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EFFECTS OF TEMPERATURE ON THE DEVELOPMENT OF *LOMA SALMONAE* AND RESISTANCE TO RE-INFECTION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

of the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Holly J. Beaman

Charlottetown, PEI

September 1998

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ABSTRACT

Loma salmonae, an economically important microsporidian parasite of farm-reared Pacific salmon, causes severe branchial infections. No treatment currently exists to control *L. salmonae* infections. Consequently, other mechanisms of control are essential. Water temperature has a regulatory effect on the life cycle and development of many pathogens and has been used to control some infectious diseases. It may be used as a means of controlling parasitic infections with *Loma salmonae*; however, little is known about the relevant pathobiology of this parasite. Objectives of this research were to determine the permissive developmental range for the life cycle of *L. salmonae*, the effect of temperature on xenoma development rate and the abundance of branchial xenomas that develop, and to examine resistance induction for controlling parasitic infections. Juvenile rainbow trout reared in freshwater were exposed to infective tissue by either intubation or feeding of infective gill material. They were then reared at water temperatures of 5°, 7°, 9°, 10°, 11°, 13°, 15°, 17°, 19°, 20° or 21°C. Fish developed branchial xenomas within the range of 9°C to 20°C, indicating the permissive temperature range for *L. salmonae*. Additionally, rate of development was significantly faster at warmer water temperatures. Since exposure time was known, a polynomial regression model and a thermal unit summation model (°C- days) were useful for predicting time of xenoma onset. Due to variation in the length of time xenomas remained in the gills, no model could be developed for predicting clearance time of the parasite from the gills. Quantification of branchial xenomas was conducted at water temperatures of 11°, 13°, 15°, 17° and 19°C. The number of xenomas per gill filament was assessed to determine if temperature affected intensity of infection after all fish received the same initial dose via gastric intubation. The number of xenomas that developed in fish at all temperatures varied considerably among fish; there appears to be no effect of temperature. Upon reinfection of fish at 15°C, few to no xenomas were detected, regardless of water temperature during initial exposure. This suggests that it is not necessary for *L. salmonae* to complete its life cycle to invoke an immune response in the fish host. The relative percent protection model developed for the re-infection trials indicated a high level of protection in fish which were previously exposed to *L. salmonae*. A potential procedure for controlling *L. salmonae* infections in farm-reared salmonids has resulted from the trials conducted herein. No prior studies have modelled development rates of fish pathogens, although the model developed in this thesis has significant potential for many fish parasites whose infective stages and development are dependent on water temperature. Fish can be exposed to the parasite at non-permissive temperatures which do not allow completion of the life cycle but induce significant protection against further *L. salmonae* infections.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	- Analysis of variance
°C	- Degrees Celsius
d	- Days
DM	- Days post-exposure moved
Fig.	- Figure
g	- Grams
H&E	- Haematoxylin and Eosin
i.e.	- Id est; that is
L	- Litre
mg	- Milligrams
min	- Minutes
ml	- Millilitres
No.	- Number
%	- Percent
P	- P-value
PCR	- Polymerase chain reaction
R^2	- Regression coefficient of determination
rDNA	- Ribosomal deoxyribonucleic acid
RNA	- Ribonucleic acid
rpm	- Rotations per minute
RPP	- Relative percent protection
SEM	- Standard error of the mean
St. Dev.	- Standard deviation
T	- Temperature
TU	- Temperature units
t	- Tonnes
Xen./Fil.	- Xenomas per filament
χ^2	- Chi-square

1. GENERAL INTRODUCTION

1.1 Aquaculture

Aquaculture generally refers to the farming, husbandry, or production of aquatic organisms including finfish, aquatic plants, molluscs, and crustaceans (Barnabé 1990; Boghen 1995). The level of human intervention depends on the species being cultured. Aquaculture production can be used in several ways: to enhance natural populations in lakes and rivers, fee-fishing ponds, hobby farms, for food production, or for the supply of ornamental fish.

World aquaculture production, based on the above definition, has undergone incredible growth over the past few decades and has become increasingly important as the capture fisheries have declined due to environmental changes and over fishing in many regions (Boghen 1995). Production volume rose from 1 million tonnes to 19.3 million tonnes between 1967 and 1992 (13.9 million tonnes when aquatic plants are excluded). Since fish and fish products are an everyday staple in many countries, the importance of this industry will likely continue to increase, providing resources that can no longer be supplied by capture fisheries (Boghen 1995; Tacon 1998).

1.1.1 History

Culturing fish for consumption began at least four thousand years ago in China. Culture also occurred thousands of years ago in Japan, Egypt, Greece and Rome (Boghen

1995; Heen et al. 1993). The second half of the 1800's recognized the development of salmonid hatcheries in Europe and North America (Laird 1996), an industry which began primarily for the purpose of wild-stock enhancement in North America (Boghen 1995). The first detailed records of aquaculture in Canada came from Quebec in 1857 where Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) eggs were studied. By 1972, salmonid species accounted for most aquaculture production, by weight, in Canada (Boghen 1995). Aquaculture in Canada began from the realization that the industry was successful in other parts of the world and led to financial benefits and employment opportunities. Aquaculture is the fastest growing agri-food business in Canada (Boghen 1995).

1.1.2 Current Status

Globally, Asia is currently the dominant producer in aquaculture, primarily in the farming of carp, tilapia, mullet, shrimp, seaweed and milkfish (Boghen 1995; Landau 1992). Ninety percent of total aquaculture production by weight was in Asia in 1995 (Tacon 1998). Salmon culture and the value of cultured fish has increased in Japan since the 1970's (Landau 1992).

In 1995, Europe produced 5.1% of total global production by weight (Tacon 1998). Salmon culture in Norway continues to develop rapidly (Landau 1992), although production in Chile has increased dramatically recently and may soon equal that of Norway (the current leader) (Heen et al. 1993). The pioneering work with floating cages in which to rear salmonids was conducted in Norway (Monahan 1993). Other cultured finfish species of importance in Europe include trout (primarily rainbow - *Oncorhynchus mykiss*), carp

(*Cyprinus carpio*), European eels (*Anguilla anguilla*), and tench (*Tinca tinca*) (Landau 1992). Mussels, oysters and crayfish are also grown commercially.

North America comprised only 2.5 percent of world aquaculture production in 1992 (Boghen 1995) and 2.2% in 1995 (Tacon 1998). This was followed by South America (1.4%), the former USSR (0.58%), Oceania (0.35%) and Africa (0.31%). Currently, more catfish (by weight) are produced than any other species in the United States. In addition to many other species of fish and crustaceans, salmon (chinook - *Oncorhynchus tshawytscha* and coho - *O. kisutch*) and steelhead trout (*O. mykiss*) are also produced in the U.S.. Canadian production has increased in the past twenty years with salmonids (trout, salmon and charr) being the most important fish cultured. In 1987 production was valued at $50 million Canadian with 11,500 t produced compared to 46,000 t with a value of $300 million in 1994 (Boghen 1995). World salmon harvests reached 325,000 t in 1991 (Folsom et al. 1992). More recently, 940,000 metric tonnes of salmon were produced in 1995, of which Canada produced 5.1% (Tacon 1998).

Canada was ranked twenty-ninth for production quantity among 154 countries conducting aquaculture in 1992 (Boghen 1995). However, despite this level of production, aquaculture has been slower to develop in Canada than other industrialized nations with similar climates (Boghen 1995). This may be partially due to the harsh climate experienced in several regions. Canada has the longest fishing coastline in the world with traditional fisheries having a long and rich history, a factor which has also marginalized the growth of the aquaculture industry in Canada (Boghen 1995).

1.1.3 Salmonid Culture

The most common finfish cultured in Canada are of the family Salmonidae. Atlantic salmon (*Salmo salar*) is the most common species cultured although chinook and coho salmon and rainbow trout are also cultured extensively. Originally found only in the northern hemisphere in Europe, North America and Japan, salmonids are now also cultured world-wide in countries such as Chile, New Zealand, and Australia (Heen et al. 1993; Smith 1993). In North America, salmonid culture is limited primarily to East and West coastal regions of the United States and Canada. However, trout production is largely centered in Idaho and Ontario, respectively. The West coast focused primarily on coho salmon until the early 1980's when farmers began rearing chinook salmon more extensively. Production of this species surpassed coho production in 1987. An increasing amount of Atlantic salmon is also being cultured on the West coast. Farmers may prefer this due to the docile nature of the species and market preference which is currently for Atlantic salmon in both Europe and North America (Monahan 1993).

There is also a large demand for rainbow trout, which occur naturally in the coastal and inland waters of British Columbia eastward into Alberta and are considered a highly valued game fish in British Columbia. The species, first introduced into Atlantic Canada in the late 1800's in Newfoundland, can adapt to varying conditions (Dubé and Mason 1995). However, juvenile rainbow trout may adversely affect Atlantic salmon smolt survival where the two species coexist. Rainbow trout may also be reared in sea water depending on the strain, where they are referred to as steelhead trout. Some are adaptable to both environments (Sedgewick 1995; Scott and Scott 1988). Both seawater and freshwater cage

culture of rainbow trout are carried out in the Atlantic provinces. Rainbow trout are used frequently in experimental physiological studies (Scott and Scott 1988).

1.1.4 Biological Constraints

Significant biological constraints are placed on any aquaculture operation, including disease, predators and parasites. Stress responses often lead to increased susceptibility to infectious disease and parasite outbreaks. Parasites, such as sea lice, that infest farmed fish cause considerable damage and losses (Laird 1996; Saunders 1995). Temperature, dissolved oxygen, current velocity and accumulation of metabolic waste are all important factors in maintaining healthy fish (Monahan 1993; Olafsen and Roberts 1993; Saunders 1995), although farm operators rarely have complete control over them. Preventing pathogens from entering a facility is the best way to reduce the risk of disease in fish but this is more difficult for vertically transmitted diseases such as infectious pancreatic necrosis virus (IPNV) and *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD) (Saunders 1995), which may be transmitted within eggs. The impact of disease can be minimized at grow-out sites through vaccinations, husbandry practices, and proper drug usage.

Seals are serious fish predators and damage salmon cages while attempting to eat the fish within them (Saunders 1995), resulting in injury and stress to fish. Birds have also been known to attack uncovered salmon cages. Injured and stressed fish are often more susceptible to infection which can then result in disease outbreaks.

1.2 Fish Health and Disease

Major constraints to the growth of intensive culture of many fish species have been the mortalities, high costs of therapeutic treatments (Laidley et al. 1988), and increasing government regulations associated with disease. Diseases are frequently triggered by adverse environmental conditions which increase the stress placed on fish, thus increasing their susceptibility to disease (Bruno and Ellis 1996; Laidley et al. 1988). Stress was defined by Brett in 1958 as "a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced" (Barton 1997). Generally, stress is a response in an organism to a stimulus or change in the environment (i.e. stressor) which may affect the fish's homeostasis (Barton 1997; Barton and Iwama 1991; Bruno and Ellis 1996). Water quality (temperature, pH, gas supersaturation, suspended solids, accumulation of metabolic waste products), husbandry practices, stocking densities and poor nutritional value of the feed may be important stressors (Barton 1997; Bruno and Ellis 1996; Dubé and Mason 1995; Saunders 1995).

Disease may be referred to as "any deviation of the body from its normal or healthy state causing discomfort, sickness, inconvenience, or death" (Piper et al. 1989). It is a condition which varies from the normal structure or function of an organism and is considered harmful to the organism (Morris 1992; Stephen and Iwama 1997). Fish disease dynamics tend to be poorly understood due to the lack of understanding of the normal state (Stephen and Iwama 1997). For disease to occur, fish which are susceptible to the disease-

causing agents must be present. Fish may be host to a disease-causing agent but not experience disease.

1.2.1 Factors Influencing Disease Onset in Cultured Fish

Numerous factors influence outbreaks of disease caused by bacteria, viruses, fungi and parasites at an aquaculture facility. Many opportunistic micro-organisms cause disease in fish when environmental conditions are sub-optimal (Laidley et al. 1988; Wedemeyer 1996, 1997) and fish are stressed (Dubé and Mason 1995). Myxobacteria do not typically cause bacterial gill disease unless fish experience gas supersaturation, overcrowding, low dissolved oxygen in the water, or high ammonia (Dubé and Mason 1995). Fish may be latent carriers of infectious pancreatic necrosis virus (IPNV), but when fish are stressed the virus attacks cells of the digestive system and destroys the gut lining and pancreas (Dubé and Mason 1995). Disease due to *Flexibacter columnaris* seldom occurs unless environmental quality deteriorates although it is present continuously in most hatchery water supplies (Piper et al. 1989). As temperatures increase, the oxygen carrying capacity of the water decreases (Landau 1992; Saunders 1995) and free ammonia content in the water increases (Landau 1992). This increases the susceptibility of fish to disease if these poor conditions are maintained for prolonged periods. In addition, pathogen development is often increased in direct relation to temperature (see section 1.5).

In addition to affecting the onset of disease, stressful environments also affect fish metabolism by altering the use of energy (Beamish et al. 1996) and have an inhibitory effect on reproduction (Pankhurst and Van Der Kraak 1997). Suboptimal conditions result in

lower food conversion efficiency and subsequently, decreased growth rates (Beamish et al. 1996; Pickering 1993).

1.2.2 Infectious Diseases of Salmonids

Many infectious agents common in salmonids can create serious disease problems when fish are exposed to sub-optimal rearing conditions (Shepherd 1988). Many diseases of salmon appear to be more severe in farmed than in feral fish. This may reflect the rearing intensity of farmed fish when compared to feral fish (which do not live in close proximity to each other), increasing the risk for spread of infection (Olafsen and Roberts 1993). In addition, farmed fish are subjected to potential stressors not present in wild populations, including transportation, grading and treatment procedures (Bruno and Poppe 1996). However, it is difficult to monitor disease status in wild populations; mortalities and moribund fish are rarely seen.

Bacterial diseases that may significantly impact cultured fish include vibriosis (*Vibrio anguillarum*), furunculosis (*Aeromonas salmonicida*), enteric redmouth (*Yersinia ruckeri*), bacterial gill disease (*Flavobacterium* sp.), and bacterial kidney disease (*Renibacterium salmoninarum*) (Shotts and Nemetz 1993).

Fungi and algae can also cause severe damage to cultured fish. Some diseases caused by these agents are saprolegniasis (*Saprolegnia* spp.), *Ichthyophonus* disease (*Ichthyophonus hoferi*), cerebral mycetoma (*Exophiala salmonis*), and phaeohyphomycosis (*Ochroconis humicola* and *O. tshawytschae*) (Chacko 1993).

In addition to fungal infections, numerous viruses affect salmonids. Salmonid

herpesvirus, infectious pancreatic necrosis virus (IPNV), viral hemorrhagic septicemia (VHS), infectious salmon anaemia virus (ISAV) and infectious haematopoietic necrosis virus (IHNV) are examples (McAllister 1993; Shepherd 1988).

Many parasites infect salmonids and can cause extensive damage to the host. They include both protozoa and metazoa and range from those which cause subclinical infections to those which are lethal. Heckmann (1993) reports an extensive list of parasites of various salmonid hosts. These include protozoans such as *Myxobolus cerebralis* (whirling disease), *Ichthyophthirius multifiliis*, *Loma salmonae* and many others. Parasitic diseases are among the most frequently observed diseases in cultured fish (Stephen and Iwama 1997).

1.2.3 Protozoan Parasites

In the aquaculture industry, infectious disease has a large impact on productivity of fish farms (Bruno and Ellis 1996) because waterborne pathogens spread easily (Saunders 1995). Parasites are organisms which obtain nutrients from other organisms (Landau 1992), at the expense of the host. Obligate parasites are incapable of surviving outside of their hosts (Möller and Anders 1986). Parasitic infections are of major concern in the industry today and fish farms often encounter various agents ranging from protozoa to parasitic crustaceans. Healthy fish in the wild may carry a small parasite load, but these can multiply rapidly in the dense population of fish farms, particularly if fish are stressed by unfavorable environmental factors or by other disease agents (Bruno and Ellis 1996; Landau 1992; Shepherd 1988). Many protozoans are found in salmonids worldwide, with most classified into several phyla: Mastigophora, Sarcodina, Microspora, Myxozoa, Apicomplexa, Ciliata and Cnidosporidia

(Bruno and Ellis 1996).

1.3 Microsporidian Parasites

The phylum Microspora comprises common obligate intracellular parasites of many groups of animals from protozoa to mammals, including humans (Cali and Owen 1990; Canning and Lom 1986; Chilmonczyk et al. 1991; Dyková 1995; Hedrick et al. 1990; Kent 1992; Leiro et al. 1994; Mullins et al. 1994). However, most species of this widespread group of parasites develop in insects and fish (Canning 1990; Canning and Lom 1986; Casal and Azevedo 1995; Kamaishi et al. 1996; Perkins 1991). Fish are the most primitive vertebrates (Kennedy-Stoskopf 1993; Zapata et al. 1996); their limited antibody repertoire may restrict their immune response to pathogens compared to that of mammals (Leiro et al. 1994), thus making them more susceptible to infections. Microsporidians have become increasingly important due to the problems attributed to them in the aquaculture industry. There have also been numerous reports of microsporidians causing problems in human patients with acquired immunodeficiency syndrome (Amigo et al. 1996; Müller 1997).

Microsporidian parasites are primitive (Baker 1994; Vossbrinck et al. 1987), and although their phylogenetic origin is not known, they represent one of the earliest lines of eukaryotes (Canning 1990; Dyková 1995; Kamaishi et al. 1996; Müller 1997; Visvesvara 1996; Weidner 1989). Characteristics of microsporidians are: they have primitive nuclear division, lack mitochondria, and possess unique RNA (Kamaishi et al. 1996; Vossbrinck et al. 1987). Those which infect fish are in the class Microspora and have an extensive

geographical distribution and numerous hosts (Dyková 1995; Schmidt and Roberts, 1989). For example, *Glugea stephani*, a cyst-forming gut pathogen, occurs in 10 species of flatfishes in the Atlantic and Pacific Oceans, and in the Mediterranean and North Seas.

The first report of a microsporidian infection was in 1857 when silkworms developed pébrine disease caused by *Nosema bombycis* infections (Canning 1990; Sleigh 1989), an outbreak that contributed to the decline of the silk industry in Europe. Most tissues in the insect are affected by this parasite (Schmidt and Roberts 1989). In addition, infection with *Nosema apis* is an important cause of mortality in honey bees and continues to threaten the economic survival of this industry (Canning 1990; Manwell 1961; Schmidt and Roberts 1989). Subsequently, there has also been rising awareness of microsporidians due to their increasing prevalence in fish (Hauck 1984; Kent et al. 1989; Morrison et al. 1990; Mullins et al. 1994) and human immunodeficiency virus patients (Didier and Bertucci 1996; Field et al. 1996) and also because of improved diagnostic techniques (Leiro et al. 1994).

1.3.1 Aquaculture Facilities

Many microsporidian species cause severe disease in fish in aquaculture facilities (Brocklebank et al. 1995; Leiro et al. 1994). Infections occur in marine (e.g. *Glugea stephani* or *Pleistophora priacanthusis*), estuarine, and fresh water (e.g. *G. plecoglossi*, *Microsporidium takedai* or *P. anguillarum*) environments (Dyková 1995). In addition to the many species which infect fish (Brocklebank et al. 1995; Dyková 1995; Hedrick et al. 1990; Kent 1992; Wongtavatchai et al. 1995), a few also infect shellfish (Bower 1995; Sindermann 1990). Similarly to insects, the risk of outbreaks in fish populations increases if the

population density is high (Canning 1990). There have been reports of microsporidian infections in fry and yearling fish in both natural and hatchery-reared populations (Dyková 1995; Hauck 1984). Hatchery conditions, such as water temperature and crowding, tend to favour transmission of the parasite and may provoke severe mortalities (Leiro et al. 1994). Microsporidian infections may limit growth of fish stocks (Bruno et al. 1995; Cali et al. 1986), causing overall reduction in productivity in wild-caught and hatchery-reared fish. They have been implicated in the collapse or decline of several natural fisheries (Matthews and Matthews 1980).

Microsporidians such as *Loma salmonae* and *Enterocytozoon salmonis* cause disease in farmed salmon leading to devastating losses. Market-size fish are often affected by these pathogens, increasing financial losses. Diseases currently emerging as farm problems may originate from wild fish in the vicinity of sea cages which act as reservoirs for many pathogens (Kent et al. 1998; Saunders 1995). After infection occurs inside a sea cage, transmission of many pathogens to other fish is facilitated by high stocking densities, optimal temperatures, and other environmental factors within the netpens.

1.3.2 Microsporidian Life Cycle

Microsporidians are characterized by the production of highly resistant spores that are infective to the host (Canning and Lom 1986; Pleshinger and Weidner 1985) and have an elaborate extrusion apparatus (Dyková 1995; Müller 1997). The spores do not have a polar capsule (Brocklebank et al. 1995) or mitochondria and the polar tube lies free in the sporoplasm (Fig. 1), unlike myxosporidians (Canning and Lom 1986; Dyková 1995; Kent,

1992; Visvesvara 1996).

The natural mode of entrance into the fish host is presumably by spore ingestion, although experimental transmission can occur via intramuscular or intraperitoneal injections (Dyková 1995; Speare et al. 1998b) (Fig. 2). More recently, Shaw et al. (1998) demonstrated that fish can be successfully infected intravascularly and by anal gavage exposure to spores. After ingestion, spores are typically activated during movement along the gut of the appropriate host (Dyková 1995; Pleishinger and Weidner 1985). In the digestive tract of the host, an appropriate stimulus causes high pressure to build up within the spore. Pleishinger and Weidner (1985) and Weidner (1989) suggest that a shift from acid to alkaline pH, calcium bound to the spore wall, and the presence of polyanions are necessary for the activation of *Spraguea lophii* spores. Weidner (1989) also stated that the glycoprotein mucin, bound to this and other microsporidian spores, is essential to activate *S. lophii* spores and is also found in the gut epithelium of most hosts. Frixione et al. (1994) suggest an increase in osmotic pressure within the spore, perhaps stimulated by the splitting of trehalose or an analogue into smaller sugars, causes an uptake of water until the spore breaks open. The apolar vacuole swells when the spore is activated to provide part of the force for the sporoplasm extrusion (Weidner 1989). The polaroplast, consisting of a highly folded membrane wrapped around part of the polar filament, swells and eventually the pressure breaks the polar tube and everts it through the spore wall (Dyková 1995). The sporoplasm is then propelled through this stiff tube into the nearest host cell, injecting the sporoplasm into the host cell cytoplasm, initiating a new infection (Amigo et al. 1996; Canning 1990; Canning and Lom 1986; Frixione et al. 1994; Pleshinger and Weidner 1985; Weidner 1989).

The sporoplasm (and host cell) is apparently engulfed by a macrophage and carried to the final site of development, although spores may develop directly in intestinal epithelial cells (Dyková 1995). Two stages occur in the host: merogony (or schizogony) and sporogony (Fig. 3). Merogony is the asexual production of numerous parasitic stages called meronts (Canning 1990; Canning and Lom 1986; Schmidt and Roberts 1989). Meronts (or trophozoites) divide by binary or multiple fission and, when not confined by a xenoma wall, may spread from one cell to another in infected tissue (Canning 1990). Spores are produced during sporogony, presumably by binary fission (Morrison and Sprague 1981). Sporonts, intermediate stages, produce sporoblasts in one of two ways. They may divide by binary fission to produce sporoblasts directly, or they may first grow into a multinucleate plasmodial sporont. This sporont then produces sporoblasts by multiple fission or fragmentation, or by producing intermediate sporoblast mother cells (Canning and Lom 1986; Dyková 1995). To complete the cycle, these produce sporoblasts that mature into spores through spore morphogenesis, which involves the formation of a thick wall consisting of a proteinaceous exospore and a chitinous endospore surrounding the spore organelles (Canning 1990). Although not definite, microsporidia may not have haploid stages in their life cycle (Loubés 1979).

