were plotted for each treatment group with the combined replicate tanks grouped as low (1), medium (5) or high (10) numbers of infected RBT (Fig. 8.1). There were no significant differences amongst the survivor curves ($p = 0.249$) and the median onset times were 63, 56 and 59 days for low, medium and high groups, respectively (Table 8.1). The proportional hazards model did not reveal any differences in the hazard ratio amongst the three treatments. Additionally, the shared frailty model did not

reveal any tank effect. An examination of the mean xenoma scores using the GEE model did not reveal any statistical significant differences in the average xenoma intensity amongst the groups, although there was a slight trend of a possible delay in the development of the peak xenoma score in the low exposure group (Fig. 8.2). Additionally, the low exposure group, with 504 hrs of FCT showed the largest median xenoma onset time, which also resulted in the lowest peak mean xenoma score (Table 8.1). The largest peak mean xenoma score was observed in naive fish exposed to ten *L. salmonae*-infected fish equaling 5040 hrs of FCT.

8.4.2 Trial II

There were no significant differences (all $p > 0.11$) between the replicate tanks within each exposure period, therefore data were combined for the remainder of the analysis. The Kaplan-Meier curves were plotted for each exposure time (Fig. 8.3) and the Wilcoxon's test revealed at least one significant difference amongst the curves ($p = 0.022$). The median survival times for the 1 hr, 12 hr, 24 hr and 96 hr exposures were calculated as 63, 56, 63 and 67 days, respectively (Table 8.1). A PH model was generated and the data were found to violate one of the model assumptions requiring that the hazard for one group to be proportional to the hazard for any other group, where the proportionality constant is independent of time. The hazard for the 12 hr exposure compared to the other exposures was not relatively constant over time (Fig 8.4). To account for the violation, a time-dependent covariate interaction term for this treatment

group was added to the model and was deemed statistical significant ($p < 0.001$). Using this interaction term, the 12 hr exposure group had an increased hazard of developing branchial xenomas until approximately day 68 PE (Fig. 8.3, Fig. 8.4). After this time, the hazard for the 12 hr treatment was no higher than the other treatment groups. An examination of shared frailty models did not reveal any tank effect ($p > 0.50$) and the residuals analysis determined the model fit the data. Although a very small percentage (5.5%) of fish died or did not develop xenomas, the influence of these censored observations was examined. To evaluate the independence between censoring and the failure event (e.g. development of xenomas), the data were examined under conditions of complete positive and complete negative correlation. The models generated from both the positive and negative censoring were not different from the original PH model, which indicates that the censoring was independent of the outcome event. Finally, the mean xenoma intensity score for each exposure time was calculated for each sample day (Fig. 8.5). From the GEE model, the mean xenoma intensity observed in the fish exposed for 12 hrs to *L. salmonae*-infected fish, for a total of 60 hrs of FCT, was significantly higher than the other three exposure periods over the duration of the entire study with a peak mean score equal to 1.14 ($p < 0.048$). Additionally, naive fish exposed to *L. salmonae*-infected fish for 1 hr, representing 5 hrs of FCT showed the lowest peak mean xenoma score of 0.535 (Table 8.1).

Table 8.1. Separately for each trial at each treatment level (Tx), the infected fish contact time (FCT), median onset time (days) until the appearance of the first xenoma and the peak mean ($\bar{x}$) xenoma score (Xn).

Tx	FCT*	Median Onset	Peak $\bar{x}$ Xn
1 infected fish	504	63	1.31
5 infected fish	2520	56	1.34
10 infected fish	5040	59	1.78
1 hour	5	63	.535
12 hours	60	56	1.14
24 hours	120	63	.583
96 hours	480	67	.750

