HOST-PARASITE RELATIONSHIPS OF *LOMA SALMONAE* AND ITS SALMONID HOST DURING MICROSPORIDIAL GILL DISEASE OF SALMONIDS

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ABSTRACT

The host-parasite relationships of *Loma salmonae* in salmonids were investigated to better understand the pathogenesis of Microsporidial gill disease of salmonids (MGDS) in order to propose treatments for the disease. The intracellular development of two variants of *L. salmonae*, OA with host specificity for rainbow trout and SV with host specificity for brook trout, was examined with transmission electron microscopy (TEM). The two variants demonstrated ultrastructural differences in their developmental stages and in mature spores. During development within the host cell the parasites appeared to use host cell endoplasmic reticulum to derive an outer parasite membrane and a parasitophorous vacuole membrane. These findings support the hypothesis that *L. salmonae* development is aided by the intracellular process of autophagy. MGDS is associated with a severe inflammatory response. To better understand the inflammatory responses and pathology in farmed Chinook salmon affected by MGDS, moribund Chinook salmon in British Columbia were sampled from a farm during an outbreak of MGDS and examined with TEM. The findings demonstrated both early parasite stages and late parasite stages within the gills of individual fish, suggesting protective immunity was not generated. Some xenomas appeared to be undergoing degeneration, although host inflammatory cells were not observed within the xenoma. Acute host responses included infiltration with neutrophils, which phagocytosed spores without subsequent degeneration, and vascular thrombosis; chronic host responses included infiltration with macrophages, which degenerated the parasite, accompanied by neovascularization. Cells that resembled mammalian dendritic cells were found within acute lesions associated with the rupture of xenomas within the farmed Chinook salmon and in experimentally infected rainbow trout. These dendritic-like cells contained unique granules that strongly resembled Birbeck granules in mammalian Langerhans cells. Further studies to identify this cell type in salmonids demonstrated that the dendritic-like cells were present in immune organs of healthy rainbow trout, brook trout, and Atlantic salmon. The cells in healthy fish were most commonly identified in the spleen, and were present in lower numbers in the head kidney. With the suggestion that farmed Chinook salmon do not have an efficient protective immune response in their second year in the oceans, it was hypothesized that farm related stress could ablate the adaptive immune response. To test this, a stress model using dexamethasone (dex) treatment in experimentally infected rainbow trout was used. Dex treatment diminished the innate immune response to the parasite resulting in 5-fold heavier infections in non-immunized fish. Dex did not affect the adaptive response though, and it was demonstrated that fish which have generated a protective immune response could not be infected by rechallenge to the parasite during treatment with heavy doses of dex. With the severe inflammatory response associated with MGDS, the efficacy of indomethacin was tested in experimentally infected rainbow trout. Chronic drug treatment caused perforations within the anterior intestine and granulomatous peritonitis. The treatment did not affect parasite clearance, healing of the tissue, and it did not seem to reduce inflammatory lesions in the rainbow trout model. In the indomethacin treated fish a more pronounced eosinophilic granular cell (EGC) response was observed.

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ABBREVIATIONS USED

BC	British Columbia
BL	Birbeck-like
CD	Cluster of differentiation
COX	Cyclooxygenase
Dex	Dexamethasone
DC	Dendritic cell
DL	Dendritic-like
EGC	Eosinophilic granular cell
ER	Endoplasmic reticulum
GI	Gastrointestinal
h	Hours
HIV	Human Immunodeficiency Virus
HRLM	High resolution light microscopy
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MGDS	Microsporidial gill disease of salmonids
MHC	Major Histocombatibility Complex
MMC	Melano-macrophage center
NK	Natural killer
NNC	Nonspecific cytotoxic cell
NSAID	Nonsteroidal anti-inflammatory drug
OA	*Oncorhynchus* variant
PO	Propylene oxide
PE	Post exposure
PI	Post infection
RER	Rough endoplasmic reticulum
SPOV	Sporophorous vesicle
SV	*Salvelinus* variant
TEM	Transmission electron microscopy
Th	T helper
TNF	Tumour Necrosis Factor

1 GENERAL INTRODUCTION

1.1 MGDS IN SALMONIDS

Microsporidial Gill Disease of Salmonids (MGDS), caused by *Loma salmonae* (Putz, Hoffman and Dunbar, 1965), accounts for mortalities in marine netpen reared Chinook salmon *Oncorhynchus tshawytscha* in British Columbia (Kent 1998). The intracellular parasite is endemic to the region and wild salmon, which often carry subclinical infections, act as reservoirs of infection for farmed fish. Farmed Chinook salmon, unlike their wild counterpart, develop clinical disease from the parasite (Kent 2000), which is likely a result of heavier infections due to the higher stocking densities in salmon farms. During infection, xenomas, hypertrophied host cells containing developing parasites, alter the general architecture of the gill with their distribution in the primary filament and secondary lamellae (Kent et al. 1995). The breakdown of xenomas is associated with fibrinoid arteritis, periarteritis, and hyperplasia of overlying epithelium (Hauck 1984, Kent et al. 1989, Speare et al. 1989, Kent et al. 1995). A hatchery with Chinook salmon reared in freshwater found 10% mortality attributed to *L. salmonae*. The organisms were systemic with the highest numbers in the gill tissue (Hauck 1984). It has been demonstrated that water temperature is an important factor for infections with the parasite; optimal water temperatures for *L. salmonae* are between 15-17°C and parasite development is inhibited outside the temperature range of 9-20°C (Beaman et al. 1999). Outbreaks of disease in netpen reared Chinook salmon are typically observed in the late summer, after the optimal parasite temperatures. With *L. salmonae* being endemic to

regions with Chinook salmon farms, MGDS remains a seasonal problem and possible control mechanisms need to be further investigated in order to avoid further losses of farmed fish.

Rainbow trout, *Oncorhynchus mykiss* are susceptible to infections with *L. salmonae*, although they do not develop infections as heavy as seen in Chinook salmon (Ramsay et al. 2002) and clear the infections faster than Chinook salmon (Kent et al. 1999). Despite these differences rainbow trout are an ideal laboratory model of MGDS (Speare et al. 1998a) and many advances in the understanding of the pathogenesis of *L. salmonae* have been made using this model. With a single dose exposure of the parasite to rainbow trout, the sequential development of the parasite was determined. Figure 1.1 demonstrates the life cycle of *L. salmonae* in laboratory infected rainbow trout. The findings demonstrate that the parasite was in the intestinal epithelium shortly after infection and early parasite stages in the heart 2-3 days post exposure (PE) (Sanchez et al. 2001a). After arrival in the gills 2 weeks PE the sequential development of xenomas up to the disintegration of xenomas at 8 weeks PE was described using transmission electron microscopy (TEM) (Rodriguez-Tovar et al. 2002, 2003). The MGDS model in the laboratory was also used to show that a cellular adaptive immune response was efficiently generated to *L. salmonae* after a primary exposure to the parasite (Speare et al. 1998b, Rodriguez-Tovar et al. 2006), and this finding was confirmed in Chinook salmon (Kent et al. 1999). These findings suggested the potential for fish vaccination as a control method for reducing MGDS outbreaks. The discovery of a *L. salmonae* strain (SV strain), which caused

heavy infections in brook trout and demonstrated low-virulence in rainbow trout proved to be a promising candidate for vaccination of rainbow trout against *L. salmonae* (Sanchez et al. 2001b, 2001c, Speare et al. 2007).

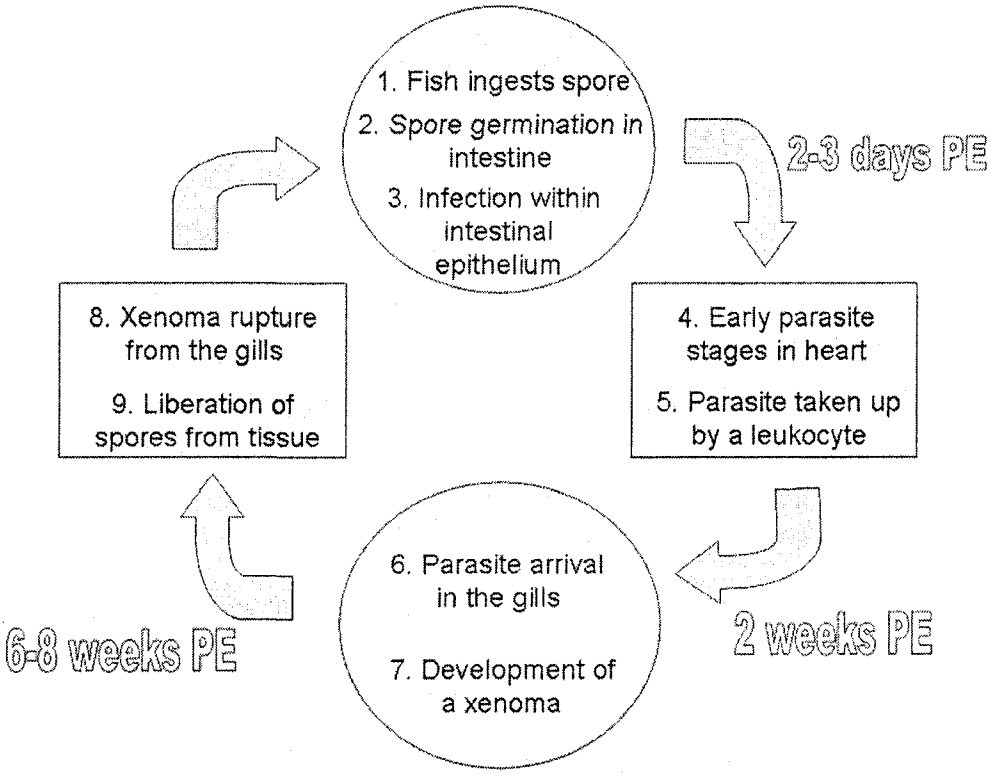

Figure 1.1. Life cycle diagram of *L. salmonae* in a laboratory infected rainbow trout from infection to 8 weeks post exposure (PE).

1.2 CHINOOK SALMON AQUACULTURE IN BRITISH COLUMBIA

British Columbia is the fourth largest salmon producer in the world after Norway, Chile, and the United Kingdom (www.env.gov.bc.ca). Although Atlantic salmon *Salmo salar* is the leader in annual production tonnage and total farmgate

value, Pacific salmon represent a large industry. In 2006 the salmon industry in British Columbia was represented by 91% Atlantic salmon and 9% Pacific salmon, of which the latter accounts for 7,000 tons of production with a farmgate value of $37.3 million. This has declined from the 2005 production totals for Pacific salmon which were 16,600 tons with a farmgate value of $66.1 million (Table 1.1). The majority of the Pacific salmon production is represented by Chinook salmon, but coho *Oncorhynchus kisutch* and other salmon account for a small percentage (Table 1.2). According to statistics from the years 1998-2002, Chinook salmon represented, on average, 77.9% of the total Pacific salmon production with 22.1% was represented by coho and sockeye salmon *Oncorhynchus nerka* (www.env.gov.bc.ca). If approximately 78% of the Pacific salmon production was represented by Chinook salmon in 2005 and 2006 then the farmgate value of Chinook salmon in British Columbia would be $51.4 and $29.1 million respectively.

The growout of Chinook salmon occurs in two major phases of production; smolt production, which consists of raising parr in fresh water at a hatchery and the grow-out phase, which is the rearing of salmonids in seawater netpens until they reach market size. There are two ecotypes of Chinook salmon, a "Stream-type", which spends one or more years in fresh water before migrating to the sea and an "Ocean- type, which migrates to the sea within three months (Healey 1991). The "Ocean-type" Chinook salmon is used in commercial culture because they can be transferred to seawater at a weight of 7 g thus reducing the time necessary in the

Year	Atlantic	Pacific	Total
1998	33.1	9.1	42.2
1999	38.8	10.8	49.6
2000	39.3	10.2	49.5
2001	58.0	10.0	68.0
2002	71.6	12.7	84.3
2003	55.6	17.1	72.7
2004	46.1	15.7	61.8
2005	53.8	16.6	70.4
2006	71.0	7.0	78.0

Table 1.1. Annual production tonnage of Atlantic and Pacific salmon (in the thousands) in British Columbia from 1998 to 2006 (Data from British Columbia Ministry of Environment, www. Env.gov.bc.ca).

Year	Chinook	Coho/other
1998	72.5%	27.5%
1999	81.5%	18.5%
2000	78.4%	21.6%
2001	75%	25%
2002	81.9%	18.1%

Table 1.2. Percent represented by Chinook salmon and coho/other salmon in total Pacific salmon production from 1998 to 2002 (Data from British Columbia Ministry of Environment, www. Env.gov.bc.ca).

fresh water phase (Clarke et al. 1996). The growout phase of salmon most commonly occurs in floating net-pen systems within sheltered areas of the ocean with adequate depths and tidal currents (Novotny and Pennell 1996). Chinook salmon have been shown to perform poorly and have reduced growth rates at relatively low stocking densities when compared to Atlantic salmon, whose growth rates are unaffected at high stocking densities (Iwama 1996). This suggests that in farms of equal sizes, more Atlantic salmon could be produced than Chinook salmon. The Chinook salmon that are commonly cultured in the farms are monosex female populations. Male Chinook salmon sexually mature at a smaller size and on average one year earlier than females. Large fish have a larger economic value per unit weight; therefore female fish are reared and marketed before reaching sexual maturity (Solar and Donaldson 1991). The female fish are maintained in the netpens for approximately two years and harvested for the market prior to sexual maturity.

1.3 MICROSPORIDIA: *LOMA SALMONAE*

Microsporidia lack typical eukaryotic organelles such as mitochondria, peroxisomes, and a typical Golgi apparatus and were once thought to be primitive eukaryotes (Cavalier-Smith 1991). Although microsporidia do not have a typical mitochondria, they contain highly reduced mitochondria which no longer synthesize ATP, but have been shown to have mitochondrial carrier proteins (Williams et al. 2008). More recent phylogenetic analysis combined with evidence suggesting a secondary loss of the mitochondrial organelle show that these organisms are likely

more closely related to fungi (Williams et al. 2002, Gill and Fast 2006). The highly reduced nature of these parasites, evidenced by the loss of several genes and the compact genome, is likely due to their adaptation to intracellular parasitism (Franzen 2004). A common feature of *L. salmonae* and other microsporidia is to induce formation of a xenoma, which is a hypertrophied host cell containing the developing parasite. After infection, microsporidia cause hypertrophic growth of the host cell (Vavra and Larsson 1999). The hypertrophied host cell increases the number of cellular organelles such as ribosomes, endoplasmic reticulum, and mitochondria, suggesting that microsporidia induce an increase in the metabolism of the cell (Didier and Bessinger 1999). Although mechanisms that the microsporidia use to induce the hypertrophic state in the host cell to develop a xenoma are unknown, it is known that various protists and myxozoans also induce xenoma-like formation (Lom and Dykova 2005). Studying the host-parasite relationship during development of *L. salmonae* within xenomas can provide information on how these highly reduced parasites interact with the host in order to develop. This information could ultimately aid in proposing treatment methods to interrupt the development of the parasite within the xenoma. Treatment of MGDS has focused on disrupting the development of the parasite in the gills and monensin, quinine hydrochloride, fumagillin, and albendazole have all had some success in minimizing or delaying the development of the parasite in the gills (Speare et al. 1998c, Speare et al. 1999, Becker et al. 2002).

The proliferative stages of *L. salmonae* within xenomas include meronts, sporonts, and sporoblasts (Rodriguez-Tovar et al. 2002). These developmental stages

are characteristic for microsporidia, although the development through these stages can vary across species and these differences are frequently diagnostic to the genus level. Spore morphology is another diagnostic feature of morphological discrimination to the species level (Cali and Takvorian 1999). The differences in developmental stages are attributed to the host-cell interface (Cali and Takvorian 1999). The host-parasite interface within the infected cell can include the parasites in direct contact with the cytoplasm or within either a host produced vacuole (parasitophorous vacuole) or parasite produced vacuole (sporophorous vesicle). As microsporidia develop and enter the sporogonic phase of development, it is likely that the needs of the parasites change and therefore it is common to observe a change in the host-parasite interface (Cali and Takvorian 1999). The host-parasite interface during development in *L. salmonae* needs to be further investigated to understand the process of development within xenomas.

1.4 HOST RESPONSES TO *LOMA SALMONAE*

Loma salmonae uses the "Trojan horse" method for evading the host immune response during development within the gills of fish. The intracellular localization of *L. salmonae* prevents the host from recognizing the parasite, as evidenced by the lack of host responses around large intact xenomas during 6 weeks PE (Rodriguez-Tovar et al. 2003). Although there is a lack of host response to intact xenomas, when disintegration of the xenoma membrane occurs there is an intense inflammatory response (Kent et al. 1989, Rodriguez-Tovar et al. 2003). The rupture phase of the

xenomas is the end of the proliferative and sporogonic phases of the parasite and this stage represents the release of pathogenic spores into the tissue and environment. From the parasite's perspective the replicative stages have ended and the parasite must seek out a new host or new host cell in which to develop in, resulting in severe insult to the host's gills. It would seem that the inflammatory response at this stage is an over reactive response that may be more detrimental to the host rather than helpful. Franzen (2005) has proposed the idea of microsporidia infecting new host cells through cellular phagocytosis. Through this method microsporidia are phagocytosed by host cells and during fusion of the phagosome with a lysosome the spore germinates and extrudes the sporoplasm into the host cell cytoplasm thus escaping the maturing phagosome. This suggests that the inflammatory response could in fact propagate microsporidial infections rather than neutralize them. It was also proposed by Rodriguez-Tovar et al. (2002) that *L. salmonae* is transported to the gills through an infected leukocyte, therefore the presence of inflammation may provide additional host cells for the parasite to develop in. The inflammatory response may facilitate the risk of further spreading the parasite, and additionally the inflammatory response has deleterious effects on the host tissue.

The host-pathogen relationship enters a new stage during the period of xenoma rupture. Previously, concepts about the parasite and how it used the host cell for development were introduced. This stage of the host pathogen relationship focuses on the host responses to the liberation of spores from their "Trojan horse" (xenoma). It is probable that the inflammatory response elicited during the rupture of

xenomas is closely linked to clinical signs associated with MGDS. The gills of fish serve multiple functions including gas exchange, osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous waste (Evans et al. 2005). The large surface area of the gill and the intricate vascular networks within the tissue make it a fragile organ and when affected by *L. salmonae* and the associated inflammatory response, normal functions are affected. The gills of fish are known to be a route of local and systemic infections with bacteria and viruses because of their direct contact within the aquatic environment and the thin epithelial layer of the lamellae that separate the blood vessels from the environment (Roberts and Rodger 2001). MGDS is a disease where the parasite does not gain entry through the gills, rather the pathogen exits through them and in doing so markedly alters the general architecture of the gill (Kent et al. 1995). The inflammatory response in fish is similar to the response seen in mammals with the similar cardinal signs as in mammals including, redness, swelling, pain, and loss of function; heat likely cannot be considered a cardinal sign in fish since they are poikilotherms (Roberts and Rodger 2001). Acute inflammatory responses with severe neutrophil recruitment and abscess formation are not common in fish; chronic granulomatous inflammatory lesions are more frequently observed (Ferguson 2006).

1.5 INFLAMMATORY CELLS IN FISH

The cells recruited during inflammation have various functions which participate in neutralizing an infectious agent and promoting tissue repair. During

this process the cells and the cytokines they secrete may induce damage to the host tissues (Kumar et al. 2005). A better understanding of the cells that participate in the inflammatory response in fish with MGDS will help in understanding the pathobiology of the disease. Mammals and fish share similar inflammatory cells with exceptions. A brief description of fish inflammatory cells follows.

1.5.1 Mast cells

Mast cells in mammals contain potent inflammatory mediators within large coarse granules and are found throughout the tissues in perivascular sites. They are most numerous in the skin, respiratory tract, and gastrointestinal tract (Bochsler and Slauson 2002). Mast cells are involved in the pathogenesis of acute inflammation by causing smooth muscle contraction and increased vascular permeability (Bochsler and Slauson 2002). In addition to initiating an acute inflammatory response they have been associated with chronic inflammation and wound healing (Noli and Miolo 2001). In fish, eosinophilic granular cells (EGCs) have been described in the gills, skin, and gastrointestinal tract of many fish species (Ferguson 2006). There is convincing evidence that the EGCs in fish are functionally similar to mammalian mast cells. EGC degranulation has been shown to occur during local insult and the degranulation was associated with neutrophil emigration (Matsuyama and Iida 1999). Additionally the staining and functional properties of the EGCs are similar to reports in mammalian mast cells (Reite and Evensen 2006). A recent study has shown that the granules in EGCs of fish belonging to the order Perciformes contain histamine, which is characteristic in mammalian mast cells (Mulero et al. 2007).

1.5.2 Eosinophils

Eosinophils are common in allergic and parasitic inflammatory diseases in mammals. They can damage and kill parasites by releasing major basic protein and eosinophil cationic protein from their granules (Bochsler and Slauson 2002). Eosinophils have been described in carp *Cyprinus carpio* (Kralj-Klobucar 1991) and in Atlantic salmon, *Salmo salar* with amoebic gill disease (Lovy et al. 2007). In the study by Lovy et al. (2007) the eosinophils contained characteristic oval shaped granules with a central core, which ultrastructurally resembled mammalian eosinophils. The granules in the cells had evidence of piecemeal degranulation, showing that the eosinophils associated with the amoebae were releasing the contents of their granules. These studies indicate that eosinophils in fish exist, but the contents of the granules have yet to be determined and thus it is not known if fish eosinophil granules contain major basic protein. The central core of the granules in carp have been shown to be positive for acid phosphatase and peroxidase, although neither of these have been demonstrated in the core of the granules in mammalian eosinophils (Kralj-Klobucar 1991).

1.5.3 Phagocytes

Fish phagocytic cells include the neutrophil and macrophage, which share many characteristics with the equivalent cells in mammals. Although fish do not typically mount an acute inflammatory response, they have neutrophils that are quickly recruited to areas of insult. Injection of the bacterial pathogen, *Yersinia ruckeri* into the peritoneal cavity of rainbow trout resulted in a rapid influx of

neutrophils that peaked at 24-48 hours post injection (PI) and reached levels 500X those in unstimulated fish and a macrophage response developed slower and peaked at day 5 PI and reached levels 7.5X those of unstimulated fish (Afonso et al. 1998). Neutrophil recruitment into sites of acute injury was also seen in a transgenic zebrafish *Danio rerio* model, which allows tracking of neutrophils by florescence (Renshaw et al. 2006). This data supports that neutrophils are likely an acute responding cell which arrive first following injury and that macrophages are a slower responding cell, which do not occur in as high numbers as neutrophils. Neutrophil granules in fish also seem to contain similar contents as mammalian neutrophils, as both are peroxidase positive and contain myeloperoxidase (Hine and Wain 1988, Castro et al. 2008). Myeloperoxidase is present in azurophil granules in mammalian neutrophils and this enzyme is considered an important component of the oxygen dependent microbicidal system (Bochsler and Slauson 2002). Fish phagocytes mount antimicrobial activity similar to mammals, by activating respiratory burst within neutrophils and macrophages, and inducible nitric oxide production in macrophages. These mechanisms of intracellular killing of pathogens are very similar to what is observed in mammalian phagocytes (Neumann et al. 2001). Primary neutrophil recruitment into lesions has been demonstrated to stimulate nitric oxide generation in macrophages. Transferrin is a serum protein that is important in inducing the nitric oxide response in fish, but in order for it to become activated for macrophages it must be cleaved. The protein first leaks into inflammatory sites by vascular leakage during the initial stage of the inflammatory response. Neutrophils are first to arrive in

inflammation and they are important in cleaving the transferrin, by production of neutrophil-derived proteases, into the active form used by macrophages. The subsequent arrival of macrophages then binds the cleaved transferrin products, which initiate the production of nitric oxide (Neumann et al. 2001). This process demonstrates, in fish, the importance of primary recruitment of neutrophils to sites of infection in order to prepare macrophages for intracellular killing of pathogens.