The host cell enlarges and absorbs nutrients from surrounding tissue to accommodate the developing parasite (Canning and Lom 1986), eventually being destroyed during the infection because it provides the necessary nutrients for the growing parasite. The host cell nucleus synthesizes new proteins, allowing the host cell to enlarge to accommodate the growing parasite which relies on host organelles (Canning 1990). Initially, some parasites

are surrounded by a membrane which is apparently of host origin, forming a parasitophorous vacuole in which the parasites divide and produce spores (Canning 1990). At the onset of sporogony a wall of unknown origin and external to the parasite's plasma membrane becomes visible (Canning 1990). This xenoparasitic complex, formed by the morphological and physiological integration of parasites and infected host cells, results in the formation of distinct entities called xenomas (Dyková 1995; Leiro et al. 1993; Morrison and Sprague 1981). Within xenomas, various stages of development occur (Canning and Lom 1986; Morrison and Sprague 1981), including meronts, sporoblasts and spores (Morrison and Sprague 1981) (See Fig. 3 for spore details). Some species of microsporidians cause infected host cells to become hypertrophic with enlarged nuclei and surface modifications (microvilli, invaginations or a thick wall).

How the sporoplasm reaches its final site of development is unknown although this may occur far from the original site of hatching in the gut (Canning and Lom 1986). Perhaps macrophages, undifferentiated mesenchyme cells and body fluids aid in distribution, after which the sporoplasm undergoes further development into meronts (Canning and Lom 1986).

Loma salmonae has uninucleate meronts, a trait common with other species of *Loma* and other microsporidians such as *Tetramicra brevifilum*, a parasite of turbot (*Scophthalmus maximus*) (Canning and Lom 1986; Hauck 1984) (Fig. 3). Merogony in *L. salmonae* is by multiple fission. The plasma membrane of this parasite is simple compared to other microsporidia that have specializations such as an electron-dense coat (*Glugea*) or an electron lucent amorphous coat (*Pleistophora*). Sporogony in *Loma* spp. and most other

microsporidian genera is polysporoblastic within a sporophorous vacuole (*L. salmonae* vacuoles each contain 2 - 4 spores). Species of *Spraguea* and *Nosema*, however, undergo sporogony free in the cytoplasm (Canning and Lom 1986). *Loma salmonae*, in addition to species of the genera *Glugea*, *Ichthyosporidium*, *Tetramicra* and *Spraguea*, forms xenomas in the host; other microsporidians, including species of *Pleistophora*, develop diffusely in various tissue of their hosts (Canning and Lom 1986).

1.3.3 Diagnosis

Identification of microsporidian infections in fish is by microscopic detection and examination of spores. The large posterior vacuole is easily identified in live spores which can be collected from xenomas or streaks within muscle tissue (Dyková 1995). Diagnosis can also be confirmed by microscopic examination of impression smears or wet mounts from infected tissues (Gratzek 1993). Microsporidian species may be differentiated by characteristics such as the size of spores, number of turns in the polar filament, the host and tissues affected, and geographical location (Kent et al. 1989; Morrison and Sprague 1983). More recently, molecular techniques involving a polymerase chain reaction (PCR) assay were developed to detect *L. salmonae* in chinook salmon (Docker et al. 1997) and other microsporidia in humans who are HIV-infected (Fedorko and Hijazi 1996).

Clinical signs of infection include inflammation, ascites, exophthalmia, haemorrhagic fins, impaired swimming and darkened tails (Hauck 1984). These, coupled with the above traits of the parasitic species, are used to diagnose a microsporidian infection. For microsporidia to be diagnosed as the cause of a syndrome or disease, several criteria must

be met: exposure to the pathogen must occur before signs of disease are evident: there must be a strong association between the pathogen and the disease (i.e. several signs of disease present); and findings must be consistent with other studies or observations. Eliminating the cause of disease should result in lower incidence of disease (Martin et al. 1987).

1.3.4 Treatment

Chemical treatments are often used for both prevention and treatment of various diseases of fish. However, chemotherapeutants are not often used for treating microsporidian infections in fish and none are approved in Canada for use in farmed fish (Brocklebank et al. 1995; Mullins et al. 1994). Therefore, we currently rely on husbandry techniques to prevent fish from becoming infected. However, this often proves to be ineffective, especially in sea water cages where there is very little environmental control.

Fumagillin DCH, an antimicrobial agent that apparently acts by inhibiting RNA synthesis, has been used experimentally to treat some microsporidians of fish (Hedrick et al. 1991; Kent and Dawe 1994) and humans (Didier 1997) and some myxosporidians (Hedrick et al. 1988; Wishkovsky et al. 1990). It is produced by the fungus *Aspergillus fumigatus* and inhibits replication of *Encephalitozoon cuniculi in vitro* (Didier 1997). The drug's effects against myxosporidial and microsporidial infections in fish were demonstrated by Hedrick et al. (1988, 1991). In the latter study, mortality of chinook salmon due to *Enterocytozoon salmonis* was arrested and the severity of infections was delayed in treated fish, indicating a temporary static effect by the chemotherapeutant rather than eliminating the parasite completely. However, later reports found adverse effects including haematopoietic cell

depletion in the kidney, vacuolation of the renal epithelium, and spleen and kidney size reduction in chinook salmon and rainbow trout (Hedrick et al. 1988, 1991; Kent and Dawe 1994; Wishkovsky et al. 1990).

Historically, fumagillin DCH has been used to treat microsporidian infections in insects and amebiasis in humans. It has been used to treat honey bees and eels for their respective microsporidial infections, *Nosema apis* and *Pleistophora anguillarum* (Kano et al. 1982; Kent and Dawe 1994; Mullins et al. 1994). A significant limitation to the use of this drug is that parasiticidal concentrations may be toxic/lethal to the host (El-Matbouli and Hoffmann 1991; Hedrick et al. 1988; Wishkovsky et al. 1990). High toxicity is also found when combinations of high concentrations and prolonged exposure are used to treat human microsporidial infections (Didier 1997), therefore restricting its use to topical infections. The drug has been tested experimentally on chinook salmon infected with *Loma salmonae (*Kent and Dawe 1994). To date, however, no chemotherapeutants have proven to be effective agents for treating microsporidia of farm-reared salmon (Kent and Dawe 1994; Kent et al. 1989).

TNP-740, an analog of fumagillin DCH, was as active as fumagillin in inhibiting tumour growth in experimental mice, and was less toxic *in vitro* (Didier 1997). Microsporidiosis in humans (Didier 1997) and perhaps cultured fish may be treated using this analog which increases proliferation of B lymphocytes. An indication that microsporidians have different metabolism than other protists is the ineffectiveness of drugs against microsporidians which work against other protistan parasites (Müller 1997). Although their energy metabolism has yet to be biochemically explored, microsporidians

lack mitochondria and have a modified Golgi apparatus (Canning and Lom 1986; Müller 1997).

1.3.5 Tissue Reactions to Xenomas

When xenomas form in a host, a 3-stage tissue reaction occurs (Canning and Lom 1986; Dyková 1995). A weak reactive stage occurs first although it may overlap later stages. An early xenoma forms and is filled with developmental stages of the parasite and there is no observed host cell reaction to the pathogen at this point (Canning and Lom 1986; Dyková 1995). Damage from the developing xenoma to surrounding tissue stimulates connective tissue proliferation which forms a layer around the xenoma. Subsequently, host inflammatory reactions are directed at isolation and elimination of spores followed by host tissue repair (Canning and Lom 1986; Dyková and Lom 1980).

Granulomas develop during the productive stage via proliferative inflammation (Dyková and Lom 1980; Dyková and Lom 1978). This stage is characterized by a mature xenoma filled with spores. Changes during this stage result in the complete disappearance of the xenoma wall which becomes a granuloma filled with spores. Mature xenomas elicit proliferative inflammation, its onset being linked to changes in the xenoma wall, which appears swollen (Canning and Lom 1986). Fibroblasts also appear within the laminar structure. This results in complete destruction of the xenoma wall. Granulation tissue invades the xenoma, turning it into a granuloma.

In the final stage where granuloma involution occurs, the xenoma wall is all that remains of the host cell components (Canning and Lom 1986; Dyková 1995; Dyková and

Lom 1980). Microsporidian xenomas are short-lived and are typically eliminated by phagocytosis of spores; there is subsequent necrosis of macrophages (Kent et al. 1989). There is gradual resolution of lesions in which the tissue is usually repaired but original function is not restored in all microsporidians (Dyková 1995; Hauck 1984). All spores will be destroyed by the host inflammatory reaction except those which are discharged to the external environment (Canning and Lom 1986; Dyková and Lom 1980; Kent et al. 1989). Xenomas that rupture at the surface of the gills are apparently released into the water, allowing for quick healing of lesions.

A *Loma* sp. described by Hauck (1984) caused infected chinook salmon to develop exophthalmos, ascites, haemorrhagic pyloric caeca and fins, petechiae on opercula and skin, and darkened tails. Focal degeneration and necrosis were also seen in the cartilage of the head. *Loma salmonae* infections in some coho salmon caused granuloma formation in the heart, spleen kidney and pseudobranch (Kent et al. 1989). However, Kent et al. (1989) also found the inflammatory reaction in these fish to be more diffuse than other studies. Apparently tissue reactions to *L. salmonae* are most severe when xenomas rupture, releasing spores (Kent et al. 1995).

1.3.6 Phagocytosis of Spores

Phagocytosis is a tissue reaction which may help eliminate spores produced during the course of infection (Dyková and Lom 1980; Hauck 1984). Phagocytic cells, including monocytic cells and polymorphonuclear cells, may contribute to host defence by eliminating spores produced during an infection (Canning and Lom 1986; Dyková and Lom 1980; Leiro

et al. 1996). When muscle disintegrates in non-xenoma forming species (e.g. *Pleistophora* spp.), migratory phagocytic cells ingest the parasitophorous vacuole surrounding the spores (Dyková and Lom 1980). Microsporidians may control the host phagocytic response, eventually decreasing the antibody response which would destroy them (Leiro et al. 1996), as a partial means of developing and transmitting between fish.

The efficiency of this defense response depends on suitable conditions, including the physiology of the host and favourable environmental conditions (Dyková and Lom 1980; Hauck 1984). Temperature affects both humoral response and phagocytosis (Dyková and Lom 1980; Finn and Nielsen 1971). Low temperatures delay or inhibit macrophage activity and response, fibroplasia and also impede parasite development. The extent of lesions probably depends on the ability of early developmental stages of the parasite to spread within the host (Dyková and Lom 1980). For example, temperatures lower than optimal affect the development of *Enterocytozoan salmonis* in chinook salmon. Infections and mortalities of salmon were severe at high temperatures whereas at low temperatures, infections were very mild and few mortalities resulted (Antonio and Hedrick 1995). Olson (1981) reported similar results with *Glugea stephani*; temperatures above 15°C are required for *G. stephani* to develop normally in English sole and few effects are seen at lower temperatures.

1.4 *Loma salmonae*

The genus *Loma* includes species of microsporidian parasites of economic importance in salmonid aquaculture. Recently, many mortalities on several fish farms have

been attributed to *L. salmonae*. Major losses of rainbow trout and sockeye salmon were attributed to *L. salmonae* infections (Bruno et al. 1995). Coho mortalities attributed to *L. salmonae* infections have been greater than 30% in some years in netpens (Kent et al. 1989).

1.4.1 Distribution

Members of the genus *Loma* are typically found in endothelial and pillar cells of the gill filaments or lamellae (Dyková 1995), but can develop to a lesser degree in internal organs including heart, spleen, kidney and pseudobranchs (Fig. 2) (Kent and Dawe 1994; Kent et al. 1995; Markey et al. 1994). Species of *Loma* are transmitted in both freshwater and seawater (Kent et al. 1995) and affect all life stages of fish hosts from hatchery fish to market-size fish. Recently, infections in farm-reared chinook and coho salmon have been detrimental to Canadian West coast aquaculture. This group of parasites occurs throughout North America, Japan, France and Scotland (Dyková 1995; Kent et al. 1989). *Loma salmonae* is widespread in wild and hatchery-reared salmonids. The parasite has caused mortality in several species, including rainbow and steelhead trout, sockeye salmon (*Oncorhynchus nerka*), chinook salmon, masu salmon (*O. masou*) and coho salmon.

Loma salmonae was first reported in Scotland by Bruno et al. in 1995 and has also been reported in England in farm-reared rainbow trout and wild brown trout (Poynton 1986). The first report of a *Loma* sp. in Western Canada was in 1987 where juvenile coho salmon in Vancouver were infected (Magor 1987). Xenomas, which are visible in the gills (Fig. 4), spleen, kidney, and pyloric caeca, contain numerous spores and vegetative stages (Bruno et al. 1995). Xenomas are surrounded by a thin epithelium and form close to or within the

pillar system (Bruno et al. 1995; Morrison and Sprague 1981). Secondary lamellar fusion and the development of hyperplastic regions often occur in infected fish.

Earlier, *L. salmonae* was considered to be a freshwater pathogen although infections could persist after seawater transfer (Hauck 1984; Kent 1992). However, recently the parasite was also transmitted in seawater (Kent and Dawe 1994; Kent et al. 1995). Epizootics may therefore occur when smolts carry infections to seawater and provide the opportunity for transmission to uninfected fish (Kent et al. 1995). Xenomas are released from dead fish during the rapid tissue disintegration that occurs after death (Kent et al. 1995). Uninfected fish may feed on mortalities, thus providing another mechanism for disease transmission.

In freshwater, *L. salmonae* infections in coho salmon are typically mild with little disease; most affected fish do not exhibit clinical signs (Kent et al. 1989). Damage is primarily caused by the inflammatory response to dispersed spores in the primary lamellae vasculature (Kent et al. 1995; Kent et al. 1989; Speare et al. 1998b). Rainbow trout with many branchial xenomas, reared in fresh and sea water, have suffered both tissue damage and mortality (Kent et al. 1989). Fish with severely damaged gills may exhibit respiratory distress. Xenomas in freshwater hosts are found primarily in secondary lamellae and are not associated with disease or tissue damage (Kent et al. 1989). Although disease is typically mild, Hauck (1984) found that severe infections can develop in chinook salmon and rainbow trout in freshwater.

In seawater, severe inflammatory lesions were observed in infected netpen-reared coho salmon (Kent et al. 1989). Xenomas developed in both the primary and secondary

lamellae, with those in the vasculature of the primary lamellae having some ruptured xenomas in their interstitium. In both environments and all host species, hyperplasia of the gill epithelium is often observed (Hauck 1984; Kent et al. 1989; Markey et al. 1994; Poynton 1986).

1.4.2 Effects on Infected Fish

During *L. salmonae* infections, gill filaments can become distorted, secondary lamellae fuse and there is epithelial hypertrophy (Fig. 5) characterized by swollen epithelial cells (Hauck 1984; Speare et al. 1989). Lamellae are often damaged by the presence of xenomas within them although little inflammation occurs when xenomas develop in pillar cells (Kent et al. 1995). Severe gill lesions have been observed in pen-reared chinook and coho salmon (Kent 1992; Kent and Dawe 1994; Speare et al. 1989). Damage done to the gills may cause functional problems such as decreased oxygen and electrolyte exchange because water does not pass closely to the blood (Poynton 1986; Speare et al. 1989). Xenomas developing in secondary lamellae of the gills cause relatively little damage and inflammation, whereas those that form within blood vessels of the primary lamellae can cause severe inflammation and tissue destruction (Speare et al. 1989). Spores released into surrounding tissue (Fig. 6) remain there until eliminated by the host immune/ inflammatory response (Kent et al. 1995).

Severity of anemia has been correlated with the intensity of *L. salmonae* infections (Dyková 1995). Thrombosis and vasculitis have been associated with xenomas (Hauck 1984; Speare et al. 1989). Other tissue alterations may include inflammation, necrosis, and

occlusion of arteries (Hauck 1984). Degeneration and necrosis of cartilage and muscle of the tail and head have also been noted which probably damage nervous tissue, causing loss of control of the posterior chromatophores and intense darkening of the tail (Hauck 1984). High mortalities in chinook salmon have been caused by systemic infections with a *Loma* sp. (Hauck 1984; Speare et al. 1989). Severe infections could impair swimming efficiency, reduce growth rate, and increase mortality.

1.4.3 Other *Loma* spp.

In addition to *L. salmonae* in salmon and in the wild sculpin *Cottus* sp. (Markey et al. 1994), there are several other species of *Loma*. *Loma fontinalis* is found in brook trout (*Salvelinus fontinalis*) in Canada and most xenomas are in the gills of infected fish (Canning and Lom 1986; Kent et al. 1989).

A species of *Loma*, appearing as white cysts, infects vascular endothelial cells of shiner perch (*Cymatogaster aggregata*) (Shaw et al. 1997). This non-salmonid fish has been suggested as a reservoir host for *L. salmonae* infections acquired by seawater-reared salmon because they are frequently found around netpens in British Columbia. The species of *Loma* found in this fish, however, was unique by geographic, morphologic, and molecular (rDNA) characters, and by host (Shaw et al. 1997).

Loma morhua develops in the gills of Atlantic cod, *Gadus morhua* (Canning and Lom 1986; Morrison and Sprague 1981) while *Loma diplodae* and *Loma dimorpha* infect fish hosts along the Mediterranean coast (Canning and Lom 1986). *Loma camerounensis* sp. nov. is found in tilapia in Africa (Canning and Lom 1986; Fomena et al. 1992).

1.5 Thermal Regulation

Temperature has a regulating effect on many aspects of fish development, immunological functions and on parasite development.

1.5.1 Effect of Temperature on Fish Development

Most fish are poikilotherms and have specific environmental temperature requirements. Water temperature regulates fish metabolism and length of developmental stages. For example, at 9°C development of rainbow trout from embryo to adult takes approximately 18 months. This time is halved when the temperature is doubled to 18°C (Dubé and Mason 1995). Rainbow trout, and other salmonids, have different optimal temperature requirements for each stage of development. Initial incubation requires temperatures of 6-8°C whereas spawning requires water at 10-13°C. The range in which this species can survive is 0.6-26°C but the optimal range is 10-16°C (Dubé and Mason 1995). Beyond this food conversion efficiency decreases although fish have an increased growth rate at 18°C. Susceptibility to disease also increases at both the higher and lower temperature extremes. Slower metabolism and a marked decrease in growth rate in rainbow trout occur as temperatures approach freezing (Dubé and Mason 1995).

1.5.2 Effect of Temperature on Immune Function

In poikilothermic fish, cellular and humoral immune responses are temperature-dependent (Finn and Nielsen 1971; Rijkers et al. 1980). Temperature limits placed on fish

development are also placed on the immune response of fish (Rijkers et al. 1980). The first phase of an immune response, antigen processing and recognition, is independent of water temperature (Rijkers et al. 1980). At low temperatures, T-helper activity is blocked (Miller and Clem 1984) or there may be an increase in T-suppressor activity and antibody production and release by plasma cells diminishes (Rijkers et al. 1980). Temperature also affects the time of appearance of macrophages within skeletal muscle with response occurring to a greater extent and earlier at warmer temperatures (Finn and Nielsen 1971). Fibroplasia onset is delayed at cooler temperatures.

Manipulation of environmental water temperature can control various diseases (Finn and Nielsen 1971). Infectious haematopoietic necrosis of sockeye salmon has been controlled in hatcheries by increasing water temperature (Finn and Nielsen 1971).

1.5.3 Effect of Temperature on Parasite Development

Water temperature affects the development of fish and their immune function, and the development of parasites. Numerous trials have outlined a common trend. Generally, as water temperature increases, the rate of parasite development increases. For example, the highest prevalence of gill infections with *Loma salmonae* in coho salmon occurred during the summer months (Kent et al. 1989). Appearance of infection and disease was most prevalent in the summer months, regardless of time of seawater entry (Kent et al. 1989). This promotion of development may be due to warmer temperatures typically found in the summer and fall.

Moreover, temperatures above 15°C are required for complete development of

Glugea stephani in English sole (*Parophrys vetulus*) (Olson 1981). This microsporidian is restricted to pleuronectid fish that occupy warmer estuarine waters; temperatures less than 10°C do not permit development of the parasite. If the temperature increases to permissive levels, development will resume if the parasite has already become established in the fish host. If fish were infected shortly before they were moved or migrated to cooler waters, the infection may not establish, preventing the parasite from continuing its life cycle.

Antonio and Hedrick (1995) also evaluated the effect of water temperature on the progression of *Enterocytozoon (=Nucleospora) salmonis*, found in the nuclei of mononuclear leukocytes of fresh and sea water reared fish, in chinook salmon (*Oncorhynchus tshawytscha*). The optimal range for parasite development in chinook salmon is 15° - 18°C. At low temperatures (9°C), progression was slow and infections were mild. When these fish were transferred to a higher temperature (15°C), infection progressed rapidly and mortality became severe. If fish underwent a smaller upward temperature shift (i.e. from 12° to 15°C), the increase in mortality was not significant. Water temperature may be affecting either the development of the parasite or the immune response of the host, or both (Antonio and Hedrick 1995).

Temperature also affects the development rate of microsporidians in hosts other than fish. Daphnids infected with *Pleistophora intestinalis* developed very weak infections at 6°C while infections became intense at temperatures of 12°, 16°, 20° and 23°C (Ebert 1995). This suggests the parasite is inhibited at low temperatures.

Additional to microsporidia which are affected by environmental water temperature in their fish hosts, other groups of parasites are also affected. Lom (1979) suggested that the

seasonal occurrence of disease caused by the flagellates *Trypansoma* spp. in fish was caused by the temperature requirements of the parasite.

The timing of proliferative kidney disease (PKD), caused by a myxozoan, is affected by water temperature, with seasonal outbreaks (Foott and Hedrick 1987). Peak prevalences occurred in July, and when the largest number of renal interstitial parasites occurred, pathology was also the most severe (Foott and Hedrick 1987). Additionally, morbidity and mortalities decline as water temperatures decrease because the myxosporidian parasite is inhibited, also suggesting the development of this parasite is temperature-dependent (Ferguson 1981). Temperature modulates the host defense response and therefore may act directly on the host-parasite relationship (Foott and Hedrick 1987). Ferguson (1981) also indicated that development of PKD is closely associated with water temperatures although recovery from the disease is less so. Clifton-Hadley et al. (1986) found fish at low water temperatures (9° and 12°C) did not develop renal swelling characteristic of this disease while fish at 15° and 18°C developed fully swollen kidneys.

Parasitic crustaceans, including sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*), are affected by water temperature. The number of eggs produced by these two species are dependent on environmental and host parameters, primarily water temperature (Johannessen 1978; Johnson and Albright 1991). Low salinities and low water temperatures markedly reduce egg hatching success. Developmental rate of eggs and the non-feeding stages are primarily controlled with water temperature, although other factors may be important. Developmental rate of attached stages for both species increases as temperature increases, ranging from 3 to 13 weeks.