*FCT = number of infected fish X number of hours of contact

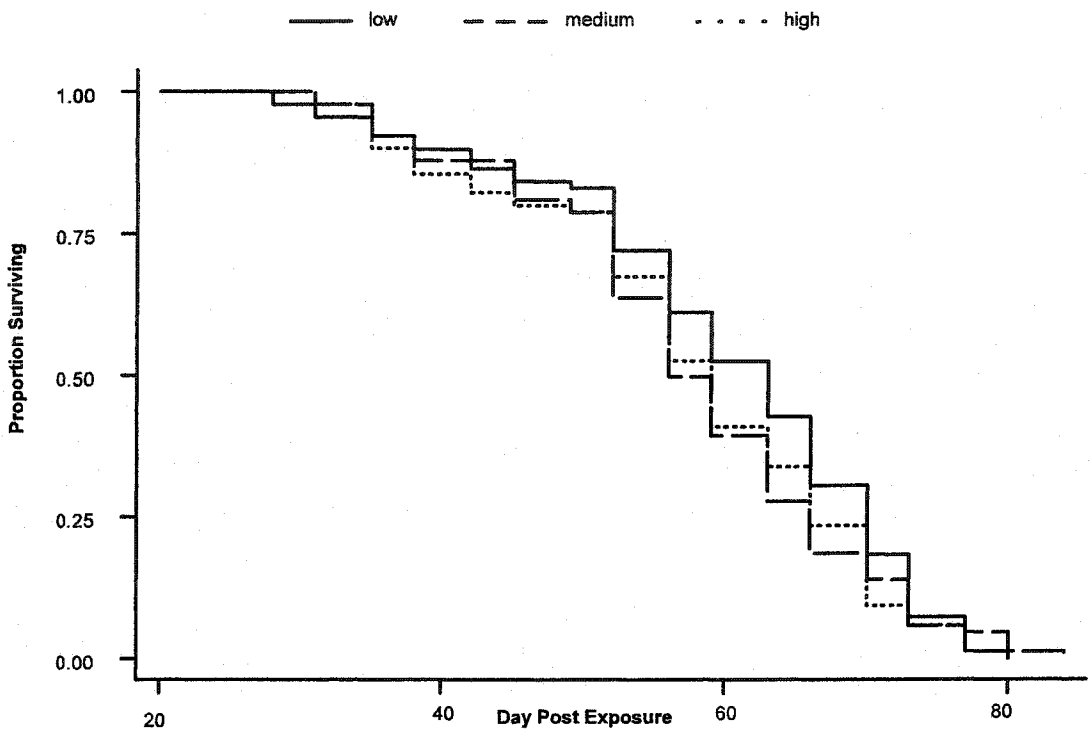

Figure 8.1. Kaplan-Meier survivor curves for rainbow trout exposed to low (1), medium (5) and high (10) numbers of *L. salmonae*-infected fish.

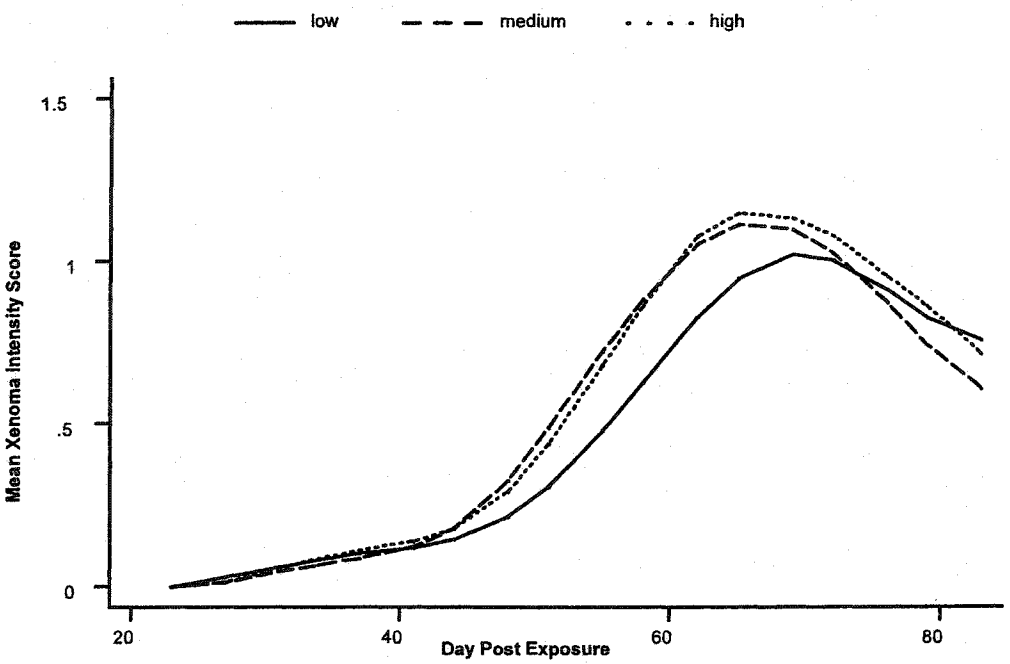

Figure 8.2. Mean xenoma scores (lowess smoothed) for rainbow trout exposed to low (1), medium (5) and high (10) numbers of *L. salmonae*-infected fish.

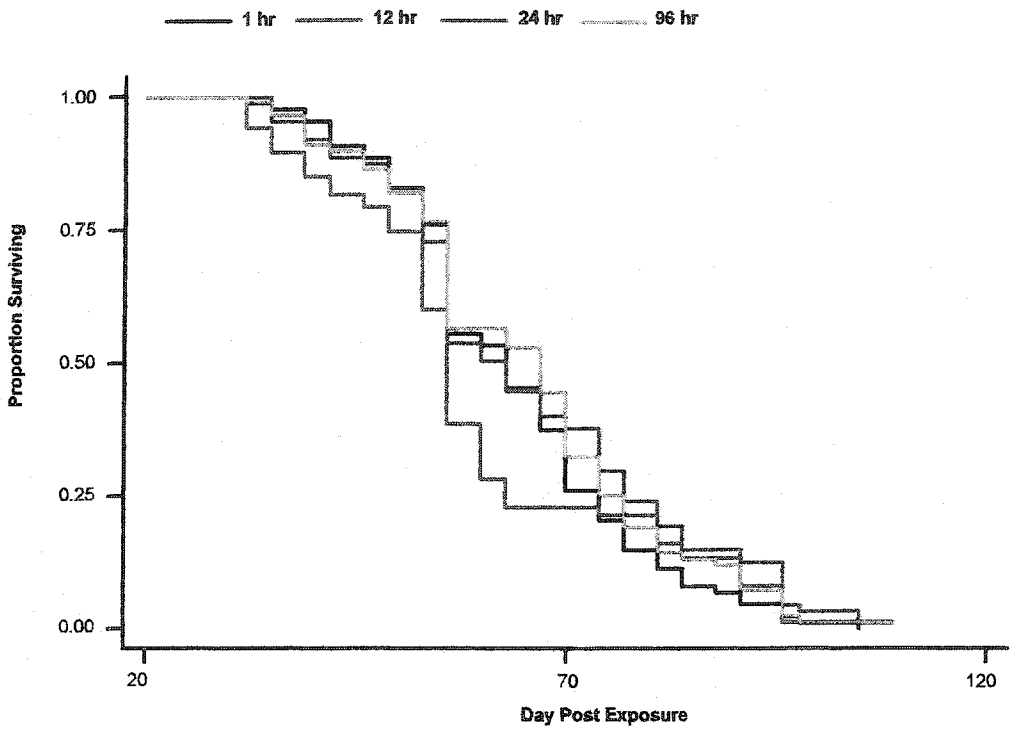

Figure 8.3. Kaplan-Meier survivor curves for rainbow trout exposed to *L. salmonae*-infected fish for various time periods.