1.5.4 Thrombocytes

Thrombocytes in fish are responsible for blood clotting by forming aggregates and are important in preventing the loss of tissue fluids from the surface of injury (Roberts and Ellis 2001, Gregory and Jagadeeswaran 2002). The cells are equivalent to platelets in mammals. While, thrombocytes are nucleated and platelets are not, the cytoplasm of platelets and thrombocytes are very similar, containing peripheral microtubules and interconnecting vacuoles throughout the cytoplasm (Ferguson 1976, Ferguson 2006). In addition to functions in blood clotting, thrombocytes in rainbow trout have also been implicated in having immune functions. This is supported by the expression of components of the MHC class Ia pathway, MHC class IIb, and various cytokines in thrombocytes (Kollner et al. 2004). Thrombocytes were also seen interacting with erythrocytes in rosettes around macrophages with the presence of neutrophils and lymphocytes leading to speculation that thrombocytes have an immunological function (Passantino et al. 2005). Thrombocytes have also been demonstrated to be phagocytic, but it remains unknown if phagocytosis in

thrombocytes occurs in the classical phagocytic pathway as seen in macrophages and neutrophils (Meseguer et al. 2002).

1.5.5 Dendritic cells

Dendritic cells (DCs) in mammals have emerged as important cell types in the induction of adaptive immunity, as they are known to control immunity and tolerance. DCs can induce a cellular immune response by processing and presenting antigen to T cells or induce a humoral response by secreting cytokines to activate B cells. Subsets of DCs in humans are divided into four major groups including, Langerhans cells and dermal interstitial DCs in the skin, and myeloid and plasmacytoid DCs in the blood (Ueno et al. 2007). Langerhans cells are located in the epidermis and other stratified squamous epithelial tissues and the cells contain characteristic racket shaped granules called Birbeck granules (Wolff 1967), while dermal interstitial DCs are located in the dermis (Mathers and Larregina 2006). Langerhans cells are efficient in inducing cellular immunity by activating high avidity cytotoxic $CD8^+$ T cells and naïve $CD4^+$ T cells, while the dermal interstitial cells are thought to activate humoral immunity by inducing naïve B cells to differentiate into IgM-secreting plasma cells (Ueno et al. 2007). Myeloid and plasmacytoid DCs come from two main pathways of DC ontogeny from hematopoietic progenitor cells. Myeloid DCs exist as resident cells in peripheral tissue, secondary lymphoid organs, and in circulating blood. The functions of circulating blood myeloid DCs are unknown, but are thought to replenish populations of DCs in the tissues and act as sensors for blood borne pathogens (Ueno et al. 2007).

Myeloid DCs of mice have been shown to form a contiguous network of cells throughout the interstitial and mesangial spaces of the entire kidney, where the cells probe the surrounding tissue with dendrite extensions (Soos et al. 2006). It has been demonstrated that dendritic cells can be locally generated within individual tissues through dendritic cell precursors and in response to inflammation (Shortman and Naik 2007). Plasmacytoid DCs are found in the blood and are known to secrete large amounts of type I IFN and other cytokines in response to viral exposure, and also can differentiate into cells with morphology and functions of typical DCs. The plasmacytoid DCs are known to induce both Th1 and Th2 responses. The various DC types are distributed throughout tissues in places where antigen may be encountered and are important cells that link the innate and adaptive immune responses.

Dendritic cells have not been identified in fish, but there is evidence for the existence of antigen presenting cells. MHC class II expression is present in antigen presenting cells and its expression has been shown to be upregulated in a macrophage-like cell line from the head kidney of Atlantic salmon when stimulated with bacterial components or virus (Koppang et al. 1999). MHC class II expressing cells were also present within the gill epithelium of Atlantic salmon, but it was unknown if these were in fact Langerhans cells or local macrophages (Koppang et al. 2003). The MHC class II immunoreactive cells were found throughout head kidney, spleen, gills, thymus, and blood leukocytes (Koppang et al. 2003). The identified cells have characteristics of antigen presenting cells, but the cell type was not

determined. Mammalian CD83 is a cell surface marker for DCs and its homolog was identified in fish (Ohta et al. 2004, Donate et al. 2007). In a gilthead sea bream, *Sparus aurata* macrophage cell culture CD83 mRNA expression was up regulated almost 14 fold 4 hours following stimulation with lipopolysaccharide (LPS) from *E. coli* (Donate et al. 2007). The upregulation was reported to be similar to the response of mammalian DCs following LPS stimulation (Donate et al. 2007). The CD83 was detected in high levels in the gills, gonads, and spleen and in low levels in brain, head kidney, heart, intestine, kidney, liver, and thymus of the gilthead sea bream (Donate et al. 2007). Cuesta et al. (2006) found MHC class II expression in acidophilic granulocytes in gilthead sea bream and suggested that they may be antigen presenting cells. It is evident that antigen presentation occurs in fish since the markers of DCs in mammals occur in cells of fish. Although it is apparent that macrophages have some ability for antigen presentation, it is still unknown if DCs exist in fish.

1.6 IMMUNE RESPONSES IN FISH

1.6.1 Innate immunity

Within salmonids *Loma salmonae* has host specificity for Chinook salmon, pink salmon *Oncorhynchus gorbuscha*, chum salmon *O. keta*, brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis*, while Atlantic salmon and Arctic char *Salvelinus alpinus* do not develop infections with the parasite (Shaw et al. 2000a). Additionally, differences in innate susceptibility to infections were observed within different strains of Chinook salmon (Shaw et al. 2000b). The differences in

susceptibility of fish species may be linked to differences in the innate immune responses of the fish species. In isolated macrophages from Atlantic and Chinook salmon the phagocytic index of *L. salmonae* spores in plasma of respective fish was greater in the Atlantic salmon macrophages. The increased uptake of spores in the Atlantic salmon macrophages may have been influenced by complement factors in the plasma, which account for the resistance to infection with the parasite (Shaw et al. 2001). It is also possible that the parasite has evolved mechanisms to avoid aspects of the innate immune response in some fish species leading to variable susceptibilities between fish species. Parasite factors have been shown to influence the susceptibility of infections to *L. salmonae* in different fish species. The *L. salmonae* strain SV has a high virulence for brook trout (*Salvelinus* genus) and a low virulence to fish in the *Oncorhynchus* genus, while the strain OA has a high virulence for fish in the *Oncorhynchus* genus and not *Salvelinus* (Sanchez et al. 2001b). Innate immunity differences may play an important role in the differences in susceptibility seen in *L. salmonae*. Differences in the innate immune responses in fish have been shown to cause dramatic differences in infection levels of parasites. The hemoflagellate parasite *Cryptobia salmositica* has been shown to infect brook trout and in these fish both *Cryptobia* resistant and *Cryptobia* tolerant fish were identified. The *Cryptobia* resistant fish were able to avoid infection by activating the alternate pathway of complement, which lysed the parasite and the *Cryptobia* tolerant fish were infected by the parasite, but countered the metalloproteases produced by the parasite with α_2 macroglobulin therefore avoiding the pathogenic mechanism of the

parasite (Woo 1998). This shows two mechanisms in which the non-specific humoral factors in the innate immune response can counter parasites during infection.

With *L. salmonae* being an intracellular parasite, it is possible that nonspecific cytotoxic cells (NCC) could participate in the innate immune resistance to the parasite. The NCCs have functional similarities to mammalian natural killer (NK) cells (Plouffe et al. 2005). NCCs are important cells in innate immune responses to intracellular pathogens and tumor cells, by detecting and eliminating infected or transformed cells (Plouffe et al. 2005). These cells have been reported to participate in the immune response to protozoan parasites by increasing in numbers in the blood of fish infected with the ciliated protozoan *Ichthyophthirius multifiliis* (Graves et al. 1985). The NCCs in rainbow trout have been demonstrated to inflict apoptotic and necrotic lesions in tumor target cells (Greenlee et al. 1991). NCCs are reported to be small (4.2-4.5 μm), agranular, mononuclear cells (Greenlee et al. 1991). In addition to the NCCs in fish a different cell type, called cytotoxic natural killer (NK)-like cells, with similar functions has been described (Shen et al. 2004). The NK-like cells predominantly employed a calcium-dependent pathway to induce apoptotic cell death in the cells targeted for elimination. These cells do not react with the common monoclonal antibody for NCCs and contain granules within their cytoplasm, suggesting that they are different from the previously described NCC. The presence of granules suggests that these cells may lyse target cells by the perforin/granzyme pathway, which is the common mechanism of killing in mammalian NK cells (Shen et al. 2004). In rainbow trout, neutrophils have been shown to induce non-specific

cytotoxicity in cells (Sasaki et al. 2002). It is evident that fish are able to mount a nonspecific cytotoxic response with the activation of multiple cells as part of the innate immune response to pathogens. Although it is still unknown, this response may be an important mechanism limiting infections with *L. salmonae* in salmonids.

1.6.2 Adaptive immunity

The innate and adaptive immune responses are often discussed separately, but it is currently understood that they are very closely connected. The innate response precedes the adaptive response and the nature of the response determines the nature of the adaptive immune response (Magnadottir 2006). It is well established that fish develop adaptive immunity through the production of specific antibodies and resistance to infections is based on this humoral response. This type of adaptive immunity has been described in fish against several fish parasites, such as the protozoans *Ichthyophthirius multifiliis* (Clark and Dickerson 1997) and *Cryptobia salmositica* (Woo 2003), and the myxozoan *Enteromyxum scophthalmi* (Sitja-Bobadilla et al. 2007). In rainbow trout there is a strong protective response generated after an initial exposure to *L. salmonae*. The protective response is induced at around 3 weeks post exposure (PE) and fully developed at 6 weeks PE. The response is attributed to a cellular response rather than to a humoral response (Rodriguez-Tovar et al. 2006).

The microsporidian *Encephalitozoon cuniculi* infects mammals and is a particular problem in immunocompromised hosts. The protective immune response to *E. cuniculi* is T cell-dependent and depletion of $CD4^+$and $CD8^+$ T cells resulted in

a 100% mortality of mice orally challenged with this microsporidian (Moretto et al. 2004). The T lymphocytes function in activating cytotoxic T cells and macrophages by releasing cytokines (Didier and Bessinger 1999). The $CD4^+$ T cells are thought to act during the acute phase of microsporidiosis by inducing macrophage-mediated elimination of the parasite and the CD8+ cytotoxic cell response, which is generated later, controls the later infections (Didier and Bessinger 1999). In mice, induction of intraepithelial lymphocytes was an important response in controlling infections following oral exposure to *E. cuniculi*, (Moretto et al. 2004). Currently the understanding of the adaptive cellular responses in fish is in its early stages. Rainbow trout have been shown to have populations of intraepithelial lymphocytes within the gut epithelium, composed mostly of T cells similar to systemic T cells seen in mammals (Bernard et al. 2006). These cells may contribute to the immune response generated against *L. salmonae* when the parasite is in the intestine during the early stages of infection.

1.7 EFFECTS OF STRESS ON THE IMMUNE RESPONSE

The techniques used for rearing salmonids within ocean netpens result in various extrinsic stressors to the fish. Fish raised in pens in the open ocean experience environmental stressors such as seasonal temperature fluctuations and exposure to endemic pathogens, such as *L. salmonae*. Additionally these fish are subjected to stressors that inevitably come from captive rearing, such as crowding and handling stress. Crowding of fish would also lead to increased transmission and

higher concentrations of pathogens. These combined factors increase the risk of netpen reared salmonids to disease by lowering the fish's resistance to pathogens (Beveridge 1996) and providing an environment for pathogen propagation. The functional significance of the stress response is to overcome stressors and increase the chance of survival by preparing the body for the flight or fight response. If the stressor becomes chronic then the chronic cortisol production may result in reduced growth and decreased resistance to disease (Wedemeyer 1996). There does not seem to be a suppressive effect of stress and cortisol on NCC in the cellular innate immune response and in fact these cells were stimulated and increased in peripheral blood during the stress response in fish (Evans et al. 2001, Esteban et al. 2004). In mammals it is hypothesized that acute stressors in the skin actually enhance both innate and adaptive immunity to prepare the immune system for the stressor, but when stress becomes chronic then it has immunosuppressive effects (Dhabhar 2003). In rainbow trout it has been demonstrated that acute stressors may enhance cellular and humoral components of innate defenses as seen by an increase in plasma lysozyme, although if the stressor becomes chronic the effect could be immunosuppressive (Demers and Bayne 1997). Ortuno et al. (2001) show that confinement stress of 2 hours in gilthead seabream results in the depression of complement and phagocytic activities. High doses of cortisol in seabream were also shown to reduce respiratory burst activity in head kidney leukocytes (Esteban et al. 2004). In channel catfish *Ictalurus punctatus* it is thought that stress–induced suppression of the innate immune response results in increased infections with the

protozoan parasite *I. multifiliis* (Davis et al. 2002). Adaptive immunity to *I. multifiliis* in carp has also been shown to be inhibited by cortisol. Fish previously immunized to *I. multifiliis* were rechallenged with the parasite and treated with corticosteroids, which resulted in 100% mortality compared to only low level mortality in the untreated immunized fish. The humoral antibody levels were relatively unaffected by the steroid treatment and it was hypothesized that the cellular adaptive immune response was affected by the treatment (Houghton and Mathews 1990). In netpen reared Chinook salmon with MGDS an understanding of how stress affects the host-pathogen relationship during infection with the parasite could help identify management techniques to avoid heavy infections in the farm environment. It is also possible that some stressors on the farm suppress aspects of the adaptive immune response in the farm reared fish.

1.8 THESIS OBJECTIVES

MGDS causes significant pathology in netpen reared salmonids and disease is thought to be attributed to an inflammatory response elicited by the rupture of xenomas. In this thesis the pathogenesis of disease is studied by examining the host-pathogen relationships in affected fish. With an understanding of the pathogenesis of *Loma salmonae* in salmonids, treatments for disease may be proposed. The objectives include (1) Examination with TEM the gills of rainbow trout experimentally infected with *L. salmonae* and netpen reared Chinook salmon during a natural outbreak of MGDS in British Columbia. The major aspects of the host-

parasite relationships that were investigated include the mechanisms that the parasite uses within the host cell during development and the host responses to the parasite after xenoma rupture.

(2) The effects that stress may have in fish infected with *L. salmonae* are investigated to determine effects of stress on the host-pathogen relationship. This is relevant to studying the pathogenesis of the disease in netpen reared fish, considering that stress may be a factor in propagating the disease in farmed fish.

(3) With a better understanding of the nature of the inflammatory response that is induced during disease, anti-inflammatory treatments to reduce mortalities are proposed. The effectiveness of anti-inflammatory treatment in fish and its effects on the parasite in gills are investigated.

1.9 REFERENCES

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2 MORPHOLOGICAL CHARACTERIZATION AND NOTES ON DEVELOPMENTAL CYCLES OF OA AND SV VARIANTS OF *LOMA SALMONAE* IN RAINBOW TROUT AND BROOK TROUT[1, 2]

2.1 ABSTRACT

Two variants of *Loma salmonae* occur in net-pen reared Chinook salmon, *Oncorhynchus tshawytscha*. The typical variant (OA) has a host specificity for salmonids in the *Oncorhynchus* genus whereas the atypical variant (SV) has a host specificity for brook trout, *Salvelinus fontinalis* and in this study, the ultrastructure of the two are compared. In fish at 8 weeks post exposure xenomas of the SV variant have a very high proportion of mature spores compared to other developmental stages, while in xenomas of the OA variant there are fewer spores and many other developmental stages. Spores of the SV variant had up to 20 turns of their polar tube whereas those of the OA variant only had 17. Furthermore the spores of the SV variant were significantly larger than those of the OA variant. In the OA and SV variants, up to 7 sporonts and 12 sporonts were observed within a single parasitophorous vacuole, respectively. The parasitophorous vacuole forms at the onset of sporogony. In the OA variant the merogonial stage is bound by a single plasma membrane in direct contact with host cytoplasm. The parasitophorous

[1] Lovy J, Wadowska DW, Wright GM, Speare DJ. Morphological characterization and notes on the life cycle of a newly discovered variant of *Loma salmonae* (Putz, Hoffman, & Dunbar) from a natural infection of chinook salmon, *Oncorhynchus tshawytscha* (Walbuam). J Fish Dis 2004; 27, 1-8.

[2] Lovy J, Wright GM, Wadowska DW, Speare DJ. Ultrastructural morphology suggesting a new hypothesis for development of microsporidians seen in *Loma salmonae* infecting the gills of trout. J Fish Biol 2006; 68: 450-457.

vacuole formation is initiated by the host cell surrounding the merogonial stages with endoplasmic reticulum (ER) as occurs in autophagy. Of the two host ER membranes surrounding the parasite, one remains in close association with the plasma membrane of the meront, while the other forms the limiting membrane of the vacuole. The sporogonial stage is bounded by two closely apposed membranes, giving the appearance of a thick electron dense plasmalemma. The observations from this study support the novel hypothesis that this microsporidian uses the intracellular process of autophagy to aid in the formation of a parasitophorous vacuole. The morphology of the SV variant is consistent with that of the OA variant suggesting that it uses the same mechanism for development.

2.2 INTRODUCTION

The microsporidium *Loma salmonae* is an intracellular parasite infecting the gills and other vascularized tissues of salmonids (Kent 1998). Within the coastal waters of British Columbia (BC) *L. salmonae* is the cause of microsporidial gill disease of salmonids (MGDS) which primarily affects net-pen reared Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (Speare 2000). Previous work has shown that cultured Chinook salmon from BC carry two variants of *L. salmonae* denoted as *L. salmonae* OA variant (which has marked preference for *Oncorhynchus* species) and *L. salmonae* SV variant (which has marked preference for *Salvelinus* species) (Sanchez et al. 2001a). Although both of the isolated variants of *Loma* can be used as the basis for vaccine preparation, the SV variant has the additional benefit

of demonstrably lower virulence against the target species (Sanchez et al. 2001b). While the OA variant has been extensively characterized, we have only just begun to characterize the SV variant. An obvious issue, based on marked host preference, is whether the SV variant is in fact *Loma fontinalis* as described by Morrison and Sprague (1983), and if this is the case, this would suggest that farmed Chinook salmon frequently carry a dual infection. However, based on the identical response of the Chinook-derived SV and the OA variants to PCR, immunohistochemistry, and *in situ* hybridization testing (Sanchez et al. 2001a), it would suggest that the OA and the SV variant are more similar than different. A reliable source of *L. fontinalis* is not available for direct comparison.

The infective agent is a spore, which is transmitted horizontally and completes its life cycle within a single host (Kent 1998). The parasite is believed to establish within a pillar cell or white blood cell within the gills, where it proliferates causing the infected cell to become severely hypertrophied (Rodriguez-Tovar et al. 2002). A cyst forms, containing multiple life stages of the parasite, and is referred to as a xenoma. Within the xenoma chronological stages of development include meronts (merogonial phase), sporonts (sporogonial phase), sporoblasts and mature spores (Rodriguez-Tovar et al. 2002). In many microsporidians sporonts develop into spores only when a vacuole forms, separating the parasite from the host cell cytoplasm. The reason for development of the vacuole is not yet understood, but it has been suggested that the vacuole is required in the later stages of development (Cali and Takvorian 1999).

In Microsporidia the vacuole is either host-derived or produced by the parasite, and referred to as a parasitophorous vacuole or a sporophorous vesicle respectively (Lom and Nilsen 2003). In some species both host and parasite contribute to the formation of the vacuole, in which case it is called a host-parasite produced vacuole. The merogonial stages of *L. salmonae* are tightly surrounded by rough endoplasmic reticulum (RER) from the host cell (Bekhti and Bouix 1985, Cali and Takvorian 1999). The meront becomes a sporont, characterized by a thickening of the plasma membrane; during this stage the vacuole forms. In *Loma* there is species variation in the origin of the vacuole; *Loma morhua* in Atlantic cod *Gadus morhua* L. forms a sporophorous vesicle (Morrison and Marryatt 1986). Bekhti and Bouix (1985) proposed that *L. salmonae* produces a vacuole membrane which fuses with vesicles from the host cytoplasm to form a host-parasite produced vacuole.

Understanding the origins of the vacuole is important because it is a useful character for speciation of the parasite. All Microsporidia fall into one of four categories: forming a parasitophorous vacuole, a sporophorous vesicle, or a host-parasite produced vacuole, or those that do not produce a vacuole and lie in direct contact with the host cytoplasm (Cali and Takvorian 1999). Achieving a complete understanding of the development may suggest ways to disrupt the life cycle for treatment of the disease caused by Microsporidia.

The purpose of the present work is to provide an ultrastructural analysis of the SV variant to determine morphologic similarities and differences to the OA variant, and to previous descriptions of *L. salmonae*, and *L. fontinalis*. Additionally the

origin of the parasite vacuole in the sporogonial stage is investigated in both variants.

2.3 MATERIALS AND METHODS

2.3.1 Source of parasite

Loma salmonae was isolated from farmed Chinook salmon from a natural outbreak of the disease in British Columbia. One variant (OA) was obtained by passage through *Loma*-naïve rainbow trout, the other (SV) was obtained by passage through *Loma*-naïve brook trout (Sanchez et al. 2001). The two variants were maintained in the laboratory by passage through the appropriate species, each time through separate groups of naïve fish.

2.3.2 Source of experimental fish

Naïve rainbow trout and brook trout were obtained from the Cardigan Fish Hatchery on Prince Edward Island. The fish were maintained at 10°C until one week prior to infection with spores of *L. salmonae* at which time they were transferred to 3 70 L tanks maintained at 15°C±0.5. The water source for the tanks was well water, which was warmed in a header tank and all tanks contained air stones for additional aeration.

2.3.3 Method of infection and sampling

Heavily infected fish identified during the serial passage procedure were euthanized using benzocaine as described by Speare *et al.* (1998) and the infected gill tissue was removed and minced with a razor blade. Two grams of minced gill tissue was introduced into each tank of 30 *Loma*-naive recipient fish, and water flow was

stopped for 30 minutes to permit the gill material to be consumed. One tank contained brook trout which received the SV variant from donor brook trout; the other tank contained rainbow trout which received the OA variant from donor rainbow trout. Five infected rainbow trout, two infected brook trout, and five control rainbow trout were sampled 8 weeks post exposure to *L. salmonae*. The length of all fish was between 150-170 mm and the weight was 30-60 g. A second experimental infection with a different inoculum was sampled during week 8 PE, where five infected rainbow trout, five infected brook trout and five controls from each were taken. Sizes of fish in the second experimental infection were approximately the same size as the previous infection. Eight weeks PE was chosen as a sampling time point because it has been shown that the xenomas in the OA variant contain a high number of parasites at various stages of development during this period (Rodriguez-Tovar et al. 2003). In previous unpublished work by Rodriguez-Tovar it has been shown that uninfected brook trout do not develop xenomas when maintained in our tanks, therefore control brook trout were not taken.

2.3.4 Sample preparation for high resolution light microscopy (HRLM) and transmission electron microscopy (TEM)

Fish were euthanized with benzocaine and the first left gill arch was removed and immediately fixed in 2% glutaradehyde in 0.1 M phosphate buffer, pH 7.2 and refrigerated for 1 h at 4°C. The tissue was removed and cut into approximately 4 pieces and transferred into fresh 2% glutaraldehyde in phosphate buffer in 5 ml vials

and stored at 4°C overnight. After washing the tissue with two changes of 1 % phosphate buffer for 15 minutes each, the tissue was post-fixed in 1% osmium tetroxide in phosphate buffer for 1.5 h at room temperature. The tissue was dehydrated through a graded series of ethanols at concentrations of 50%, 75%, and 95% respectively. Tissue was maintained in each ethanol concentration for 30 minutes and two exchanges were done for each concentration. The tissue was then put through two 1 hour exchanges of anhydrous ethanol and propylene oxide (PO) respectively. After tissue was cleared with PO it was embedded in Spurr's resin (Marivac Limited, Halifax, N.S. Canada) by submersing into a mixture of Spurr's resin and PO at a ratio of 1:1, 2:1, and finally 100% Spurr's resin, after each change vials were maintained on a rotator for 2 hours. Lastly, samples were embedded in pure Spurr's resin at 60°C for 24h overnight.