1.6 Disease Resistance

Epizootics of many fish diseases have resulted from intensive culture of many fish species (Woo 1987). Few chemical treatments are available against many of these and therefore, alternative control mechanisms are necessary.

Rainbow trout that recovered from proliferative kidney disease (PKD) were resistant to reinfection (Clifton-Hadley et al. 1986; Foott and Hedrick 1987). Those fish which survived the initial infection also carried sporogonic stages of the parasite but not fully formed spores. PKD is caused by a myxosporidian parasite, PKX (unclassified) and is of importance to salmonid fish farmers in Europe and North America (Foott and Hedrick 1987). Infected fish exhibit kidney hypertrophy, and histologically show hyperplasia, tubular atrophy, leukocytic infiltration, and granulomatous nephritis (Foott and Hedrick 1987). Ferguson (1981) also found that rainbow trout were resistant to PKD after recovery from the initial infection. Foott and Hedrick (1987) suggest that the majority of the observed protection in these resistant fish may arise from cell-mediated immunity against the PKX parasite.

Fish develop acquired immunity in response to parasites from various major protozoan groups, including microsporidians (Estevez et al. 1992; Leiro et al. 1993). They have both specific and non-specific responses against pathogens (Woo 1987). Monocytes, granulocytes and thrombocytes are non-specific phagocytic cells in fish (Woo 1987). Responses by these cells occur before the specific immune system is activated (Woo 1987). The elicitation of both primary and immune memory responses is affected by temperature,

unlike the immune system of mammals (Woo 1987). Catfish infected with *Ichthyophthirius multifiliis* are protected from further infections for up to 8 months if they recover from the initial infection (Woo 1987) and immunity appears to be against the cilia of the parasite. Similar results were found in rainbow trout that recovered *Cryptobia salmositica* (haemoflagellate) infections (Laidley 1988). Fish vaccinated against *C. salmositica* were protected 24 months after vaccination (Li and Woo 1995). Although results obtained on vaccination against *C. salmositica* are encouraging, there are currently no live attenuated or killed vaccines for use against protozoal diseases in fish (Woo 1987).

1.7 Research Objectives

Diseases are one of the largest concerns facing the fish farming industry today. Fish farmers must find every mechanism possible to decrease production costs in a competitive industry. One way to achieve this is by disease control. However, to control many diseases, biological characteristics of the pathogen must be determined.

Little is known about the pathobiological characteristics of *Loma salmonae*, making control and prevention of outbreaks difficult. This study was completed to reveal some essential biological facts necessary for controlling infections in farm-reared salmonids.

The specific objectives of this study include:

1. Investigation of the permissive temperature range of *L. salmonae* in rainbow trout and the effect of temperature on developmental rate of the parasite.
2. Investigation of the effect of prolonged cool temperatures on the development of *L.*

salmonae infections in rainbow trout.

3. Determination of the effect of temperature on induced resistance to *L. salmonae* in rainbow trout.

4. Development of a summation equation based on degree-days to predict *L. salmonae* xenoma onset.

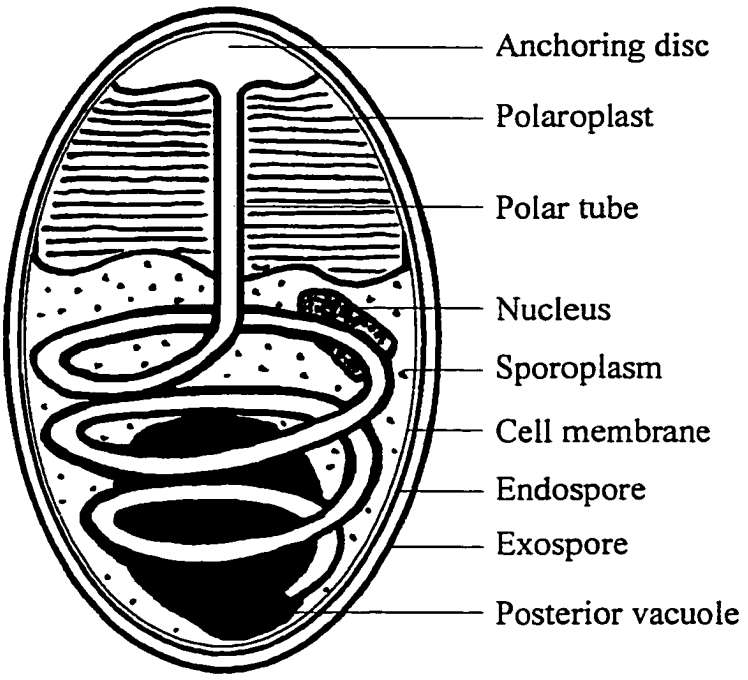


Fig. 1 Generalized diagrammatic representation of a microsporidian spore with contents: anchoring disc, polaroplast, polar tube, nucleus, sporoplasm, cell membrane, endospore, exospore and posterior vacuole.

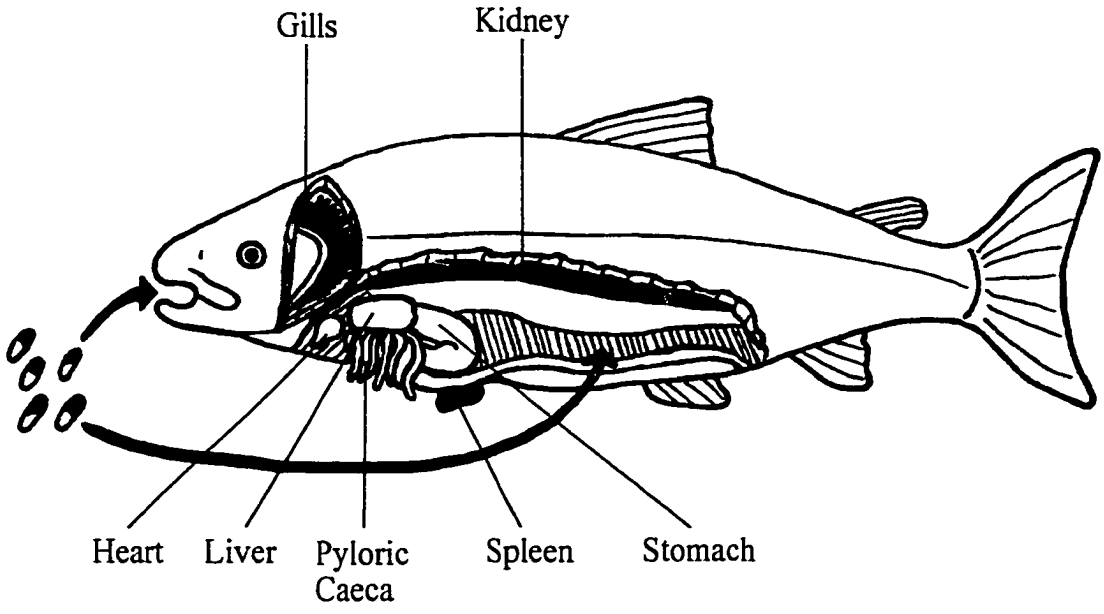


Fig. 2 Diagram of a fish receiving microsporidian spores via oral ingestion (natural route), intraperitoneal injection or intramuscular injection. Several organs which may become infected with microsporidians include the heart, liver, pyloric caeca, spleen, stomach, kidney and gills. (Adapted from Ellis et al. 1989).

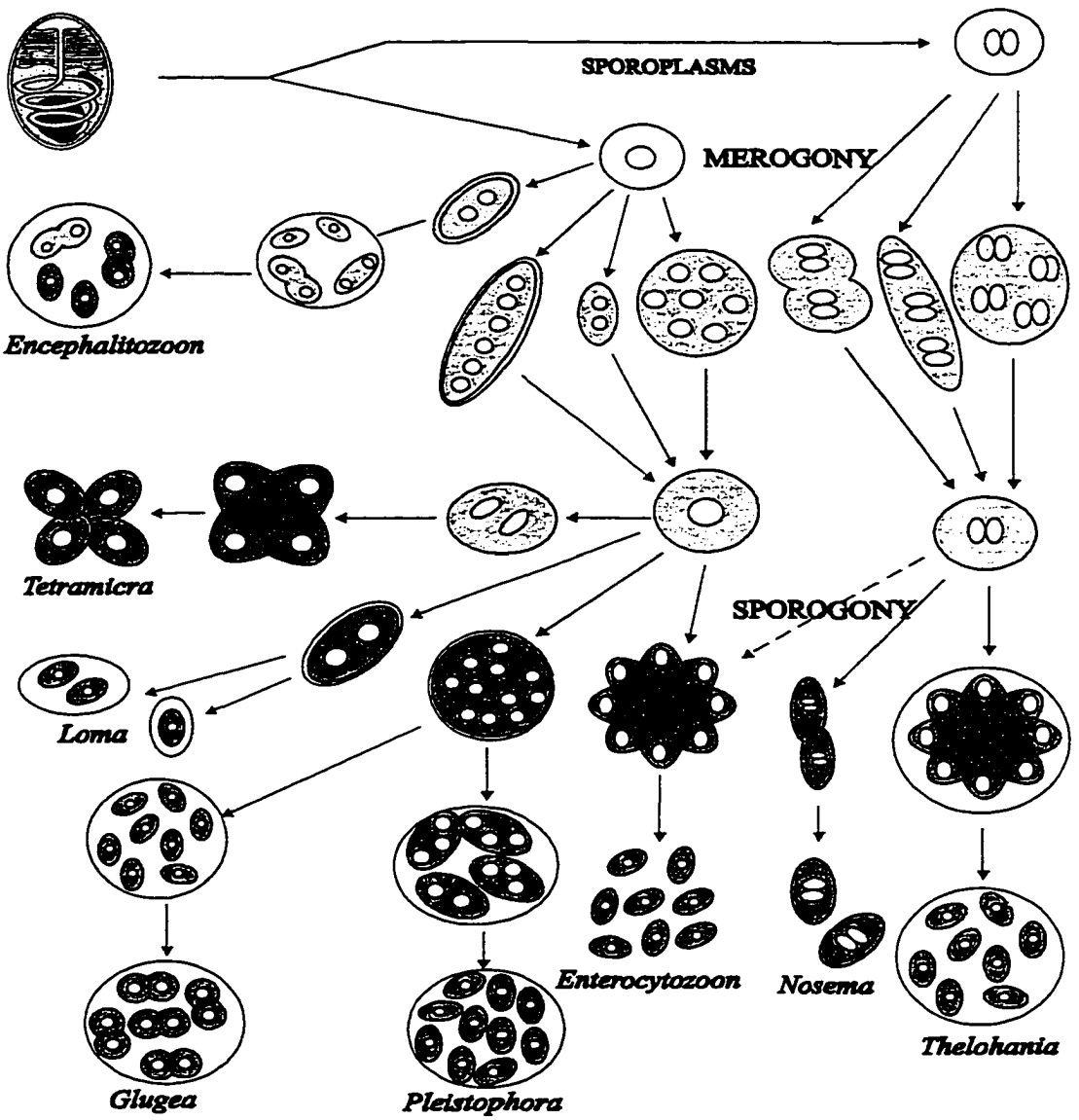


Fig. 3 Diagram of the life cycle of several microsporidians that infect vertebrates. Sporoplasms are released from the spore through the polar tube. Light shading represents merogonic stages and dark shading represents sporogonic stages. (Adapted from Canning and Lom 1986).

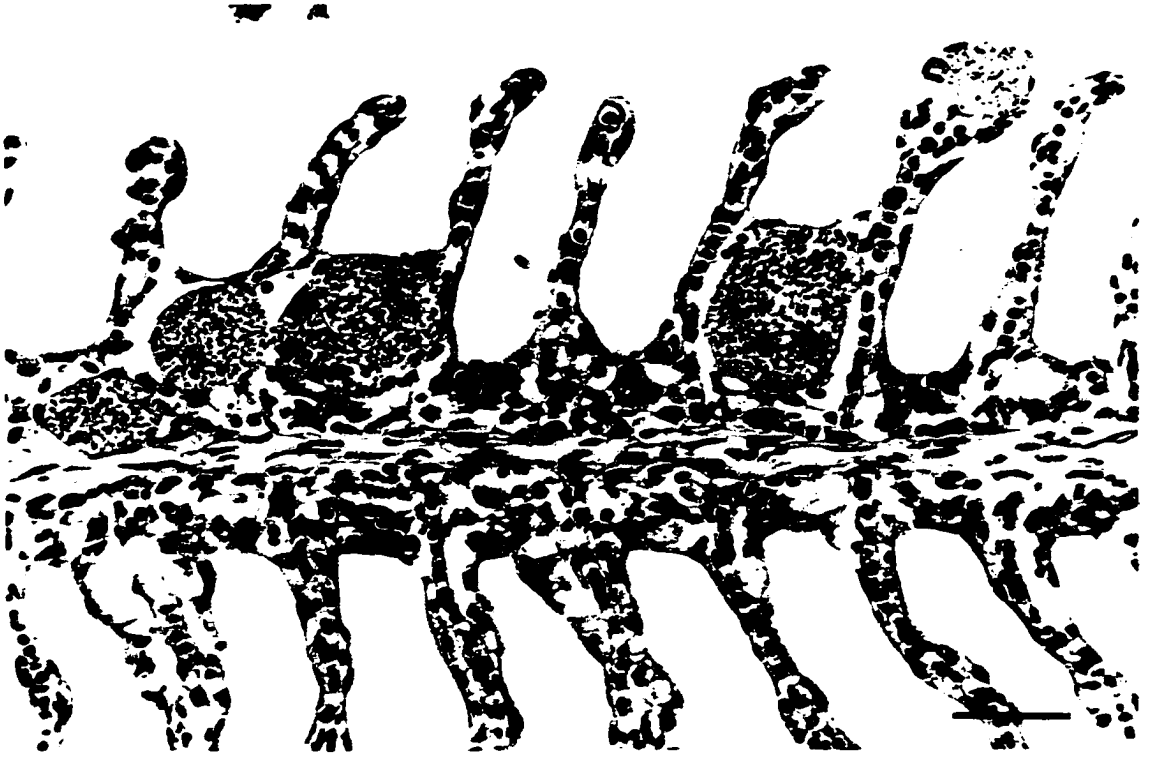


Fig. 4 Histological section of rainbow trout gill infected with *Loma salmonae*. Shown are several large parasitic xenomas containing many spores. H&E. Scale bar = 50 μm

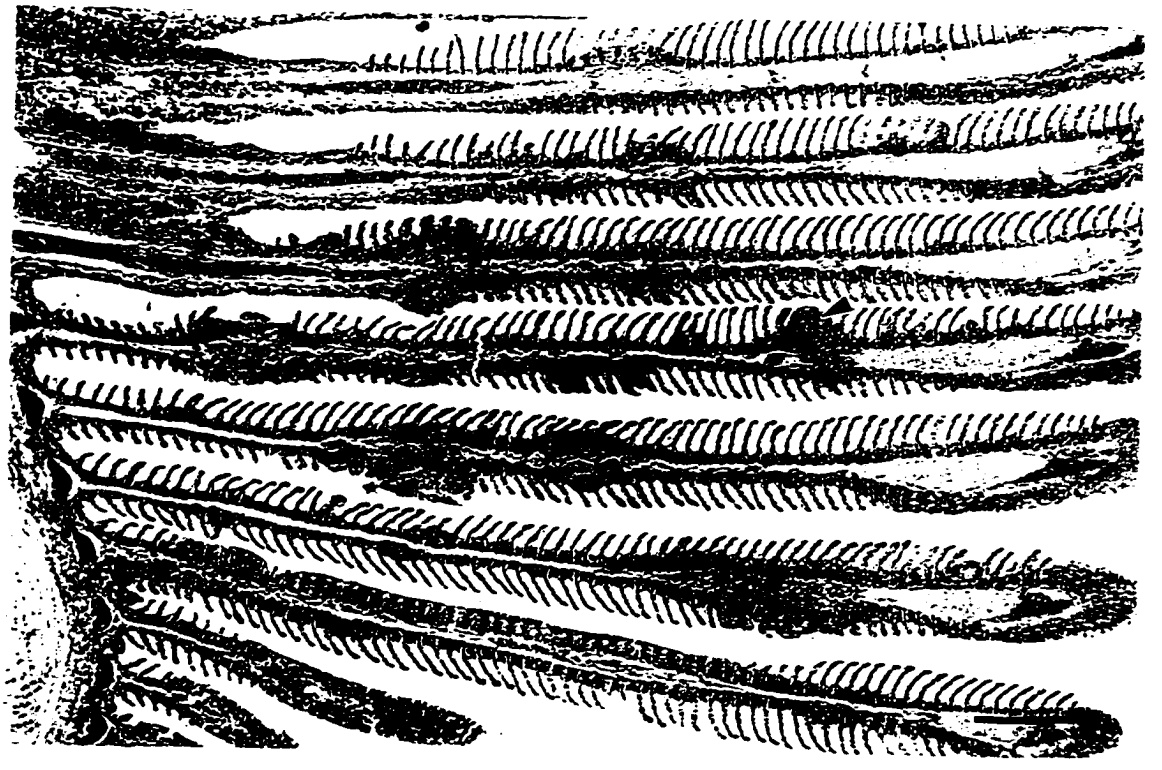


Fig. 5 Histological section of a *Loma salmonae*-infected rainbow trout gill showing epithelial hyperplasia. Lamellae show several thickened areas of epithelial tissue (typical example shown by arrowhead). H&E. Scale bar = 350 μm

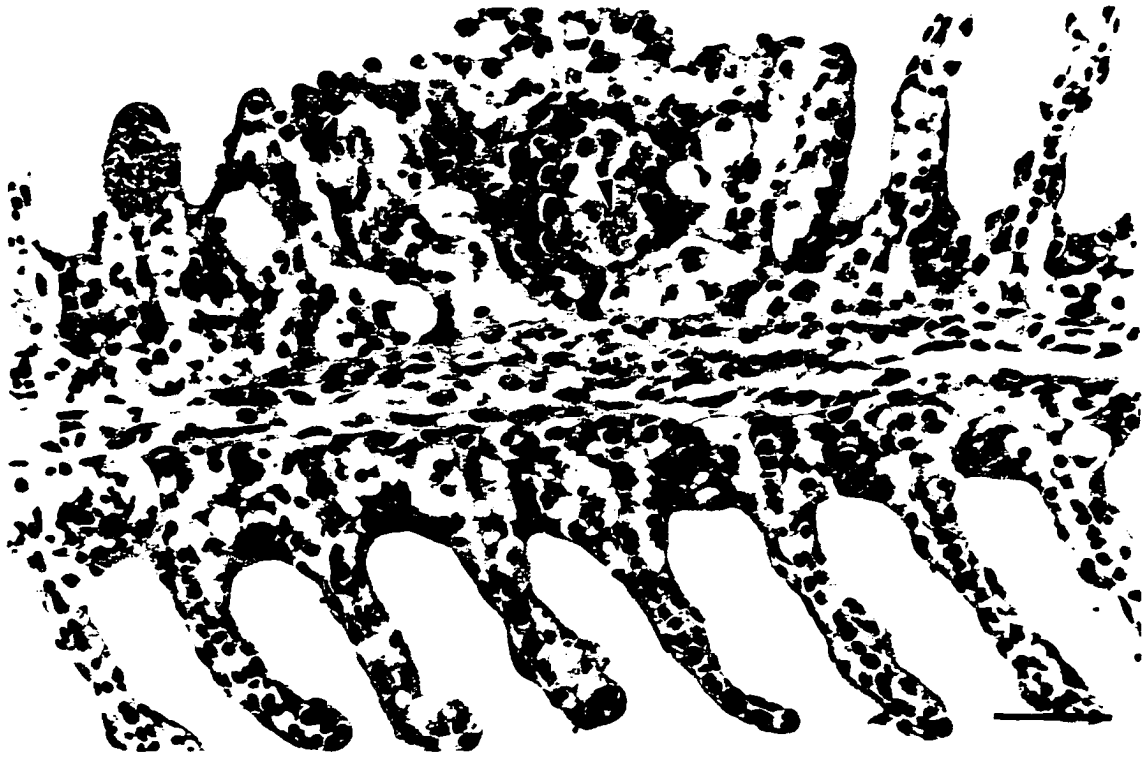


Fig. 6 Histological section showing a ruptured *Loma salmonae* xenoma in the gills of an infected rainbow trout (arrowhead). H&E. Scale bar = 50 μm

2. THE REGULATORY EFFECTS OF WATER TEMPERATURE ON *LOMA SALMONAE* (MICROSPORA) DEVELOPMENT IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

2.1 ABSTRACT

Temperature regulates the life cycle of many organisms, including fish pathogens, by either accelerating or delaying developmental rates. Experimental infection was established to determine the permissive temperature range for *Loma salmonae* development in rainbow trout (*Oncorhynchus mykiss*) and the effects of temperature on developmental rate of the parasite and on intensity of infection. Juvenile fish, either fed or intubated with infective gill material, were held at 11 water temperatures from 5° to 21°C, over three trials, for several weeks. Fish were assessed weekly for the presence of branchial xenomas, which represents the final stage of *L. salmonae* development. Xenomas developed at all temperatures between 9°C and 20°C, defining the permissive range for *L. salmonae* in rainbow trout. The absence of visible xenomas at the upper and lower temperatures suggested the parasite did not complete its life cycle. An average of 30 and 63 to 70 days were required before xenomas became visible at 19° and 11°C, respectively. The data was used to develop a degree-days model, based on thermal unit calculations, which may be useful for fish farmers to predict disease onset in infected fish. In another trial, the number of visible branchial xenomas in fish held at five water temperatures (11°, 13°, 15°, 17°, and 19°C) following gastric intubation with similar doses was also assessed. The number of xenomas varied considerably among fish (e.g. $\bar{x} = 1.67 \pm 1.53$ at 11°C) at all temperatures (i.e. no correlation), indicating water temperature does not affect the quantity of xenomas that develop.

2.2 INTRODUCTION

Loma salmonae, a microsporidian pathogen affecting the gills of farm-reared Pacific salmon and trout (Bruno et al. 1995; Kent 1992), induces substantial branchial inflammation when spore-filled xenomas rupture at maturation (Kent et al. 1995). Fry and parr of both wild and hatchery-reared salmonids can be infected (Dyková 1995; Hauck 1984; Markey et al. 1994) and hatchery conditions (i.e. crowding and water quality) often favour transmission of parasites, provoking severe pathological changes and mortalities (Leiro et al. 1994). The parasite is also transmitted in sea water (Kent et al. 1995). Mortalities greater than 30% of fish on some grow-out sites have also been attributed to *L. salmonae* (Kent et al. 1989), and infections are more severe in market-size fish, resulting in millions of dollars in losses to farmers. Infections are chronic (occur in 10-30% of fish stocks) and fish remain infected for several months. For this and several other important protozoan diseases of farmed salmonids (i.e. the myxosporidian agent responsible for proliferative kidney disease), no effective treatments are licensed in Canada (Brocklebank et al. 1995; Mullins et al. 1994). In addition, vaccines have yet to be developed, although studies suggest effective resistance develops after infection (Speare et al. 1998b) in rainbow trout regardless of whether xenomas form.