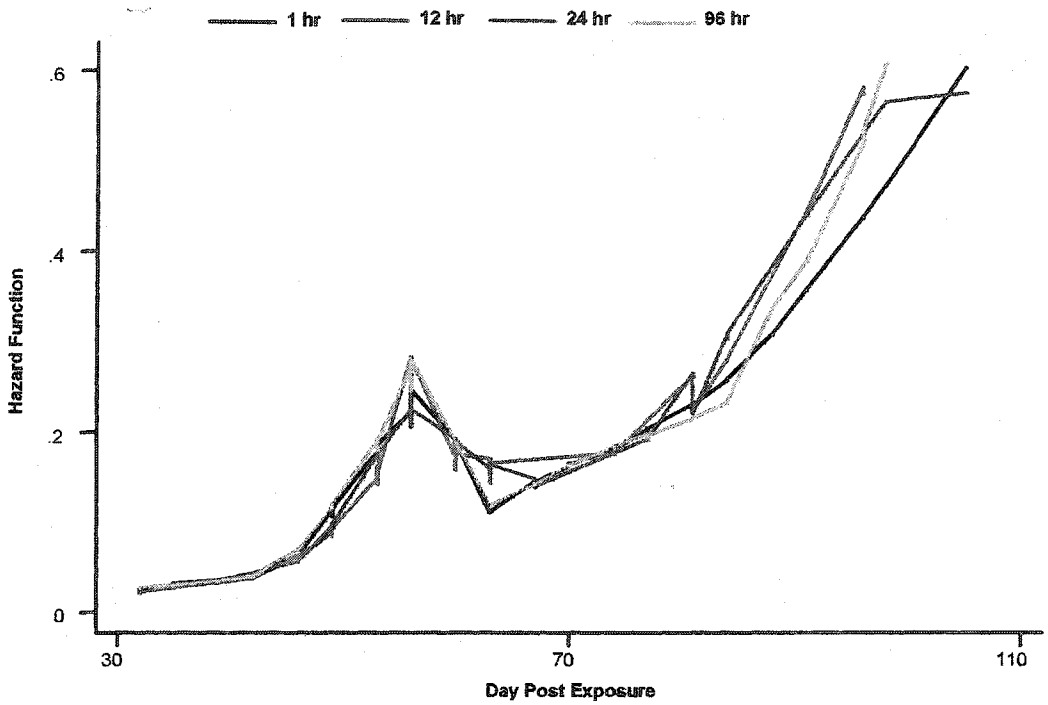

Figure 8.4. Lowess smoothed (bandwidth = 0.4) hazard function estimates for rainbow trout exposed to *L. salmonae*-infected fish with various exposure periods.

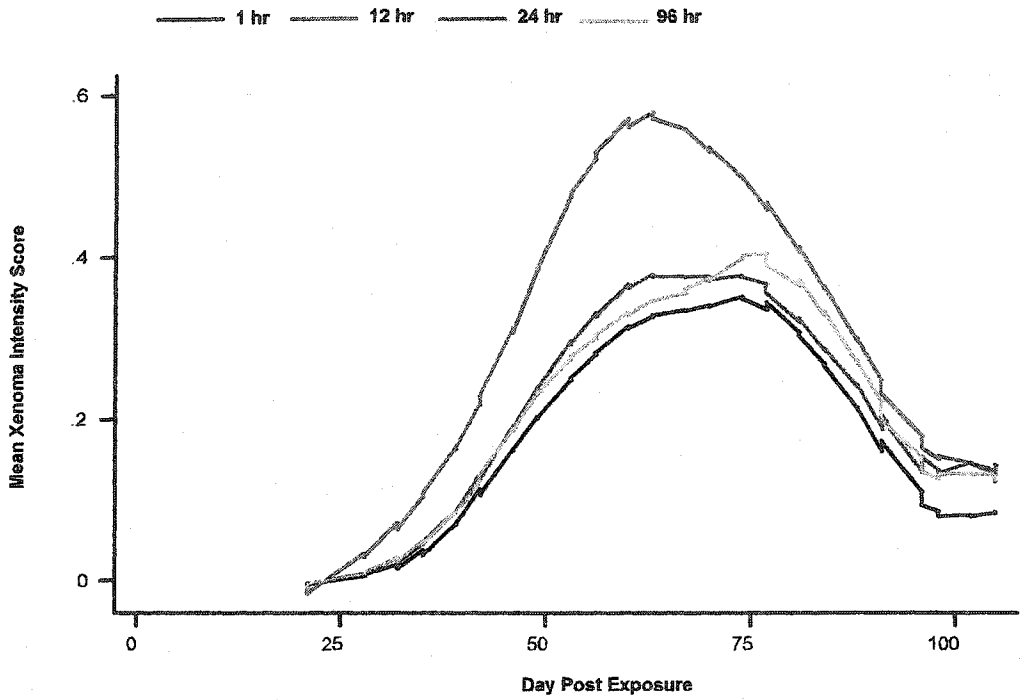

Figure 8.5. Mean xenoma scores (lowess smoothed) for rainbow trout exposed to *L. salmonae*-infected fish with several exposure periods.