Semi-thin sections (0.5 μm) were cut from five blocks of tissue from each infected fish and one tissue block from each of the controls. The sections were stained with 1% toluidine blue in 1% sodium tetraborate solution and viewed with the light microscope. All samples that contained xenomas were recut to generate ultra-thin sections (90 nm). The ultra-thin sections were retrieved onto copper super grids (200 mesh) and stained with uranyl acetate and Sato's lead stain. The sections were examined and photographed using a Hitachi H7500 transmission electron microscope operated at 80 kV.

2.3.5 Spore measurements

Measurements of mature spores were made on ultra-thin sections of fixed specimens using the transmission electron microscope and imaged using a CCD digital camera (Hamamatsu). The computer program, AMT Advantage software v 3.20.1 was used to take measurements of spores sectioned along their longest axis from the digital image. Lengths along the long axis and widths were recorded. In total fifty-eight measurements of the SV variant were taken from three different xenomas and 50 measurements of the OA variant were taken from four different xenomas.

2.3.6 Statistical analysis

Data were analyzed using a two sample student's T-test at the .05 level of confidence using the Minitab software.

2.4 RESULTS

2.4.1 Comparison of the OA and SV variants

A very high proportion of mature spores compared with the earlier developmental stages are seen in all xenomas of the SV variant (Fig. 2.1.A). In all xenomas examined from the OA variant there appeared to be a higher proportion of earlier developmental stages of the parasite (meronts and sporonts) compared with mature spores (Fig. 2.1.B).

2.4.1.1 Spore measurements

The mean (± standard error) spore length for the SV variant was 3.71 μm

(±0.43) and the mean length of the OA variant was 4.02 μm (±0.41). This difference is highly significant with a $P<0.0005$. The range in the lengths between the two is quite similar; in both variants the minimum length is 3.0 μm and the maximum length is 5.0 μm. The mean (± standard error) width of all the mature spores from both strains was 1.83 μm (±0.231) for OA variant and 1.83 μm (±0.199) for SV variant.

2.4.1.2 Number of coils in the polar tube

The OA variant exhibited 14-17 coils of the polar tube and the SV variant exhibited 14-20 coils (Fig. 2.2). Table 2.1 summarizes the number of spores and associated polar tube coils.

Number of coils in polar tube	OA variant	SV variant
14	4	2
15	6	5
16	8	5
17	2	6
18	0	3
19	0	3
20	0	2
total	20	26

Table 2.1. Frequency of the number of polar tube coils in two variants of *Loma salmonae*

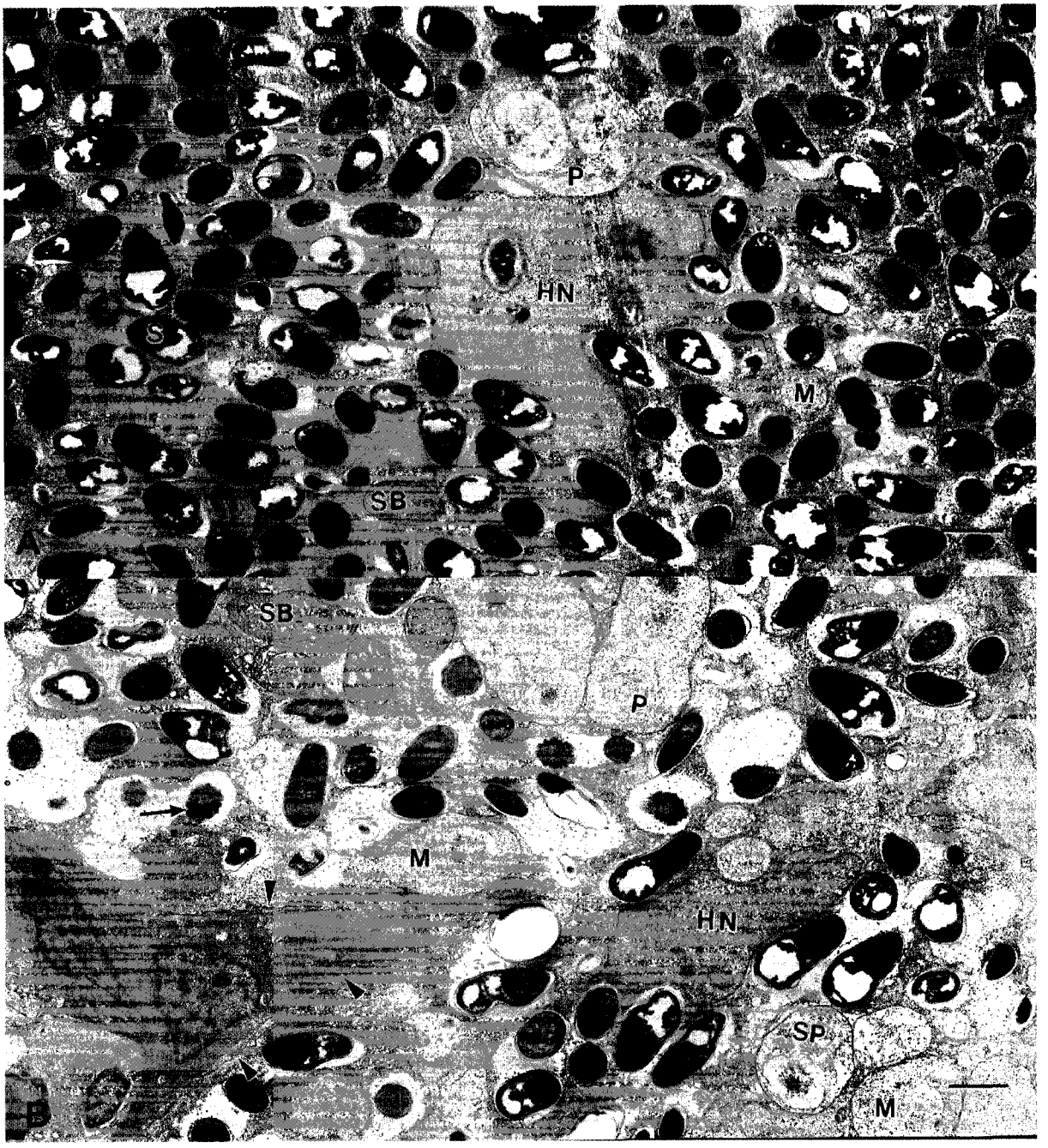
2.4.1.3 Developmental stages

Both variants possess typical meronts which have free ribosomes and a variety of small vesicles and cisternae of rough endoplasmic reticulum. In the OA variant plurinucleate plasmodia with up to six nuclei were seen and in the SV variant plasmodia with three nuclei were seen in a section plane. Sporonts were characteristic in both variants, having an electron dense plasma membrane and a cytoplasm containing more stacks of cisternae than seen in the meront.

The plasmodia of the OA variant become surrounded by two host-derived membranes and begin to accumulate electron dense material on the plasma membrane (Figs. 2.3.A and B). The SV variant also shows a thickening of the plasma membrane (Figs. 2.3.C and D). A parasite vacuole in both variants began to form around a plasmodia; the vacuole is evident when the plasmodia undergoes cytokinesis due to the parasite plasma membrane retracting away from the vacuole membrane (Figs. 2.4.A and B). This results in numerous sporonts within the vacuole, which either form directly into spores or further divide into sporoblasts to produce mature spores (Figs. 2.5.A, B and 2.6.). In the vacuole of the SV variant up to 12 developing parasites were seen in a single section plane (Fig. 2.5.B) and in the OA variant 7 sporoblasts are apparent (Fig. 2.6.).

Figure 2.1. Comparison of xenomas from the SV variant and the OA variant of *Loma salmonae*. **A.** Xenoma of SV variant; note the large proportion of mature spores (S) compared to other developmental stages: meronts (M), sporogonial plasmodium (P), and sporoblasts (SB). The host nucleus (HN) is located centrally. (Bar=3.0 μm) **B.** Xenoma of OA variant showing an apparent higher proportion of developmental stages: meronts (M), sporogonial plasmodia (P), sporonts (SP), and sporoblasts (SB) than spores. The xenoma wall is seen (arrowheads) and the host cell nucleus (HN). (Bar=2.7 μm)

Figure 2.2. A mature spore of the SV variant showing 19 coils in the polar tube (arrowheads). The exospore (arrow) is adjacent to the electron lucent endospore. (Bar=0.36 μm)


P
HN
S
M
SB
A
SB
P
M
HN
SP
M
B

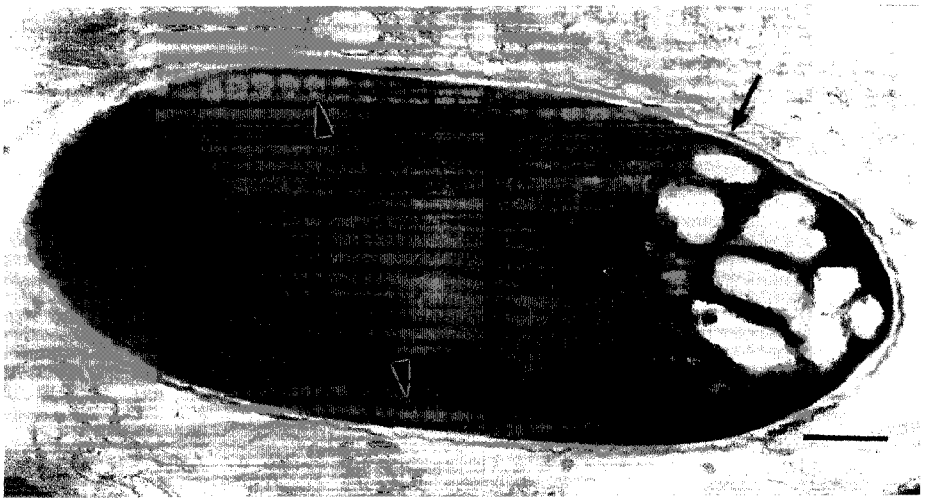


Figure 2.3. Stages of development in both variants of *L. salmonae*. **A.** A sporogonial plasmodium (P) with 3 nuclei (N) in the OA variant. Arrows show plasma membrane of the parasite. (Bar=0.60 μm) **B.** Higher magnification of the plasmalemma (arrows) seen in contact with the parasite cytoplasm (PC). Membranes (arrowheads) originating from the host cell cytoplasm (HC) are seen tightly abutting the plasma membrane of the parasite. (Bar=0.26 μm) **C.** A sporogonial plasmodium (P) of the SV variant showing 3 nuclei (N). (Bar=0.88 μm) **D.** A higher magnification of the thickened plasmalemma (arrows) of the parasite. (Bar=0.44 μm)

HG
PC
B
P
N
A
B
N
P
C

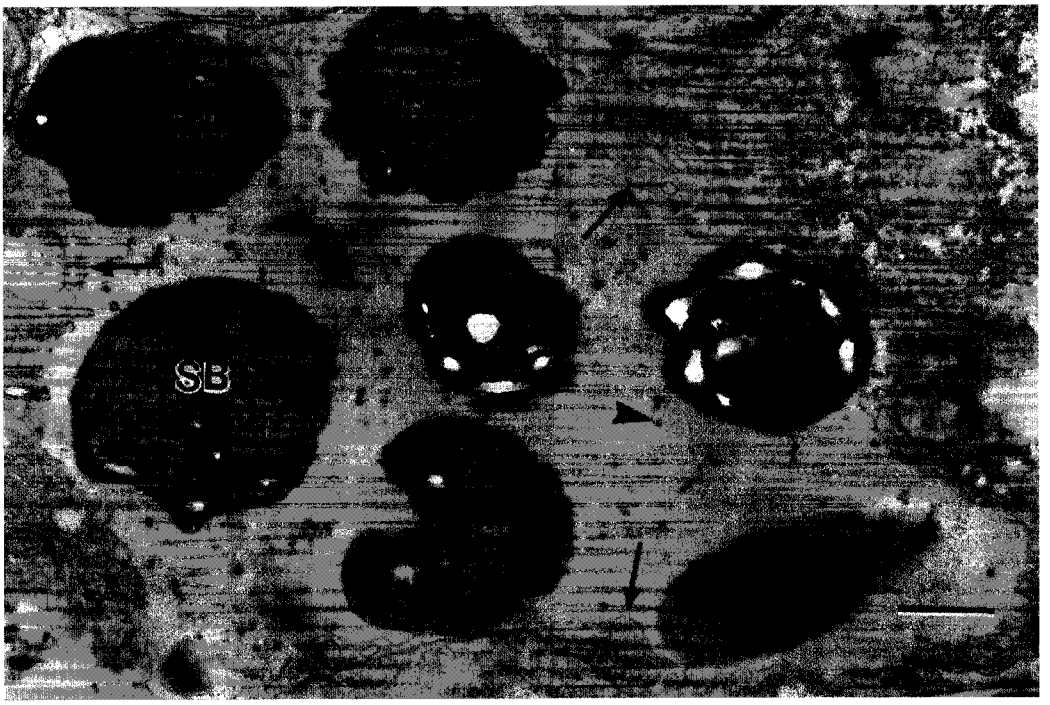
Figure 2.4. Sporogonial plasmodium in two variants of *L. salmonae*. **A.** The parasite in the SV-variant (P) with the start of the sporophorous vesicle (arrowheads). Two nuclei (N) are apparent. (Bar=0.83 μm) **B.** Sporogonial plasmodium in OA variant undergoing cytokinesis. Note the thickened plasma membrane (arrows) and the lumen of the parasite vacuole (arrowheads) in areas of division. (Bar=0.56 μm)

Z
I
P
I
A
Z

Figure 2.5. Multiple parasites are formed within a single parasite vacuole. **A.** The OA variant with 4 sporonts (SP) formed within the lumen of a single vacuole (arrowheads), the sporonts appear uninucleated (N). (Bar=0.77 μm) **B.** Twelve sporonts (SP) seen within the lumen of a single parasite vacuole (arrow heads) in the SV variant. (Bar=1.11 μm)

Figure 2.6. A parasite vacuole in the SV variant with 7 sporoblasts (SB) maturing into spores. The arrows show the membrane that separates the vacuole from the host cytoplasm; arrowheads show tubules associated with the parasite vacuole. (Bar=0.63 μm)


SP
N
A
SP
SP
B


SB

2.4.2 Origins of the sporogonial parasite vacuole

2.4.2.1 OA variant

All xenomas examined contained *L. salmonae* in many stages of development. Merogonial (early) stages of the parasite were in direct contact with host cytoplasm. They were bounded by a single plasma membrane and surrounded by cisternae of host ER. Many cisternae of the host ER had ribosomes attached (Fig. 2.7.A and B). All sporogonial stages were within a parasitic vacuole delimited by a single membrane. Sporogonial plasmodia observed undergoing cytokinesis clearly showed the parasitic vacuole in the areas of division (Fig. 2.7.C). The sporogonial stages were bounded by two membranes: an inner membrane adjacent to the sporogonial cytoplasm and an outer membrane adjacent to the lumen of the parasitic vacuole (Fig. 2.7.D). The two membrane structure bounding the parasite was referred to as a sporogonial wall. The host cytoplasm around the parasite and developing vacuole was rich in vesicular elements. Intermediate stages between the merogonial and sporogonial phase were observed. The host ER membrane adjacent to the parasite (ER1) remained in close association with the parasite plasma membrane, while the outer ER membrane (ER2), which was decorated with ribosomes, was separated from the ER1 by the ER lumen. The ER luminal space was variable in size around the parasite (Fig. 2.7.E). The parasite cytoplasm in both the merogonial and the sporogonial stages appeared similar containing numerous cisternae of ER and free ribosomes (Fig. 2.7. A and C). No vesicles within the parasite cytoplasm were found associated with the bounding membranes.

Figure 2.7. Development of the OA variant of *L. salmonae* **A.** A merogonial plasmodium, parasite plasma membrane (arrow), surrounded by cisternae of host ER, some of which are rough ER (small arrowheads), (N) is nucleus of parasite. (Bar=1 μm) **B.** A higher magnification of the boxed in area of (A) showing the parasite plasma membrane (arrow) with two membranes of ER closely associated, an inner membrane closely associated with the parasite membrane (small arrowhead) and a membrane adjacent to it (large arrowhead). (Bar=170 nm) **C.** A sporogonial plasmodium undergoing cytokinesis, leaving three sporonts (S) within the parasitic vacuole (large arrowheads), note the electron dense sporogonial wall (arrow). A merogonial stage (M) is seen in the lower right. (Bar=1.25 μm) **D.** The sporogonial wall with two distinct membranes; an inner membrane (arrow) and an outer membrane (small arrowhead). The single membrane of the parasitic vacuole (large arrowhead) is also seen. (Bar=100 nm) **E.** A merogonial stage (M) with the ER1 membrane (small arrowhead) closely associated with the parasite plasma membrane (arrow) and the ER2 membrane decorated with ribosomes (large arrowhead). The space between the two membranes is the lumen of the ER (L). (Bar=200 nm)


A
N
B
C
S
M
D
L
M

2.4.2.2 SV variant

Xenomas of the SV variant contained the parasite in multiple stages of development, but appeared to have a higher proportion of mature spores compared with early developmental stages. Merogonial stages were in direct contact with the host cell cytoplasm and bound by a single plasma membrane with cisternae of host ER in close proximity. Similar to that observed in the OA variant from rainbow trout the sporogonial stage was bounded by two membranes and was always within a single membrane bound parasitic vacuole. Cytoplasmic composition of merogonial and sporogonial stages appeared similar to each other and also similar to that in the OA variant.

2.5 DISCUSSION

2.5.1 Comparison of OA and SV variants

The results of this study show that mature spores of the OA variant of *Loma salmonae* are morphologically consistent with previous reports for this species. The spores are within the appropriate size range and exhibit the appropriate number of polar tube turns. However unlike previous observations of *Loma* species a feature of the OA variant used in this study was the common occurrence of 4-7 spores forming within a parasite vacuole in a single section plane. Morrison and Marryatt (1986) report that only one to three spores are seen in each vacuole in *Loma*. Morrison and Sprague (1983) and Cali and Takvorian (1999) observed in *Loma* one or two spores

developing in a vacuole and the parasite vacuole forming around a sporont with a single nucleus that proceeds through sporogony to yield 1-3 spores within the vacuole. The results of this study suggest that the parasite vacuole forms around a sporogonial plasmodium, which undergoes cytokinesis to produce numerous sporonts within a vacuole. Some of these sporonts may further divide in sporogony to produce 2-3 sporoblasts in which case they would be referred to as sporoblast mother cells. Other sporonts may directly develop into sporoblasts and mature spores (Lom and Nilsen 2003). The outcome is a varying number of spores (from one to many) forming in a parasite vacuole.

Our results show that the SV variant is morphologically distinct from the OA variant and from the species *Loma salmonae*. The number of polar tube turns in the SV variant (14-20) are no longer within the range used as a criteria for the morphological identification of *L. salmonae*. It was common to see 18-20 polar tube coils in spores of the SV variant (Fig. 2.2.). Previous studies have reported that polar tube coils in *L. salmonae* do not exceed 17 coils. *L. salmonae* in farmed rainbow trout in Scotland are reported to have 13-17 polar tube coils (Bruno et al. 1995). *L. salmonae* described from brook, brown, and rainbow trout from the Budford Trout Hatchery, Georgia, USA all have 12-15 turns in the polar tube (Bader et al. 1998). The dimensions (length & width) of the SV variant are within those reported for the species *L. salmonae*; ranging from 3.0 μm (± 0.3) by 1.1 μm (± 0.3) to 4.37 μm (range 2.84-6.16; ± 0.61) long and 2.26 μm(range 1.42-2.84; ±0.34) wide (Bruno et al. 1995, Bader et al. 1998), although some variation when compared to the OA-

variant. It was interesting to see 12 sporonts developing within a single parasite vacuole of the SV variant (Fig. 2.5.B). This again is likely due to the vesicle developing around a sporogonial plasmodium. Morrison and Sprague (1983) isolated a species of *Loma* from brook trout, which had 14-15 coils in the polar tube and typically developed individually in a sporophorous vesicle. Based on the small differences in morphology from *Loma salmonae* and host specificity to brook trout this *Loma* isolate became the species *Loma fontinalis* (Morrison and Sprague 1981). The SV variant is clearly morphologically distinct from *L. fontinalis* based on the difference in number of polar tube coils and number of spores developing within a parasite vacuole.

One could speculate that the morphological differences among the variants are a result of host induced differences rather than differences in the parasite. However, based on previous work with *Loma salmonae* and microsporidia in general this is not likely the case nor can it be directly studied through a factorial infection design because of host specificity of the variants. A microsporidium morphologically consistent with *L. salmonae* was isolated from the Buford Trout Hatchery, Georgia, USA from rainbow, brook, and brown trout. The morphology of the microsporidium was consistent in all three species of fish showing that the same parasite forms similarly in three different hosts (Bader et al. 1998). There are examples of microsporidian species that occur in a variety of hosts. *Enterocytozoon bieneusi* is a common microsporidium infecting humans suffering from acquired immunodeficiency syndrome (AIDS). This microsporidium has been isolated from

monkeys, *Macaca mulatto*, and is indistinguishable both genetically and morphologically to the human isolate (Chalifoux et al. 1998). Though the possibility of the changes in the parasite being host induced exists there is more evidence against it. Historical morphological studies of the microsporidia compare the developmental stages and spore morphology amongst an array of host species including insects, fish, and mammals.

Our study presents a morphological distinction between the two variants of *L. salmonae* and suggests that the SV variant may belong to a separate species. The morphology of mature spores from the OA variant is consistent to the previous reports for *L. salmonae*, however the life cycle differs from that described by Morrison and Sprague (1983). This variation could reflect an evolutionary change in the parasite development over the past 20 years. Unfortunately species descriptions for *Loma* are problematic because designation as a species has been based on host specificity, spore morphology, and geographic location alone. The species *L. fontinalis* and *L. morhua* may be conspecific to *L. salmonae* and *L. branchialis* respectively (Shaw and Kent 1999). Molecular studies along with morphological work are needed to verify the taxonomy of this genus.