The pathologic response to *L. salmonae*, the lack of suitable treatments and the suggestion of a protective immune response are findings similar to those attributed to PKD. A disease management strategy has been developed to control PKD (Clifton-Hadley et al. 1984; Ferguson 1981) which involves exposing naive fish to the infectious stage of PKD as water temperatures are declining. This minimizes renal damage by regulating parasitic

development and the host inflammatory reaction (Foott and Hedrick 1987). Ferguson (1981) also concluded that decreasing water temperatures may accelerate the recovery of infected fish which appear resistant to re-infection the following year as temperatures increase. A similar model may be useful for the control of *L. salmonae* infections after several critical aspects of the parasite's life cycle are investigated. Data pertaining to the effect of water temperature on the kinetics of *L. salmonae* infections is incomplete (i.e. permissive temperature range, rate of xenoma formation and dissolution). Earlier findings suggested xenoma formation and dissolution following experimental infection are not necessary for triggering a protective response to further exposures to *L. salmonae* spores (Speare et al. 1998b). Therefore, at some temperatures in which *L. salmonae* does not develop into xenomas, protective immunity may be elicited.

Water temperature regulates development of many pathogens in their fish hosts. Generally, decreasing water temperatures result in longer developmental rates; however some pathogens develop more rapidly as temperatures decrease. Temperature-affected pathogens include the salmonid bacteria *Flexibacter columnaris* (columnaris disease) (Holt et al. 1975; Sawyer 1976; Wakabayshi 1991), *Aeromonas salmonicida* (furunculosis) (Marsden et al. 1996; Ortega et al. 1995), and *Renibacterium salmoninarum* (bacterial kidney disease - BKD) (Ortega et al. 1995); viruses including infectious pancreatic necrosis virus (Ortega et al. 1995; Wolf 1988), infectious haematopoietic necrosis virus (Wolf 1988), and viral haemorrhagic septicaemia virus (Wolf 1988); fungi, i.e. *Saprolegnia* spp. (Waterstrat 1997); protozoans, i.e. *Glugea stephani* (Olson 1981) and *Enterocytozoon salmonis* (Antonio and Hedrick 1995); and metazoans, i.e. the parasitic copepods *Lepeoptheirus salmonis* and

Caligus elongatus (sea lice) (Johannessen 1978; Johnson and Albright 1991). Temperature-pathogen and temperature-host response interactions are frequently used practically for predicting disease onset through predictions of pathogen numbers or interference with host protective immunity. A difference in disease severity is seen between coho salmon transferred to sea water in the spring versus those transferred in the fall; more severe disease caused by *L. salmonae* occurs in spring entries when temperatures are rising (Kent et al. 1989). This and other evidence (Speare et al. 1998a,b) suggest temperature affects development of *L. salmonae*. However, difficulties arise when using water temperature to predict the onset of xenoma formation or clinical disease in infected fish after exposure to spores because fluctuating conditions are often present at hatcheries and in sea water cages.

The current study used a rainbow trout model to conduct *in vivo* challenges (Speare et al. 1998a,b) to investigate the interaction between water temperature and the pathobiology of *Loma salmonae*. Our objectives were to: a) determine the upper and lower permissive temperatures for branchial xenoma development; b) determine the effects of water temperature on rate of xenoma formation, dissolution and the abundance of branchial xenomas that develop in fish after standard challenges with spores; and c) develop a mathematical model to describe the effect temperature has on the timing of xenoma onset.

2.3 MATERIALS AND METHODS

Four trials with the following objectives were designed to evaluate the effects of water temperature on critical aspects of *L. salmonae* development in rainbow trout:

Trial 1: to determine an upper temperature threshold for *L. salmonae* development and the effect of temperature on *L. salmonae* developmental rate.

Trial 2: to more precisely define the upper temperature threshold observed in Trial 1.

Trial 3: to determine the lower lethal temperature for *L. salmonae* development and to examine the relationship between rate of parasite development and water temperature.

Trial 4: to determine whether water temperature affects the number of xenomas that develop in the gills of rainbow trout.

2.3.1 Sample Population

Juvenile rainbow trout, purchased from a certified disease-free (notifiable pathogens) commercial hatchery on Prince Edward Island with no previous history of *Loma salmonae*, were used in all trials. All procedures were conducted according to the guidelines of the Canadian Council on Animal Care.

2.3.2 Experimental Design

Trial 1: Six identical circular fibreglass tanks with a habitable volume of 78.0 L of flow-through fresh water were used (Appendix A). Water temperatures in each tank were 11°, 13°, 15°, 17°, 19°, and 21°C, respectively; flow rates in all tanks were 3.0 L/min. The tank of fish at 15°C in this trial was used as a positive control group because fish are susceptible to infection at this temperature (Speare et al. 1998a,b). One hundred and eighty size-graded rainbow trout with an average weight of 80 g were acclimated in six other tanks at 15°C for one week prior to the start of this trial. This trial encompassed 13 weeks.

Trial 2: Four circular fibreglass tanks, identical to those used in Trial 1, were used. Water temperatures were 17°, 19°, 20°, and 21°C respectively; flow rates were 3.0 L/min. One hundred and forty-four size-graded rainbow trout with an average weight of 10 g were acclimated at 15°C from 11°C for one day prior to the start of this experiment which continued for 15 weeks.

Trial 3: Six circular fibreglass tanks identical to those described in previous trials were used. This was a cool-water study; water temperatures in each tank were 5°, 7°, 9°, 10°, 11° and 15°C respectively. Two hundred and twenty-five size-graded rainbow trout with an average weight of 15 g were held at 11°C for several weeks prior to the commencement of this trial which encompassed 26 weeks.

Trial 4: Five circular fibreglass tanks, identical to those above, were used. Water temperatures were 11°, 13°, 15°, 17°, and 19°C respectively. Seventy-five size-graded rainbow trout with an average weight of 25 g were acclimated at 15°C from 11°C for one day prior to starting this trial. 10 weeks were used to complete this trial.

2.3.3 Temperature Control and Monitoring

To ensure system precision, temperature was monitored in all trials. In Trials 1, 2 and 4 described above, two header tanks supplied the system. 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 each of the six tanks to provide the desired temperatures (±0.3°C) (Appendix A).

To obtain the cold temperatures required in Trial 3, a chiller was used to cool water to 4.5°C. Two header tanks supplied the system, one containing chilled well water, the other containing ambient well water. Chilled water was mixed with ambient water just before entering each tank for individual control (±0.3°C).

Due to seasonal variation in well water temperatures, the tank originally held at 11.0°C in Trial 3 decreased in the latter part of the trial to 10.0°C. This variation in header tank temperature resulted in the need for manual control of individual tank temperatures.

Temperature was monitored in all trials using a Fluke electronic thermometer and the Campbell Scientific Datalogger which monitors temperature parameters in the tanks and header tanks. The system records 10 minute averages within these tanks.

2.3.4 Methods of Infection

Infected fish with numerous mature xenomas were killed and their gills prepared for either intubation or feeding of naive fish, as described by Speare et al. (1998b), with minor modifications. In both infection methods, gill material was always used the day it was harvested from infected fish. Macerated gills prepared for intubation were diluted with distilled water and fish in all trials received 0.2 ml of prepared tissue through a 1 ml syringe with attached tubing. The inoculum was inverted rigorously after every second syringe to ensure a more consistent inoculum.

Trial 1: Gastric intubation was used as described by Speare et al. (1998b). Five fish were randomly selected from one of the six tanks at 15°C, manually restrained, intubated and assigned to one of the 6 trial tanks until there were 30 fish per tank (Appendix B). No

anaesthetic was used in this trial.

Trial 2: Gastric intubation was used to infect juvenile rainbow trout as previously described (Speare et al. 1998b). Fish were anaesthetized in benzocaine (60 mg/L water), intubated and transferred randomly (in groups of 5) into one of the 4 experimental tanks, until there were 36 fish per tank.

Trial 3: Two hundred and twenty-five naive rainbow trout were placed in a large circular fibreglass tank (760 L volume) and fed infective gill material. Water flow was stopped while the fish were feeding to prevent loss of infective tissue into the outflow drain. After 1.5 h the fish were transferred randomly in groups of 5 to the experimental tanks at 5, 7, 9, 10, 11 and 15°C until 35-37 fish were in each tank.

Trial 4: Gastric intubation was used to infect 75 rainbow trout. Groups of 5 fish were anaesthetized in benzocaine (60 mg/L water), intubated, and then placed randomly in the 5 experimental tanks (Appendix C) until there were 15 fish per tank.

2.3.5 Dose Quantification

Several mechanisms were used to estimate the number of spores ingested by fish in the trials.

After intubating fish in Trial 1, *L. salmonae* spores were counted in a sample of the gill homogenate using a fluorescent antibody technique (Judy Sheppard, pers. comm.). Two million spores/ml (400,000 spores per fish) were counted; however, this may be an underestimate since the material was not homogenous and some spores may have been obscured with clumps of material.

Trials 2 and 4 used the method of Speare et al. (1998b) to quantify the number of spores ingested by each fish. In Trial 2, the mean number of spores given to each fish was 95,000. In Trial 4, each fish received approximately 101,000 spores. However, material was not homogenous and some spores may have been obscured and not counted.

To determine the dose of spores that each fish consumed in Trial 3, a spore purification technique was used (Jones, unpublished, Appendix H). This method of quantification assumes that all the infective tissue fed to the fish was consumed and that all fish in the tank consumed equal amounts of the infective tissue. The dose in this trial was estimated at 675,000 spores per fish.

2.3.6 Sampling and Infection Assessment

During all four trials, fish were assessed weekly for the presence or absence of branchial xenomas using methodology described by Speare et al. (1998b). Fish were anaesthetized using 60 mg/L benzocaine. Using a dissecting microscope, all visible gill lamellae were examined. This included all filaments of the second gill arch and tips of the primary lamellae on all other visible arches. Subsequent to becoming positive (i.e. xenomas seen in the gills), fish were labelled for individual identification.

Trial 1: After xenomas became evident in a fish, a visible implant (VI) tag was implanted in the superficial dermis of that fish just caudal to both eyes. Additionally, a different fin clipping sequence was used for each week new fish became positive (i.e. xenomas present in the gills). All fish at 21°C and 11°C that did not develop xenomas after 13 weeks (91 days) had their water temperature adjusted to 17°C for 46 days, during which they were

examined weekly to determine if the parasite had been killed at the high and low temperature extremes, or if parasite development was inhibited while the fish were at non-permissive temperatures.

Trial 2: After xenomas were found in the gill filaments of a fish, the fish was tagged by sewing tags into its epaxial muscle just posterior to the dorsal fin.

Trial 3: Dorsal fin tags were sewn into fish when they became positive to monitor the temporal course of individual infections. After 16 weeks (112 d), all fish from tanks held at 5 and 7°C were moved to 17°C to determine whether cold temperatures had killed or slowed the development of *L. salmonae*. These fish were screened weekly for xenomas for an additional 9 weeks (63 d).

Trial 4: Each tank of fish was euthanised at the week when branchial xenomas were well developed in most fish in the tank (this was dependent on water temperature). Fish were killed using an overdose of benzocaine (100 mg/L), after which the second gill arch was removed from either side of the fish. The number of filaments and xenomas were counted on one side of each arch and a xenoma index (= mean number of xenomas per gill filament) was calculated.

2.3.7 Data Analysis

For Trials 1, 2, and 3 statistical comparisons were made between data recorded for each temperature used in the trial, with the unit of study being the individual fish rather than the entire tank of fish. Environmental influences (i.e. tank effect) were considered to be of little importance in this study because they have not been proven to affect the development

of *L. salmonae* within the fish. In addition, fish were exposed to infective material prior to being placed in treatment tanks, thus eliminating the effect of dose on treatment groups. Two parameters were recorded throughout the trial: days to onset of xenomas in the gills and days (from observed onset) to total clearance of xenomas from the gills. The mean, associated standard deviation and standard error of the mean were calculated for each parameter at each temperature. The percentage of fish which developed xenomas at each temperature was also recorded.

A one-way analysis of variance (ANOVA) was used to test the null hypotheses that water temperature has no significant effect on the developmental rate of *Loma salmonae* in the gills of rainbow trout and that it has no effect on the length of time xenomas remain in the gills. Tukey's pairwise comparisons were used when ANOVA results were significant to find specific differences between temperatures. If zero was found in the confidence interval, the temperatures were not significantly different. The non-parametric Kruskal-Wallis test was also used to confirm ANOVA results. This test, based on ranks, is used when three or more treatment groups exist with different individuals and when we cannot assume observations are from normally distributed populations with similar variances (Glantz 1997).

Regression analysis of the xenoma onset and clearance data was used to develop a descriptive mathematical model for predicting these events at fixed water temperatures within the permissive range of the parasite. To compare linear with polynomial models, the goodness of fit (the percent of variation explained by the fitted equation) was assessed through the R-squared value (Ryan and Joiner 1994). Alternatively, since variations in water

temperature occur in most fish rearing facilities, examination of alternative prediction models including the thermal unit model described for fish egg hatching (Piper et al. 1989) would be useful. Thermal units (TU) are measured in degree-days (°C above 0°C [the "no development temperature" for eggs] x days). From our first three trials, a model based on degree-days was derived and statistically assessed through a one-way ANOVA. In developing a final (useful) model for predicting xenoma formation, the original degree-days approach was modified from egg hatching prediction models, in that the "no development temperature" zeroing points were selected from results in Trial 3.

In Trial 4, statistical comparisons between data recorded for each temperature used were made. Before combining data collected from the left and right gill arches, data from the two sides was compared at each temperature using a t-test. No differences were detected; the two sides were combined into a single group. Comparisons were then made between each temperature and multiple comparisons used to determine specific differences. Comparisons between temperatures, using ANOVA, was also done for the left and ride sides individually, before the data was combined.

All statistical analyses were carried out using commercial software (MINITAB™ Inc.). Differences were considered significant at the α=0.05 level of probability.

2.4 RESULTS

2.4.1 Trial 1

Development of branchial xenomas occurred more rapidly at warmer temperatures

(i.e. 17° and 19°C) than at lower temperatures (i.e. 11°C) (Appendix D,1; Fig. 7). Since branchial xenomas did not develop at 21°C, this temperature was excluded from statistical analyses. Time of onset (= time to first appearance of xenomas) at each temperature was significantly different among temperatures ($p<0.001$). Tukey's pairwise comparisons indicated that all temperature comparisons were significantly different except 17° and 19°C (Table I). The Kruskal-Wallis test also indicated the same differences among the temperatures ($p<0.001$).

Time of xenoma onset in the gills was modelled by regression analysis. A quadratic equation proved to be most useful for prediction purposes compared to a simple linear regression (Appendix E,1), as the R^2 value is higher (88.3% compared to 70.5%) (Table II).

Comparison of the length of time xenomas remained in the gills before rupturing indicated an overall significant relationship among temperatures ($p<0.001$) (Appendix D,1; Fig. 8). Xenomas ruptured from the gills more rapidly (from the time of first appearance) at warmer temperatures than at cooler temperatures. Tukey's pairwise comparisons found that although the overall relationship was significant, not all temperatures were significantly different from one another (Table I). For example, the periods xenomas remained in the gills at 11°C and 13°C were not significantly different, whereas significant differences were noted between 11 and 15°C. The Kruskal-Wallis test confirmed these findings ($p=0.008$).

The data obtained for time to total clearance of xenomas (i.e. xenoma duration) from the gills could not be modelled by regression although there was an overall general trend which indicated that the duration of persistence in gills was reduced at warmer water temperatures. The spread of the data in this trial was quite large, giving a low R^2 value and

making it difficult to model by regression analysis (Appendix E,2; Table III).

Xenoma-negative fish that were moved to 17°C from the high (21°C) and low (11°C) temperature extremes did not become positive after 7 weeks at this temperature.

When the unmodified degree-days approach was used to describe the cumulative thermal units (TU) necessary to induce xenoma appearance in the gills, significant differences were present among the different temperatures at which fish were held ($p<0.001$) (Table IV,A). The TU equation was then modified to reflect the apparent lethal temperature threshold for *L. salmonae* (Trial 3) in which 5°C was identified as this threshold (i.e. TU = days x °C above 5°C). Using this model, TU values were much closer than in the original model; however significant differences still existed from the high and low ends of the temperatures used ($p<0.001$) (Table IV,A). The third and final equation derived is based on the temperature in Trial 3 which appeared to non-lethally stall parasite development (i.e. TU = days x °C above 7°C). Using this approach, the TU values derived from the trials in the temperature range of 11-17°C were very similar and not significantly different. However, TU values for trials run at 19°C were significantly different from the others.

2.4.2 Trial 2

Analysis by the one-way ANOVA comparing time of onset of xenoma development at each temperature indicated an overall significant difference among temperatures ($p=0.002$) (Appendix D,2; Fig. 9). Xenoma development in this trial was comparable to rates obtained in Trial 1. Data from 21°C is not included in the analyses because only 1 fish developed branchial xenomas at 63 days post-exposure, making it an outlier that would skew the data.

Tukey's pairwise comparisons determined that xenoma onset at 19°C and 20°C were significantly different (Table V). The Kruskal-Wallis test results ($p=0.009$) again confirmed this.

Due to the close temperature range used, xenoma onset in the gills could not be modelled by regression analysis. No significant difference was found in the duration of time xenomas remained in the gills (Appendix D,2; Fig. 10) with either the one-way ANOVA ($p=0.598$) or the Kruskal-Wallis test ($p=0.611$).

Cumulative temperature units were again calculated based on observed data (Table IV,B). With the unmodified degree-day approach, TU values significantly varied from 558 to 709°C-days ($p<0.001$). Similarly, when adjusted to either 5°C or 7°C as the zero point for TU calculations, differences between temperatures were still significant ($p<0.001$).

2.4.3 Trial 3

Time of xenoma onset varied significantly among the temperatures used in this study ($p<0.001$ for both the one-way ANOVA and Kruskal-Wallis test) (Appendix D,3; Fig. 11). Time of onset ranged from 33.9 days at 15°C to 88.2 days at 9°C. An overall significance did result ($p<0.001$) and Tukey's pairwise comparisons revealed that times to onset at all temperatures differed significantly from each other (Table VI). Results obtained at temperatures in this trial corresponding to those in Trial 1 are comparable (see Fig. 1).

In this trial, xenoma onset was modelled by a linear regression model (Table VII; Appendix F,1), although (as in Trial 1), a quadratic model was more accurate for predicting and gave a higher R-squared value.

The one-way ANOVA for time to total xenoma clearance from the gills in this trial was significant ($p<0.001$) (Appendix D,3; Fig. 12). Generally, xenomas remained in the gills for longer periods at cooler temperatures. Almost all temperatures were significantly different when Tukey's pairwise comparisons were completed (Table VI). This was again confirmed with the Kruskal-Wallis test ($p<0.001$).

Duration of infection could not be modelled by regression analysis although there was a trend indicating xenomas clear the gills more rapidly as temperature increases (Table VIII; Appendix F,2).

Among fish moved from 5°C and 7°C to 17°C after the first 16 weeks of the study, none of those originally at 5°C subsequently developed branchial xenomas at the warmer temperature, and only 10% (3/31) at 7°C subsequently became positive at 17°C. The mean cumulative thermal units calculated for these fish using the modified equation (i.e. zeroing point adjusted to 7°C) was 350°C-days, similar to fish at 17°C in Trial 2 (Table IV,B).

Original TU's calculated in this study decreased as water temperature increased (Table IV,B). Although there was an overall significant difference ($p<0.001$), temperature units calculated at 10 and 11°C did not differ significantly. Adjusting the zeroing point to 5°C narrowed the differences between temperatures but there was still an overall significant difference ($p=0.013$). Cumulative TU calculations using the final iteration (i.e. 7°C) were comparable to Trial 1 although the range of thermal units was greater.

2.4.4 Trial 4

Fish at 17° and 19°C were euthanised at week 6, fish at 13° and 15°C were harvested

at week 8, and fish at 11°C were harvested at week 10. There were no significant differences between the number of xenomas on the left and right sides of each fish or among temperatures for each side (t-test analyses). Data from both sides of the fish were then combined and an ANOVA showed no significant difference between the number of xenomas found at any temperature (p=0.223) (Table IX).

2.5 DISCUSSION

The thermal range for full development of *L. salmonae* was defined in this study to be 9°-20°C which contrasts with previous reports (Speare et al. 1998a,b) where no development was observed in fish infected at 10°C. In our trials, 7°C was permissive in that it allowed the parasite to survive but not develop xenomas until fish were transferred to a higher temperature. Therefore, perhaps those fish at 10°C in the study conducted by Speare et al. (1998a) would have developed xenomas had the fish been moved to a more permissive temperature soon after being infected at the low temperature. Other factors that may have contributed to the conflicting results are fish size or age or dosage received by each fish.

Our results, summarized in Figs. 13 and 14, also demonstrate the trend that an increase in water temperature results in an increase in xenoma development rate within an optimal temperature range. Beyond this, water temperature has varying effects. This regulation of development rate by water temperature is similar to other protozoan pathogens (*Enterocytozoon salmonis* - Antonio and Hedrick 1995; *Sphaerospora* sp. [proliferative kidney disease] - Ferguson 1981; *Loma salmonae* - Kent et al. 1989; *Glugea stephani* -

Olson 1981), with more rapid parasite development occurring at warmer water temperatures.

We conclude that water temperatures at and below 7°C do not permit full development of *L. salmonae*; specifically, xenoma production does not occur. In fish held at 5°C, incomplete development is caused by death of the parasite (xenomas did not form after transfer of fish to 17°C). In contrast, *L. salmonae* reared in fish at 7°C, survived and developed into xenomas after transfer of fish to a permissive temperature. This is similar to findings by Olson (1981) with *Glugea stephani* development in English sole. In Trial 1, xenoma formation took longer and the percent of fish infected was lower at 11°C than at warmer temperatures, perhaps because the parasite was approaching the lower threshold for full development and expression in the gills. Results similar to this were reported previously (Speare et al. 1998a). The proportion of fish that developed xenomas in Trial 3 was not significantly different among temperatures although the developmental rate was significantly slower at lower temperatures. Fish in Trial 3 were smaller than in Trial 1 and perhaps these were more susceptible to infection at 11°C. Also, infections did not appear as heavy and were associated with less gill damage at 11° and 13°C (Trial 1). Although these parameters were not quantified, one might expect a milder inflammatory response with lighter infections. This may explain the observations of Kent et al. (1989), who found in that infections (with *L. salmonae*) in coho salmon are more prevalent in the summer, when the water temperature are warmer than in early spring or late fall. Low temperatures also affect developmental rate, hatching success and the quantity of eggs that develop in sea lice (parasitic copepods) (Johannessen 1978). In addition to the parasite being affected by different water temperatures, physiological processes of poikilotherms are directly affected

by temperature as they respond to sensory input from peripheral and central thermoreceptors when temperature changes (Hazel 1993). Antibody production, feeding, digestion and growth are all adversely affected by unfavorable water temperatures (Wedemeyer 1996).

Loma salmonae did not develop fully (i.e. xenoma production) at temperatures above 20°C; this appeared to be the upper lethal threshold for development, as was demonstrated by the lack of xenoma formation after fish were transferred to 17°C for several weeks. At 19 and 20°C, the rate of xenoma development was variable. Perhaps the parasite was not developing normally because it was close to the upper lethal threshold. A similar trend occurred with the development of the monogenean *Diplectanum aequans* on sea bass (Cecchini et al. 1998); larval hatching time was increased near the upper permissive temperature. A polynomial equation was also used to model this parasite's development.