8.5 DISCUSSION

This study demonstrated that *L. salmonae* transmission was possible with a minimum of one hour exposure time between cohabiting infected and naive RBT. Adding five *L. salmonae*-infected fish (at week 7 PE) to a group of naive RBT for a period of one hour resulted in 95% disease prevalence. This was the shortest exposure duration in both trials with a total of 5 hours of FCT between infected fish and naive RBT (Table 8.1). Moreover, it was interesting to note that the effect of adding one, five or ten infected fish to a tank of 45 naive RBT, representing 504, 2520 and 5040 hours of FCT resulted in similar hazards of developing branchial xenomas with the resulting infections leading to similar xenoma intensities. From trial I, the quantity of spores released from a single cohabiting *L. salmonae*-infected fish was sufficient to cause over 95% disease prevalence in a group of 45 naive fish. Overall, mean xenoma intensities observed in trial II at all treatment levels were lower compared to those observed in trial I. Typically, the cohabitation challenge model produces significantly lower numbers of branchial xenomas with a lower disease prevalence compared to the per os challenge model (Speare et al. 1998, Ramsay et al. 2001, Ramsay et al. 2003, see chapter 4).

The key to success for all microsporidia including those infecting fish, lies in the diversity and flexibility of their transmission strategies and life cycles (Dunn and Smith 2001). Many transmission studies have demonstrated that *L. salmonae* was readily transmitted through many challenge models (e.g. per os,

cohabitation, intra-peritoneal, effluent water) using different host species (rainbow trout, chinook and coho salmon) using both freshwater and seawater environments (Kent et al. 1995, Speare et al. 1998, Shaw et al. 1998, Ramsay et al. 2001, Ramsay et al. 2002, Ramsay et al. 2003, Becker and Speare 2004). Although Becker and Speare (2004) readily transmitted *L. salmonae* using only effluent water in a non-contact horizontal transmission model, this current study was aimed at investigating the minimum exposure time required amongst naive and infected fish. This study demonstrated that in a net-pen scenario, a few *L. salmonae*-infected pen-mates will probably transmit MGD to the entire pen. Moreover, these few infected fish have the potential to transmit *L. salmonae* to neighbouring pens or farms because fish-to-fish contact is not required for horizontal transmission (Becker and Speare 2004). This is the first study to focus on the minimum fish-to-fish contact required to initiate a disease cycle resulting in a high prevalence and the development of xenomas. The minimal 5 hrs of FTC required to propagate a disease cycle demonstrated the phenomenal transmission ability for this microsporidial pathogen.

The amount of available contact between infected and naive fish, either as a function of time or the number of infectious cohorts added has been noted as significant transmission factors in other important infectious diseases to the salmonid aquaculture industry in Canada, including infectious pancreatic necrosis (IPN) (Bebak-Williams et al. 2002), infectious salmon anemia (ISA) (Jones and Groman 2001) and furunculosis (Nordmo and Ramstad 1999). Bebak-Williams et al. (2002) investigated the effect of the number of infected

and naive RBT density on the transmission rate of infectious pancreatic necrosis virus (IPNV) using survival analysis and generating Cox PH models. It was reported that as the number of infected fish increased from one to three, the hazard curve shifted to the left, which represented an increased hazard for mortality in tanks containing high numbers of infected fish. However, the current study revealed that the effect of adding one or ten infected fish produced similar disease prevalence and intensity. Additionally, Bebak-Williams et al. (2002) investigated the effect of adding one to three infected fish to populations of naive RBT with low, medium and high stocking densities. It was found that the effect of increasing naive fish density resulted in increased mortality when one infected fish was added but when three infected fish were added, the effect of density diminished (Bebak-Williams et al. 2002). For *L. salmonae*, the combination effect of naive fish density and the number of infected RBT has not been investigated and will be a subject for future study.

A study investigating infectious salmon anemia virus (ISAV) transmission reported increased mortality associated with increasing the number of infected fish from two to six in naive Atlantic salmon (*Salmo salar*) populations (Jones and Groman 2001). Jones and Groman (2001) suggested that the infection dose was related to the number of injected fish and presumably, therefore to the number of ISAV-shedding fish. In the current study, the assumed dose of *L. salmonae* spores reported as FCT, was a combination of the number of infected fish and the number of hours of contact time with the naive RBT. This integrated infection dose allowed the two trials with differing challenge protocols to be

placed on the same scale. Additionally, a previous study indicated that handling stress experienced by the infected fish following transfer to the tanks containing naive fish does not appear to initiate a wave of xenoma rupture, even with a shift in water temperature (Becker and Speare 2004). Finally, Nordmo and Ramstad (1999) reported that increased mortality was observed in naive Atlantic salmon during cohabitation with increasing numbers of fish inoculated with *Aeromonas salmonicida*, the causative agent for furunculosis. The authors also noted that the impact of the number of infected fish added (10, 20 or 50 to 100 naive fish) disappeared after 28, 35 and 48 days in tanks held at 12°, 10° and 8°C, respectively (Nordmo and Ramstad 1999). The reduced effect of the number of infected fish was attributed to the fact that the incubation period for *A. salmonicida* was five days and as time progresses, the challenge pressure set up by the initial infected fish will be masked over time by the naive fish becoming infected and shedding bacteria themselves (Nordmo and Ramstad 1999). Similarly, this current study reported an increased hazard for fish exposed to five *L. salmonae*-infected cohorts for 12 hrs or 60 hrs of FCT, however this effect diminished by day 68 PE. Using a cohabitation challenge model, the incubation period for RBT exposed to *L. salmonae* ranges from 3 to 10 weeks. Due to longer and variable incubation periods, it is impossible to differentiate if the late infections were due to the initial challenge from the infected fish or from a naive fish that become infected and began to release spores. Additionally, the reduced survival and increased xenoma intensities observed in the 12 hr

exposure group may be an anomalous outcome as no biological interpretation has been identified.