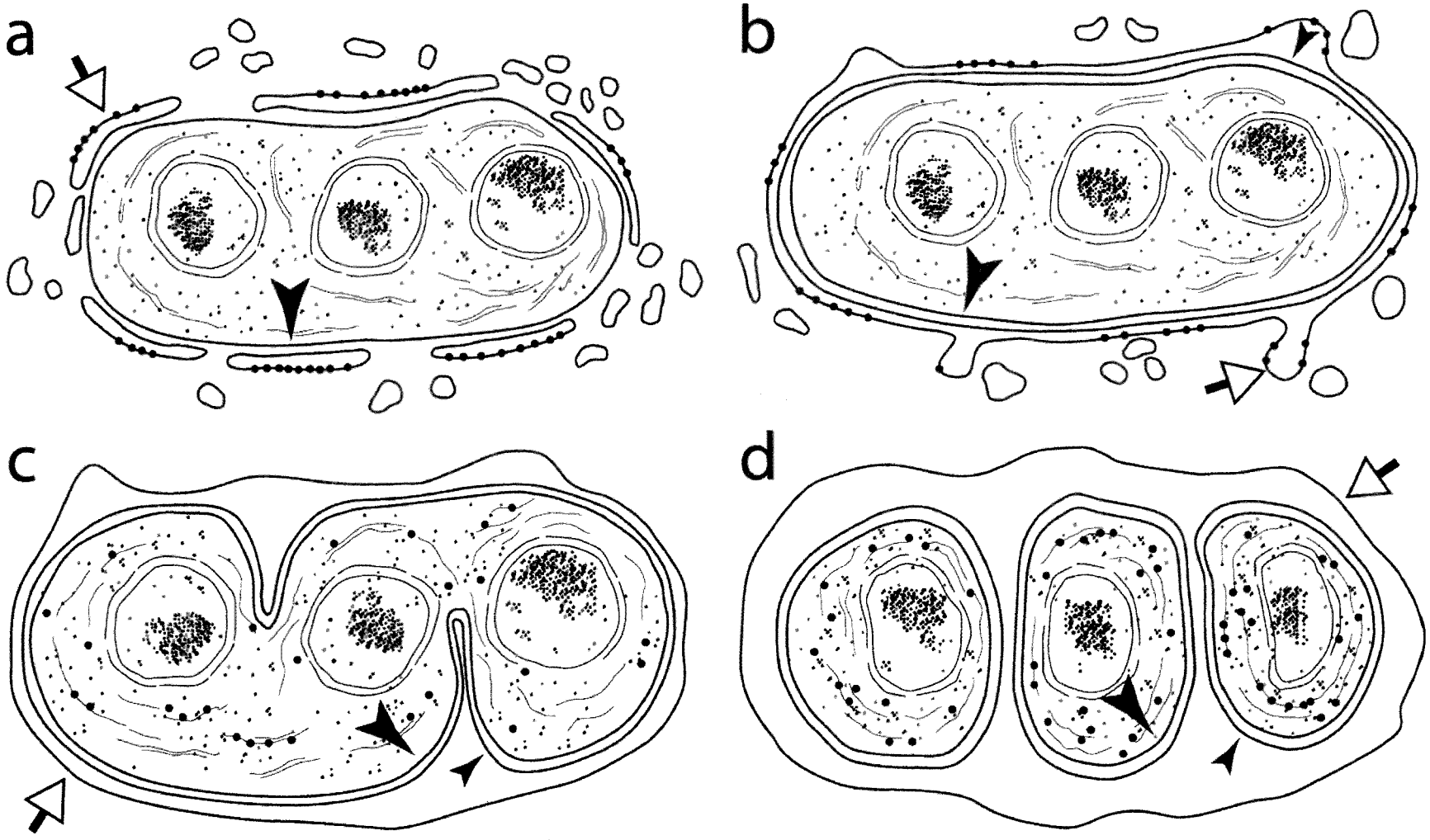
2.5.2 Origins of the parasite vacuole

Mechanisms that Microsporidia use to develop vacuoles during the sporogonial phase vary between species. Cali and Takvorian (1999) discussed several types of vacuoles that can be encountered in Microsporidia. Parasitophorous vacuoles may be formed where the host ER forms a double membraned vacuole wall

as in the genus *Endoreticulatus*. In *Pleistophora* the parasite produces a sporophorous vesicle where the parasite plasma membrane secretes the vesicle membrane, from which the parasite plasma membrane withdraws. The vacuole can also be formed with both the parasite and the host contributing; according to the literature the genus *Loma* falls into this category (Bekhti and Bouix 1985, Cali and Takvorian 1999). The morphological features observed in this investigation are not consistent with those already reported on *L. salmonae* vacuole development. Cali and Takvorian (1999) state that the host ER closely abuts the parasite plasma membrane but the parasite plasma membrane forms the vacuole by producing blisters. Bekhti and Bouix (1985) propose a different hypothesis for development of *L. salmonae* in coho salmon *Oncorhynchus kisutch* (Walbaum). They show that the merogonial stage is surrounded by host ER, which breaks down into small vesicles. Simultaneously the parasite produces the limiting membrane for the vacuole, which separates from the parasite plasma membrane. The host ER-derived vesicles in the cytoplasm then fuse with the vacuole membrane to enlarge it. During this time the parasite plasma membrane becomes thicker, but remains as a single membrane. The present investigation showed that *L. salmonae* developing within rainbow trout gills acquired their parasitic vacuole in a manner not described previously in Microsporidia. On the basis of the morphological findings in the present study, a new hypothesis is proposed to explain the formation of parasitophorous vacuoles by *L. salmonae* in *O. mykiss* (Fig. 2.8.). The merogonial stage of *L. salmonae* is surrounded by cisternae of host ER (Fig. 2.8.A) and in section the following is

observed: (1) the parasite bounded by a single plasmalemma, (2) host cytoplasm, (3) the host ER membrane (ER1), (4) the lumen of the host ER and (5) the host ER membrane adjacent to the host cytoplasm (ER2). The cisternae of host ER surrounding the merogonial stage fuse together and the lumen of the host ER enlarges forming the parasitophorous vacuole, with the host ER2 becoming the limiting membrane of the vacuole (Fig. 2.8.B). Vesicular elements in the host cytoplasm may fuse with the ER2 to enlarge the vacuole. The host ER1 remains in close association with the parasite plasmalemma and together they form the double membrane of the sporogonial wall. During cytokinesis of the sporogonial plasmodium the ER1 membrane would divide with the parasite and the ER2 remains as the vacuole membrane (Fig. 2.8.C). This results in numerous sporonts within a single parasitophorous vacuole (Fig. 2.8.D). The results of this study clearly demonstrate that *L. salmonae* in rainbow trout develops a parasitophorous vacuole, which is strictly host-derived. The host cell surrounding the parasite with ER resembles the first step in the process of autophagy. Autophagy is a physiologically important process that eukaryotic cells use to degrade excess or injured organelles (Yoshimori 2004). This process is required for removal of cellular debris and is often associated with cell stress or starvation of amino acids. Within the cell, cisternae of endoplasmic reticulum surround whatever is to be degraded and form an autophagosome, which fuses with a lysosome to accomplish degradation (Yoshimori 2004). Although contained within an autophagosome, *L. salmonae* may survive by blocking the fusion of the lysosomes with the phagosome, or the activation of

Figure 2.8. Diagrammatic representation of *L. salmonae* developing in salmonids according to the proposed theory. **A.** Merogonial stage is surrounded by cisternae of host ER. **B.** Cisternae of host ER fuse together and fully surround the parasite, while cytoplasmic vesicular elements fuse with the ER2 membrane to enlarge the ER lumen. **C.** During cytokinesis of the parasite, the ER1 membrane divides with the parasite and the ER2 membrane remains as the parasitophorous vacuole membrane; the ER lumen becomes the lumen of the parasitophorous vacuole. **D.** Numerous double membraned sporonts are formed within the parasitophorous vacuole. Host ER2 membrane (arrow), host ER1 membrane (small arrowhead), parasite plasma membrane (large arrowhead).


a
b
c
d

oxidizing enzymes subsequent to fusion. This is not unique to intracellular organisms and indeed there are species of intracellular bacteria, such as *Brucella abortus*, *Legionella pneumophila* and *Porphyromonas gingivalis*, which develop within the autophagosomes in order to avoid the host's defence mechanisms and obtain nutrients for development (Dorn et al. 2002). After avoiding the autophagolysosome *L. salmonae* benefits by using the host ER lumen and membranes for development. It would be advantageous for the parasite to develop within the lumen of the host ER during the later stages of development because it contains many proteins and enzymes that are not found directly in the host cytoplasm. The lumen of the ER can provide the parasite with the nutrients required to develop organelles characteristic of mature spores, such as the polar tube and the polaroplast. As Microsporidia are highly evolved parasites lacking mitochondria and a typical Golgi apparatus (Vavra and Larsson 1999), it is likely that they would evolve to utilize the host as efficiently as possible. This is the first time that parasitophorous vacuole formation by a microsporidium is suggested to be connected to the intracellular process of autophagy by the host cell. If indeed this is true it may represent a host response to cell stress caused by the presence of the parasite and an attempt to eradicate a foreign body from the cell, but instead of removing the parasite the process accommodates the pathogen and may be critical for continued development.

In the SV variant of *L. salmonae* in brook trout the single membraned parasitic vacuole develops during the onset of sporogony and the sporogonial stages

display the double membraned wall. The merogonial stages are bound by a single plasma membrane and cisternae of host ER are in close proximity. Based on these similarities it is probable that the vacuole formation in the SV variant is similar to that of the OA variant. The parasite cytoplasm of both variants in merogonial and sporogonial stages showed no evidence of new plasma membrane formation. The cytoplasm consisted of ribosomes and cisternae of ER and there was no abundance of membranous vesicles peripherally, which might be expected if the parasite was producing a new membrane. The morphological evidence from this study supports the idea that the membranes are of host origin and both variants form parasitophorous vacuoles. Descriptions of *L. salmonae*, and other species such as *L. acerinae*, *L. camerounensis*, *L. myrophis*, *L. boopsi* and *L. morhua*, show sporogonial stages having a single membrane that is thickened by electron dense deposition (Bekhti and Bouix 1985, Morrison and Marryatt 1986, Fomena et al. 1992, Faye et al. 1995, Lom and Pekkarinen 1999, Azevedo and Matos 2002). An important observation from this study is that the sporogonial stage, which has been described previously as having a thickened plasma membrane with electron dense deposition, is composed of two membranes. This second membrane is always present simultaneously with the formation of the parasitic vacuole. The present finding is important in that the thickening of the plasma membrane of the sporogonial stage is shown to be a result of the addition of a second membrane of host origin. This is clearly evident at a higher magnification although it may appear to be a single membrane at lower

magnifications. The double membrane of the sporogonial stage has not been previously described and may be unique to *L. salmonae*.

2.6 REFERENCES

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3 ULTRASTRUCTURAL EXAMINATION OF THE HOST INFLAMMATORY RESPONSE WITHIN GILLS OF NETPEN REARED CHINOOK SALMON WITH MICROSPORIDIAL GILL DISEASE[3]

3.1 ABSTRACT

The sequence of host changes following the rupture of spore-laden xenomas of the microsporidian *Loma salmonae* during Microsporidial Gill Disease of Salmonids was deduced from ultrastructural examination of the gills of naturally infected, moribund, Chinook salmon from a commercial aquaculture site. The gills contained many stages of parasite development suggesting fish were chronically exposed to the parasite. Intact xenomas were generally found beneath the endothelium in arteries and arterioles and were encapsulated by a layer of collagen containing fibroblasts sometimes joined by desmosomes. Spores of *L. salmonae* germinated within intact xenomas and within phagocytic cells suggesting the possibility for autoinfection. Some xenomas contained an environment that triggered spore germination where the cytoplasm was electron dense or contained many coated vesicles. Xenoma dissolution was characterized by neutrophil infiltration and loss of the xenoma plasma membrane and encapsulation. The inflammatory responses associated with ruptured xenomas ranged from acute lesions, denoted by a marked neutrophil infiltration and vascular thrombosis, to chronic lesions with a macrophage-rich infiltrate variously accompanied by neovascularization and vascular remodelling.

[3] Lovy J, Wright GM, Speare DJ. Ultrastructural examination of the host inflammatory response within gills of netpen reared chinook salmon, *Oncorhynchus tshawytscha* with microsporidial gill disease. Fish Shellfish Immunol 2007; 22: 131-149.

Dendritic-like cells and plasma cells were characteristic throughout. Basement membrane damage of the filament epithelium and subsequent transepithelial expulsion of spores were associated with severe inflammation. An unusual previously undescribed multifocal change, in which epithelial cells invaded deeply beyond the normal boundaries of the basement membrane, affected areas of gill filament epithelium with basement membrane damage. Some neutrophils that contained *L. salmonae* spores, or spore polar tube, displayed morphological changes that included irregular cell shape, cytoplasmic darkening associated with an abundance of free ribosomes, lysis of neighbouring cells, and type II nuclear clefts. Fusion of apparently intact neutrophils occurred in other areas of the lesion, where close contacts between neighbouring cells were established and in some areas plasma membrane fusion occurred. Closely associated neutrophils with intact plasma membranes were observed to contain type II nuclear clefts, abundant granules and rough endoplasmic reticulum. Other neutrophils in the lesion displayed type I nuclear pockets, which is suspected to be an early stage of apoptosis.

3.2 INTRODUCTION

Over the last decade, Microsporidial Gill Disease of salmonids (MGDS) has emerged, and is now seasonally endemic within the Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum 1792), marine aquaculture industry along coastal British Columbia. With respect to treatment and control, previous work has defined the parasite's life cycle dynamics- particularly the rate of xenoma development,

determined its susceptibility to several classes of therapeutic antiparasitic compounds, and elucidated some aspects of host protective immune response (Speare et al. 1998, Speare et al. 2000, Becker et al. 2002, Rodriguez-Tovar et al. 2002, Rodriguez-Tovar et al. 2003a). Despite observations that clinical disease is temporally linked to the onset of xenoma rupture and release of spores into tissue, primarily within the gills, the nature of the host inflammatory response has not been thoroughly examined. Although previous research has focused on developing treatments to reduce parasite numbers by blocking parasitic development within the host, defining the host responses to the parasite may provide information on ways to modulate the host symptoms during disease. Anti-parasitic compounds are only effective in the early stages of infection when applied before the onset of xenomas in gills (Becker et al. 2002), whereas modulation of host symptoms during xenoma rupture would have the benefit of a treatment which can be applied during disease when clinical signs are observed.

To date little ultrastructural work has been done in describing host responses to pathogens in fish. MGDS provides a useful *in vivo* model for the study of inflammatory responses in naturally infected netpen reared salmon. Histopathology using light microscopy has revealed numerous pathological alterations in *Loma* infected tissues. Xenomas that have undergone degeneration have been associated with fibrinoid arteritis, periarteritis, and hyperplasia of the overlying epithelium. The inflammatory response and multifocal distribution of the xenomas in fish with heavy infections markedly disrupts the general architecture of the gills (Kent et al. 1995).

Ultrastructural studies using rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) experimentally infected with *Loma salmonae* showed leukocytes around xenomas at 7 weeks post exposure (PE) and neutrophils within xenomas actively ingesting the spores at 8 weeks PE (Rodriguez-Tovar et al. 2003a). There is no ultrastructural work describing cellular responses and associated pathology that follow xenoma degeneration. The intent of this investigation was to examine gill ultrastructural pathology associated with MGDS in moribund netpen reared Chinook salmon. Attention was particularly given to describing the cellular responses to *L. salmonae* within the primary gill filament during xenoma degeneration and the associated tissue pathology.

3.3 MATERIALS AND METHODS

3.3.1 Sample fish

Chinook salmon were sampled from a netpen site that has historically had severe mortalities attributed to MGDS. Site data for 2002 shows that MGDS was attributed to a 13% cumulative mortality. The monosex female population of fish sampled were in their second growing season and weighed approximately 3 kg. The particular site was also chosen because according to 2002 site data MGDS resulted in 86% of the total mortalities and there had not been previous problems with other pathogens. Peak mortalities for 2004 were in August and all fish were sampled during that time. Eight samples of 5-8 moribund fish were taken over the month giving a total sample size of 47 fish. The moribund fish were removed from multiple

pens on site. Moribund fish displayed signs of respiratory distress and difficulty with swimming. These fish were collected with a dip net and organs were immediately dissected and fixed for light microscopy and transmission electron microscopy.

3.3.2 Tissue processing for microscopy

Organs sampled for light microscopy included gills, spleen, head kidney, trunk kidney, heart and liver. The samples were fixed in Davidson's solution, dehydrated in ethanols, cleared and embedded in paraffin wax. Five μm sections were cut and stained with haematoxylin and eosin (H & E). Gills from the respective fish were also processed for transmission electron microscopy. The first left gill arch was sampled from each fish, cut in half and immediately fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 2 h on ice. Following initial fixation individual gill filaments were dissected from the gill arch and transferred to fresh glutaraldehyde solution and maintained overnight at 4°C. All glutaraldehyde fixed samples were then washed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer for 2 h at room temperature. Samples were dehydrated in ethanol to propylene oxide, infiltrated and embedded in Spurr's resin. A more detailed tissue processing protocol is provided in section 2.3.4. Gill filaments from individual fish were distributed into 10 resin blocks.

3.3.3 Sample selection for TEM

Sections stained with H & E were screened for all 47 fish sampled and xenomas per gill filament and degree of inflammation was visually assessed. Twenty fish, which had the most xenomas and the most significant inflammatory lesions were

selected to be examined with HRLM and TEM. For HRLM, 0.5 μm sections were cut from 200 resin blocks of gill tissue, stained with toluidine blue, and examined with light microscopy. In areas of interest ultrathin sections (90 nm) were recut, stained with uranyl acetate and lead citrate, and examined and photographed with a Hitachi 7500 electron microscope. Serial sections were taken of several lesions where ultrathin sections were harvested every 6 μm through approximately 36 μm of the area of interest.

3.4 RESULTS

3.4.1 Parasites in the gills

The gills contained xenomas in both the gill filament associated with the endothelium of arteries and arterioles and within the lamellae. The parasite was observed in many stages of development from a host cell containing a single meront to large xenomas containing mature spores. The parasite life stages were similar to those described for *Loma salmonae* in rainbow trout and brook trout, where meronts were single membraned and sporonts were double membraned (see chapter 2). Development appeared to be aided by autophagy induced by the host cell as a response to the parasite, where endoplasmic reticulum was observed surrounding meronts as was described for *L. salmonae* in rainbow and brook trout in chapter 2.

Sections of polar tube were seen within intact xenomas, outside xenomas within the extracellular matrix, and within surrounding host cells (Fig. 3.1.A). In some xenomas a large proportion of spores were empty and sections of polar tube

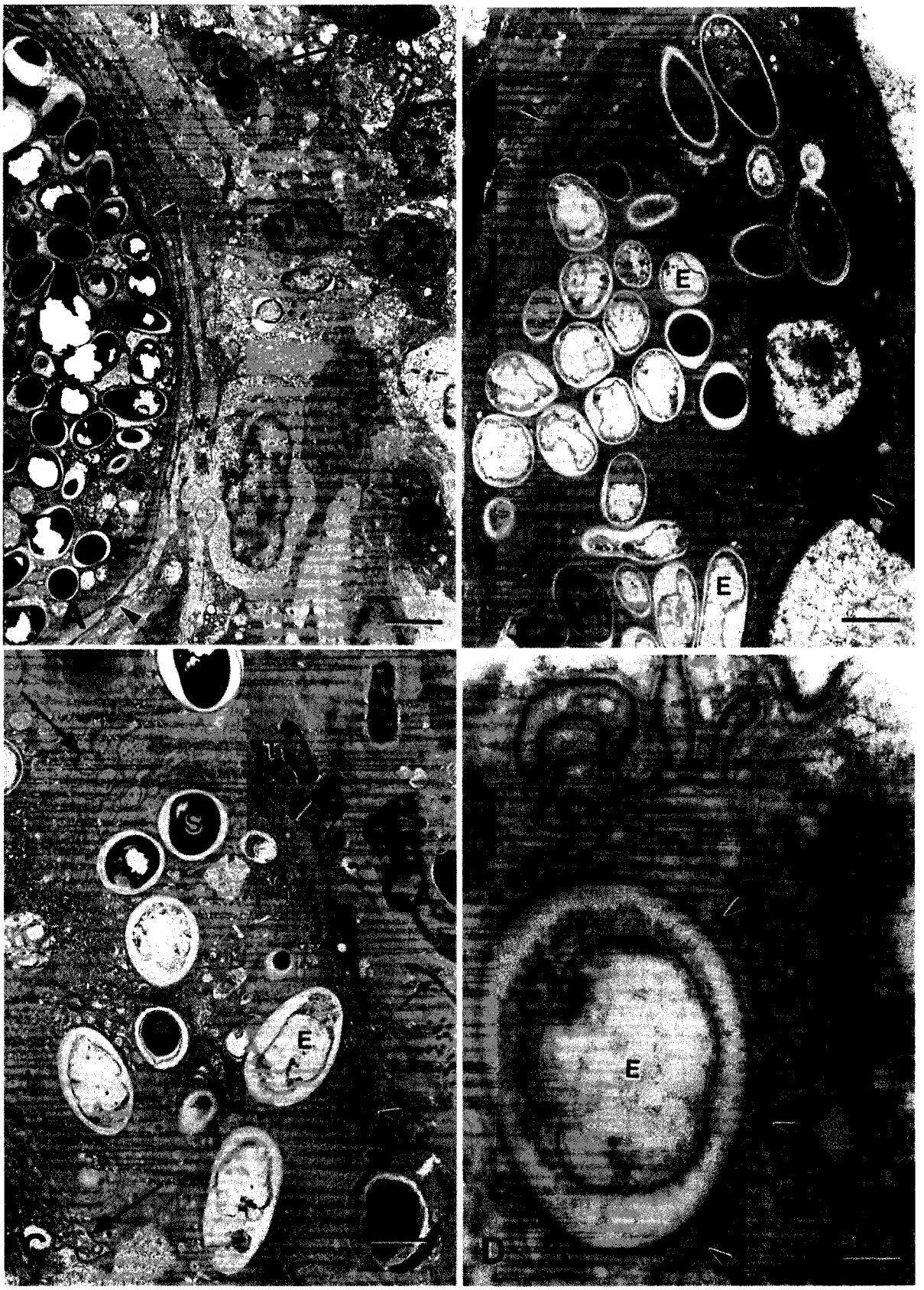
were present within the cytoplasm that was electron dense and contained membranous elements (Fig. 3.1.B). Xenomas with a collapsed appearance were observed where the plasma membrane displayed a very irregular shape and was difficult to follow in a single section plane. Many spores were empty likely due to germination within these collapsed xenomas and remnants of spore walls were found with irregular shape and at times discontinuous (Figs. 3.1.C and D). Serial sections of collapsed xenomas failed to reveal infiltration with host inflammatory cells. Spore exospores appeared thickened and had an electron dense fuzzy appearance. Partially surrounding the thickened exospore was a density similar in morphology to but separated from the exospore (Fig. 3.1.D). Closely associated with the density were many variable sized vesicles and tubular elements some of which had an electron dense coating similar to the exospore and surrounding density (Figs. 3.1.D and 3.2.A). In addition to a cytoplasm containing coated vesicles, tubular elements and endoplasmic reticulum, many mitochondria appeared intact (Fig. 3.2.B).

3.4.2 Host responses to parasites in the gills

Within the gill filament, xenomas were located primarily beneath the endothelium of arteries and arterioles. A host response to intact xenomas was the formation of a collagenous wall containing fibroblasts encapsulating the xenomas (Fig. 3.3.A). An unusual feature of the fibroblasts was the presence of desmosome-

like junctions consisting of electron dense plaques and associated tonofilaments on the cytoplasmic side of both cells (Fig. 3.3.B). Collagenous walls surrounded xenomas containing spores that had everted their polar tubes. The

Figure 3.1. **A.** Xenoma within the gill filament surrounded by fibroblasts (arrowheads) and collagen (*). Sections of polar tube (small arrows) are found within host cells and extracellular matrix surrounding the xenoma, one cell with a section of polar tube has a type 2 nuclear cleft (large arrow). (Bar= 3.5 μm) **B.** Xenoma containing many empty spores (E) within an electron dense cytoplasm surrounded by fibroblasts (arrowheads). (Bar= 1.6 μm) **C.** Xenoma containing spores (S), empty spores (E), and irregularly shaped spore walls (large arrows) within a cytoplasm containing many coated vesicles. Sections of polar tubes are found outside the xenoma (small arrow). Thrombocytes (T) are seen outside the xenoma plasma membrane (arrowheads). (Bar= 1.7 μm) **D.** An empty spore (E) with a closely associated electron dense band (arrowheads). Near the band are coated vesicles with a similar electron density. An empty spore with an irregular shape (large arrow) is also seen. (Bar= 266 nm)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everted tubes penetrated these walls, and sections of polar tube were found within a variety of peripheral host cells including fibroblasts, neutrophils, and plasma cells. Dendritic-like cells, identified by their characteristic rod-shaped granules containing a square lattice structure (Fig. 3.4.A), were found peripheral to encapsulated xenomas but never contained sections of polar tube. A further characterization of dendritic-like cells is provided in chapter 4.