Water temperature also affects host immune functions. The efficiency of the fish host's defense response depends partially on environmental conditions (Finn and Nielsen 1971); low temperatures delay or inhibit macrophage function. The extent of lesions may depend how early stages of the parasite spread within the host. This may explain why xenomas take longer to develop at cooler temperatures. Apparently, macrophages are used for transport of sporoplasm of other microsporidians to the host cell in which sporulation occurs (Dyková 1995). If macrophages react more slowly at colder temperatures, then it would take longer for the parasite to reach the gills, thus extending the time required to develop into xenomas.

Temperature did not affect the abundance of branchial xenomas that developed in Trial 4. The number of fish that develop xenomas and the number of xenomas that develop

in the gills appear to be independent of water temperature; values were highly variable at each temperature. This is contrary to observations made in Trial 1 where more xenomas developed at higher temperature.

Very rarely do water temperatures remain constant throughout the entire production cycle of salmonids. The derived regression equations for predicting xenoma onset and dissolution which assume constant temperatures, have restricted predictive application. However, the use of thermal units (degree-days) may be very useful in predicting the timing of xenoma formation in the gills, similar to determining salmonid egg hatching (Billard and Jensen 1996). The original thermal units calculated in this study were based on the assumption that 0°C was the temperature above which development of the parasite would proceed, which occurs with salmonid eggs in hatcheries (Bromage 1988; Piper et al. 1989). However, from our trials, we determined that *L. salmonae* development is restricted until at 7°C; temperature units were adjusted to reflect this. The adjusted thermal unit calculations for those fish which were moved from 7°C to 17°C were similar to those calculated for fish in Trial 2 at 17°C. This model may be of use to farmers that want to predict when to harvest fish or be prepared for mortalities.

Knowing xenoma formation and dissolution, events causing disease in infected fish, do not occur if fish are held at 7°C for prolonged periods, and that these fish are resistant upon re-exposure to spores (Chapter 4), indicates we may be able institute methodology presently used to control PKD infections, on *L. salmonae*-infected farms. This management practice may be useful when fish are in hatcheries, when rearing temperatures are usually quite low (Piper et al. 1989). Exposing fish at low temperatures may allow the infection to

establish within the host, without complete development. Alternatively, fish may be exposed in the fall before transfer to seawater cages where the water temperature will drop, slowing down or inhibiting development of *L. salmonae*.

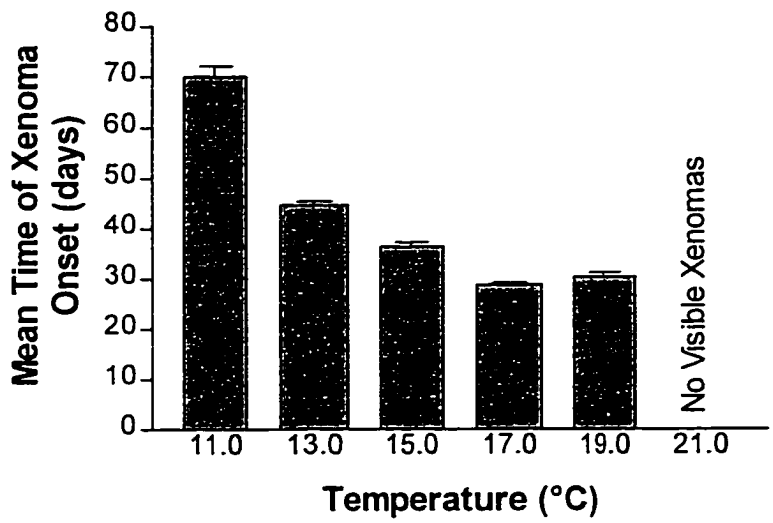


Fig. 7. Trial 1. Effect of water temperature on mean time post-inoculation to first appearance of branchial xenomas (+SEM) in the gill of rainbow trout.

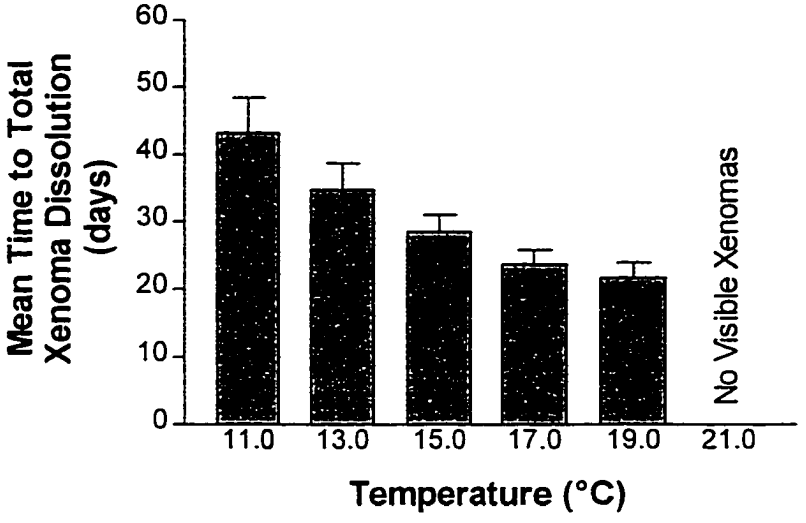


Fig. 8. Trial 1. The effect of water temperature on mean time (+SEM) to xenoma clearance from the gills of rainbow trout.

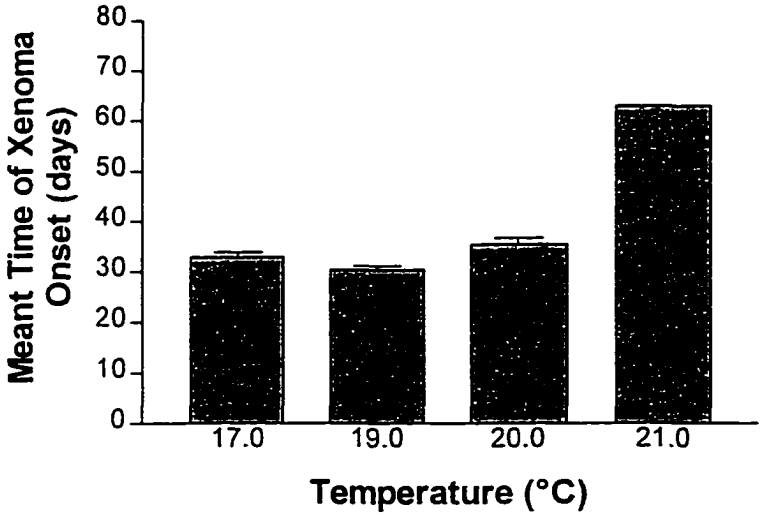


Fig. 9. Trial 2. Effect of water temperature on mean time of onset (+SEM) of branchial xenomas in rainbow trout in the upper temperature range for *L. salmonae* development.

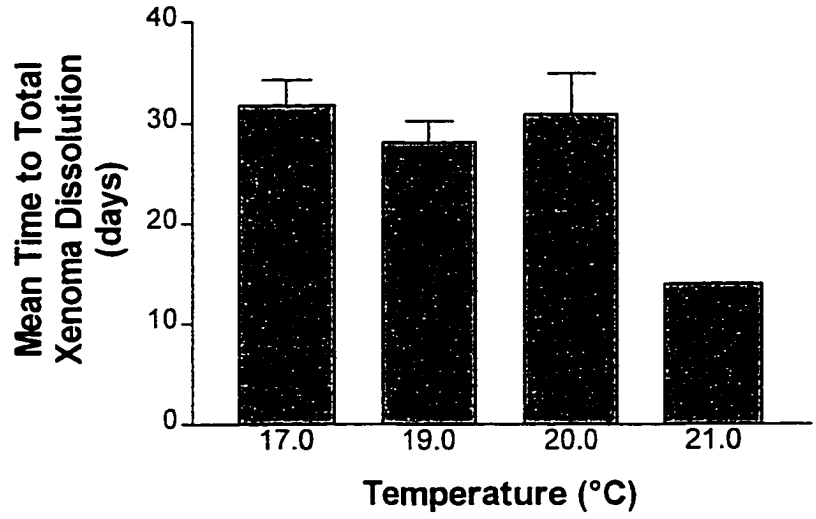


Fig. 10. Trial 2. Mean time (+SEM) from first appearance to xenoma clearance from the gills in the upper temperature range for *L. salmonae* development.

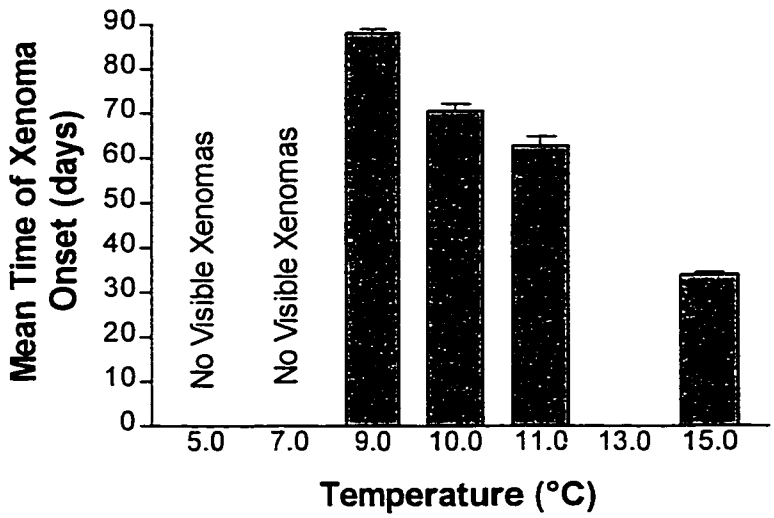


Fig.11. Trial 3. Mean time of xenoma onset (+SEM) at water temperatures used to determine the lower developmental range for *L. salmonae*.

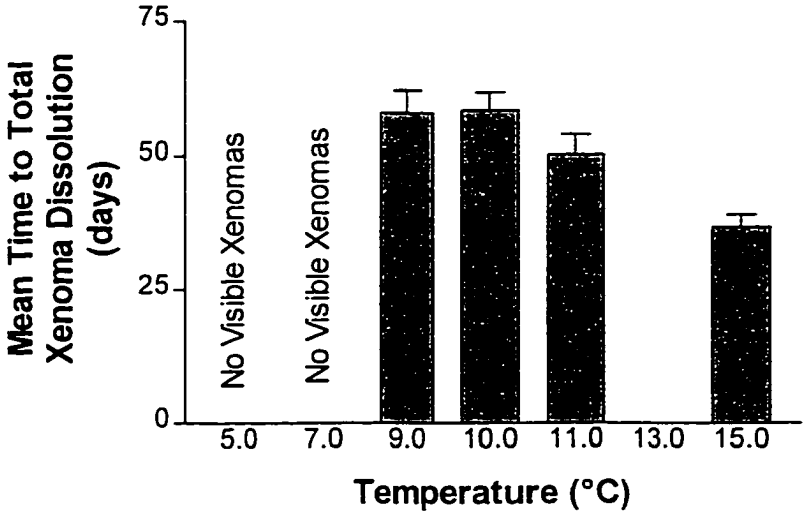


Fig. 12. Trial 3. Mean time to clearance (+SEM) of branchial xenomas during the cold-water study used to determine the lower permissive temperature range for *L. salmonae* development.

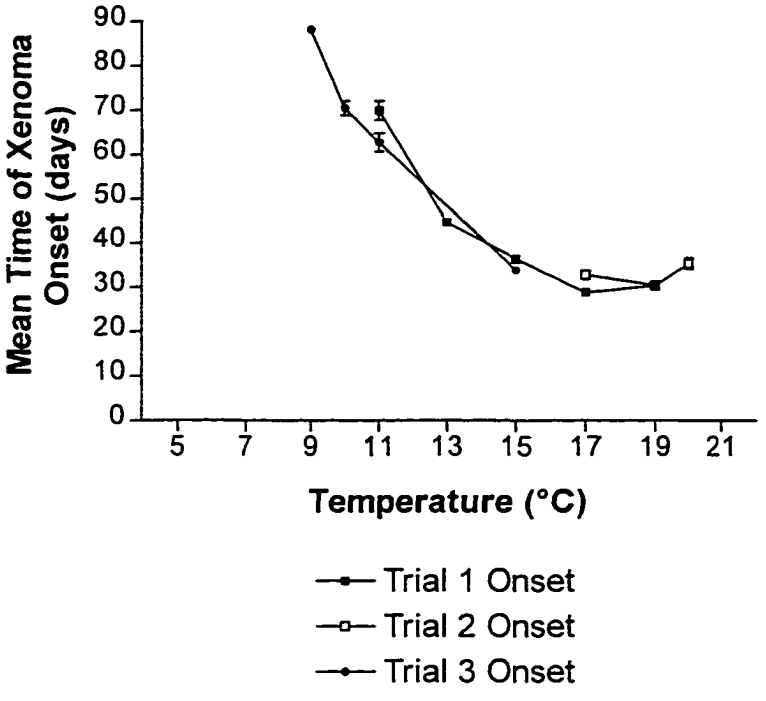


Fig. 13. Summary graph indicating the mean time of *Loma salmonae* branchial xenoma onset (±SEM) during each of three trials. This graph spans all permissive developmental temperatures of the parasite. Note: A single fish developed one xenoma after 9 weeks at 21°C and 3 fish from 7°C developed a few xenomas, but only after they were moved to 17°C for 9 weeks after a period of 16 weeks at 7°C.

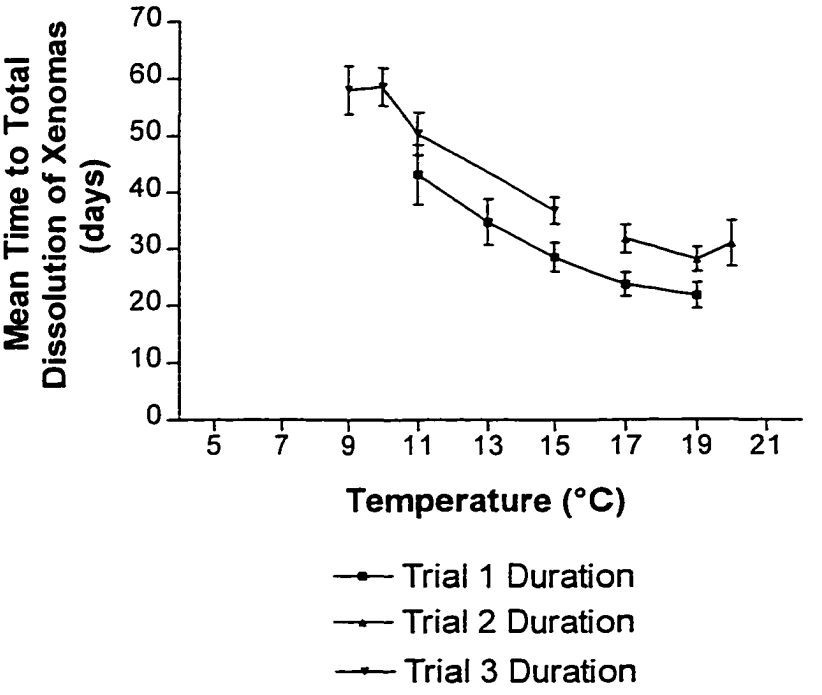


Fig. 14. Summary graph indicating mean time to total xenoma clearance (±SEM) of *Loma salmonae* from the gills of infected rainbow trout during three trials. Note: Data for fish which developed xenomas at 7°C and 21°C were excluded.

Table I. Tukey's pairwise comparisons for xenoma onset and duration in Trial 1.

ONSET:

	11	13	15	17
13	20.956 29.711			
15	29.293 37.791	4.136 12.280		
17	37.014 45.371	11.861 19.857	3.793 11.508	
19	35.277 43.854	10.119 18.345	2.047 10.00	-5.528 2.274

Family Error Rate = 0.0500; Individual Error Rate = 0.00651

DURATION:

	11	13	15	17
13	-5.08 21.89			
15	1.28 27.96	-6.59 19.02		
17	6.37 32.55	-1.49 23.60	-7.55 17.23	
19	7.97 34.94	0.09 26.00	-5.98 19.64	-10.55 14.54

Family Error Rate = 0.0500; Individual Error Rate = 0.00652

Table II. Regression analyses for xenoma onset in the gills of rainbow trout in Trial 1.

Regression Equation	P	R^2 (%)	Data Set Used
Onset = 110 - 4.58T	<0.001	70.5	11-19°C inclusive
Onset = 320 - 33.4T + $0.954T^2$	<0.001	88.3	11-19°C inclusive
Onset = 134 - 6.37T	<0.001	80.9	11-17°C inclusive, 19°C data removed
Onset = 340 - 36.4T + $1.06T^2$	<0.001	88.3	11-17°C inclusive, 19°C data removed
Onset = 122 - 5.59T	<0.001	86.6	11-17°C, outliers 15-18 removed, 19°C data removed

T is water temperature; P is the p-value; R^2 is the amount of variation explained by the relationship.

Table III. Regression analyses on duration (i.e. time to total clearance) of branchial xenomas from rainbow trout in Trial 1.

Regression Equation	P	R^2 (%)	Data Set Used
Duration = 70.4 - 2.68T	<0.001	19.7	11-19°C inclusive
Duration = 126 - 10.3T + 0.254T^2	<0.001	20.7	11-19°C inclusive
Duration = 77.2 - 3.19T	<0.001	16.9	11-17°C inclusive, 19°C removed
Duration = 120 - 9.4T - 0.222T^2	<0.001	17.1	11-17°C inclusive, 19°C removed
Log (Duration+1) = 4.45 - 0.078T	0.004	9.7	11-17°C inclusive, 19°C removed
SqrtDuration = 9.00 - 0.251T	0.001	13.4	11-17°C inclusive, 19°C removed

T is water temperature; P is the p-value; R^2 is the amount of variation explained by the relationship; SqrtDuration is the square root of duration data.

Table IV. Temperature units (°C-days) (±St.dev.) for time of xenoma onset in Trials 1(A), 2(B), and 3(C). Significant differences were found between groups with all three sets of calculations.

A.

Temperature (°C)	Original TU (°C x days)	TU-5°C	TU-7°C
11.0	770.0 ±102.3[1,2,3,4]	420.0 ± 55.8[1]	280.0 ± 37.2[1]
13.0	580.7 ± 45.3[1,5]	357.3 ± 27.9[1,2]	268.0 ± 20.9[2]
15.0	546.9 ± 61.8[2,6]	364.6 ± 41.2[1,3]	291.7 ± 32.9[3]
17.0	489.7 ± 38.8[3,5,6,7]	345.7 ± 27.4[1,4]	288.1 ± 22.8[4]
19.0	578.3 ± 76.2[4,7]	426.1 ± 56.1[2,3,4]	365.2 ± 48.1[1,2,3,4]

B.

Temperature (°C)	Original TU (°C-days)	TU-5°C	TU-7°C
17.0	557.9 ± 96.5[1]	393.8 ± 68.1[1]	328.2 ± 56.8[1]
19.0	577.6 ± 82.3[2]	425.6 ± 60.7[2]	364.8 ± 52.0[1]
20.0	709.2 ± 125.6[12]	531.9 ± 94.2[12]	461.0 ± 81.7[1]

C.

Temperature (°C)	Original TU (°C-days)	TU-5°C	TU-7°C
9.0	793.8 ± 43.8[1,2,3]	352.8 ± 19.5[1]	176.4 ± 9.7[1,2,3]
10.0	705.8 ± 95.2[1,3]	352.9 ± 47.6[2]	211.8 ± 28.6[1,2,3]
11.0	690.9 ± 134.0[2,3]	376.9 ± 73.1[3,4]	251.2 ± 48.7[2,3]
15.0	508.6 ± 47.0[3]	339.1 ± 31.4[4]	271.3 ± 25.1[3]

[1,2,3,4,5,6,7] Superscripts which are the same within a column, for a specific trial [1(A), 2(B), or 3(C)], are significantly different; Original TU are the temperature units calculated using 0°C as the lowest temperature at which xenomas will develop; TU-5°C are the temperature units calculated when 5°C is used as the lowest permissive temperature; TU-7°C are the temperature units calculated when 7°C is used as the lowest permissive temperature.

Table V. Tukey's pairwise comparisons for xenoma onset in Trial 2.

ONSET:

°C	17	19
19	-0.705 5.542	
20	-6.019 0.732	-8.395 -1.729

Family Error Rate = 0.0500; Individual Error Rate = 0.0193

Table VI. Tukey's Multiple Comparisons for xenoma onset and duration for Trial 2.

ONSET:

°C	9	10	11
10	12.42 22.81		
11	20.23 30.55	2.65 12.90	
15	48.94 59.65	31.36 41.99	23.62 34.19

Family error rate = 0.0500; Individual error rate = 0.0103

DURATION:

°C	9	10	11
10	-13.45 12.25		
11	-5.12 20.40	-4.52 21.00	
15	7.93 34.45	8.53 35.05	0.38 26.73

Family error rate = 0.0500; Individual error rate = 0.0103

Table VII. Regression analyses for xenoma onset in the gills of rainbow trout for Trial 3.

Regression Equation	P	R^2 (%)	Data Set Used
Onset = 159 - 8.47T	<0.001	80.9	9-15°C inclusive
Onset = 304 - 33.3 T + 1.02T^2	<0.001	83.5	9-15°C inclusive

T is water temperature; P is the p-value; R^2 is the amount of variation explained by the regression model.

Table VIII. Regression analyses for time to total clearance of xenomas from the gills of rainbow trout.

Regression Equation	P	R^2(%)	Data set used
Duration = 93.4 - 3.77T	<0.001	14.3	9-15°C inclusive
Log (duration) = 4.68 - 0.0761T	<0.001	12.2	9-15°C inclusive

T is water temperature; P is the p-value; R^2 is the amount of variation explained by the regression model.

Table IX. Mean number of xenomas per gill filament (± St. Dev.) on the left and right sides of fish at different water temperatures. P = 0.223 for one-way ANOVA on temperature for all data (with left and right sides combined at each temperature).

Temperature (°C)	Left Xen./Fil.	Right Xen./Fil.	P-value	Combined Xen./Fil.
11	1.71±1.51	1.62±1.59	0.88	1.67±1.53
13	1.21±1.12	1.32±1.21	0.80	1.27±1.15
15	0.94±1.26	1.09±1.25	0.75	1.01±1.23
17	2.00±2.46	1.86±2.34	0.88	1.93±2.35
19	1.39±1.48	1.43±1.55	0.94	1.41±1.49

Left Xen./Fil. is the mean number of xenomas per filament on the left side of infected fish; Right Xen./Fil. is the mean number of xenomas per filament on the right side of infected fish; P-value indicates the relationship between the left and right side; Combined Xen./Fil. is the mean number of xenomas found overall, with left and right sides combined.

3. THE EFFECT OF TEMPERATURE MANIPULATION ON THE RATE OF *LOMA SALMONAE* XENOMA DEVELOPMENT *IN VIVO*

3.1 ABSTRACT

The effect of manipulating water temperatures on *Loma salmonae* development in rainbow trout (*Oncorhynchus mykiss*) was examined to determine effects on timing and degree-day predictions of xenoma formation. When infected fish were maintained at cool water temperatures (i.e. 11°C) for prolonged periods, xenoma development took longer than when fish were moved to warmer water within eight days of being exposed to *L. salmonae*. Juvenile rainbow trout were experimentally intubated with infective gill material at 11°C. Randomly selected groups of 30 fish were moved at time intervals of 0, 2, 4, 8, 16, and 32 days post-intubation to 15°C, presumably at different stages of the parasite's life cycle. Fish were then assessed weekly for the presence of branchial xenomas. On average, 39 days were required before seeing branchial xenomas in fish moved to 15°C immediately following infection whereas 65 days were required for those fish which remained at 11°C throughout the study. A thermal unit (TU) model (developed in Chapter 2) for predicting xenoma formation was used to assess predictability of xenoma formation. The model using 7°C as the "no development" zeroing point gave mean degree-day values of 311, 316, 311, 317, 332, and 285 at movement intervals of 0, 2, 4, 8, 16, 32 days post-exposure, respectively. For fish that remained at 11°C for the duration of the trial, 261 °C-days were required for xenoma formation. The TU model proved effective at predicting the time of onset of xenoma formation, even when water temperatures change during the course of infection, indicating that all stages of the life cycle are similarly affected by water temperature.