Effective integrated fish health management programs are built on strategies that emphasize prevention and control of infectious disease outbreaks (Bebak-Williams et al. 2002). Ramsay et al. (2001) noted the usefulness of further investigations into transmission potential of *L. salmonae* so that researchers can predict, control and possibly prevent outbreaks of microsporidial gill disease. This study acknowledged the impressive transmission potential generated from a small group of *L. salmonae*-infected fish during cohabitation exposure with a larger naive population of RBT. It would be useful to investigate the outcome of a brief cohabitation exposure under the influence of other significant transmission factors including water temperature, host species and fish size (Beaman et al. 1999, Ramsay et al. 2002, Becker et al. 2003, Becker et al. 2004).

8.6 ACKNOWLEDGMENTS

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9 GENERAL DISCUSSION

The goal for this thesis was to identify transmission factors associated with microsporidial gill disease (MGD) caused by *Loma salmonae* with specific aims designed to explore practical and realistic disease solutions for the salmon industry. In addition, the results generated herein bridged gaps amongst several satellite areas of research that encompass the pathobiology of this important pathogen. Project *Loma*, which is approximately at the midway point through a second consecutive strategic grant from NSERC, has supported a diverse array of research centered on *L. salmonae* including, the development of challenge models, transmission and ultrastructure studies, the localization of the parasite within the host using immunologic methods and the identification of potential drug therapies. These past results have been not only necessary for the formulation of this thesis but have also greatly advanced the knowledge surrounding MGD caused by *L. salmonae*. Most notably, the development of robust and reliable challenge models using several exposure methods in marine and freshwater environments with several fish hosts, is the primary reason for the success of scientific research surrounding *L. salmonae*. With an effective challenge model, the permissible temperature range for *L. salmonae* in rainbow trout (RBT) was determined to be between 9° and 20°C (Beaman et al. 1999). These data were then used to demonstrate increased resistance to challenge at an optimal temperature (15°C) among RBT first exposed to spores below permissible temperatures (Speare et al. 1998).

Using the optimum water temperature for *L. salmonae* infections, the investigations shifted to examine the life cycle for this pathogen using polymerase chain reaction (PCR) and to follow the movement of the parasite within the host from the site of spore germination in the gut to the appearance of a visible branchial xenoma using *in situ* hybridization (ISH) (Sánchez et al. 2000, Sánchez et al. 2001a, Sánchez et al. 2001b). *Loma salmonae* was detected in the gut mucosal epithelium as early as 24 hours post exposure (PE) and it localized in the lamina propria of the intestine within this time period (Sánchez et al. 2001b). Dividing merogonic stages in infected cells were detected using ISH in the heart as early as 2 days PE (Sánchez et al. 2001b), subsequently *L. salmonae* DNA was found in the gills beginning at 2 weeks PE (Sánchez et al. 2001a). A series of trials then examined the ultrastructural features of gills from *L. salmonae*-infected rainbow trout to investigate the mechanisms of infection in the gill lamellae (Rodríguez-Tovar et al. 2002). Meronts were the earliest parasitic stage observed in the secondary lamellae beginning at week 3 PE (Rodríguez-Tovar et al. 2002). At week 5 and 6 PE, mature spores were peripherally distributed within the xenoma and eventually occupied the entire xenoma (Rodríguez-Tovar et al. 2003).

Although related to the life cycle localization and ultrastructure studies, a distinct stream of research investigated the host-parasite-environment relationship for *L. salmonae* through manipulation of potential transmission factors. A comparison between per os and cohabitation challenge models was

examined with respect to transmission potential, measured by the number of weeks with visible xenomas, the intensity of xenomas and the ability of recovered *L. salmonae*-infected fish to infect a group of naive RBT (Ramsay et al. 2001). The per os challenge model produced significantly higher xenoma intensities and infection prevalence compared to the cohabitation model. Exposure to *L. salmonae*-infected fish at week 3 PE, using either challenge model was unable to elicit xenoma development in naive RBT (Ramsay et al. 2001), although the parasite can be detected in the gills at this stage (Sánchez et al. 2001a, Rodríguez-Tovar 2002). Subsequently, an investigation into the possibility of differences in host species revealed that chinook salmon were most susceptible to *L. salmonae* using the per os challenge model (Ramsay et al. 2002). Chinook salmon showed significantly higher numbers of xenomas, which persisted for longer time periods in both fresh- and seawater compared to rainbow trout and coho salmon, demonstrated an intermediate level of disease between these two fish hosts (Ramsay et al. 2002).

The ultimate goal the preceding research aids in achieving is the ability to predict, control and manage microsporidial gill disease caused by *L. salmonae*. The generation of scientific knowledge from the *L. salmonae*-centered research has created the necessary stepping stones required to complete the studies profiled in this thesis. The overall research goal for this thesis was to identify important host, pathogen and environmental factors associated with the transmission of microsporidial gill disease caused by *Loma salmonae* in rainbow

trout. Studies were designed to examine the impact of water temperature and flow rate as potentially influential environment transmission factors on *L. salmonae* transmission as well as studies involving feeding rate, fish size and drug therapy as important host factors. A non-contact horizontal transmission model was developed and the minimum exposure period required between naive and *L. salmonae*-infected tank-mates was investigated under the umbrella of pathogen related factors.