Loss of the xenoma plasma membrane and liberation of spores within the interstitium of the primary filament was associated with a heavy infiltration of neutrophils, which frequently contained undegraded spores (Fig. 3.4.B). The marked infiltration of neutrophils was associated with a disruption of the basement membrane and the presence of inflammatory cells and spores in the epithelium (Fig. 3.4.C). Some areas of congestion included blisters within the epithelium that contained inflammatory cells, spores, and edematous fluid (Fig. 3.4.D). In other regions epithelial cells had infiltrated the connective tissue of the filament in areas where basement membrane was missing (Fig. 3.5.A). Many cells within the neutrophil infiltrate containing sections of spore polar tube or whole spores within their cytoplasm displayed a unique morphology. The cells had an electron dense cytoplasm, containing an abundance of free ribosomes and filamentous inclusions, which resemble neutrophil granules (Fig. 3.5.B) as well as rough endoplasmic reticulum, intact mitochondria, and tubular elements. The plasma membrane of the cells appeared discontinuous in areas and neighbouring cells appeared fused together

Figure 3.2. **A.** A spore with an increased electron dense exospore surrounded with similar electron dense material (arrowheads) where coated vesicles (arrow) are seen close to the electron dense material. (Bar= 380 nm) **B.** Intact mitochondria (M) seen in close proximity to the coated vesicles (arrow). (Bar= 220 nm)

Figure 3.3. **A.** An intact xenoma with a hypertrophied nucleus (N), spores (S), and meronts (M) is encapsulated by fibroblasts (arrowheads) and collagen. (Bar= 4.5 μm) **B.** Portion of the capsule surrounding an intact xenoma showing two fibroblasts joined with a desmosome-like junction (arrow). Rough endoplasmic reticulum (R), plasma membrane (arrowheads), collagen fibrils (*). (Bar= 150 nm)

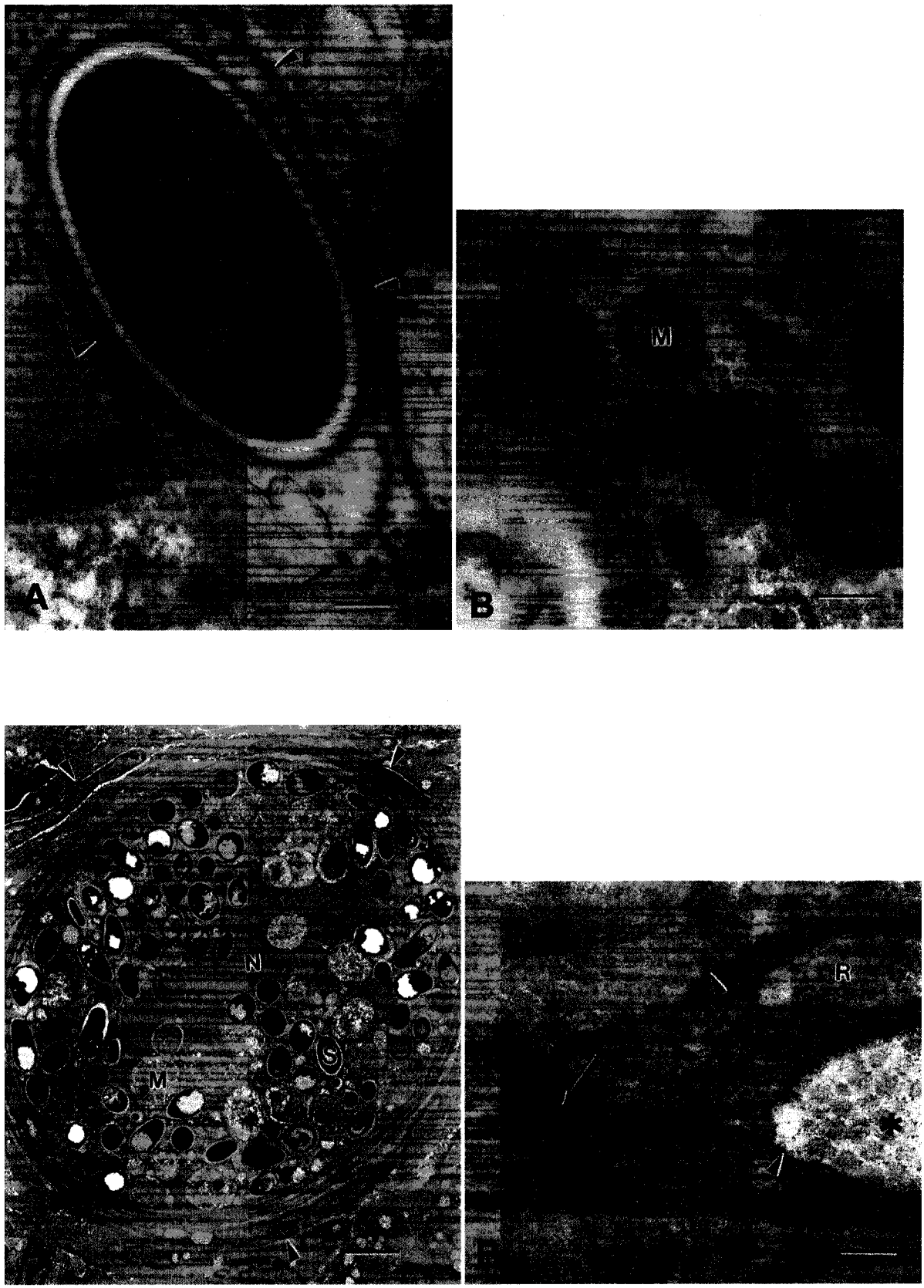
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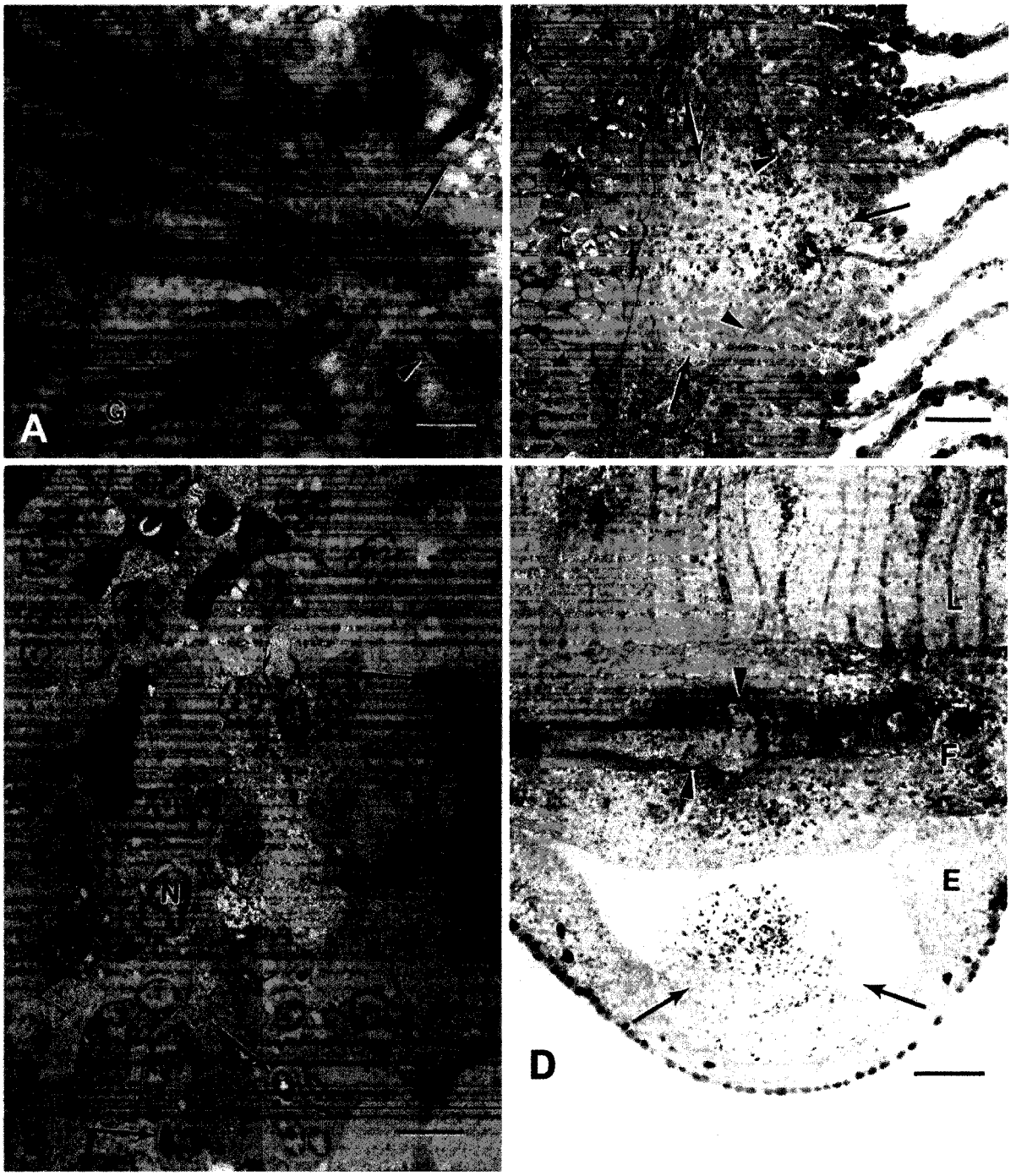
Figure 3.4. **A.** Cytoplasm of a dendritic-like cell containing characteristic granules (G) located near a centriole (arrow) and microtubules (arrowhead). (Bar= 200 nm) **B.** Lesion containing predominantly neutrophils with segmented nuclei (N) where intact spores are seen within the cells (arrows). (Bar= 5.3 μm) **C.** A high resolution light micrograph (HRLM) showing a neutrophil rich lesion (arrows) in the primary filament that has spread into the lamellar epithelium where there is a break in the basement membrane (arrowheads). (Bar= 200 μm) **D.** A HRLM showing a primary gill filament (F) with an infiltration of inflammatory cells and an artery (arrowheads) surrounded with fibrin. A blister full of spores, neutrophils, red blood cells, and edematous fluid (arrows) is seen in the epithelium (E). Lamellae (L). (Bar= 400 μm)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forming an irregular shaped mass of dense cytoplasm containing many nuclei. The dense cytoplasmic mass containing parasitic spores or polar tube filled the space between other intact cells (Fig. 3.5.C). The nuclei within the cytoplasmic mass displayed margination of heterochromatin and an infolded inner nuclear membrane producing a nuclear cleft, within which pockets containing cytoplasm were frequently observed. The outer nuclear membrane was densely covered with ribosomes (Fig. 3.5.D). Within the cytoplasmic mass near engulfed spores were accumulations of tubular structures (Fig. 3.6.A). Neutrophils observed outside the electron dense cytoplasmic mass contained similar nuclear clefts and abundant fibrillar granules with an intact plasma membrane (Fig. 3.6.B). Aggregates of intact neutrophils within the lesion developed close contacts with each other, and the plasma membranes of neighbouring cells were closely apposed (Fig. 3.6.C). In some areas of the closely apposed cells the plasma membrane was continuous and the cytoplasm of the two cells appeared to be fused (Fig. 3.6.D). Other neutrophils within the lesion contained thin chromatin bands that enclosed pockets of cytoplasm within the nucleus (Fig. 3.7.A). Rupture of xenomas localized within blood vessel walls and the associated inflammation resulted in a number of morphological changes within the vasculature. Thrombosis was commonly observed within arteries and arterioles of the filament. Thrombocytes were present in the connective tissue among spores; an extensive search revealed no thrombocytes containing spores. Thrombi in the main filament artery obstructed the width of the luminal space in section plane.

Figure 3.5. **A.** A HRLM showing a gill filament (F) with patches of epithelial cells (arrows) originating from the epithelium (E) where there is a break in the basement membrane (arrowheads). Lamellae (L). (Bar= 200 μm) **B.** A cell closely associated with a xenoma containing a condensed nucleus (N), electron dense cytoplasm, filamentous inclusions (arrowheads), and numerous sections of spore polar tube (arrows). (Bar= 580 nm) **C.** Electron dense cytoplasmic material (arrowheads) between intact cells contains multiple nuclei, two of which have type II nuclear clefts (arrows) and many spores (S). (Bar= 3 μm) **D.** A type II nuclear cleft showing outer nuclear membrane (arrowheads) decorated with ribosomes and the inner nuclear membrane (arrows) folded into the nucleus. Intranuclear cytoplasmic channels (large arrow) are observed within the cleft. (Bar= 185 nm)

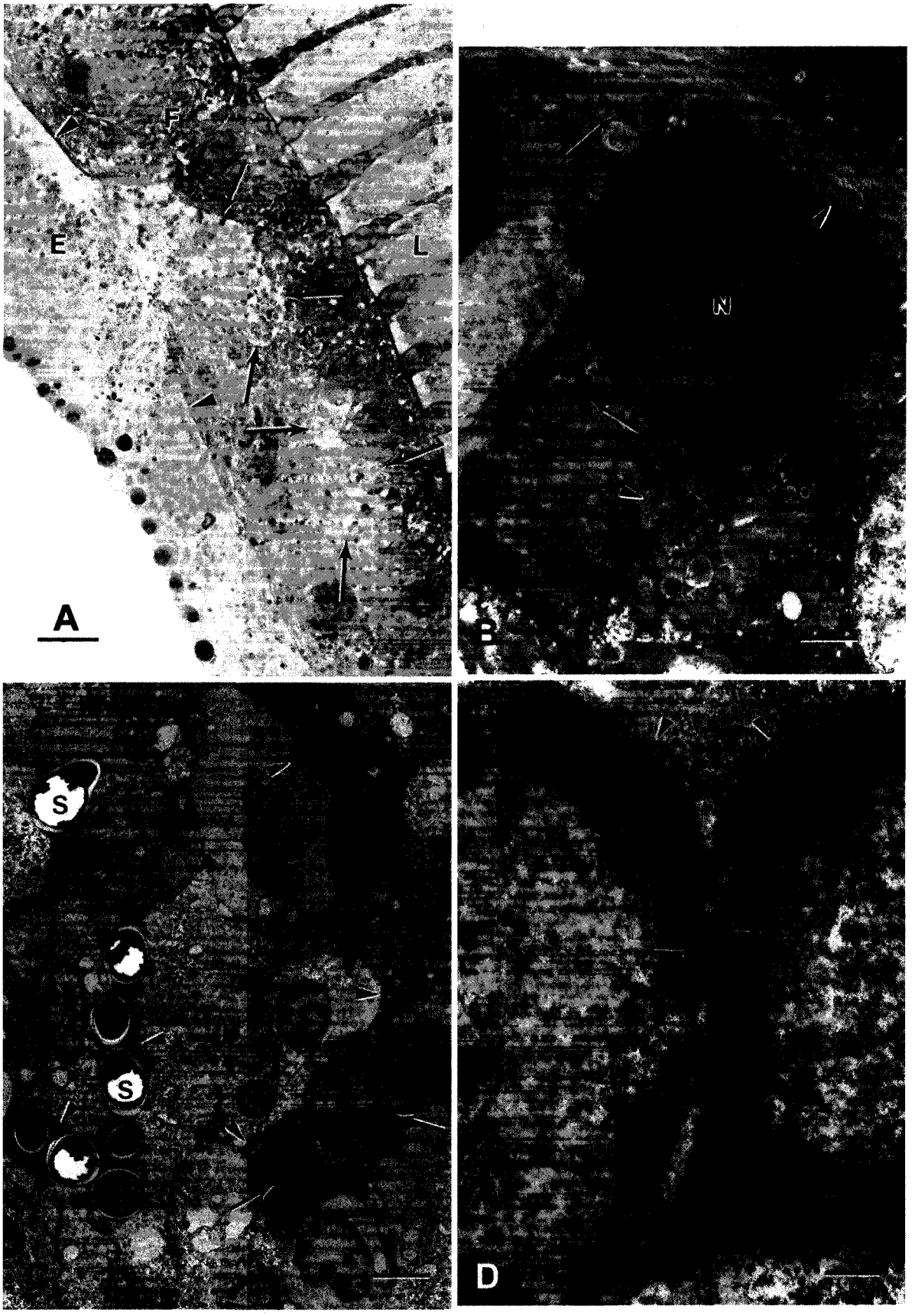
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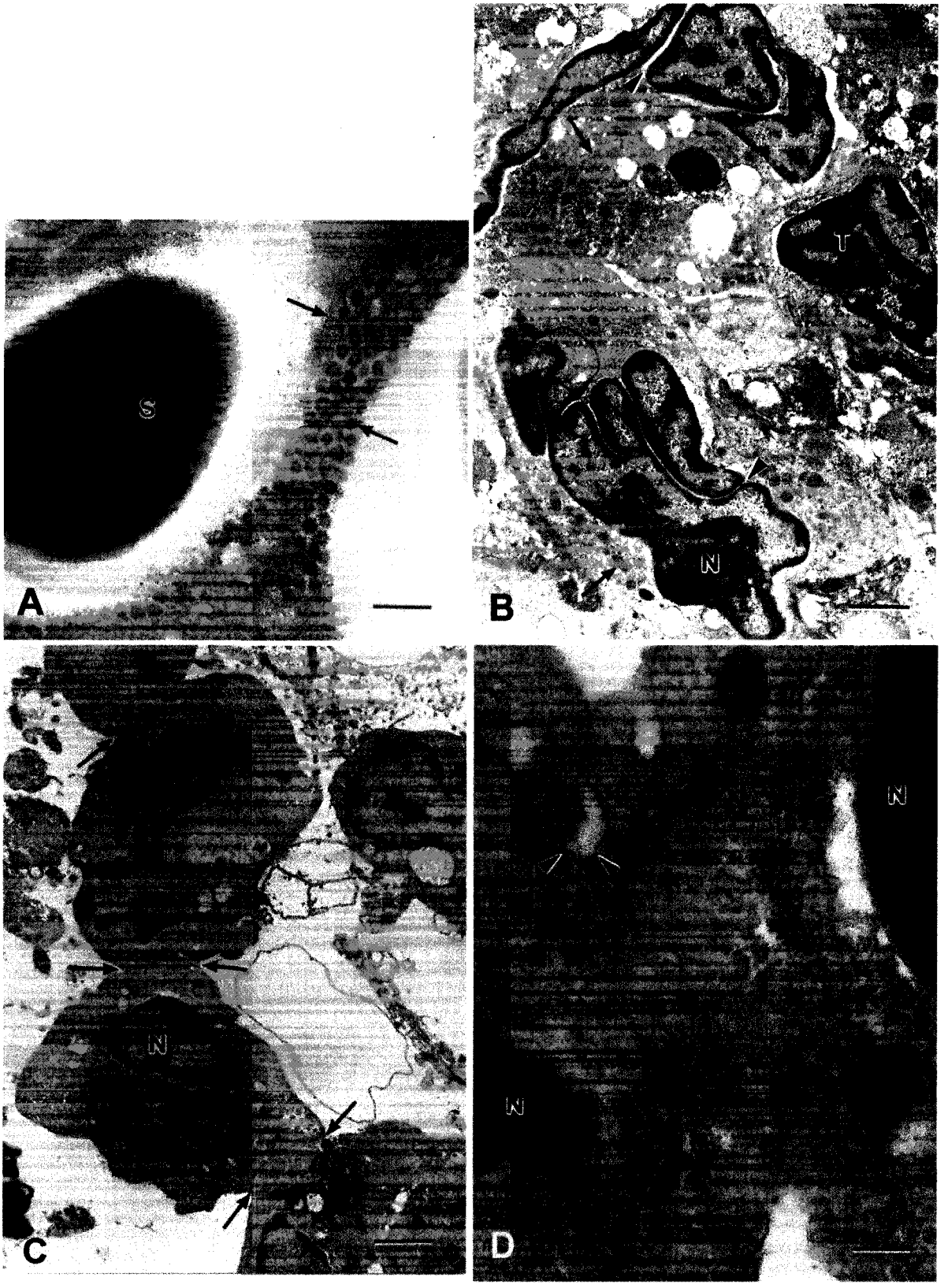
Figure 3.6. **A.** Tubular elements (arrows) within the electron dense cytoplasm surrounding spores (S). (Bar= 185 nm) **B.** Neutrophils with intact plasma membranes containing type II nuclear clefts (arrowheads), abundant granules (arrows), and plentiful rough endoplasmic reticulum. Neutrophil nucleus (N). Closely associated with the neutrophils is a thrombocyte (T). (Bar= 1.6 μm) **C.** A neutrophil aggregate where the cells develop close contacts with each other (arrows). Neutrophil nucleus (N). (Bar= 1.4 μm) **D.** A higher magnification of a close contact point between neutrophils from C. Nuclei (N) from apposing cells. In one area the plasma membrane appears continuous between the two cells (arrowheads), linking the cytoplasm (*). (Bar= 200 nm)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Interruptions in the arterial endothelium were observed, adjacent to which was deposition of fibrin and a group of thrombocytes walling off the injured area (Fig. 3.7.B). Large aggregations of neutrophils were adjacent to the thrombi (Fig. 3.7.B). Arterioles were enlarged and contained thrombi composed of thrombocytes and fibrin which fully obstructed the lumen (Fig. 3.7.C). Some arterioles were extremely enlarged and contained mitotic cells within fibrin near the vascular wall, but did not contain thrombocytes within the lumen. The enlarged arterioles occupied most of the interstitium of the filament and caused a bulge of the epithelium (Fig. 3.7.D).

Lesions were observed within the gill filament where the inflammatory infiltrate contained neutrophils and an increased number of macrophages. Multiple spores were seen within macrophages and were being degraded. Scattered throughout the inflammatory cell infiltrate were circular arrangements of cells (Fig. 3.8.A). Higher magnification revealed these structures to be tubules with a small lumen lined with a cuboidal epithelium and partially surrounded by an elongated cell (Fig. 3.8.B).