3.2 INTRODUCTION

Severe disease associated with the microsporidian gill pathogen, *Loma salmonae*, occurs in several farmed salmonids (Bruno et al. 1995; Hauck 1984; Kent et al. 1989; Speare et al. 1998a,b), with most severe infections occurring in sea water-reared chinook salmon (*Oncorhynchus tshawytscha*). Although preliminary reports suggest that practical control of disease caused by this pathogen may be possible through pharmacological intervention (Kent and Dawe 1994) or induction of protective immunological mechanisms (Speare et al. 1998b), therapeutic agents and vaccines may not become available soon. There are currently no effective therapeutic pharmacological methods for controlling this and many other microsporidian infections (Brocklebank et al. 1995; Mullins et al. 1994). Since xenoma formation and dissolution is the likely cause of *Loma*-related morbidity (Kent et al. 1995), fish health professionals must be able to predict the timing of xenoma formation of this pathogen. Management strategies currently rely on attempting to prevent infections or minimize the effect of disease.

Preventing *L. salmonae* infections in sea water netpens is difficult due to the lack of control over transmission from naturally infected species. Marine reservoirs (feral salmon within the genus *Oncorhynchus*) of *L. salmonae* likely occur in proximity to netpen culture (Kent et al. 1998), and if a small portion of the farmed population becomes infected, horizontal transmission, as previously demonstrated (Speare et al. 1998a), is likely to ensure further distribution of the parasite.

Little research has been directed at finding methods to minimize the effects of *L.*

salmonae after a population is infected. Severe branchial inflammation occurs during the final stage of development when xenomas form and subsequently release spores (Hauck 1984; Kent et al. 1995; Speare et al. 1998b). Heavy gill infections may interfere with respiration (Morrison and Sprague 1981; Poynton 1986). However, fish may survive if husbandry practices are used which avoid increasing the oxygen demand by fish and excessive handling and feeding. In contrast, as a preventative measure, farmers could use a technique used for the control of proliferative kidney disease (PKD) to control *L. salmonae* infections. In this method, sea water entry occurs in the fall as temperatures are declining; fish become infected but never fully develop disease and are subsequently immune to further infections (Clifton-Hadley et al. 1984; Ferguson 1981). To accomplish these aims with *L. salmonae* infections, we need to predict the approximate timing of xenoma formation after exposure to spores has occurred.

A previous study (Chapter 2) modelled the effect of temperature on the development of *L. salmonae* using a thermal unit (TU) approach, which predicts the time of xenoma formation after fish are exposed to spores. This model, similar to that used for salmon egg hatching (Piper et al. 1989), uses degree-day (°C-days) calculations and functions within the limits of thermal tolerance for the organism. However, the previous study assessed developmental rate based on constant temperatures. This may be practical in land-based aquaculture settings using groundwater (with minimal temperature fluctuations); however, sea water cages in the ocean often experience varying temperatures. We cannot assume fluctuating water temperature affects all stages of *L. salmonae* development similarly to some parasites (e.g. sea lice are affected by water temperature during all stages - Johannessen

1978; Johnson and Albright 1991). If changing water temperatures affect only parts of the parasite's life cycle, then the current model may have limited application.

This study was designed to determine if subjecting fish to different water temperatures (within the permissive range for *L. salmonae*) at various time intervals post-infection, affects timing and degree-day prediction of xenoma formation or the proportion of fish that develop xenomas.

3.3 MATERIALS AND METHODS

3.3.1 Sample Population

Juvenile rainbow trout were obtained from a certified disease-free (specific pathogens) commercial hatchery on Prince Edward Island, with no previous history of *L. salmonae*. All procedures were carried out according to the guidelines of the Canadian Council on Animal Care.

3.3.2 Experimental Design

Six circular fibreglass tanks were used, identical to those described in Chapter 2. The mean temperature for each tank was 14.9±0.4°C and the flow rate in each tank was approximately 3.0 L/min. The habitable volume of each tank was 78.0 L (Appendix A). 210 size-graded rainbow trout, with an average weight of 80 g, were randomly selected from a larger population of fish reared at 11.0°C. Fish were maintained at 11°C until the day they were moved to 15°C. This trial was carried out over a 14 week period.

3.3.3 Temperature Control and Monitoring

Water temperature for this study was controlled in the single header tank for the system. Both ambient (11°C) and heated (27°C) water were supplied to this tank, in proportions that gave the desired temperature. Temperature in the header tank was monitored using the Campbell Scientific Datalogger, a computerized system that monitors temperature parameters and records 10 minute averages.

3.3.4 Method of Infection

Methods described by Speare et al. (1998a,b) were used to prepare infected gill tissue for infecting fish by intubation, with minor modifications. Gill tissue was used the same day it was harvested from infected fish. The tissue was macerated, diluted with distilled water and all fish received 0.2 ml of the prepared inoculum, which was shaken routinely to ensure homogeneity.

Fish were then restrained without anaesthetic and intubated with the inoculum in groups of 5 fish. At time intervals of 0, 2, 4, 8, 16, and 32 days post-intubation with *L. salmonae* spores, groups of 30 randomly chosen fish were moved from the initial water temperature of 11.0°C to a tank at 15.0°C. An additional group of 30 fish was kept at 11°C for the duration of the trial. Those fish moved to 15°C at day 0 were standard controls used for comparison to previous trials (see Chapter 2).

3.3.5 Dose Quantification

From the inoculum, a representative sample was removed and assessed to quantify

the spores received by each fish. Several xenomas remained intact, making quantification of spores for dosage inaccurate. Therefore, the tissue was digested in 0.6% pepsin solution (60 min at 37°C), which released the spores without causing any apparent damage. The count was completed using a sample placed on a hemocytometer under a compound microscope, giving 1.9 x 10^6 spores per ml (380,000 spores per fish).

3.3.6 Sampling and Infection Assessments

Weekly throughout the study, fish were assessed for the presence or absence of branchial xenomas using previously described methodology (Speare et al. 1998b) whereby fish were anaesthized in benzocaine (60 mg/L water) and examined under a dissecting microscope. Fish were considered positive when xenomas were seen in the gills upon examination.

3.3.7 Data Analysis

Statistical comparisons were made between data recorded for each interval at which fish were moved from 11.0°C to 14.9°C, with the unit of study being the individual fish rather than the entire tank. The time to xenoma onset from the time of inoculation was the primary factor examined in this trial. An ANOVA was used to test the null hypothesis that the time interval of movement from cooler to warmer temperatures has no significant effect on the timing of degree-day prediction (as defined in Chapter 2) or xenoma formation. Tukey's pairwise comparisons were used to find specific differences between the intervals.

Regression analysis was used to develop a mathematical model for predicting

xenoma onset at the various movement intervals, with the goodness of fit being assessed through the associated R-squared value (Ryan and Joiner 1994).

The proportion of fish which developed xenomas in each group was recorded and compared using a chi-square table.

3.4 RESULTS

Based on the ANOVA, the time for xenomas to develop from the time of inoculation was affected significantly by the period that fish remained at 11°C before being moved to 15°C ($p<0.001$). Branchial xenoma formation occurred more rapidly in fish which were moved sooner to warmer water after exposure to *L. salmonae* spores (Appendix H; Fig.15). There was no difference in xenoma development rate among those fish moved up to 15°C on days 0, 2, 4, and 8 post-exposure. However, fish moved 8 days post-exposure or earlier had more rapid xenoma development than those fish moved at 16 or 32 days or those which remained at 11°C throughout the trial (Tukey's pairwise comparisons - Table X). Xenomas appeared after 40 to 44 days in the earlier intervals, compared to 50 to 65 days for those moved to the warmer temperature in later intervals and those which remained at 11°C for the duration of the trial.

Xenoma onset in the gills was modelled by linear regression ($p<0.001$), giving a correlation, the R^2 value, of 62.6%. The degree of correlation did not increase substantially when either polynomial models were applied or log transformations were completed with the data (Table XI).

The one-way ANOVA for timing of xenoma formation based on cumulative thermal unit (TU) calculations (Table XII) indicated the unmodified model (TU = days x °C above 0°C) had significant differences among the time intervals used for moving fish from 11 to 15°C. No significant differences existed when the model was adjusted to 5°C (TU = days x °C above 5°C), an apparently lethal temperature for *L. salmonae*. All TU were the same except for one comparison using the final model which is based on 7°C (TU = days x °C above 7°C), a temperature which is not lethal but inhibits parasite development until a more permissive temperature is obtained (Chapter 2).

There was no significant difference in the proportion of fish which developed xenomas at each movement interval (Table XIII). However, the variation ranged from 63.0% infected of those fish that remained at 11°C throughout the trial to 88.9% infected of those fish moved to 15°C the same day they were exposed to *L. salmonae*.

3.5 DISCUSSION

The timing of *L. salmonae* xenoma formation in rainbow trout was delayed in fish that were maintained at 11°C for prolonged periods before transfer to 15°C. After fish were moved to the warmer temperature, development progressed more rapidly than it did in fish maintained at 11°C for the entire life cycle. Antonio and Hedrick (1995) found that chinook salmon infected with *Enterocytozoon salmonis* developed infections slower at 12°C than at 15°C and that when fish were moved to warmer temperatures, development progressed more rapidly. Delayed development of many pathogens occurs when water temperatures are sub-

optimal or close to lethal extremes for the pathogen (Antonio and Hedrick 1995; Cecchini et al. 1998; Holt et al. 1975; Johannessen 1978; Johnson and Albright 1991; Olson 1981; Wakabayashi 1991). Our study also demonstrated similar results in the development of *L. salmonae* in rainbow trout (Chapter 2).

As previously outlined (Chapter 2), a TU model can be used to predict timing of xenoma formation when temperatures are held constant. Less variable water temperatures may be found at hatcheries using ground water. The water temperature could be reduced to delay xenoma formation in this setting. Previous work has indicated that resistance to *L. salmonae* infections can be induced in fish by previous infections (Speare et al. 1998b) and that xenoma formation is not required for this protection to develop. Therefore, to induce protection, temperature can be reduced below that required for full development of *L. salmonae*, as defined in Chapter 2. The TU calculations in this trial, which ranged from 261 to 332°C-days using the model with an adjusted zeroing point to 7°C, are similar to those calculated in Chapter 2 (268-365°C-days in Trial 1, 328-461°C-days in Trial 2 and 176-271°C-days in Trial 3). Looking more specifically at those fish in this study which remained at 11°C and 15°C for the duration of the trial, the °C-days calculated for each of these groups overlapped with those calculated in the same groups in Chapter 2.

From the current study, we demonstrated that the same model applied in Chapter 2 also applies when temperatures are changing, a situation often experienced by fish during the grow-out phase of their life cycle. This type of mathematical model may be applied to other fish pathogens to help fish health professionals more accurately predict disease onset, reducing economic losses to fish farmers. Losses may be limited through early harvesting

of fish before they actually die from disease, or by treating fish with chemotherapeutic agents if they exist for other pathogens affected by water temperature.

Although there was a trend in the data for the proportion of fish that became positive (Table XIII) in each group, differences were not significant among movement intervals. This finding is similar to those of by Antonio and Hedrick (1995) who found that in fish moved from 12° to 15°C; disease onset is delayed but it progresses and cumulative mortalities are similar to fish maintained at the same temperature throughout the trial. For both our study and that of Antonio and Hedrick, this may reflect the temperature range of the trial being within the permissive developmental range. Perhaps fewer fish would have developed branchial xenomas if part of the trial been conducted at non-permissive temperatures. Also, the proportions positive for fish that remained at 11 and 15°C (63.0% and 88.9% respectively) for the entire trial were similar to fish in those groups in Chapter 2 (62.1% and 82.8% of fish became positive in these groups respectively).

There is relatively little knowledge on the timing of each stage of the *L. salmonae* life cycle and on the affects of temperature on all stages of the life cycle. From the current study, apparently all stages of the life cycle are affected somewhat homogenously by water temperature. This is because the thermal unit model applies, regardless of the time intervals fish were moved from cooler to warmer water, which were presumably at different stages of the life cycle. Thus the described TU model is useful in most practical aquaculture settings, whether fish are in hatcheries, or in marine grow-out cages.

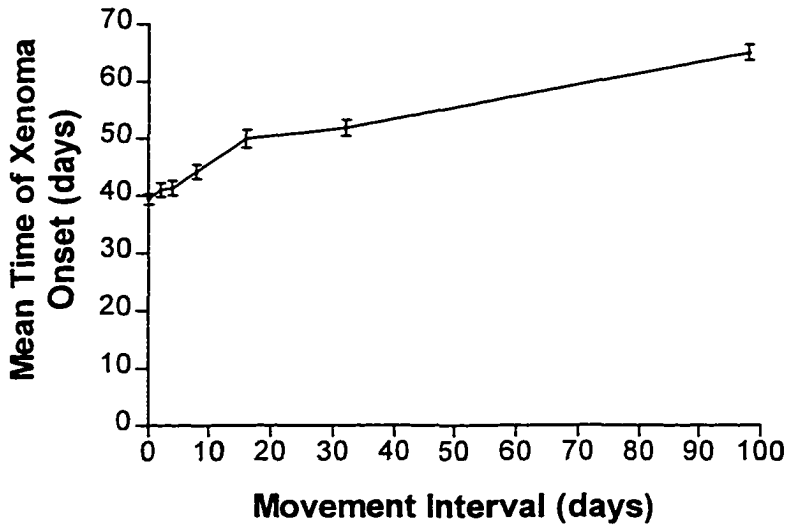


Fig. 15 Effect of movement interval on mean time of onset of branchial xenomas (±SEM). Movement interval (0, 2, 4, 8, 16, and 32 days) is time spent, following infection, at 11°C before transfer to 15°C. Data at 98 d are the fish which remained at 11°C for the duration of the trial.

Table X. Tukey's pairwise comparisons for xenoma onset at movement intervals.

MI	0	2	4	8	16	32
2	-6.845 3.695					
4	-7.195 3.345	-5.855 5.155				
8	-9.835 0.324	-8.503 2.142	-8.153 2.492			
16	-15.718 -5.441	-14.383 -3.636	-14.033 -3.276	-11.015 -0.663		
32	-17.684 -7.281	-16.346 -5.468	-15.996 -5.118	-12.981 -2.473	-7.213 3.408	
Never (98)	-31.379 -20.342	-30.028 -18.543	-29.678 -18.193	-26.673 -15.537	-20.902 -9.659	-19.057 -7.699

MI is the movement interval (days) when fish were moved from 11°C water to 15°C; Family Error Rate = 0.0500; Individual Error Rate = 0.00328

Table XI. Regression analyses for xenoma onset in the gills of rainbow trout.

Regression Equation	P	R^2 (%)
Onset = 41.3 + 0.301DM	<0.001	62.6
Onset = 40.0 + 0.502DM + 0.00243DM^2	<0.001	64.6
[1]Onset = 3.74 + 0.00576 DM	<0.001	59.6

[1] Regression analysis based on log-transformed data; DM is the days post-exposure that fish were moved from 11 to 15°C; P is the p-value; R^2 is the amount of total variation explained by the regression model.

Table XII. Temperature units (°C x days) (±St. Dev.) for *Loma salmonae* time of xenoma onset using three models developed based on different lower permissive temperatures.

MI (days)	Original TU (°C x days)	TU-5°C	TU-7°C
0	586.7±67.5[157]	389.81±44.8[1]	311.06±35.8[17]
2	602.4±77.7[257]	397.60±51.6[2]	315.71±41.2[27]
4	599.8±82.2[357]	393.27±54.6[3]	310.67±43.6[37]
8	626.3±85.7[47]	405.69±57.0[4]	317.43±45.5[47]
16	681.9±108.2[5]	432.15±71.9[5]	332.24±57.4[567]
32	647.9±97.9[6]	388.59±65.0[6]	284.87±51.9[6]
Never (98)	717.6±60.1[7]	391.41±32.8[7]	260.94±21.8[7]

[1234567] Superscripts which are the same within a column are significantly different; MI is movement interval from 11 to 15°C post-intubation; Original TU are the temperature units calculated using 0°C as the lowest temperature at which xenomas develop, $p<0.001$; TU-5 are the temperature units calculated when 5°C is used as the lowest temperature at which xenomas develop, $p=0.135$; TU-7 are the temperature units calculated when 7°C is used as the lowest temperature at which branchial xenomas develop, $p<0.001$.

Table XIII. Proportion of fish which developed xenomas in each group.

% Positive	Day Moved
88.9	0
71.4	2
76.9	4
82.1	8
71.0	16
77.8	32
63.0	77

Day moved is the movement interval post-infection at which fish were moved from 11°C to 15°C. Proportions have been converted to percentage for each group.

4. THE EFFECT OF TEMPERATURE ON INDUCED RESISTANCE IN JUVENILE RAINBOW TROUT TO *LOMA SALMONAE* (MICROSPORA)

4.1 ABSTRACT

Loma salmonae causes severe branchial disease in farm-reared Pacific salmon which is not normally evident until after fish are transferred to seawater netpens. Since no effective treatments exist to control *L. salmonae* infections, other mechanisms of control are essential. Preliminary studies indicated that a protective response against the parasite may be elicited in fish following exposure to *L. salmonae* and this study supports those findings. Juvenile rainbow trout were initially exposed to infective *L. salmonae* spores via oral intubation or feeding of infective gill material, with trials conducted at 10 different controlled water temperatures ranging from 5° to 21°C. Branchial xenomas developed in fish at all temperatures except 5°, 7° and 21°C. All fish were transferred to 15°C and re-challenged 16 to 26 weeks after the initial exposure to *L. salmonae*. Branchial xenomas were rarely detected following the second challenge, regardless of the water temperature at which the initial exposure was conducted. Thus, development of resistance in rainbow trout to *L. salmonae* appears to be independent of water temperature during the initial challenge, including those temperatures which do not permit the parasite to complete its life cycle. This was evident when fish at 5°, 7° and 21°C did not develop xenomas during an initial exposure but were protected when re-challenged with *L. salmonae* at temperatures typically ideal for the parasite. Relative percent protection (RPP), commonly used to examine vaccine efficacy, was high in all groups of fish. Consequently, the potential for the development of a vaccine against *L. salmonae* should be examined more closely.

4.2 INTRODUCTION

Loma salmonae is a significant pathogen of farm-reared salmonids, causing severe branchial damage in infected hosts. As with other microsporidian pathogens, there are currently no chemotherapeutic agents to control or prevent outbreaks. Kent and Dawe (1994) suggest *L. salmonae* may be controlled with fumagillin DCH, a drug used to control microsporidian infections including *Enterocytozoon salmonis* in chinook salmon (Hedrick et al. 1991), *Nosema apis* in honey bees, and *Pleistophora anguillarum* in eels *Anguilla japonica* (Kano et al. 1982), and several myxosporidian infections (Hedrick et al. 1988; Wishkovsky et al. 1990). However, toxic side effects occurred in several studies (Hedrick et al. 1988; Laurén et al. 1989; Wishkovsky et al. 1990). Fumagillin and its alternatives are not currently licensed for use in salmonid aquaculture in Canada. In addition, many chemotherapeutants, including fumagillin, become toxic to fish rather than therapeutic with only a small change in concentration (Dunn et al. 1990). Therefore, research on other approaches to disease control is warranted.

Development and use of efficient vaccination programs is an emerging tool for preventing various infectious diseases of fish (Dunn et al. 1990; Leong and Fryer 1993; Lillehaug 1989; Newman 1993). Manipulation of the immune system of fish to encourage resistance to pathogens has many advantages over the use of therapeutic agents within aquaculture situations. Vaccination programs against endemic bacterial pathogens are emerging (Dunn et al. 1990; Hjeltnes et al. 1989; Lillehaug 1989; Lillehaug et al. 1992; Newman 1993; Nordmo and Ramstad 1997; Rorstad et al. 1993). Their use has been shown

to be cost-effective relative to disease-associated expenses (Lillehaug 1989; Newman 1993). Additional benefits can include improved growth and feed conversion (Lillehaug 1989), and in some cases dramatic declines in antibiotic use (Iwama 1991; Lillehaug 1989; Newman 1993).

Practical advances in vaccine development and use have been directed primarily at bacterial pathogens. In Canada, bacterins are used to protect farmed salmon against several species of pathogenic vibrio agents, in addition to *Yersinia ruckeri* (agent of Enteric Red Mouth), and *Aeromonas salmonicida* (agent of furunculosis). The only commercial viral vaccine that currently exists is for infectious pancreatic necrosis (IPN). However, no commercial vaccines are currently available for parasitic pathogens of fish despite their impact on aquaculture production. This seems paradoxical, considering the ability of fish to develop effective protection to re-infection against agents such as the myxosporidian responsible for Proliferative Kidney Disease (Clifton-Hadley et al. 1986; Foott and Hedrick 1987), the ciliate *Ichthyophthirius multifiliis* (Wahli and Meier 1985) and the flagellate *Cryptobia salmositica* (Woo 1987).

Preliminary studies indicate that rainbow trout which recover from infections with *L. salmonae* are protected against experimental re-challenge (Speare et al. 1998b). More importantly, trout develop protection after initial infections in which xenomas do not develop, thereby evading the xenoma-induced branchitis typical of *L. salmonae*. Thus, exposure of naive fish to *L. salmonae* spores at temperatures which are outside the range necessary for xenoma formation (see Chapter 2), may result in sufficient protective immunity generated by the fish. This strategy could be superimposed on the production cycle of

salmonids. For example, an initial exposure of naive fish to infective material could be carried out during winter months in hatcheries when water temperatures block the parasite's ability to complete its life cycle (and hence the infection may be completely subclinical). Later, in the spring or early summer, when these fish are transferred to marine netpens and encounter marine reservoirs of infection, induced resistance obtained during the hatchery exposure could protect them from disease.

The purpose of the present study was to develop more complete data pertaining to the ability of fish to develop resistance to *L. salmonae*. Specifically, we addressed the effect of a wide range of water temperatures during the initial exposure on the ability of fish to develop a protective response against re-exposure to the pathogen at a permissive water temperature.

4.3 MATERIALS AND METHODS

Three trials were conducted in this study using fish previously infected with *L. salmonae* (Chapter 2). The initial infection challenges for each of the following trials are illustrated in this chapter; however, for more details, refer to Chapter 2. During the initial challenges, fish were exposed to *L. salmonae* by either feeding of infective gill material or gastric intubation. Before re-infection trials began, all fish that developed xenomas in the initial challenges were given sufficient time to clear the infection (i.e. xenomas were no longer visible in the gill lamellae upon examination under the dissecting microscope). All procedures were carried out according to the guidelines of the Canadian Council on Animal

Care.