Water temperature is considered to be an important environmental variable in the transmission of many fish diseases because it can act directly on the development of the pathogen, on the immune system of the fish or both (Antonio and Hedrick 1995). Typically, an increase in water temperature leads to a reduction in the number of days until disease onset, increased disease prevalence and intensity of disease for many key pathogens to the salmonid industry. This was readily demonstrated in Atlantic salmon (*Salmo salar*) cohabited with fish infected with *Aeromonas salmonicida*, the causative agent for furunculosis, which showed increased mortality rates with increasing water temperatures from 8° to 12°C (Nordmo and Ramstad 1999). As a result of it's overall importance to fish disease modeling, water temperature was the subject of investigation in three of the seven research chapters described in this thesis.

The first study (chapter 2, Becker et al. 2003) examined the impact of water temperature (within the permissible range for the pathogen) on *L. salmonae* transmission using a cohabitation challenge model. Naive rainbow trout were

held at 11°, 15° and 19°C and allowed to cohabitate with infected fish for 21 days. Similar to the results reported using a per os exposure (Beaman et al. 1999), fish held at 19°C had the least number of days to the development of branchial xenomas. However, unlike the per os exposure results, there was no difference in the time to onset for the moderate and cold water fish in the cohabitation model. The fish held at 11°C were expected to have a lagged onset time because of the delayed parasite development reported by Beaman et al. (1999). It was hypothesized that a change in water temperature (particularly a decline in water temperature) may cause an accelerated dissolution of pre-formed xenomas, which was the main objective for chapter 3 (Becker and Speare 2004a). The second objective for chapter 3 was to determine whether the rate of xenoma formation and dissolution at three constant permissible temperatures of the laboratory strain of *L. salmonae* presently in use was similar to the historical data.

To study these two objectives, two cohorts of fish were created; (1) rainbow trout per os exposed were held at 15°C until peak xenoma formation and then were transferred to tanks held at 11°, 15° or 19°C and (2) satellite fish were held at 15°C, exposed per os and subsequently transferred to either 11°, 15° or 19°C the day after exposure. All of the fish in the first cohort were individually tagged to monitor xenoma onset and dissolution. As the water temperature of the transfer tank increased, the amount of time required for the dissolution of all branchial xenomas decreased. This indicated that the temperature during the exposure period or during the early developmental stages of merogony did not

fix the development rate of this parasite. Additionally, fish shifted to either 11° or 19°C at peak xenoma development did not reveal an altered rate of xenoma dissolution due to the temperature change. This result did not support the hypothesized differential rate of xenoma rupture with sudden temperature change. Additionally, data generated from the satellite fish did not suggest that the associated temperature dependency of the life cycle of *L. salmonae* is a result of strain differences. Notably, the defining role of temperature in the life cycle of *L. salmonae* now includes the disease recovery period and this study was the first to accurately measure the impact of a temperature shift on the xenoma dissolution. However, the disparity observed between the per os and cohabitation challenge models with respect to the effect of water temperature on xenoma onset during a *L. salmonae* infection was still unexplained.

The objective in chapter 4 was to re-evaluate the regulatory effects of water temperature on xenoma development using a high dose per os challenge model and a presumed low dose cohabitation challenge model. The results suggested that the regulatory effects of water temperature on xenoma onset during a *L. salmonae* infection were dependent on experimental challenge model. The overall impact of water temperature on disease pathogenesis was greater when the RBT were fed *L. salmonae*-infected macerated gill material for the per os challenge model compared to the cohabitation model. The effect of temperature appeared to be dampened when using the cohabitation exposure model, which is considered to give a low dose of spores compared to the per os challenge.

The dependency of the effect of water temperature on the challenge model used was presumed to be related to the fact that per os exposures lead to significantly higher numbers of xenomas compared to a cohabitation exposure.

Water temperature has been noted as a key transmission factor for another microsporidian parasite, *Nucleospora salmonis*, infecting chinook salmon (Antonio and Hedrick 1995). It was determined that the optimal range for this parasite was between 15° and 18°C, which is quite similar to optimal temperatures reported for *L. salmonae* (Antonio and Hedrick 1995; Beaman et al. 1999). Similar to *L. salmonae*, *N. salmonis* exhibited delayed development at lower temperatures (e.g. 12°C), however a strong disease response was elicited with almost 74% mortality. Predicting *L. salmonae* disease events using a water temperature based model was first reported by Beaman et al. (1999) and successfully tested to accurately predict xenoma onset (Speare et al. 1999b). In the open net-pen environment, disease events associated with *L. salmonae* are often reported to occur in late August, coinciding with peak water temperatures. Remembering that the ultimate goal is the ability to predict actual disease events occurring in a net-pen, the three studies investigating the impact of water temperature throughout a *L. salmonae* disease cycle will assist in determining the transmission potential amongst the various cohorts of infected and susceptible fish (e.g. wild salmon, moribund pen-mates, neighbouring net-pens). For example, the impact of water temperature will be less severe for transmission between farmed salmon in neighbouring cages or with migrating