3.5 DISCUSSION

3.5.1 Parasites in the gills

Several methods of autoinfection have been described for *Loma* spp. and microsporidia in general and are suggestive in this investigation. Spores of the *Loma* species in Atlantic cod, *Gadus morhua* and *L. myrophis* of Amazonian fish, *Myrophis*

Figure 3.7. **A.** A neutrophil with few cytoplasmic organelles, fibrillar granules (arrowheads) and nucleus (N) with a type I nuclear pocket that contains cytoplasm (C) separated with a thin chromatin band (arrows). (Bar= 420 nm) **B.** Section of a damaged primary filament artery where the endothelium (bottom) is disrupted. The lumen containing neutrophils (*) is separated from the endothelium (arrow) by a wall of thrombocytes (arrowheads) and fibrin (F). (Bar= 7.6 μm) **C.** Two enlarged arterioles (large arrows) within the gill filament fully obstructed with thrombocytes (T), many of which contain sections of polar tube (small arrows). Adjacent to the arterioles are inflammatory cells containing spores (arrowheads). (Bar= 6 μm) **D.** A HRLM shows a severely enlarged arteriole (small arrows) containing red and white blood cells that fills the width of the gill filament and causes a bulge of the epithelium (large arrows). Note the size of normal arterioles (arrowheads). (Bar= 200 μm)

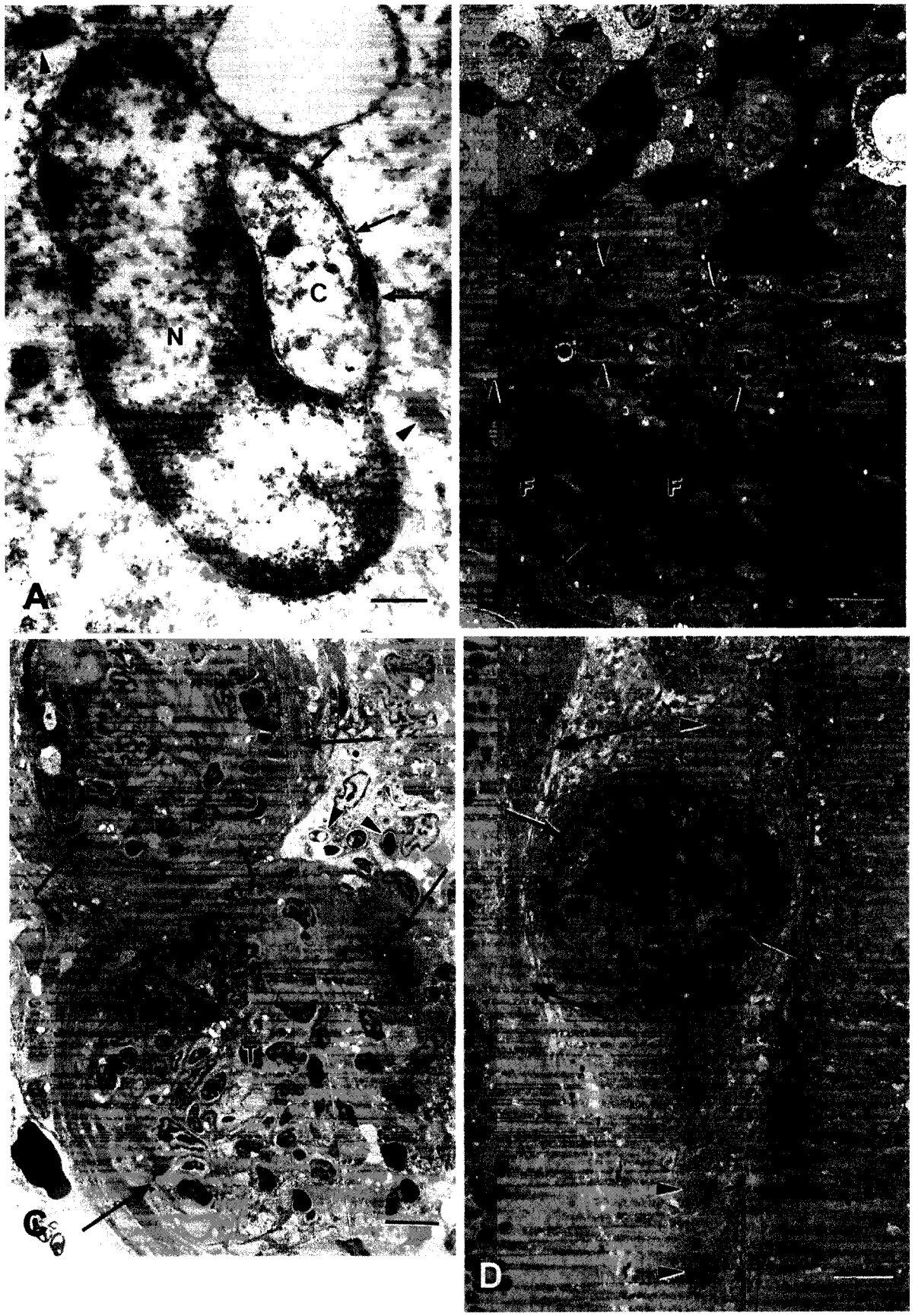
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Figure 3.8. **A.** A HRLM showing a gill filament containing an infiltrate of leukocytes with abundant macrophages and cells arranged in circular structures (arrows). (Bar= 200 μm) **B.** A circular structure showing cuboidal epithelial cells (E) with desmosomes (arrows) arranged around a lumen (L) and an elongated cell (arrowheads) surrounding a large portion of the epithelium. (Bar= 1.8 μm)

A
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platyrhynchus have been shown to evert their polar tubes within xenomas, pierce the xenoma plasma membrane and penetrate into surrounding host cells (Matos et al. 2003, Rodriguez-Tovar et al. 2003b). In this investigation it was common to see germinated spores within intact xenomas and polar tube sections in various white blood cells that were surrounding the xenoma. Since a white blood cell is suggested to carry the infection to the gills (Rodriguez-Tovar et al. 2002), autoinfection mayoccur upon infection of this cell type. With the rupture of xenomas within the walls of arteries and arterioles spores are liberated into the blood, leading to another possibility for autoinfection. Intravascular injection of spores into Pacific salmon has shown to induce infection (Shaw et al. 1998). Lastly, microsporidia can invade cells through phagocytosis, where a spore is phagocytosed and it evades the phagosome by germinating and using its polar tube to infect the cytoplasm of nearby cells (Franzen 2004). The presence of empty spores within macrophages and neutrophils in this study lends support to this theory. Multiple pathways of autoinfection are likely and if fish are immunosuppressed then it is more likely that the autoinfections would occur.

In addition to spores germinating in intact xenomas, two other degenerative types of xenomas that were not infiltrated with inflammatory cells contained a high proportion of empty germinated spores (Figs. 3.1.B and 3.1.C). Figure 3.1.B shows a xenoma where nearly all the spores appear empty and the cytoplasm of the xenoma is increasingly electron dense. It's possible that encapsulation of the xenoma by fibroblasts and collagen compromises the supply of oxygen and nutrients to the

xenoma, causing a change in the xenoma microenvironment, which may stimulate spore germination. Figures 3.1.C, D, and 3.2.A show irregularly shaped xenomas with cytoplasm containing mature spores and empty spores surrounded by coated vesicles and tubular elements. The intact mitochondria suggest that the xenoma is not in a degenerative state. The presence of collapsed and broken spore walls in the cytoplasm suggests that the host cell is degrading the parasite. The coated vesicles may represent fragmentation of the parasite or the host cell surrounding the parasite with early lysosomal vesicles in order to degenerate the spore. Although the function of the coated vesicles is unknown it is an important observation that some host cells harbouring the parasite are creating an environment where a majority of the spores are undergoing germination.

3.5.2 Host responses to parasites in the gills

Previous ultrastructural studies have examined the pathology of MGDS from tissues of rainbow trout which had received a single large experimental exposure of spores, and therefore the parasite development and associated tissue changes were relatively synchronous. In contrast, the current study has examined material from a natural case of MGDS in Chinook salmon and therefore provides the advantage of examining multiple developmental stages of the parasite and the related range of host reaction within single specimens. From this case material, based on finding the co-existence of later and early stages of the parasite within tissue, it would appear that the immune protection that robustly protects experimentally infected fish from re-infection, apparently fails to protect fish during natural outbreaks of MGDS. This

suggests that disease factors and co-factors within the farm environment may override the development or effect of protective mechanisms. However, it may also partially be explained by species differences. While work by Rodriguez-Tovar et al. (2006) demonstrates that the onset of resistance in rainbow trout occurred at three weeks (PE) and was complete by six weeks (PE), we have yet to define the onset time for Chinook salmon. If substantially delayed, this could explain our findings. Differences between rainbow trout and Chinook salmon have already been documented for susceptibility and persistence of MGDS, with Chinook salmon being both more susceptible, and much slower to clear the xenomas (Ramsey et al. 2002). Another possible explanation that cannot be overlooked is that salmonids do not acquire sterile immunity to *Loma*. If challenged with high concentrations of the parasite, which may be present in the farm environment, then partial protection may still result in significant infections.

Although fibroblasts were previously shown to be closely associated with xenomas at week 6 PE in rainbow trout (Rodriguez-Tovar et al. 2003a), a full encapsulation of intact xenomas by fibroblasts and collagen has not been previously reported for *Loma salmonae*. Encapsulation is seen in other xenoma forming microsporidia in fish when xenomas are infiltrated with inflammatory cells (Amigo et al. 1998, Canning and Curry 2005). A unique observation in this study was the presence of reinforcing desmosomes between fibroblasts. Desmosomes are not normally present in fibroblasts, but they have been reported in human periodontal ligament fibroblasts, although they lacked the inserting tonofilaments described

herein (Yamaoka et al. 1999). Examples of unexpected desmosomal cellular coupling during the formation of granulomas have been described for teleost epithelioid macrophages (Gauthier et al. 2004, Koehler et al. 2004).

In this study dissolution of the xenoma plasma membrane and encapsulation was not directly observed, however it is believed that host inflammatory cells participate in its degradation. As demonstrated in rainbow trout during the late stages of xenoma maturation, host inflammatory cells surround the xenoma (Rodriguez-Tovar et al. 2003a); this may be explained by diffusion of weak signals across the xenoma plasma membrane. Later phagocytic cells infiltrate the xenoma (Rodriguez-Tovar et al. 2003a). In this study, during spore germination across the xenoma wall, an increase in neutrophils surrounding the xenoma was observed. Damage to the xenoma wall resulting from spore germination may result in leakage of signals, further stimulating recruitment and infiltration of neutrophils into the xenoma. A loss of the xenoma plasma membrane and encapsulation seen during a heavy infiltration of neutrophils suggests that damage caused by large numbers of neutrophils entering the xenoma may be responsible for the xenoma wall dissolution. Neutrophils actively phagocytosed spores, but interestingly spore degradation was not observed.

The acute neutrophilic infiltration in the gill filament was severe enough to cause destruction of the basement membrane in the epithelium of both the filament and the lamellae. Although it was previously thought that spores within the gill filament would be trapped in the tissue and dealt with exclusively by phagocytic cells, this investigation shows that transepithelial expulsion of spores, inflammatory

cells, and exudate occurs. Reducing the number of spores and inflammatory cells within the filament may reduce the host inflammation in addition to giving the parasite access to the environment, however the destruction of basement membrane may have its own consequences. Areas of damaged basement membrane contained lesions comprised of epithelial cells infiltrating the interstitium of the filament.

Within the neutrophil infiltrate associated with xenoma destruction and spore release some suspected neutrophils that contained spores or sections of spore polar tube underwent interesting morphological changes. These included, darkening of the cytoplasm associated with an increase in free ribosomes, and type II nuclear clefting, characterized by deep infolding of the inner nuclear membrane (Stekhoven and Holland 1986). The presence of filamentous inclusions within the cytoplasm that resemble fibrillar granules of neutrophils and the presence of these cells within a neutrophil aggregate suggests that they are neutrophils. The membrane integrity of the cells had been compromised and neighbouring cells had fused together producing a large cytoplasmic mass containing multiple nuclei, where empty and intact spores were seen within. These morphological changes are not consistent with normal features of cell necrosis or apoptosis, but likely represent a response to the parasite since all the cells with these features contained the parasite. The cytoplasm of these cells did not experience an influx of fluid or contain swollen mitochondria, which is often associated with plasma membrane damage during necrosis. It was interesting to observe type II nuclear clefts, sometimes referred to as intranuclear annulate lamella, in these cells. Reports of these are scarce and have been associated with

mainly neoplastic cells (Stekhoven and Holland 1986) or germ and embryonic cells (Ghadially 1988). Although the significance of these clefts remains unknown they have been suggested to represent a heightened metabolic activity (Ghadially 1988). Neutrophils containing these clefts prior to fusion with other neutrophils contain an abundance of fibrillar granules and rough endoplasmic reticulum, suggesting that the type II nuclear cleft could be associated with heightened metabolic activity in hypergeneration of granules. These neutrophils may later fuse with each other in the presence of the parasite, producing an electron dense cytoplasmic mass with abundant granules and numerous clefted nuclei. Multinucleate giant cells are common in chronic inflammation and are derived from the fusion of neighbouring macrophages initiated by contact with certain pathogens and cytokine stimulation (Gasser and Most 1999). Nuclei of these cells exhibit folds and deep indentations and the cytoplasm is crowded with free ribosomes, dense mitochondria, and rough endoplasmic reticulum (Sutton and Weiss 1966). The cytoplasmic makeup of the fused neutrophils in this study resemble that described for multinucleate giant cells. Intact neutrophils in this study were also observed to form aggregates and fuse with each other. Although previously undescribed, our results show that fish neutrophils may use a similar mechanism of giant cell formation, which commonly occurs in macrophages during chronic inflammation. Mammalian neutrophilic responses to fungal spores have been demonstrated to induce a type of extracellular killing where several neutrophils can associate with each other and surround spores, enveloping them in an extracellular vacuole where degradation occurs (Rozental et al. 1996,

Fernanda et al. 2004). This interaction with fungal spores and neutrophils led to the destruction of neutrophils within 30 minutes (Rozental et al. 1996). Another possibility is that neutrophils in this study could associate with each other in order to surround spores. After surrounding the spores, attempts at spore degradation may cause plasma membrane damage of neutrophils leading to lysis of the neighbouring cells. Other neutrophils appeared to be undergoing degeneration as supported by the observation of cytoplasm with fewer organelles and nuclei with type 1 nuclear pockets, which were identified by cytoplasm being enclosed by a nuclear heterochromatin bridge (Stekhoven and Holland 1986). Nuclear pockets in neutrophils are characteristic of stage I in apoptosis and have been reported in numerous pathological alterations in mammals (Sasaki et al. 1995).

Thrombosis was a common finding within arterioles and arteries. The roles of thrombocytes in fish have previously been shown to include thrombosis, phagocytosis, and recently have been suggested to behave as antigen presenting cells (Kollner et al. 2004). In this investigation thrombocytes were seen in repair of injured blood vessels where the response was similar to that seen in mammalian platelets. Figure 3.7B shows the classical response of thrombocytes in thrombosis, where thrombocytes and fibrin form a thrombus wall near the damaged endothelium. Adjacent to the thrombus wall within the lumen of the blood vessel a large aggregation of neutrophils appeared attracted to the site. During the coagulation cascade in mammals activated platelets can recruit leukocytes to the site of vascular injury and activated leukocytes release substances that further activate platelets.

Neutrophils can accumulate within a growing thrombus to help with blood clotting and activated platelets can substitute for endothelial cells in recruitment and migration of leukocytes through the damaged vessel (De Gaetano et al. 2003). Phagocytosis in thrombocytes was not observed in this study; thrombocytes appeared limited to sites of vascular injury. Dendritic-like cells were frequently present in lesions within the inflammatory infiltrate near thrombus formation. The origin of these dendritic-like cells is still unknown, but their presence along with thrombocytes is likely a consequence of both cell types being involved in an acute inflammatory response.

Lesions appear to develop chronicity where a higher number of macrophages actively degrading spores are present at the site. This macrophage rich infiltrate contains newly formed blood vessels (Figs. 3.8.A and B), which are likely the product of neovascularization. Neovascularization is a characteristic feature in mammalian chronic inflammatory diseases such as asthma and chronic bronchitis and morphologically comprises a single layer of endothelial cells which are often completely surrounded by pericytes (Bian et al. 2004). The immature endothelial cells in this study appear as cuboidal cells and the desmosomes are not well defined likely because they are in the process of development. Neovascularization can provide the leukocytes with oxygen and nutrients, but with activated leukocytes releasing cytokines and proteolytic enzymes tissue damage can lead to excessive angiogenesis and persistence of the inflammatory response (Bian et al. 2004). Another vascular change seen during the chronic inflammatory response was the

severe enlargement of arterioles without the presence of thrombocytes and occasionally the presence of mitotic cells near the vascular wall, which resembles vasodilation, although the presence of mitotic cells suggests vascular remodelling. Remodelling is when angiogenesis occurs within arterioles, capillaries, or venules and the blood vessel enlarges, but new blood vessels are not formed (McDonald 2001). Neovascularization and vascular remodelling are often seen together in chronic inflammation of mice with chronic airway inflammation (Thurston et al. 1998). This is the first report of neovascularization and possibly vascular remodelling within inflammatory lesions of fish.

3.6 REFERENCES

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4 COMPARATIVE CELLULAR MORPHOLOGY SUGGESTING THE EXISTENCE OF DENDRITIC CELLS IN SALMONIDS[4, 5]

4.1 ABSTRACT

This is the first morphological description of cells that resemble dendritic cells, which are observed in the gills of netpen reared Chinook salmon with Microsporidial Gill Disease of Salmonids (MGDS), and in rainbow trout with experimentally induced MGDS. The cells contain characteristic granules with a racket shape, which strongly resemble Birbeck granules in mammalian Langerhans cells. The racket shaped granules, frequently localized near centrioles, had a vacuolated end and a rod portion that measured about 150 nm in diameter. These dendritic-like cells also appeared to form resident populations within the spleen and anterior kidney of healthy salmonids. Based on an examination of three salmonid species including, rainbow trout, brook trout, and Atlantic salmon, the cells were most abundant in the spleen, although they were always present in the anterior kidney. The cells appeared diffusely distributed, often near blood vessels of the spleen and kidney of healthy fish and within the epithelium, connective tissue, and blood vessels of rainbow trout gills with experimentally induced MGDS. The cells were about 6 μm in diameter and contained Birbeck-like (BL) granules localized near centrioles. Although the dendritic-like cells in the three salmonid species shared

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[5] Lovy J, Wright GM, Speare DJ. Comparative cellular morphology suggesting the existence of resident dendritic cells within immune organs of salmonids. Anat Rec 2008; in press

many similarities, morphological differences were found in the fine structure of the rod portion of the BL granules. Rainbow trout BL granules contained amorphous material, while the other salmonid species contained particulate material arranged in a square-lattice arrangement. The BL granules in the cells of Atlantic salmon had a narrow diameter and contained 4 layers of particulate material when sectioned longitudinally; two layers enveloped by the granule membrane and two central layers making up a central lamella. The presence of central lamellae within Birbeck granules is characteristic in mammalian Langerhans cells.

4.2 INTRODUCTION

Microsporidial Gill Disease of salmonids (MGDS) is a significant disease of net-pen reared Chinook salmon, *Oncorhynchus tshawytscha* within the coastal waters of British Columbia (Speare 2000). In MGDS the microsporidian, *Loma salmonae* becomes established within the endothelium of the gills and forms a parasitic cyst termed a xenoma (Rodriguez-Tovar et al. 2002). It appears that the xenomas are undetected by the host until the rupture phase where severe inflammation and tissue destruction is seen. These lesions show individual spores within phagocytic cells (Kent 1998).

Fish with a single dose exposure to *L. salmonae* spores have been shown to induce a protective immune response against later infections (Sanchez et al. 2001). Work by Vallejo et al. (1992) have shown that channel catfish, *Ictalurus punctatus* process and present antigen during induction of their immune response. With a

monocyte catfish cell line endocytosis of protein antigen, intracellular processing, and reexpression of immunogenic peptides on the surface of the cell has been demonstrated (Vallejo et al. 1992). Cell mediated cytotoxicity occurs during the adaptive immune response in mammals and requires key molecules on the surface of leukocytes and target cells; these similar expression patterns and genes have now been identified in fish leukocytes (Fischer et al. 2006). Additionally MHC class II + cells have been identified in the gills of Atlantic salmon *Salmo salar* affected by amoebic gill disease (Morrison et al. 2006). With this evidence there can be speculation that the teleostean fish immune system is equipped with a similar antigen presenting cell as seen in mammals.

Dendritic cells (DC) function to capture antigen, process it into immunogenic peptides, and present the processed antigen to lymphocytes in order to initiate clonal immunity (Makala and Nagasawa 2002). DCs are classified into a number of subpopulations based on origin and location in the body; for example Langerhans cells are found in the epidermis and peripheral blood dendritic cells are in circulation (Makala and Nagasawa 2002). There is evidence to believe that these cells may be found throughout immunologically important organs of salmonids. It has been shown that CD83, a standard marker for Langerhans cells and activated or differentiated DCs, is expressed in various tissues of rainbow trout and expression is up-regulated during viral infections (Ohta et al. 2004). As gills are a vulnerable organ to infectious agents from the environment, the spleen and the kidney of fish are the two major filtering organs in the vascular system and the high trapping abilities of

these organs result in an accumulation of blood-borne pathogens (Ellis 1980, Noga 2006). These organs are also known to contain melano-macrophage centers (MMCs), which are thought to be evolutionary precursors of mammalian germinal centers, and are important in immune surveillance (Agius and Roberts 2003, Vigliano et al. 2006). Considering the importance of the spleen and kidney in filtering the blood and initiating immunity to pathogens we hypothesized that these two organs would contain dendritic cells.

To date little ultrastructural work has been done with host responses to pathogens and tissue inflammation in fish. The ultrastructure of various inflammatory cells of fish have been described by Ferguson (2006), but characterization of inflammatory cells during disease states have not been described well and dendritic cells have not been morphologically identified in fish. The purpose of this study was to use TEM to demonstrate the presence of dendritic cells in the gills of moribund net-pen reared Chinook salmon and laboratory maintained rainbow trout with MGDS. Various immunologically important organs of healthy salmonids are also examined to determine the presence and location of DCs.

4.3 MATERIALS AND METHODS

4.3.1 Netpen reared Chinook salmon

Moribund Chinook salmon, *Oncorhynchus tshawytscha* were sampled with a dip-net from a farm site with a known history of *Loma salmonae* during peak mortalities (August) in 2004. Gills from 20 heavily infected fish out of 47 were

selected for viewing with HRLM and TEM, these were the same fish that were described in sections 3.3.1-3.3.3.

4.3.2 Experimentally induced MGDS in rainbow trout

Naïve rainbow trout were acquired from the Cardigan fish hatchery on Prince Edward Island and maintained at 10°C ±1°. One week prior to infection with *Loma salmonae* 30 fish were moved into 70 L tanks and maintained at 15°C. The fish (weight 40-50 g) were infected with *L. salmonae* by feeding infective gill material from previously infected fish that had parasitic xenomas in the gills, similar as described in section 2.3.3. Fish were fed infective gill tissue daily for a period of 3 weeks and fish were maintained in the tanks for 8 weeks to allow for the disease to progress. Control fish were maintained in a separate 70 L tank with the same source water at 15°C. At this time 5 infected fish and 3 control fish were euthanized with an overdose of benzocaine and the first left gill arch was dissected and immediately fixed for transmission electron microscopy (TEM).

4.3.3 Spleen and anterior kidney sampling from healthy fish

Three species of salmonids, rainbow trout (weight 40-50 g), brook trout, *Salvelinus fontinalis* (weight 100-130 g), and Atlantic salmon parr (weight 10-20 g), were maintained in the laboratory in separate 70 L tanks at 15° ±1°C. The source water came from a well, which was warmed with a heat exchanger and mixed in a header tank to adjust temperature. Three fish from each of the species were overdosed in benzocaine and examined grossly, externally and internally, to ensure

that fish had no lesions. From each fish the spleen and head kidney was dissected, cut into 5 mm^3 pieces and immediately fixed for TEM.

4.3.4 Sample preparation for HRLM and TEM

Samples were processed as described in Chapter 2.3.4. Semi-thin sections (0.5 μm) were cut from all the gill tissue from rainbow trout with experimentally induced MGDS and each of the spleen and kidney from all three species of salmonids. The sections were stained with 1% toluidine blue in 1% sodium tetraborate solution and viewed with a light microscope. Sections of gill that contained ruptured xenomas with an inflammatory response in MGDS affected fish, three sections of control rainbow trout gill, and one block for spleen and one block for kidney from each of the 3 rainbow trout, brook trout, and Atlantic salmon was re-cut to generate ultra-thin sections (90 nm). The ultra-thin sections were retrieved onto copper super grids (200 mesh) and stained with uranyl acetate and Sato's lead stain. The sections were examined and photographed using a Hitachi H7500 transmission electron microscope operated at 80 kV. One ultrathin section per organ per fish was examined by scanning the entire section with TEM and dendritic-like (DL) cells were counted to get a rough estimate of the numbers of cells within a 1 mm by 1 mm section of the organ. The counts do not fully represent that area because of areas blocked by the grid bars.

4.4 RESULTS

4.4.1 Tissues containing dendritic-like (DL) cells

4.4.1.1 Gills of netpen reared Chinook salmon

Lesions in the filament of the gills had individual spores, some enclosed by phagocytic cells, within a heavy infiltrate of inflammatory cells. The lesions contained predominantly neutrophils, characterized by a segmented nucleus and fibrillar granules and red blood cells. Some of the neutrophils appeared to have phagocytosed individual spores. To a lesser degree macrophages and thrombocytes were also present; thrombocytes were identified by their extensive peripheral microtubule arrangement. Throughout the lesions within the connective tissue there were a small number of DL cells.