4.3.1 Experimental Design

Trial 1: After all xenomas had cleared the gills of infected fish at 11°, 13°, 15°, 17°, 19°, and 21°C (Fig. 16), 124 remaining fish were tagged with beads and stamped with liquid nitrogen to identify the tank from which they originated. All fish were placed in a single, round flow through tank at 15°C with a volume of 760 L and a flow rate of 3.0 L/min. Two groups of positive controls were added to the tank, one consisting of 10 naive fish which were approximately the same size as the other fish in the tank (140 g), the second including 20 smaller (30 g) naive rainbow trout.

Trial 2: After 15 weeks, all fish in the trial at 17°, 19°, 20°, and 21°C that had developed xenomas in the gills had cleared the infection (Fig 17). In total, 62 fish from 17° and 21 °C were divided into two identical flow through tanks with similar flows (2.9 L/min), each at 15°C, thus mixing the two groups of fish together. Five fish were moved simultaneously into each of the tanks until there were similar numbers in each tank. Ten naive fish were added to each tank for use as controls. There was a 24 h acclimation period at 15°C.

Trial 3: After 26 weeks of the initial infections at 5°, 7°, 9°, 10°, 11° and 15°C, xenomas had cleared from the gills of all fish (Fig. 18). With the exception of fish from 15°C, all fish were placed in a single tank with a volume of 760 L. Thirty control fish, with an average weight of 45 g, were added to this tank and there was a one week acclimation period. After being exposed to *L. salmonae*, 156 fish were then moved via systematic

randomization to 6 identical flow through tanks (Appendix A), each at a water temperature of 15°C. The flow rate was similar in all tanks (3.0 L/min).

4.3.2 Methods of Infection

Gill tissue containing numerous xenomas was prepared for intubation by removing the arch cartilage from filaments, macerating the gill filaments using razor blades, and diluting the resulting tissue with water. Fish received 0.2 ml of this dilution directly into the stomach via a flexible tube connected to a syringe. Spores were counted using a haemocytometer under a microscope with polarized light. The initial sample was diluted by a factor of 10 to facilitate counting. If fish were fed infective gill tissue, the entire arch, including the cartilage, was chopped into many small pieces. These were mixed with a small amount of tank water to prevent clumping. Before this mixture was poured into the tanks and during feeding, water flow was stopped to prevent the loss of infective tissue down the outflow drain. Using this methodology, the spores were counted by removing the arch from the filaments, macerating the filaments, diluting them with distilled water, and counting as above.

Trial 1: One week after fish were tagged and stamped, fish were anaesthetized in 60 mg/l of benzocaine and challenged with *L. salmonae* via gastric intubation. The dose level of this mixture was estimated at approximated 35,000 spores per fish.

Trial 2: Fish were fed infective gill material after being off feed for 2 days. Dosage was approximately 438,180 spores per fish. However, this assumes that all fish ate equally and that all the infective gill material was consumed.

Trial 3: Feed was withheld from fish for 2 days prior to feeding with infective gill material. Assuming all fish ate equal amounts and that all tissue was consumed, each fish received approximately 262,500 spores.

4.3.3 Sampling and Infection Assessment

During each trial, fish were assessed non-lethally for the presence of branchial xenomas using methodology previously described (Speare et al. 1998a,b). Filaments of the second gill arch and visible tips of other arches were examined. Fish were anaesthetized (60 mg benzocaine/L water) and the gills were examined with a dissecting microscope, by gently lifting the operculum.

Trial 1: Three and four weeks post-intubation, a subsample of 30 fish was screened non-lethally for the presence of xenomas in the gills. Between weeks 5 and 8, all fish (124) were examined weekly for the presence of xenomas.

Trial 2: Beginning at 4 weeks post-exposure and continuing until week 9, the gills of all 62 fish were examined non-lethally for xenomas.

Trial 3: Between weeks 4 and 9 post-exposure to *L. salmonae*, all 156 fish were non-lethally examined for the presence of xenomas.

4.3.4 Data Analysis

This study tested the null hypothesis that there was no difference in the proportion of fish that developed branchial xenomas, regardless of original water temperature or whether fish had prior exposure to *L. salmonae*. The cumulative proportion of fish which

developed xenomas in each group was examined through a series of Chi-Square (χ^2) or Fisher's exact tests. Relative percent protection (RPP), a modification of the relative percent survival (RPS) statistic used to test the effectiveness of vaccines (De Kinkelin et al. 1995; Rorstad et al. 1993), was calculated to assess how well fish were protected from *L. salmonae* after having been previously exposed to the parasite. However, this technique assesses mortalities in a trial. Rainbow trout infected with *L. salmonae* do not typically experience the clinical disease or mortality often seen in other salmonids. Therefore, in our study, this statistic described the proportion of fish which developed branchial xenomas in the following formula:

$$RPP = \left(1 - \frac{\%\text{ previously exposed with xenomas}}{\%\text{ naive with xenomas}} \right) \times 100\%$$

In all statistical analyses, a confidence level of 95% ($P<0.05$) was considered significant. All statistical analyses were completed using commercial software (MINITAB™ Inc. and GraphPad InStat™).

4.4 RESULTS

No differences were seen in the proportion of control fish that developed branchial xenomas among trials ($p=0.3094$) and the relative percent protection in each trial was similar.

4.4.1 Trial 1

Xenomas developed in one of 124 previously exposed fish following the second exposure to *L. salmonae*. The positive fish was negative in the original trial at 13°C and very few xenomas were present in the second infection challenge. In contrast, significantly more control fish (13 of 16) were positive 7 weeks after the second challenge (Table XIV). There were no significant differences within experimental groups. In addition, the RPP's were similar among groups of fish originating from a different water temperature in the initial exposure study. All previously exposed fish were combined into one group and all control fish were combined into another group to compute a mean level of protection (=RPP) which was 99.0%.

4.4.2 Trial 2

After 9 weeks, xenomas were detected in 19 of 20 control fish. In contrast, no fish originally from 17°C were positive and only 2 of 29 originally from 21°C developed xenomas. No significant difference in proportion infected was found between control fish in either tank (χ^2 =0.3049, p=1.000). Consequently, they were grouped together for the purpose of RPP calculations. The RPP for fish originating from 17°C and 21°C were 100.0% and 92.7%, respectively (Table XIV). The mean level of protection for this trial was 96.4%.

4.4.3 Trial 3

When this trial terminated after nine weeks, xenomas were seen in 27 of 29 (93%)

control fish. No fish developed xenomas from tanks originally at 7°, 10° or 11°C although some fish did develop xenomas from the tanks originally at 5°C (9 of 25) and 9°C (1 of 32). The proportion of infected control fish differed significantly from that of fish previously infected at any temperature. The relative percent protection seen in all previously infected groups was similar (96.7% to 100.0%) except for fish originally reared at 5°C (61.3%), in which RPP was significantly lower (Table XIV). An overall RPP value was calculated in two ways: one calculation included data from fish originally at 5°C while the second calculation excluded it since it was significantly different in previous calculations. Values obtained were 93.1% and 99.2%, respectively.

4.5 DISCUSSION

Control of *L. salmonae* infections in farmed salmon through the methods of therapy or prevention is essential due to devastating losses attributable to the parasite. Since no therapeutants are licensed in Canada for treating internal protozoans such as *L. salmonae*, alternatives are required. Rainbow trout develop resistance to *L. salmonae* following recovery from experimental laboratory infections (Speare et al. 1998b). The present study clearly supports the previous evidence, additional to indicating that water temperature during the initial exposure of fish to the parasite does not affect resistance which develops in fish to subsequent challenges. The observed protection in our study is similar to that found in rainbow trout against the haemoflagellate *Cryptobia salmositica* (Jones and Woo 1987). This adaptive immunity to pathogens typically persists for relatively long periods (Ellis

1988). Fish have also shown the ability to develop resistance against agents such as PKX, the causative agent of proliferative kidney disease (Clifton-Hadley et al. 1986; Foott and Hedrick 1987) and the ciliate *Ichthyophthirius multifiliis* (Burkart et al. 1990; Valtonen and Keränen 1981; Wahli and Meier 1985).

Similar methodology to that employed here is used to test efficiency of vaccines in that fish are exposed to a pathogen, develop an immune response, and are subsequently re-challenged with the same pathogen (Hjeltnes et al. 1989; Kawakami et al. 1997; Lillehaug et al. 1992; Magariños et al. 1994). Relative percent survival (RPS) or relative percent protection (RPP) are often used as an indication of vaccine efficacy. In our study, RPP was high in all three trials where fish were exposed to *L. salmonae* prior to the current challenge. The only outlier in this study was the group of fish which were initially exposed to *L. salmonae* at 5°C. These fish had significantly lower RPP than all others in the same trial. However, other vaccine trials, either laboratory studies or field trials, typically have lower RPS or RPP values (De Kinkelin 1995; Hjeltnes et al. 1989; Kawakami 1997; Lillehaug et al. 1992; Magariños et al. 1994; Rorstad et al. 1993) than those in the current study. The similarity among groups in our study indicates that water temperature upon initial exposure to *L. salmonae* had little effect on the level of protection generated by fish when re-challenged (except at 5°C).

The major difference between vaccine trials described above and those conducted in the current study is that those vaccines are rendered non-pathogenic before being administered (Ellis 1988). The antigens derived from pathogenic organisms stimulate the immune system to increase resistance upon subsequent exposures to the pathogen. Vaccines

composed of inactivated pathogens or extracts are considered dead while those which are attenuated are considered to be live vaccines and have little or no virulence (Ellis 1988). Using a vaccine composed of live tomites resulted in 100% protection when channel catfish were challenged with *I. multifiliis* immunized using live tomites (Burkart et al. 1990).

Since protection against *L. salmonae* is high despite the high proportion of fish that did not develop xenomas during the initial challenge, a strategy could be employed to infect fish in hatcheries at a lower temperature that does not permit full parasite development (i.e. xenoma formation), but induces significant resistance to further infections. For example, when re-challenged at 15°C, fish originally infected at 7°C were completely resistant to re-infection although a very small percentage of fish developed branchial xenomas in the initial challenge (Fig. 18). Although the fish immune system is not likely functioning optimally at this temperature (Newman 1993), if fish are vaccinated early enough, they should have sufficient time to develop resistance to exposures which may occur after transfer to marine netpens. A similar strategy currently used to control PKD, results in limited renal damage during initial exposure to the myxosporidian agent at low temperatures. Fish developed protection against re-infection or recrudescence when water temperatures were favourable to the parasite.

How long fish remain protected against *L. salmonae* is unknown, hence further studies are required to determine if the initial infection has a long-lasting effect. Some vaccination trials testing adjuvanted furunculosis vaccines have shown that RPS declines after a period of time following vaccination (Lillehaug et al. 1992) and other trials have shown booster vaccinations against *Vibrio salmonicida* increase RPS (Hjeltnes et al. 1989).

The current study was compared to vaccination trials because the initial challenge of fish to *L. salmonae* was similar to using a live vaccine which typically induce high levels of protective immunity (Newman 1993). However, to ensure this is a safe mechanism of control for Pacific salmon, the parasite antigen would likely be either attenuated or killed. Further trials are required to determine if the same protection is induced in chinook and coho salmon, species experiencing large losses due to this parasite and whether the presence of other pathogens affects the development of protection.

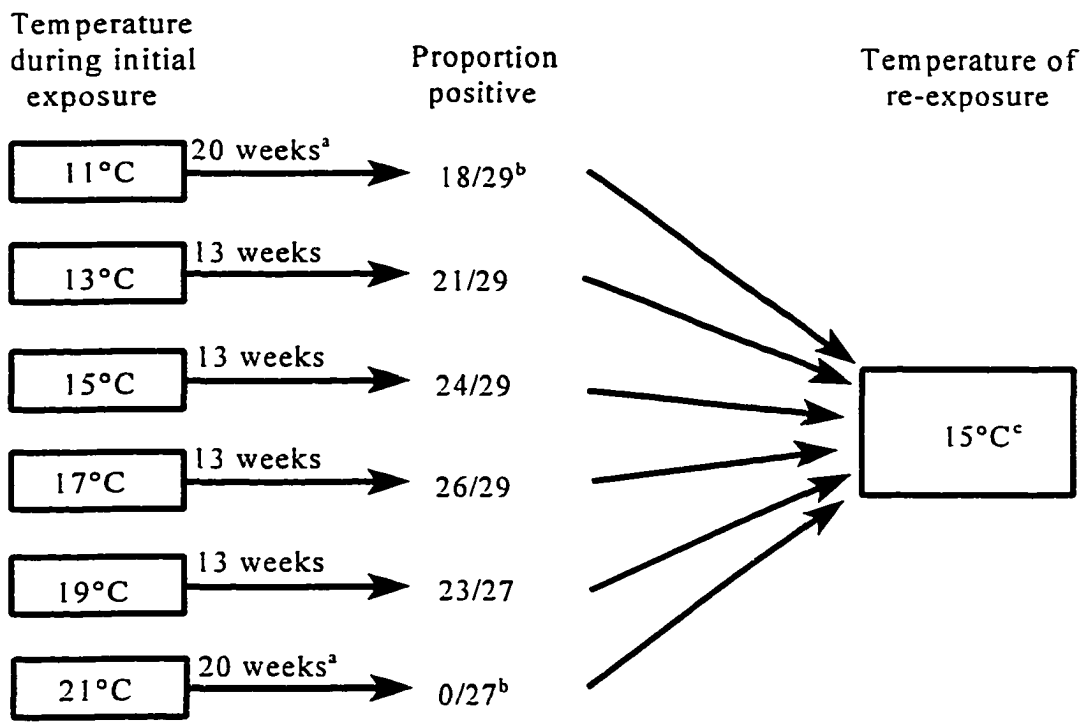


Fig. 16 Flowchart describing infection process for fish in Trial 1, Chapter 2.
[a] 13 weeks at 11°C plus 7 weeks at 17°C
[b] No new positive fish after movement to warmer temperature
[c] 30 control fish also added to this tank

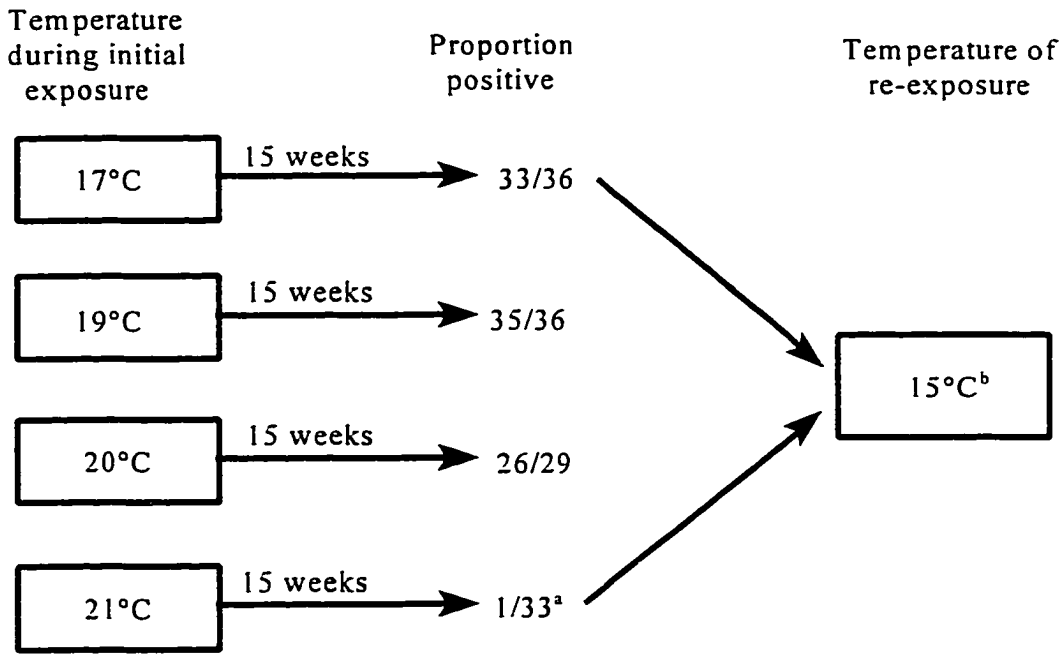


Fig. 17 Flowchart of initial infection challenge for Trial 2, Chapter 2 (verification of upper lethal temperature).

[a] Positive fish only after 9 weeks, 1 visible xenoma in gill lamellae

[b] Fish divided into two tanks, 10 control fish added to each tank

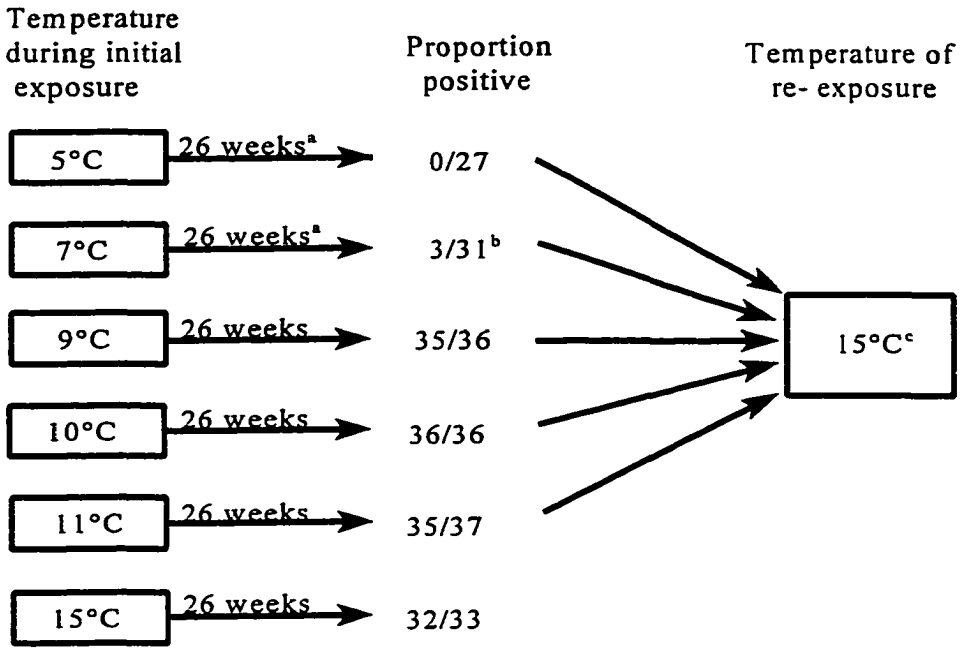


Fig. 18 Flowchart describing initial infection challenge for cool water study.
[a] 16 weeks at low temperature followed by 10 weeks at 17°C
[b] fish positive only after being moved to warmer temperature
[c] 30 control fish also added to tank holding fish from all other temperatures

Table XIV. Effect of water temperature during initial exposure to *Loma salmonae* on subsequent exposures at 15°C.

Trial No.	Temp. at first exposure (°C)	No. of fish per group	No. with xenomas	% with xenomas[a]	RPP[b]
1	11	22	0	0.0	100.0
	13	25	1	4.0	95.1
	15	21	0	0.0	100.0
	17	19	0	0.0	100.0
	19	16	0	0.0	100.0
	21	21	0	0.0	100.0
	Small controls[c]	7	7	100.0	-
	Large controls[c]	9	6	66.7	-
2	17[d]	33	0	0.0	100.0
	21[d]	29	2	6.9	92.7
	Controls[d]	20	19	95.0	-
3	5	25	9	36.0	61.3
	7	29	0	0.0	100.0
	9	32	1	3.1	96.7
	10	34	0	0.0	100.0
	11	36	0	0.0	100.0
	Controls	29	27	93.1	-

[a] Percent with xenomas during second challenge (except for naive controls)

[b] Relative Percent Protection =

$$\left(1 - \frac{\%\text{ previously exposed with xenomas}}{\%\text{ naive with xenomas}} \right) \times 100\%$$

[c] Controls were not included in the initial challenge with *L. salmonae* but were naive to this challenge study. Since no significant difference was found between the two groups of controls ($p = 0.2125$), they were combined for further analyses.

[d] Although two tanks were used in Trial 2, no differences were found between tanks for each group, therefore, they were each combined into one group for each temperature.

5. GENERAL DISCUSSION

Microsporidians have adapted for survival within their hosts by becoming intertwined with host physiology, thus making them difficult to treat. Most available treatments do not work well or have side effects which can be detrimental to the host (Hedrick et al. 1988; Laurén et al. 1989; Wishkovsky et al. 1990). Effective treatments that do exist (Hedrick et al. 1991; Kano et al. 1982; Kent and Dawe 1994) are not likely to be licensed for use in Canada in the near future. Therefore, alternatives for control are necessary. Understanding the thermal preferences of some parasites has led to methods of parasitic control in infected hosts, best exemplified by the control strategy for the myxosporidial agent causing proliferative kidney disease (Clifton-Hadley 1984; Ferguson 1981).

Temperature affects the life cycle of many parasites, including the ciliates *Cryptocaryon irritans* (Diggles and Lester 1996) and *Ichthyophthirius multifiliis* (Noe and Dickerson 1995), the nematodes *Hyostrongylus rubidus* and *Oesophagostomum* spp. (Fossing et al. 1995) and heterorhabditids (Mason and Hominick 1995), the monogenean *Polylabroides multispinosus* (Roubal and Diggles 1993) and the digenean *Crassicutis cichlasomae* (Scholz et al. 1995). *Loma salmonae* is also affected by water temperature (Speare et al. 1998a,b), creating an opportunity, explored in this thesis, to exploit the relationship between the thermal requirements of *L. salmonae* and one of its hosts, the rainbow trout.

Although infections with *L. salmonae* have been described for many years, its basic biology remains poorly understood. Although the xenoma-stage of the parasite has been

morphologically described (Canning and Lom 1986; Kent 1992; Kent et al. 1989; Morrison and Sprague 1981, 1983; Speare et al. 1989), less is known about experimental transmission (Kent et al. 1995), or epidemiology (Bruno et al. 1995; Gandhi et al. 1995; Magor 1987; Markey et al. 1994). The thermal biology of *L. salmonae* is largely unexplored beyond some preliminary findings (Speare et al. 1998a,b) even though thermal manipulation may offer the best odds through which to control or limit infections.

The upper and lower critical temperature thresholds for *L. salmonae* development to its xenoma stage were 21°C and 7°C respectively if the parasite was maintained at those temperatures for prolonged periods (Chapter 2). Low temperatures limited xenoma formation but may not have killed the parasite; a small percentage of fish that were originally held at 7°C (for 16 weeks) developed xenomas after they were moved to 17°C. No fish at 5°C developed xenomas when moved to the warmer temperature after 16 weeks although studies in our laboratory (Speare 1998, unpublished) indicate fish will develop xenomas if fish are moved to warmer water sooner. Those fish at 21°C never developed xenomas, indicating the parasite was likely killed.

Within the permissive temperature range, and with temperature held constant, the rate of development of *L. salmonae* is dependent on the water temperature at which it is reared, and is defined by the regression equation presented in Chapter 2. Similar trends occur with other microsporidians such as *Glugea stephani* (Olson 1981) and *Enterocytozoon salmonis* (Antonio and Hedrick 1995), although the exact relationships to temperature have never been modeled for other teleost microsporidians. The current findings are therefore important because we have determined the range in which this parasite develops and the timing of

development given a constant temperature. Chapter 3 also demonstrates the effects of variable water temperature on parasite development, a situation likely to be encountered on fish farms. This chapter also demonstrated the robustness of results among trials since rate of xenoma formation was similar in Chapter 2 and 3 for those portions of the trials conducted at the same temperatures, indicating results are not dependent on trial. Chapter 4 then demonstrated the ability of rainbow trout to resist *L. salmonae* infections when they had previously been exposed to the parasite, regardless of whether xenomas formed during the initial challenge. Combining information from all three chapters may provide the strategy required to control *L. salmonae* outbreaks through water temperature manipulations. In aquaculture, these manipulations may be possible in hatcheries, land-based grow out sites, and where cooler, deeper water may be pumped into marine netpens, a strategy used during diatom bloom periods.