wild salmon, which only share the ocean environment, compared to the transmission that is possible with naive pen-mates feeding on moribund or dead *L. salmonae*-infected salmon. Furthermore, water temperature was related to xenoma dissolution time so that as water temperature decreased, the number of days until rupture increased. This provided evidence that trout held at 11°C could harbour low levels of xenomas for extended periods of time, therefore significantly increasing their transmission longevity. Additionally, chinook salmon, compared to rainbow trout have a much longer natural course of disease with the majority of fish clearing branchial xenomas by week 20 PE (Kent et al. 1999). For example, chinook salmon in a net-pen are identified as having numerous branchial xenomas on September 1, 2004, which assumes that the exposure occurred approximately 5 weeks earlier at the end of July. With constant water temperatures at 15°C, the xenomas will be cleared from the gills by week 20 PE or mid-December 2004. However, with the onset of autumn and winter, decreasing water temperatures could presumably extend the xenoma dissolution time by many weeks. The delayed rupture time may be sufficient for the over-winter xenomas to infect newly transferred smolts the following spring. The impact of constant and fluctuating water temperature on xenoma dissolution in chinook salmon should be considered in future investigations.

In addition to water temperature, the other major environmental transmission factor studied was flow rate (chapter 2, Becker et al. 2003). Increasing flow rates is a routine husbandry suggestion to increase overall fish health and until now was not studied during *L. salmonae* infections. Flow rate is easily manipulated in

a laboratory setting as demonstrated in chapter 2 by reducing the habitable volume of the tanks from 70 L to 50 L to achieve water turnover rates of 1, 2 or 3 exchanges per hour. Fish held in a low flow tank developed xenomas the fastest with consistently higher intensity levels. Although flow rate manipulation is not feasible in an ocean net-pen aquaculture situation, it could be relatively practical if the industry moves towards land-based rearing facilities. Additionally, *L. salmonae* was initially identified as a hatchery-related disease (Magor 1987) and subsequently has been reported to cause severe disease with high mortalities at rainbow trout hatcheries (Markey et al. 1994, Bader et al. 1998), where increasing flow rates can be a practical approach in this rearing situation.

Investigations under the domain of host factors centered on the effects of various feeding rates, the dependency of the weight of the fish at the time of exposure and further studies investigating the efficacy of oral monensin therapy. Although feeding rate did not alter the onset or resulting intensities of branchial xenomas, fish size was found to be a significant host-related factor (chapter 6, Becker et al. 2004). Rainbow trout ranging from 17 to 23 g had a significantly faster rate of development of xenomas with the median onset time ranging from 7 to 11 days sooner compared to the two larger size groups. Moreover, generally the smallest group had significantly higher numbers of xenomas observed on the gill filaments with a peak scores equal to 1.5 compared to a peak score of 0.75 for other two size groups. Size-related susceptibility has been reported in other significant salmonid pathogens. Notably, it has been demonstrated that small (1.7 g and 0.2 g, respectively) RBT and kokanee (*O.*

nerka) are more susceptible to infectious hematopoietic necrosis virus (IHNV) compared with larger sizes (7.4 g and 7.2 g, respectively) (Lapatra et al. 1990, Lapatra 1998). Horizontal transmission of IHNV occurs readily in fresh- and seawater and the virus infects several salmonid species and recently has caused numerous outbreaks at Atlantic salmon farms in British Columbia (Saksida 2003). Highest mortality rates greater than 70% were observed in farmed Atlantic salmon weighing less than 1 kg compared to 50% and 40% mortality reported in fish weighing 1 - 2 kg and more than 2 kg, respectively (Saksida 2003). For MGD caused by *L. salmonae*, the identified host risk factors are (1) host species with chinook salmon (*O. tshawytscha*) being most susceptible (Ramsay et al. 2002); (2) host strain differences seen in chinook salmon from British Columbia (Shaw et al. 2000), and from this thesis (3) fish size at the time of exposure to *L. salmonae.*

Another host factor considered was the potential use of monensin therapy to treat MGD by investigating the minimum dose and treatment time required for therapeutic success (chapter 5, Becker et al. 2002). Monensin-treated fish (at 1000 ppm) with a per os exposure to spores showed reduced xenoma intensity of 69% and 85% at weeks 7 and 8 PE, respectively, compared to the similarly exposed non-treated fish. The 1000 ppm treatment group satisfied the criteria set for the acceptable dose in that fish consumed all the feed offered, the weight gain was normal and xenomas reduction at peak formation was well over 50%. The dose level was carried over to a subsequent trial, which demonstrated the impact of prophylactic treatment on xenoma formation. Prophylactic treatment

with monensin reduced the xenoma formation on the gills of rainbow trout exposed to *L. salmonae*. When treatment was started at the time of exposure or one week before, significantly lower mean numbers of xenomas were produced than if the fish were not treated. As described above, *L. salmonae* is localized in the gut and heart during the first week of the parasite life cycle, which was in concordance with most beneficial therapy period, indicating that once the parasite has localized in the gills, the therapy was no longer effective. To further investigate monensin as an effective therapeutic for MGD, a follow-up study to reduce the dose range would be beneficial because significant, although not consistent reductions in xenoma counts were observed in 100 and 10 ppm groups. Additionally, the viability of the xenomas that did form in the monensin treated fish should be considered in future research.