4.4.1.2 Gills of rainbow trout with experimental MGDS

The gills of rainbow trout with experimentally induced MGDS had intact parasitic xenomas and focal areas of inflammation containing microsoporidian spores within the connective tissue of the gill filament and within the lamellar epithelium. TEM revealed that both macrophages and neutrophils were phagocytosing the spores within the lesions. DL cells were observed in the lesions within the inflammatory infiltrate within the connective tissue of the filament, in the epidermis, and in one case in a pillar channel within gill lamellae (Fig. 4.1.). In control fish DL cells were rarely observed in the gills, but in one instance a cell was seen within a cellular infiltrate of the epidermis of the lamellae.

4.4.1.3 Spleen and kidney of healthy salmonids

The spleen and head kidney of all three salmonid species contained DL cells, but of the two organs the spleen had the highest number of DL cells in all species examined. The average DL cell counts per ultrathin section for each species examined was 10 for rainbow trout, 15 for brook trout, and 50 for Atlantic salmon in the spleen, while cell counts in the head kidney were 1 for rainbow trout, 5 for brook trout, and 10 for Atlantic salmon.

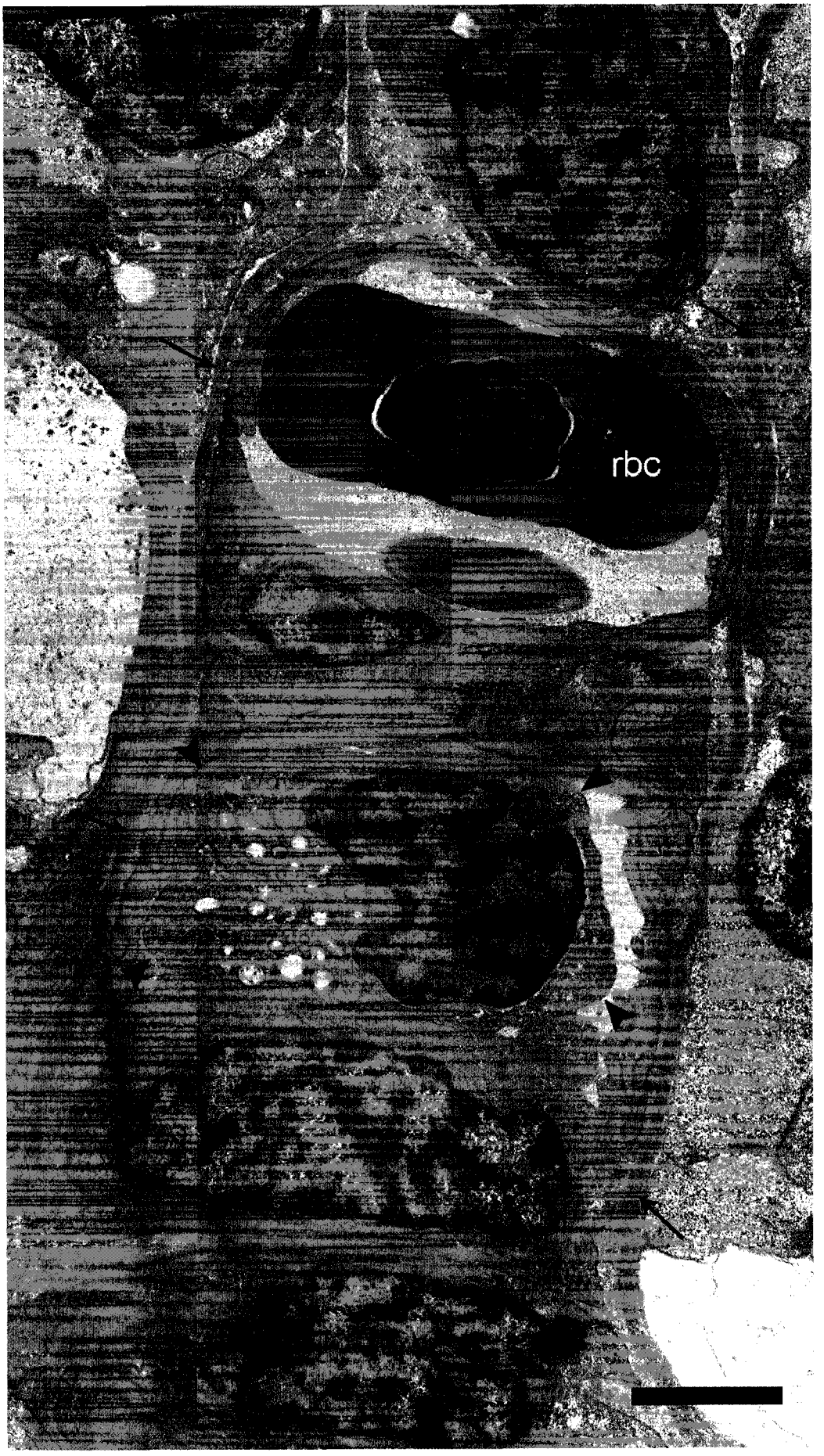
4.4.2 Ultrastructure of DL cells

Dendritic-like cells in all three salmonid species measured approximately 6 μm in diameter, contained an unsegmented nucleus, and did not possess dendritic processes. The cells all contained Birbeck-like (BL) granules frequently found surrounding the centriole and never observed to be in continuation with the cell membrane. Occasionally the granules were seen near a Golgi apparatus. Although the BL granules shared some common ultrastructural details in that they consist of a rod portion and a vacuolated portion giving them their racket shape, differences in their fine detail were noted across the salmonid species.

4.4.2.1 Chinook salmon DL cells

The cells had a large rounded nucleus with numerous mitochondria and ribosomes in addition to the granules within their cytoplasm (Fig. 4.2.A). The membrane bound granules appeared as rods when sectioned longitudinally and as spherical structures in cross section. One end of the granule was frequently observed

Figure 4.1. *Oncorhynchus mykiss*, with a dendritic-like cell (arrowheads) within the lumen of a pillar channel of the lamella of the gill. A red blood cell (rbc) can be seen in a nearby pillar channel and the basement membrane (arrows) of the lamella is seen. Scale bar = 3 μm.


rbc

to be vacuolated to give the granule a tennis racket shape. The lumen of the vacuolated portion was lined with an amorphous material and square lattice structure (Fig. 4.2.B). Measurements of 20 granules through the rod shaped region give an average diameter of 150 nm. In some sections large vacuoles were seen with the same amorphous coat and square lattice structures lining the lumen (Fig. 4.2.C). The lumen of both the vacuolated portion of the granule and the large vacuoles contained loosely arranged membranous material (Figs. 4.2.C and D). The lumen of the rod-shaped portion of the granule contained a square lattice structure. In certain sections this lattice structure filled the entire lumen, but sometimes incontinuities were observed (Fig. 4.2.C). When cut in a clean cross section the rod-shaped portion of the granule showed a structuring of particles arranged in concentric rings within the lumen (Fig. 4.2.D). The position of the granules in the cytoplasm was in the cell body frequently associated with microtubules and centrioles (Fig. 4.2.D).

4.4.2.2 Rainbow trout DL cells

Birbeck-like granules were localized near centrioles (Fig. 4.3.A). The rod portion of the BL granule had a diameter ranging from 69 nm to 200 nm and contained amorphous material within the lumen (Fig. 4.3.B). In one instance a mitotic DL cell containing vacuolated BL granules was seen in the spleen of rainbow trout (Fig. 4.3.C). These cells were distributed throughout the connective tissue of the spleen and frequently localized near blood vessels (Fig. 4.3.D).

4.4.2.3 Brook trout DL cells

In brook trout the DL cells contained a type of BL granule with the rod portion ranging in diameter from 50 nm to 150 nm and the lumen filled with material in a square lattice arrangement (Figs. 4.4.A and B). The rod portion in the granules with narrow diameters, measuring about 50 nm, contained electron dense amorphous material instead of square lattice structured material (Fig. 4.4.C).

4.4.2.4 Atlantic salmon DL cells

Birbeck-like granules in Atlantic salmon were frequently vacuolated and had a long rod portion with a diameter from 40 nm to 70 nm, which contained 4 layers of particles within the lumen when sectioned longitudinally; two layers of dense particulate material adjacent to the granule membrane on the luminal side and a central lamella made up of two layers of particulate material which were spaced slightly apart giving a striated appearance (Fig. 4.5.A) along the center of the granule. In some of the granules, square-lattice structured particulate material was seen instead of the four layers of particulate material within the rod portion (Fig. 4.5.B). Granules in cross section, which appear circular, were observed in Atlantic salmon DL cells. These granules had a diameter of about 100 nm and contained an electron dense layer of material within the granule forming a concentric ring (Fig. 4.5.C).

Figure 4.2. Dendritic-like cells from naturally-infected gill tissue of *Oncorhynchus tshawytscha* **A.** A DL cell with characteristic granules within the cytoplasm. (M) mitochondria, (N) nucleus, (arrows) cell membrane. (Bar= 1 μm) **B.** A BL granule within the cytoplasm of a DL cell. Membrane bound rod (arrow) with a vacuolated region containing amorphous and lattice structured material (arrowheads) lining the luminal side of the membrane. (Bar= 150 nm) **C.** A BL granule showing the square lattice arrangement of particles within the lumen of the rod, which appears interrupted in a portion (arrow). Also a large vacuole is seen with amorphous and lattice arranged material lining the membrane (arrowheads) that extends into the vacuole lumen (V). (Bar= 160 nm) **D.** BL granules concentrated around a centriole (large arrow) with microtubules (arrowheads). Granules are cut in longitudinal section showing rods and in cross section showing spherical structures containing particles arranged in concentric lines to the limiting membrane of the granule (small arrow). (Bar= 170 nm)


M
N
A
B
V
C
D

Figure 4.3. Dendritic-like cells from the spleen of uninfected/healthy *Oncorhynchus mykiss*. **A.** DL cell with an unsegmented nucleus (N) and cytoplasm with BL granules (arrowheads) surrounding a centriole (arrow). (Bar= 1 μm) **B.** A racket shaped granule near a centriole (arrow), the granule is vacuolated on one end and the rod portion (arrowhead) contains amorphous electron dense material. (Bar= 300 nm) **C.** A mitotic DL cell (arrows) containing vacuolated BL granules (arrowheads) and a mitotic nucleus (MN). (Bar= 1 μm) **D.** Spleen tissue showing two DL cells (arrowheads) near a blood vessel, endothelial cells (arrows). (Bar= 4 μm)

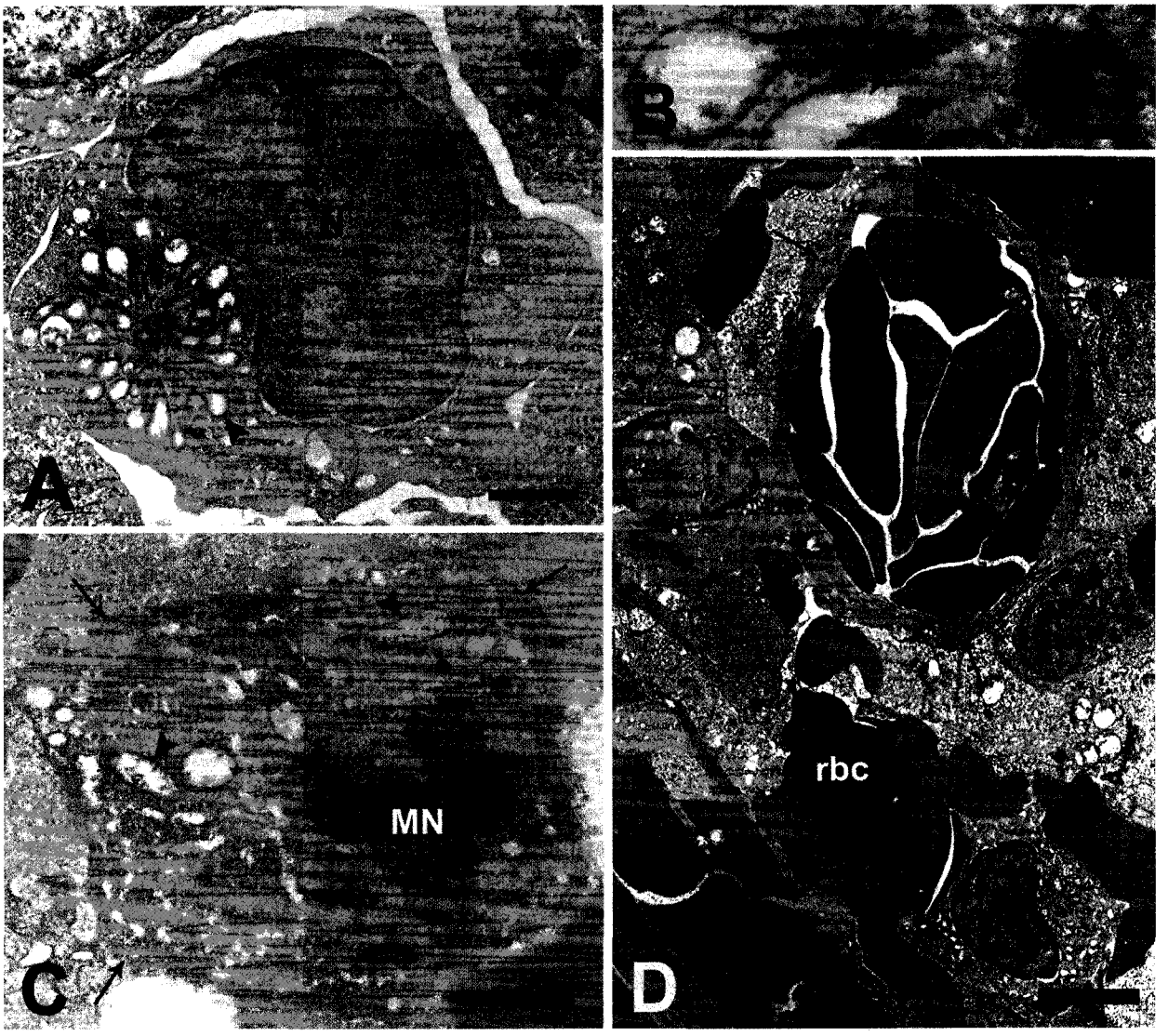

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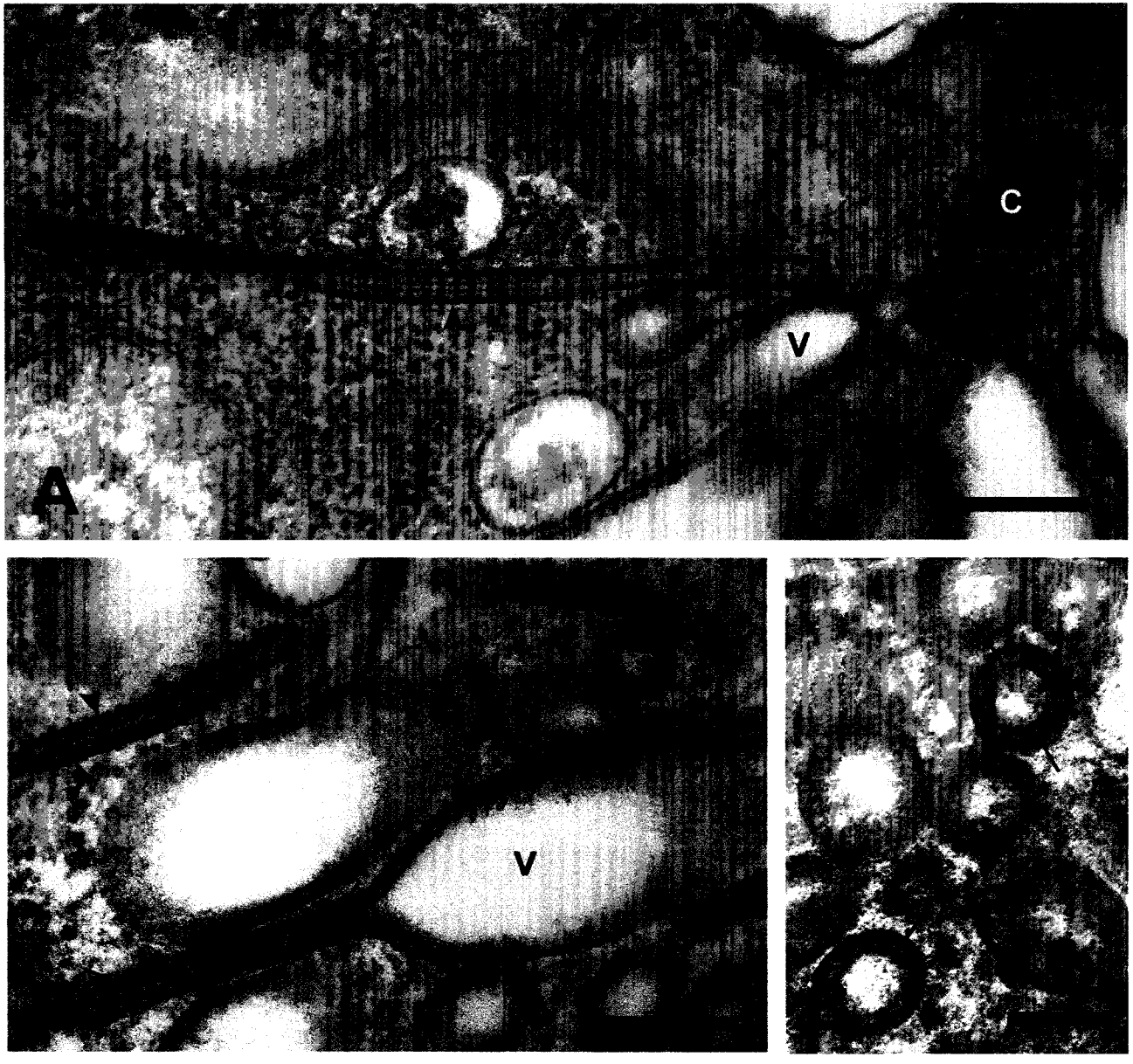
Figure 4.4. Birbeck-like granules in cells of *Salvelinus fontinalis*. **A.** Granules centered around a centriole (arrow), the rod portion of the granules (arrowhead) contains electron dense material of various densities. (Bar= 500 nm) **B.** A single BL granule showing a square-lattice arrangement of particles within the lumen of the rod portion (arrowheads). (Bar= 150 nm) **C.** Sections of granules containing a square-lattice arrangement of particles (arrows) and a narrow rod portion of the granule containing amorphous material (arrowheads). (Bar= 300 nm)


B
C

4.5 DISCUSSION

Ultrastructurally the granules of the cells observed in this study resemble the Birbeck granules found in Langerhans cells of many other vertebrate species. These BL granules are rod-shaped and commonly vacuolated at one end giving the appearance of a tennis racket. Similarly to the description given by Wolff (1967), the vacuolated area of the granule contains amorphous material lining the luminal side of the membrane appearing as a "fuzzy coat". However, square lattice structures are also found in addition to the 'fuzzy coat'. In this study the BL granules in all species except for Atlantic salmon lacked the electron dense striated central line (central lamella) that is common in Birbeck granules (Wolff 1967), and instead, the rod was filled with particles that form a square lattice arrangement. Interestingly, in Wolff's description of the cross section of a granule, particles are shown in a square lattice arrangement identical to those seen in longitudinal section in this study. The central lamella observed in the granules of DL cells in Atlantic salmon very closely resembles the central lamella in human Birbeck granules. The diameter of the rod structure in Chinook salmon is approximately 150 nm, but narrow rod structures were observed in rainbow trout (69-200 nm), brook trout (50-150 nm), and Atlantic salmon (40-70 nm). Birbeck granules in human Langerhans cells are approximately 45 nm wide (Grassi et al. 1998), 43 nm in mice, and 13-22 nm in chickens (Perez-Torres and Ustarroz-Cano 2001). The low end of the range in diameters of the BL granules in fish are similar to those seen in human Langerhans cells, but there appeared to be a large range in the thickness of the BL granules in fish. Some

Figure 4.5. Birbeck-like granules in cells of uninfected *Salmo salar*. **A.** A longitudinal section of a granule, which has four layers of particulate material; two layers are enveloped by the granule membrane (arrowheads) and in the center is the central lamella composed of two layers of particulate material with a striated appearance (arrows), vacuolated portion of a granule (V). (Bar= 200 nm) **B.** A racket shaped granule with one side vacuolated (v) and the rod portion containing a square-lattice arrangement of particles. Another granule is seen (arrowheads) with a central lamella. (Bar= 200 nm) **C.** Circular granules containing material arranged in a concentric ring (arrows). (Bar= 100 nm)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Birbeck granules in the chicken epidermis were seen to have an increased thickness, absence of the striated central line, and a square lattice arrangement (Perez-Torres and Ustarroz-Cano 2001). The square lattice arrangement has been interpreted as a result of an unzipping of the striated central line and this may explain the morphology of some of the granules examined in this study since all of the wider diameter rod sections lacked the central lamella. It has been theorized that the unzipping of the Birbeck granule occurs simultaneously with the fusion of an endosomal vesicle, which would form the head of the tennis racket shaped structure (Bartosik 1992). The unzipping may reflect the first step in the degradation of the Birbeck granule (Bartosik 1992). The large vacuoles and vacuolated regions of granules with the "fuzzy coat" lining the membrane seen in this investigation were also described by Wolff (1967) when defining the fine structure of the Langerhans cells. A major ultrastructural difference in the BL granules of rainbow trout when compared to the cells of brook trout, Atlantic salmon, and Chinook salmon, was the material within the rod portion of the granule. In rainbow trout the material was always amorphous, while in all the other fish species examined the material often made up a square-lattice arrangement of particles. Monocyte derived DC contain a granule that is morphologically distinct from the Birbeck granule. These granules are described as ring-like structures measuring approximately 60 nm that comprise two concentric membranes separated by a lattice with a variably striated line (Grassi et al. 1998). These closely resemble the cross sections of the rod-shaped portion of granules seen in Chinook salmon (Fig 4.2) and Atlantic salmon (Fig 4.5).

The location of the BL granules within the cells of this study was within the cell body frequently associated with microtubules and centrioles. Birbeck granules in Langerhans cells of human lymph nodes from patients with "atypical hyperplasia" were also commonly seen near centrioles and microtubules (Shamoto et al. 1976). Two theories have emerged in the origins of the Birbeck granule; one theory describes the granule forming from invagination of the cell membrane through an endocytic process (Bartosik 1992) and another theory proposes that Birbeck granules form in the pericentriolar area and are subdomains of the endosomal recycling compartment (Mc Dermott et al. 2002). In this study the granules were never observed to be continuous with the cell membrane. The Birbeck granules could serve as a loading compartment for antigens before DC maturation (Mc Dermott et al. 2002). In order for the antigen filled granules to be transported throughout the cell during antigen processing functional microtubules are necessary. Microtubules allow for the movement of vesicles within cells and may be necessary for antigen uptake to the Golgi complex and movement to the cell membrane (Peachmann et al. 2004). The theory of Birbeck granules forming in the pericentriolar zone and acting as an antigen loading compartment that would need to be transported by microtubules to the cell membrane would explain the relationship of the granules to the centrioles and microtubules in this study.