A curious finding regarding *L. salmonae* is that although it is adapted to its salmonid hosts, it is not adapted to the entire temperature range of these fish, thus creating an opportunity for potential control (i.e. resistance induction). This was demonstrated when xenomas did not form at 5°, 7°, or 21°C although fish at each of these temperatures developed significant protection against the parasite during subsequent exposures.

Prior studies indicated that fish which recover from *L. salmonae* infections are able to resist re-infection (Speare et al. 1998b). The same study also highlighted the possibility that xenoma-formation during initial exposure was not required for resistance to be elicited to subsequent exposures. This is important because formation and dissolution of xenomas corresponds to clinical disease in chinook salmon (Hauck 1984) and growth suppression in

rainbow trout (Speare et al. in press). This concept was explored further within this thesis, and elucidated a practical possibility for controlling *L. salmonae* infections using thermal manipulations. Specifically, fish exposed to *L. salmonae* at a water temperature which did not permit xenoma formation were still protected when re-challenged with infective spores, even when the re-challenge was conducted at a water temperature highly favourable to the parasite. The relative percent protection in previously exposed fish ranged from 61.3% to 100% in the three trials conducted in Chapter 4, whereas 81.2% to 95% of the previously naive fish developed xenomas in these trials. The high level of protection observed and the consistency between trials indicates this may be a very practical mechanism for preventing *L. salmonae* outbreaks since the relative percent protection observed was higher than that observed in many vaccine trials (Hjeltnes et al. 1989; Kawakami et al. 1997; Lillehaug et al. 1992; Margariños et al. 1994; Rorstad et al. 1993). The application of this approach would not be difficult and would simply involve exposure of hatchery-stock to *L. salmonae*-infected material during winter months when water temperatures are in the range of 5°C to 7°C. Protection established from this would protect the fish when transferred to sea water net pens where endemic infections are likely to be encountered.

Although the findings regarding resistance are very exciting, several areas of research need to be assessed before attempting to implement this strategy. Specifically, we do not known whether our findings in rainbow trout also apply to chinook salmon or if resistance persists after transfer to sea cages, and if so, how long it persists. Finally, studies to uncover the mechanisms of resistance are warranted for a better understanding of how environmental and production factors may augment or diminish the acquired protection.

The relationship between temperature and development of ectotherms has been widely studied, resulting in mathematical models, based on regression analyses and thermal unit calculations, which are used to predict a range of phenomena. They are a useful tool for predicting disease onset in many parasite groups and the timing of fish egg hatching (Piper et al. 1989; Bromage and Shepherd 1988), although they are not commonly used in dealing with fish parasites. The ability to predict disease onset, when exposure time to the pathogen is known, would allow predictions of outbreaks or severe periods of the infection process, allowing farmers an opportunity to treat fish before disease becomes a major problem (if therapeutic agents are available). With *L. salmonae* infections, it would allow fish farmers time to manipulate water temperatures in efforts to decrease the severity of infections often seen in farmed chinook and coho salmon (Hauck 1984; Kent et al. 1989). Where drugs are used, the window of time in which drugs are used to treat diseases would decrease, thus decreasing money spent.

Thermal unit calculations, measured in degree-days, are a common approach used in examining the emergence of several parasites into the environment and are calculated based on a lower critical temperature threshold for parasite development. This model was used to predict the optimum temperature for spore production of the microsporidian *Edhazardia aedis* in its host, the mosquito, *Aedes aegypti* (Becnel and Undeen 1992). Also, developmental rate of various stages of the dipteran *Parasarcophaga* (*Liopygia*) *ruficornis* are assessed through the use of degree-day calculations (Amoudi et al. 1994). Similar methods are used when examining stages of development of the worm *Angiostrongylus cantonensis*, of the flesh fly *Wohlfahrtia nuba*, and the western black-legged tick *Ixodes*

pacificus. Degree-day calculations are also used to predict outbreaks of the nasal bot fly, *Oestrus ovis*, in small stock such as sheep and goats (Flasse et al. 1998) since high temperatures accelerate larval maturation and reduces the fly's puparial period. It is used to warn farmers when to treat their stocks for this parasite, just before the most severe period of fly strike (Flasse et al. 1998). This method is also used to determine the time of aerial spraying in Colorado for mosquitos such as *Culex* and *Aedes* (McCallister 1992).

The lower critical threshold (7°C) used in degree-day calculations in this thesis was determined based on the presence of xenomas at each water temperature tested. This temperature was chosen as the final zeroing point because it was the temperature below which no xenoma formation occurred. This approach is very practical, even when water temperatures are changing during the developmental cycle of the parasite, demonstrated in Chapter 3.

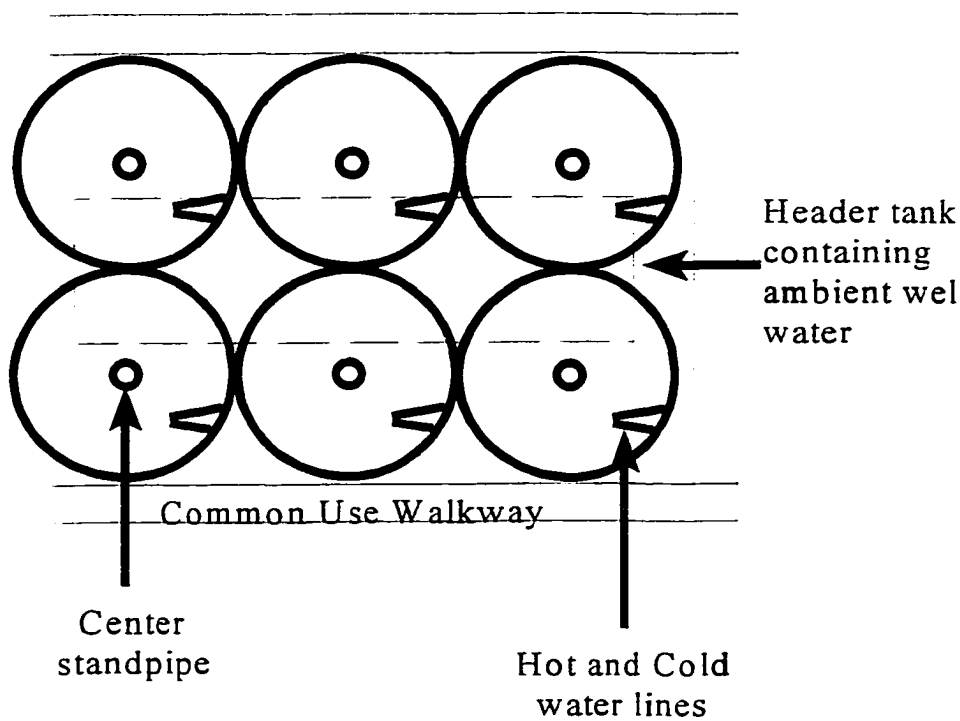
When temperatures are held constant throughout the parasite's life cycle, a regression equation is appropriate for predicting time of onset, but only for the optimal developmental temperature range. Beyond this, prediction accuracy begins to decline as developmental times vary, a pattern frequently observed with other parasite developmental cycles (Cecchini et al. 1998; Pandey et al. 1989; Stables and Chappell 1986). Within the range, predictions are close to actual data recorded in all three trials conducted using the quadratic model. Regression equations have also been used previously to calculate critical low temperatures for oviposition, egg hatching and larval and nymphal moulting of the tick, *Haemaphysalis longicornis* (Yano et al. 1987). This study occurred at stable, controlled temperatures. However, temperatures rarely remain constant for extended periods either in hatcheries or

in sea water netpens. Therefore, it may be useful to examine more closely the degree-day approach for prediction which accommodates changing water temperatures.

Surprisingly, there are no previous studies which have modelled the development rates of fish pathogens this way, although the current method has significant potential for other microsporidians, myxosporidians or other fish parasites whose infective stages are dependent on water temperature. Such a predictive model would be particularly useful for *L. salmonae* since clinical manifestations of the disease are attributable to a particular stage of parasite development, whose temperature-linked onset was clearly amenable to modelling, both at constant and varying temperatures, as shown herein. Assuming that enzootic reservoirs of infection exist within a marine environment and that fish become exposed to infective spores soon after marine transfer, assessment of water temperatures and use of our predictive model should provide insight to farmers as to when xenoma formation should be occurring within the gills of farmed stock.

APPENDIX A

Tank Design for Trials used in all trials. A common use walkway was present on both sides of the system and on one end. The header tanks supplying the system in Trials 1,2 and 4 were located above the tanks. In Trial 3, one header tank (contained ambient water) was above the system, the other was on the floor beside the system (contained chilled water). In any case, the two tanks mixed just before entering the tanks to give the desired temperatures in each tank.



APPENDIX B

Randomization protocol for Trial 1. Fish were moved from one set of six tanks held at 15°C to a second set of six tanks, each with a different water temperature (1=11°C, 2=13°C, 3=15°C, 4=17°C, 5=19°C and 6=21°C).

Remove	Replace
1	2
6	5
3	6
2	3
5	4
4	1
2	3
5	4
6	1
3	5
4	6
1	2
3	4
4	1
1	5
5	6
6	2
2	3
4	5
1	2
5	4
6	1
2	3
3	6

Remove	Replace
5	6
2	3
4	2
1	4
3	1
6	5
6	1
3	6
2	3
4	2
1	5
5	4

APPENDIX C

Randomization Protocol for Trial 4. Fish were moved from a common tank at 15°C to five tanks, each at a different water temperature (1=11°C, 2=13°C, 3=15°C, 4=17°C, and 6=19°C). Fish were placed in the five tanks in groups of 5 in the following order:

3
1
4
2
6

1
6
3
4
2

2
4
6
1
3

APPENDIX D

1. Trial 1. Mean onset and duration of branchial xenomas with associated standard deviations and standard errors of the mean. One-way ANOVA on onset $p<0.001$. One-way ANOVA on duration $p<0.001$.

Temp. (°C)	% with Xenomas	Mean Time of Onset (days)	St.Dev.	S.E.M.	Mean Time to Total Clearance (days)	St.Dev.	S.E.M.
11	62.1	70.0	9.3	2.2	43.2	22.4	5.3
13	72.4	44.7	3.5	0.8	34.8	18.5	4.0
15	82.8	36.5	4.1	0.8	28.6	12.0	2.6
17	89.7	28.8	2.3	0.4	23.7	10.4	2.1
19	85.2	30.4	4.0	0.8	21.7	10.4	2.3
21	0.0	-	-	-	-	-	-

2. Trial 2. Mean time of branchial xenoma onset and duration with associated standard deviations and standard errors of the mean. One-way ANOVA on onset $p<0.001$. One-way ANOVA on duration $p = 0.598$.

Temp. (°C)	% with Xenomas	Mean Time of Onset (days)	St.Dev.	S.E.M.	Mean Time to Total Clearance (days)	St.Dev.	S.E.M.
17	91.7	32.8	5.7	1.0	31.8	14.1	2.5
19	97.2	30.4	4.3	0.7	28.1	11.6	2.2
20	89.7	35.5	6.3	1.2	31.0	6.3	1.2
21	3.0[1]	63.0	-	-	14.0	-	-

[1] One fish developed a single visible xenoma after 9 weeks.

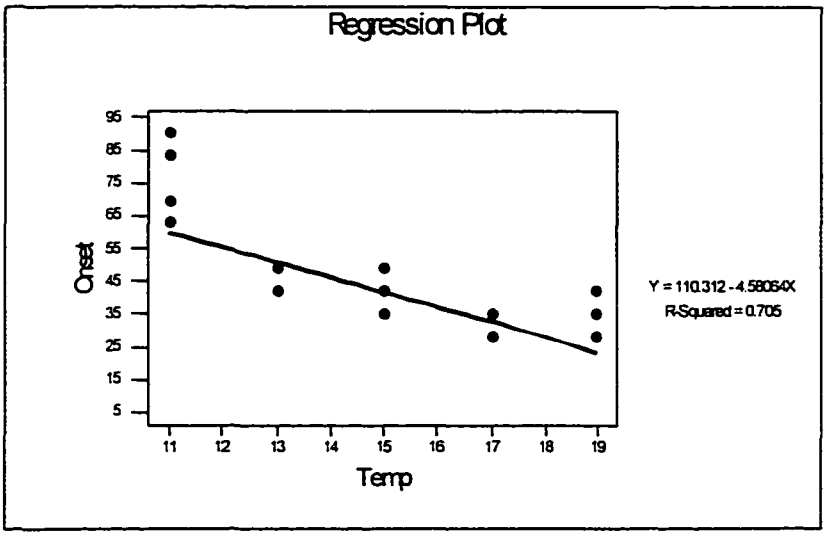
3. Trial 3. Mean onset and duration of branchial xenomas with associated standard deviations and standard errors of the mean. One-way ANOVA on onset $p<0.001$. One-way ANOVA on duration $p<0.001$.

Temp. (°C)	% with Xenomas	Mean Time of Onset (days)	St.Dev.	S.E.M.	Mean Time to Total Clearance (days)	St.Dev.	S.E.M.
5.0		-	-	-	-	-	-
7.0	9.7[1]	-	-	-	-	-	-
9.0	97.2	88.2	4.9	0.8	58.0	25.0	4.2
10.0	100.0	70.6	9.5	1.6	58.6	19.5	3.3
11.0	94.6	62.8	12.2	2.0	50.4	22.3	3.7
15.0	97.0	33.9	3.1	0.6	36.8	13.2	2.4

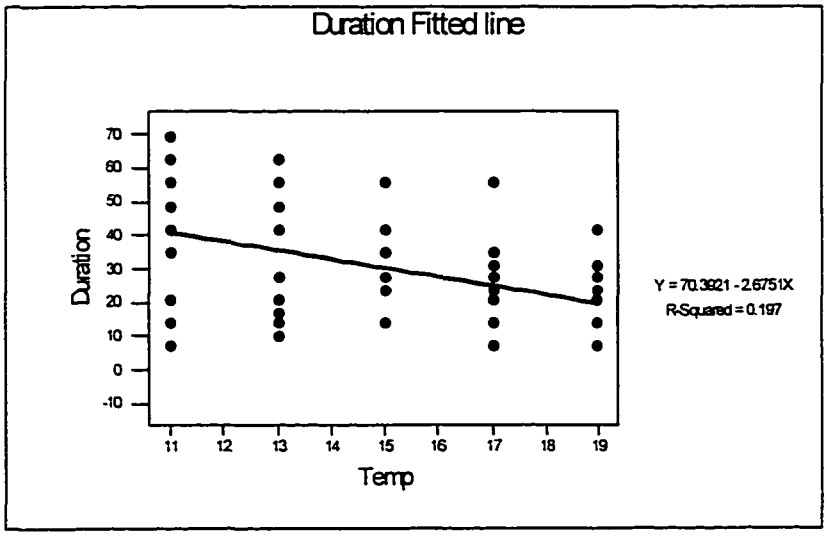
[1] Three fish became positive only after fish were moved from 7°C to 17°C, 16 weeks post-exposure to *Loma salmonae*.

APPENDIX E

1. Fitted linear regression line for xenoma onset in Trial 1.

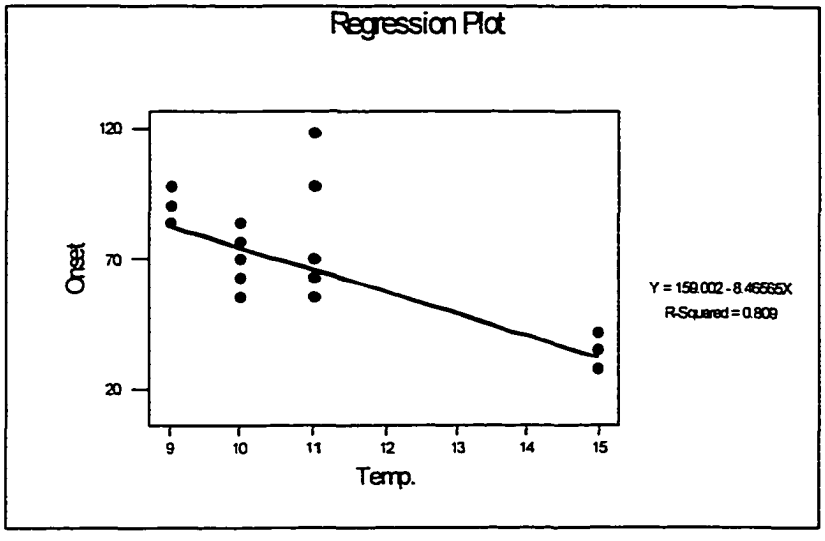


2. Linear regression fitted line for duration of xenomas in Trial 1.

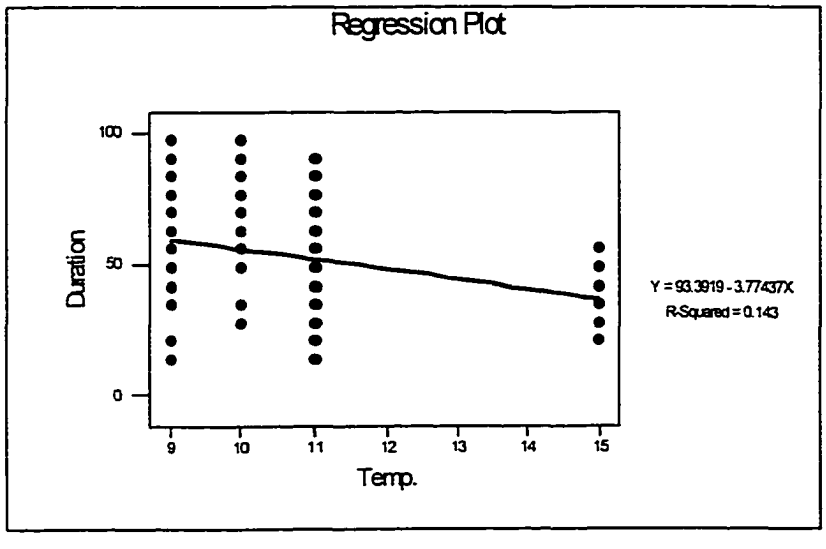


APPENDIX F

1. Linear regression fitted line for xenoma onset during cold-water study (Trial 3).



2. Linear regression fitted line for xenoma duration during cold-water study (Trial 3).



APPENDIX G

Protocol for Spore Purification Technique:

1. Rinse infected gill arches with saline and place in large Timbrock
2. Grind up tissue for several minutes, collect homogenate in a tube
3. Rinse grinder with 5 ml of saline
4. Filter resulting liquid through a syringe barrel packed with 2 gauze pads
5. Once complete, rinse the gauze with approximately 30 ml of saline
6. Collect resulting product in a centrifuge tube and spin at 2,000 rpm (325 g forces) for 30 minutes
7. Resuspend the pellet in 5 ml of saline
8. Use a 1:10 dilution of this to count the spores with the hemocytometer to give a general idea of how many spores were exposed to each fish

APPENDIX H

Mean onset of branchial xenomas with associated standard deviations and standard error of the means. One-way ANOVA on xenoma onset $p<0.001$.

Movement Interval (days post-exposure)	Mean Time of Onset (days)	St. Dev.	S.E.M.
0	39.38	4.53	0.92
2	40.95	5.22	1.17
4	41.30	5.52	1.23
8	44.13	5.75	1.20
16	49.95	7.26	1.55
32	51.86	6.57	1.43
Never (98)	65.24	5.46	1.32

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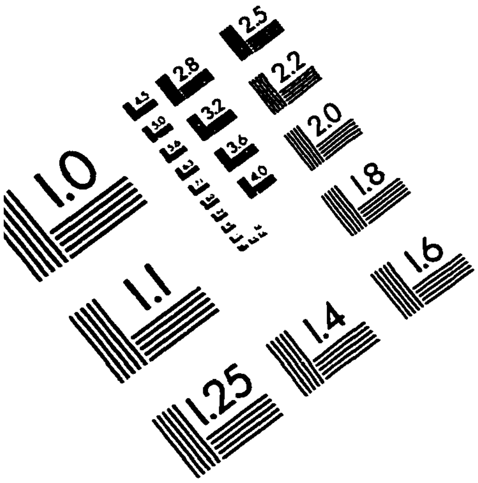
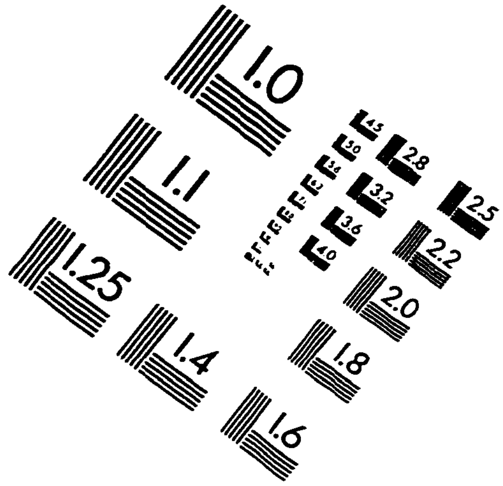
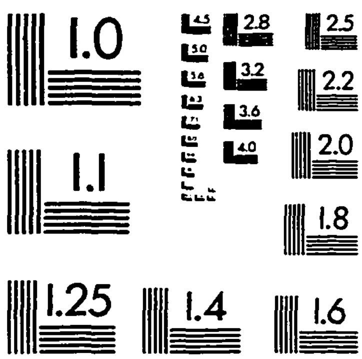
IMAGE EVALUATION TEST TARGET (QA-3)







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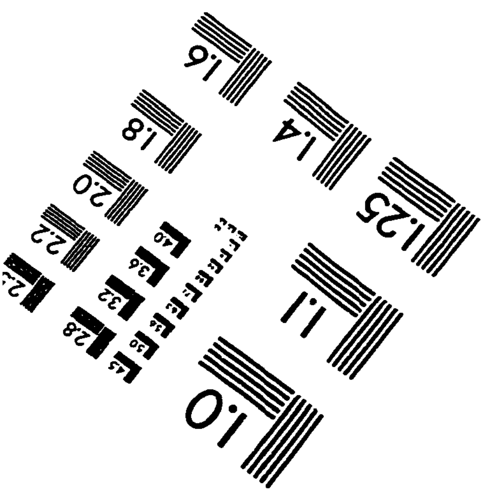

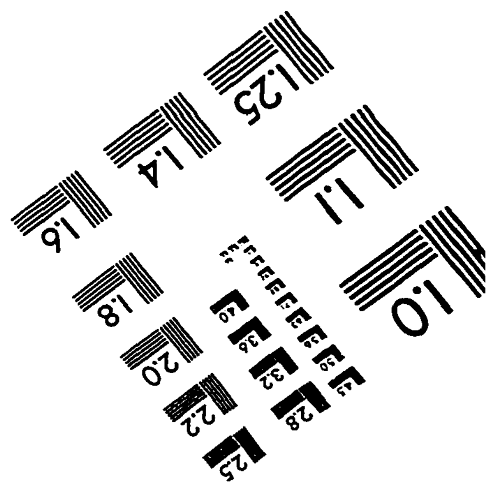

APPLIED IMAGE. Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989