Finally the third domain of the disease triad involves those factors that directly pertain to the pathogen. Typically, pathogen related transmission factors involve the infective dose, the duration of exposure, the delivery method for the pathogen and virulence (which was not studied due to a lack of virulence variable strains). As noted above, the previous development of many robust experimental challenge models were imperative to the formulation of this thesis. However, one model for this pathogen that had not been described was that of a non-contact horizontal transmission model using only effluent water. *Loma salmonae* was transmitted to naive RBT without the need for physical contact with the infectious fish, with the median xenoma onset time being delayed by approximately one week compared to the contact cohabitation model (chapter 7,

Becker and Speare 2004b). Subsequently, another experiment was conducted with the effluent water from the upper tanks containing the infectious fish traveling through an UV light sterilizer before entering the lower tanks containing naive RBT. After 80 days of being challenge with UV-treated, *L. salmonae*-infected water, none of the naive rainbow trout developed branchial xenomas. A similar non-contact model was developed for infectious salmon anemia (ISA), a severe pathogen affecting Atlantic salmon aquaculture industry in New Brunswick, Canada (Jones and Groman 2001). Non-contact models were identified as important factors to assess the potential of transmission within and amongst farms. The results from initial experiment indicated the potential for *L. salmonae* transmission between fish that share the same ocean environment, although they are not in direct contact (e.g. migrating wild salmon and reared salmon, within a salmon farm with several net-pens). The second experiment emphasized the importance of biocontrol of incoming water to a salmon or rainbow trout hatchery and that UV sterilization can increase this control.

The last area of study was designed to investigate the minimum infective dose required for disease transmission between naive rainbow trout and *L. salmonae*-infected cohorts by varying exposure time. This was initially evaluated based on adding one, five or ten infectious cohorts into a tank of naive fish for a period of 21 days and secondly, it was studied by limiting the cohabitation period amongst five infectious cohorts and naive fish to 1, 12, 24 and 96 hours, with subsequent evaluations on xenoma development. This study demonstrated the ability for this pathogen to initiate a full disease cycle with the minimum infective

dose of one hour exposure time between cohabiting infectious and naive RBT. Adding five *L. salmonae*-infected fish (at week 7 PE) to a group of naive RBT for a period of one hour resulted in 95% disease prevalence. This was the shortest exposure duration investigated in this experiment and that has been reported for MGD. Additionally, the 21 day cohabitation period with one *L. salmonae*-infected fish was sufficient to cause over 95% disease prevalence in a group of 45 naive fish. In contrast, Jones and Groman (2001) reported increasing mortality rates during experimental ISA cohabitation challenge with increasing numbers of infectious cohorts. Presumably, there was an increase in the infective dose with an increase in the number of infectious cohorts added to a naive population (Jones and Groman 2001). Applying the results reported from the current study, there are indications that in a net-pen scenario, a few *L. salmonae*-infected pen-mates will probably transmit MGD to the entire pen. Moreover, these few infected fish have the potential to transmit *L. salmonae* to neighbouring pens or farms because fish-to-fish contact is not required for horizontal transmission.

The research described in this thesis has advanced the body of scientific knowledge surrounding the host, pathogen and environment disease triad for microsporidial gill disease caused by *Loma salmonae*. The contributions contained herein were partly novel observations identifying previously unexplored transmission factors and partly studies furthering knowledge involving known transmission factors. Although the importance of identifying specific transmission factors that alter the disease cycle is emphasized throughout the entire thesis, there are two key issues that also should be

highlighted; (1) *L. salmonae* is a very effective parasite and (2) the need to investigate the development of a *L. salmonae* disease surveillance program for both wild, feral and farmed salmon. Firstly, all microsporidians infect a host with an extrusion apparatus including the coiled polar tube contained within an environmentally resistant spore. The ability of microsporidians to release massive quantities of small resistant spores into the environment, to infect a variety of hosts and as demonstrated herein, the low infective dose are key characteristics to describing this phylum as having a diverse and flexible life cycle. This diversity and strong transmission potential demonstrates their importance to human and veterinary medicine, especially surrounding the health and welfare of immunosuppressed patients and animal hosts with agricultural importance (e.g. honey bees and salmon). Secondly, this thesis has highlighted the significant potential for disease transfer between wild and farmed salmon.

Laboratory-based transmission studies are often downplayed due to claims that they are too rigid and do not reflect the interaction of many variables that field research has the capability to study and explore, although both types of studies have advantages and disadvantages. Laboratory-based studies are essential in ascertaining the pathobiology for the parasite and host and the interaction of the two organisms. All of the transmission factors identified in this thesis were studied individually and consistently, which has created practical stepping stones to formulate field-based research projects. The next phase for this research is to incorporate the identified transmission factors (e.g. water temperature during the entire parasite life cycle, flow rate, fish size, strain and species) into surveillance programs recording epidemiological data to assess

these factors at a chinook salmon farm. Studying the epidemiology of MGD could identify new variables significant to *L. salmonae* transmission that have not been considered, or it may determine that a significant factor during an experimental challenge may not convey significance in the field. Furthermore, field-based studies are imperative to determining the transmission potential amongst the wild, feral and farmed salmon populations found in and around salmon cages in British Columbia. Subsequently, the identified risk factors for MGD can be used to change animal husbandry practices, industry management and to identify areas in need of scientific research. Although *L. salmonae* was first reported at a hatchery on Vancouver Island in 1987, it was not until the mid 1990s that it was declared a major pathogen to the chinook and coho salmon aquaculture industry. Concurrently, since 1998 the production of farmed chinook salmon has risen from 6 600 tonnes to over 11 000 tonnes in 2002 (MAFF 2002). Presumably, the observed increase in MGD outbreaks is indirectly related to the near doubling in the production of chinook salmon and this upward production trend will maintain *L. salmonae* as an important pathogen to the future salmon industry in Canada.

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