Another interesting observation from this investigation is the location of these cells within the connective tissue of the gill filament within a marked inflammatory infiltrate. The experimental model of MGDS in rainbow trout showed that this

disease model causes an infiltration of DL cells into the gills during the rupture of parasitic xenomas. Dendritic-like cells were also present, although rarely, in the gills of Atlantic salmon affected by amoebic gill disease (Lovy et al. 2007). Although a DL cell was observed in the gills of uninfected controls, the numbers of cells in the infected fish were much higher. Considering the gills of fish are in constant contact with the environment and the unavailability of truly pathogen free fish, it seems logical to find small numbers of this cell type in the gills of uninfected fish possibly acting as sentinels for pathogens entering through the gills. The presence of DL cells in a blood vessel of the gills suggests that this cell may travel via the blood and be recruited to areas of insult, similar to Langerhans cells during epidermal insult (Merad et al. 2002), or the cells may be exiting the tissue into the blood on their way to perform antigen presenting functions. *In vitro* human peripheral blood monocytes can mature into dendritic cells in the presence of granulocyte macrophage colony stimulating factor, interleukin-4, and other cytokines (Grassi et al. 1998, Makala and Nagasawa 2002). The origin of these DL cells in fish gills is speculative, but it seems more logical based on their location that these cells would have matured from blood monocytes. This is supported by the presence of a DL cell in a blood vessel of the gill in rainbow trout and the fact that control fish did not commonly have these cells in the gills. The inflammatory lesion caused by the rupturing xenoma may contain cytokines that would stimulate blood monocytes to develop into DCs.

The finding of DL cells in the spleen and anterior kidney of various healthy salmonid species in this study shows that the DL cells have a broad distribution in a

variety of tissues other than the gill. In healthy fish, the spleen was the organ that contained the most DL cells in all salmonid species examined in this study, although they were also present in the anterior kidney. The spleen and kidney of fish overlap in function in that they are the two organs responsible for filtering the blood and they both contain MMCs, which accumulate antigens and are thought to be the areas where immune responses are generated to antigens (Agius and Roberts 2003). In salmonids MMCs are not distinct compartments as in the more advanced bony fishes; instead melanomacrophages are randomly distributed throughout the tissue (Agius and Roberts 2003). Considering fish lack a well developed lymphatic system with lymph nodes as seen in mammals (Noga 2006), the roles of lymph nodes in antigen retention and antigen processing would likely be taken up by the spleen and kidney of fish. MMCs are hypothesized to be evolutionary precursors of germinal centers that are seen in many birds and mammals (Vigliano et al. 2006) and if true then they would have to contain DCs. In this study DL cells were found randomly distributed throughout the spleen and kidney and sometimes found in close association with blood vessels. In one case a mitotic DL cell was observed in the spleen of rainbow trout, suggesting the possibility of this cell type to proliferate *in situ*. It has been shown that blood-derived DCs develop within the spleen itself from pre-DC precursors that are seeded within the spleen directly from the bone marrow (Villadangos and Heath 2005). It may be possible in fish that even when these cells are fully differentiated then they are still capable of proliferation. It has been shown that Langerhans cells in the normal epidermis undergo mitosis *in situ* to maintain

their populations and during all phases of mitosis Birbeck granules remain within the cytoplasm (Miyauchi and Hashimoto 1989; Oota 1999; Vaigot et al. 2005).

The ultrastructure of the DL cells described in this study strongly resemble Langerhans cells of mammals with Birbeck granules. At least seven distinct populations of DCs have been described in mouse spleen and lymph nodes (Villadangos and Heath 2005), but Birbeck granules are unique to Langerhans cells, which are a DC subset found in the epidermis. Human epidermal Langerhans cells may originate from various bone marrow derived cell precursors including monocytes and upon activation they migrate to regional lymph nodes to present antigen to T cells (Koch et al. 2005). Interestingly the cells described in this study ultrastructurally fit the criteria for Langerhans cells, but they are found in various locations including the blood, epidermis and connective tissue of gills, and randomly dispersed throughout the spleen and anterior kidney. It is possible that these cells originated in the epidermis, migrate through the blood, since fish don't have a lymph system, and end up in the kidney or spleen where they would present the antigen to T cells, which would occur in a regional lymph node in mammals. This theory is unlikely though, because all the cells contain BL granules and thus suggest that they are immature DCs. It has been shown that freshly isolated Langerhans cells have Birbeck granules, but after stimulation of cells in culture they lose the Birbeck granules and resemble lymphoid DCs (Schuler and Steinman 1985; Romani et al. 1989). Langerhans cells would likely become activated when they enter lymph nodes and loose the Birbeck granules, therefore the DL cells in the spleen and anterior

kidney with BL granules seen in this study likely did not originate from the epidermis and likely represent a resident population of cells in these organs.

The cell type described in this study strongly resembles Langerhans cells in mammals and is commonly found in lymphoid tissues of salmonids and inflammatory lesions in gills of rainbow trout with MGD. One other study identified a granulocyte with BL granules in the thymus of mandarin fish (Xie et al. 2006), but these granules lack some of the fine structures common to Birbeck granules. Further work needs to be done to isolate this cell type and further characterize it. This investigation presents evidence suggesting that DL cells are a resident cell type abundant in the spleen and also in the anterior kidney of salmonids and these organs can be targeted for studies in the isolation and further characterization of these cells.

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5 EFFECTS OF DEXAMETHASONE ON THE HOST INNATE AND ADAPTIVE IMMUNE RESPONSES AND PARASITE DEVELOPMENT IN RAINBOW TROUT INFECTED WITH *LOMA SALMONAE*[6]

5.1 ABSTRACT

The effects of dexamethasone (dex) treatment on infections with the microsporidian parasite, *Loma salmonae* and the effects of dex on initiation of the adaptive immune response are investigated in rainbow trout, *Oncorhynchus mykiss* experimentally infected with the parasite. Dex treatment resulted in significantly higher infections with the parasite in the gills ($P<0.001$) and other internal organs, suggesting that dex inhibits aspects of the innate immune response to *L. salmonae*; the heavier infections in the gills and organs of rainbow trout resembled infections seen in Chinook salmon. Mean (± SE) xenoma counts per microscope field in the gills of fish infected with *L. salmonae* treated with dex or left untreated were 169 (± 29) and 30 (± 5.9), respectively. Although higher numbers of xenomas were observed in dex treated fish, the xenomas were generally smaller in size than in infected control fish. The xenomas in dex treated fish showed morphological signs of degeneration including loss and degeneration of early parasite stages, accumulation of amorphous material in xenomas, and infiltration with phagocytic cells containing degenerated parasites. The xenomas in infected control fish had larger xenomas with a more uniform size and contained identifiable parasite stages in

[6] Lovy J, Speare DJ, Stryhn H, Wright GM. Effects of dexamethasone on innate and adaptive immune responses and parasite development in rainbow trout, *Oncorhynchus mykiss* with *Loma salmonae*. Fish Shellfish Immunol; submitted

the cytoplasm. However, once fish have developed an adaptive immune response to the parasite following previous exposure, then fish have 100% protection to reinfection even when treated with heavy doses of dex. Fish immune to *L. salmonae*, which were either treated or untreated with dex during reinfection with the parasite, did not develop xenomas in the gills 6 weeks post reinfection. These results indicate that once the innate cellular immune response to *L. salmonae* is primed, then dex related immunosuppression does not reduce the effectiveness of the adaptive immune response.

5.2 INTRODUCTION

Microsporidial gill disease of salmonids (MGDS) is a serious economic problem within netpen reared Chinook salmon, *Oncorhynchus tshawytscha*, of the Pacific Northwest. The disease is caused by the microsporidian *Loma salmonae*, which is endemic to that region. The parasite is found intracellularly predominantly within the gills of fish, where cysts termed xenomas, which contain multiple life stages of the parasite, are formed (Kent and Speare 2005). While there is little host response to the development of the xenomas, there is dramatic granulomatous branchitis during their rupture and release of mature spores (Hauck 1984, Kent et al. 1989, Chapter 2). Clinical disease is most frequently observed in fish in the late summer of the second year in the netpens when fish are reaching market size. Cumulative mortalities in one year can reach over 10% (Hauck 1984, Chapter 2).

This is a devastating loss to the salmon farm because at this stage of production these fish have the most time and money invested.

Rainbow trout, *Oncorhynchus mykiss*, are susceptible to infections with *Loma salmonae* and are used as a laboratory model of MGDS (Speare et al. 1998a). Many aspects of the sequential development of the parasite have been determined using the MGDS model in rainbow trout. During infection the parasite is first detected in the lamina propria of the intestine and within a few days early merogonic stages are found in the endocardium (Sanchez et al. 2001a). Host cells containing parasites in early developmental stages, termed meronts, appear within the endothelium of the gills at about 2 weeks post exposure (Rodriguez-Tovar et al. 2002). The infected host cells become severely hypertrophied during parasite multiplication and thus xenomas are formed. The xenomas continue to increase in size and parasite numbers until their rupture at about 6-8 weeks post exposure (Rodriguez-Tovar et al. 2003) which causes a granulomatous branchitis. The parasite is cleared from the gills at around 12-14 weeks post exposure. After clearance of the parasite, strong resistance to reinfection with the parasite has been shown for both rainbow trout and Chinook salmon (Speare et al. 1998b, Kent et al. 1999). Using a vaccine preparation it has been shown that onset of resistance occurs at about 3 weeks post exposure and by 6 weeks post exposure there is complete resistance (Rodriguez-Tovar et al. 2006). Similar to many other intracellular pathogens, this resistance is attributed to cellular immunity rather than humoral immunity (Sanchez et al. 2001b, Rodriguez-Tovar et al. 2006). Although resistance has been proven in the laboratory, it remains unclear

why Chinook salmon in their second year in the netpens are primarily affected by disease when they likely encounter the pathogen in the first year in seawater and should have generated a protective immune response going into their second year. Other evidence that Chinook salmon within their second year in the netpens do not generate an efficient immune response is the presence, in individual fish, the parasite in both very early stages of development and late stages where spores are released from xenomas (Chapter 2).

There are a number of farm factors and individual fish factors that could contribute to inefficient generation of an adaptive immune response in netpen reared fish to *Loma salmonae* compared with fish maintained in the laboratory. The differences may be linked to two major factors; (1) the extrinsic factors that affect fish on a farm versus fish that are maintained in the laboratory and (2) the methods in which fish are infected, natural exposure in netpens versus a single dose exposure in the laboratory model. Stressful events encountered in a farm setting may affect the interaction of the pathogen and its host through host immunosuppression. Stress in fish is difficult to avoid in an aquaculture setting and it has been known to contribute to diseases. The stress response consists of an initial alarm reaction that activates the pituitary-interrenal axis causing a release of corticosteroid hormones and catecholamine, which initiate changes in the vascular system. If the stressor is not severe then the individual's physiological systems compensate and acclimate, reaching a stage of resistance, but if the stressor continues or is severe then a stage of exhaustion occurs and immune protection becomes impaired (Wedemeyer 1996). It

has been shown that chronic elevation of plasma cortisol levels in fish increases susceptibility to a wide range of pathogens (Pickering and Duston 1983, Pickering and Pottinger 1985, Woo et al. 1987). The synthetic steroid, dexamethasone (dex), has similar effects as cortisol and has been shown to block cortisol response by acting upon the pituitary-interrenal axis in brown trout after oral administration at a concentration of 3 μg/g of fish (Pickering et al. 1987). Dex treatment in rainbow trout and Atlantic salmon has been shown to increase susceptibility to infections with the ectoparasite *Gyrodactylus derjavini* (Lindenstrom and Buchmann 1998, Nielsen and Buchmann 2003). Immunosuppression in humans is also known to be a major factor for infections with microsporidia, with *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi* being the major species infecting humans (Didier 2005). Dex treatment has been demonstrated to result in heavy microsporidial infections in mice experimentally inoculated with *E. cuniculi* (Lallo et al. 2002) and although *E. bieneusi* has been shown to occur in healthy people (Nkinin et al. 2007), it is commonly associated with individuals with human immunodeficiency virus (HIV) (Didier 2005). The purpose of this study is to examine the effects of dex on the interactions of the host, rainbow trout, with the pathogen, *Loma salmonae* during innate immunity and during activation of the adaptive immune response.

5.3 MATERIALS AND METHODS

5.3.1 Source of *Loma salmonae*

Infective gill material was obtained from clinically affected Chinook salmon during a natural outbreak of *Loma salmonae* in British Columbia. Gills were minced and fed to *Loma*-naive rainbow trout to initiate an infection. The rainbow trout were maintained in 70 L plastic tanks set up as flow-through systems fed with well water. Infections were maintained in the laboratory by sequential passage of infective gill material through *Loma*-naive rainbow trout every seven weeks.

5.3.2 Source of Rainbow trout

Loma-naive rainbow trout were acquired from a local trout hatchery (Ocean Trout Farms Ltd; Brookvale, PEI, Canada). Fish were maintained at 10°C and one week prior to experimental trials were transferred to 70 L flow-through tanks maintained at 15°C. The water supply was from a well and water was warmed with a heat exchanger and collected into a header tank where warm and cool water was mixed to adjust the temperature. Each tank contained an air stone for supplemental aeration.

5.3.3 Dex coated feed

Three mm extruded pellets (Corey Aquafeeds; Fredericton, New Brunswick, Canada) were coated with pure dex (ICN Biomedicals Inc; Aurora, Ohio, USA), which was mixed into gelatin (Oxoid; Basingstoke, Hampshire, England). For every kilogram of feed, 4 g of gelatin was dissolved into 70 ml of boiled distilled water and allowed to cool to about 25°C. Dex was slowly added to the cooled gelatin mixture

at a concentration of 300 mg/kg of feed with constant mixing until the dex appeared evenly dispersed throughout the mixture. The mixture of gelatin with dex was slowly poured onto the feed with constant mixing using a rubber policeman to evenly coat the feed. Feed was immediately spread over a tray to dry at room temperature for one hour. The feed was stored at 4°C until use. Dex was used in the feed for oral delivery rather than in a form for administration via injection because feeding was a convenient and noninvasive method to chronically administer the drug over a five week period. It was considered likely that with the immunosuppressed condition of fish that repeated injections may cause secondary infections to occur at the injection points.

5.3.4 Experiment 1. Effects of dex on the innate immune response

120 rainbow trout (20-40 g each) were distributed into two 70 L tanks containing 60 fish per tank, and allowed to acclimate for 1 week. One tank of fish was fed 32 grams of *Loma* infective gill material (infection tank) and left undisturbed for 6 hours. The fish were then redistributed into four groups each including 30 fish in four separate 70 L tanks. Two groups included a random allocation of the fish from the infection tank and the other two groups included *Loma*-naive fish. The following day a daily dex treatment regime began for one group that was infected with *Loma* and one group of *Loma*-naive fish. The treatment regime included feeding fish at 1% body weight with dex coated feed at a concentration of 300 mg/kg of feed per day for five weeks. This concentration was chosen because it was previously demonstrated that dex fed in a single meal at 1% body weight at a

concentration of 300 mg/kg of feed caused immunosuppression and it blocked the cortisol response for up to three days in brown trout (Pickering et al. 1987). The amount of feed was adjusted weekly assuming a 1:1 growth in body weight to feed ratio. The other two groups of fish were given the same amount of feed, which was neither covered in gelatin nor coated with dex. Mortalities were monitored throughout the trial and at 6 weeks post infection with *Loma* the fish were sampled. A schematic of the experimental design can be seen in Fig. 5.1.A.

5.3.5 HRLM and TEM

Gills of fish from experiment 1 were examined with high resolution light microscopy (HRLM) and transmission electron microscopy (TEM). Fish were euthanized with an overdose of benzocaine and a single left holobranch was removed from 15 trout which had been infected with *L. salmonae* and treated with dexamethasone, 9 trout which had been infected with *L. salmonae* without treatment, and 6 trout that were *L. salmonae* naive and treated with dexamethasone. The holobranch was submersed into cooled 2% glutaraldehyde in phosphate buffer and maintained at 4°C for two hours. The tissue was removed and cut into 4 pieces and transferred into fresh 2% glutaraldehyde in phosphate buffer in 5 ml vials and stored at 4°C overnight. After washing with phosphate buffer, the tissue was post-fixed in 1% osmium tetroxide in phosphate buffer for 1.5 hrs. at room temperature. The tissue was dehydrated through a graded series of ethanols, cleared in propylene oxide (PO), and embedded in Spurr's resin. About 3 tissue pieces from each fish were individually transferred to BEEM capsules with 100% Spurr's resin and allowed to

polymerize at 60°C overnight. From each block, 0.5 μm thick sections were cut and stained with toluidine blue for HRLM. Measurement of xenomas were made by capturing a light microscopy image with an Optronics analog camera and measuring the area with the Bioquant Nova Prime (V6.90.10) image analysis software. Other HRLM images were captured using a Zeiss Axiocam Ultra High Resolution color digital camera. After examining sections with light microscopy, areas of interest were re-trimmed on the embedded tissue blocks and ultrathin (90 nm) sections were cut and stained with uranyl acetate and lead citrate. Ultrathin sections were mounted on copper super grids and examined and photographed with a Hitachi 7500 transmission electron microscope operated at 80 kV.

5.3.6 Experiment 2. Effects of dex on the generated adaptive immune response

A total of 233 rainbow trout (weight 20-40 g) were used for this experiment. 130 naive rainbow trout were evenly distributed into two 70 L flow-through tanks and maintained at approximately 15°C. Fish were infected with *Loma salmonae* by feeding about 40 g of minced infective gill material to each of the two tanks. The fish were maintained for 6 weeks and a random sample of 15 fish was taken from each tank. The 30 fish were euthanized with an overdose of benzocaine. To establish that the fish had been infected with the parasite, a gill arch was examined by preparing a wet mount on a microscope slide and xenoma counts were made. The remaining gill material from the 30 fish was dissected and fed to a group of 55 naive rainbow trout in a third 70 L tank; the gills of these fish were used as the next infective innoculum. The remaining 100 fish were maintained until 12 weeks post

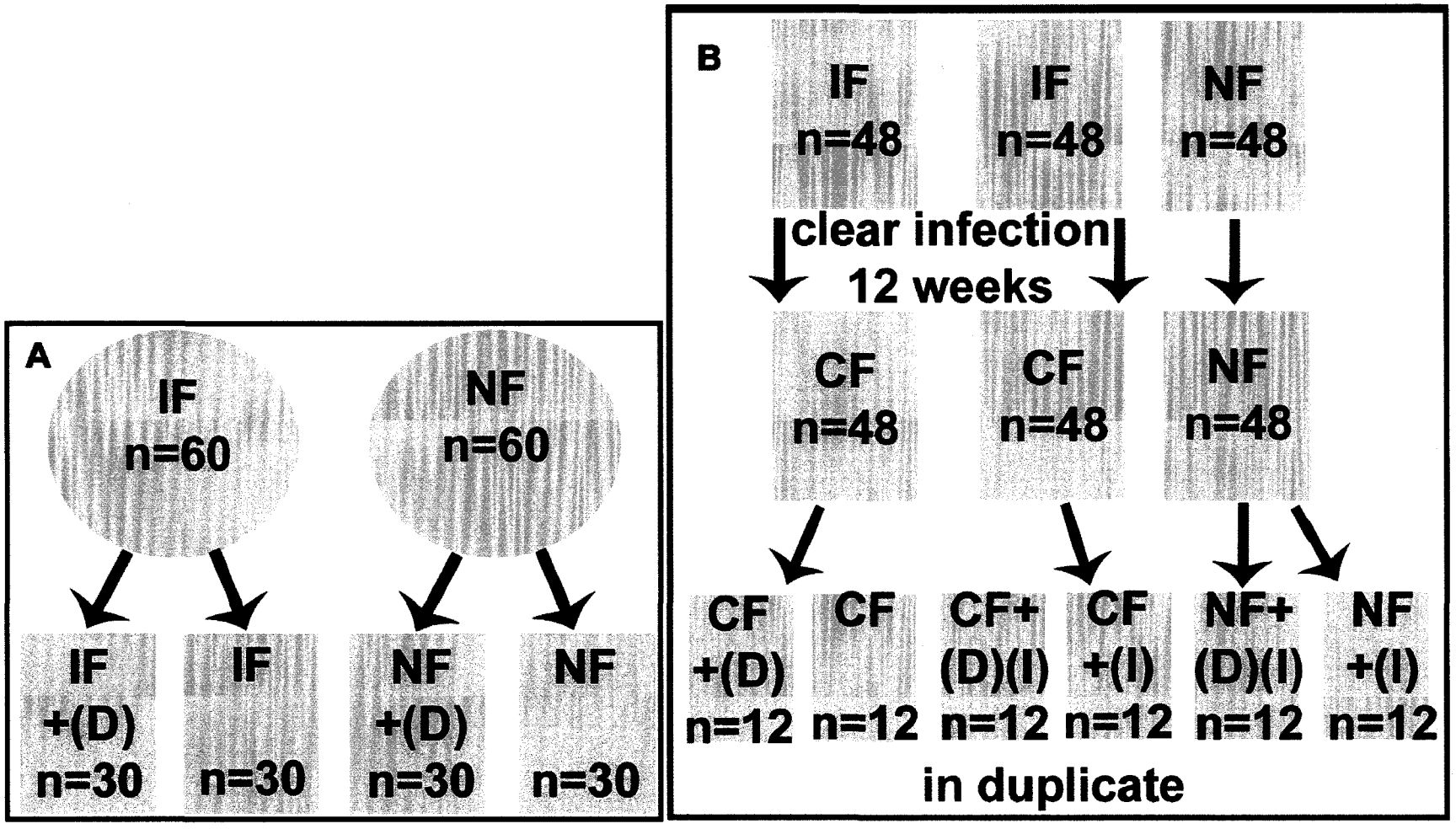
infection, weighed and 96 fish were redistributed into eight separate 70 L tanks. In addition, 48 *Loma*-naive rainbow trout were weighed and distributed into four 70 L tanks. In summary, a total of 12 tanks were utilized each containing 12 fish; eight groups were previously infected with *Loma salmonae* and four groups were *Loma*-naive. The group of 55 rainbow trout, which were infected with *Loma* 6 weeks earlier, were euthanized with an overdose of benzocaine and all the gill material was removed and minced. The minced infective gill material was mixed using a plastic policeman and divided into eight equal portions. This material was fed two days after the first dex treatment to four groups of fish which had been previously infected with *Loma* and four *Loma*-naive groups. Two of the four groups of fish which had been rechallenged with *Loma*, two of the four groups of fish that were infected once with *Loma* 12 weeks earlier, and two of the four groups which were *Loma*-naive infected with *Loma*, all received dex treatment. The concentration of dex given to fish was reduced from the first experiment because daily treatment with feed at 300 mg/kg of dex caused mortalities in the fish. Fish were fed the same dex coated feed at 300 mg of dex/Kg of feed twice per week at a 2% body weight for three weeks. The study design consisted of a total of 12 tanks with 6 treatment groups run in duplicate. The treatment groups included; *Loma*-cleared/non-treated and *Loma* cleared/dex-treated groups which were infected with *Loma* once, allowed to clear the infection, then fed with untreated or dex-treated feed; *Loma*-rechallenged/non-treated and *Loma*-rechallenged/dex-treated which were infected with *Loma* once, allowed to clear the infection, and infected with *Loma* a second time while fed either untreated

or dex-treated feed; *Loma*-infected/non-treated and *Loma*-infected/dex treated which were infected with *Loma* once and concurrently fed untreated or dex-treated feed. Six weeks post infection all fish were sampled. A schematic of the experimental design can be seen in Fig. 5.1.B.

5.3.7 Sampling procedure

Mortalities were recorded throughout the experiments. At the end of the trials all fish were euthanized with an overdose of benzocaine and for each fish a wet mount of the gills was examined under a light microscope. Wet mounts were prepared by removing a holobranch, cutting away the gill arch, adding a few drops of water to the tissue on a glass slide, and placing a cover slip over the tissue. The tissue was examined under a phase contrast light microscope with the 4X objective and xenomas were counted on the visible hemibranch. Due to the heavy infections encountered in experiment 1, xenomas were counted within a single microscope field, while in experiment 2 the counts were done over the entire gill hemibranch in order to detect light infections. Organs including liver, heart, spleen, head kidney, and trunk kidney were removed and fixed in formalin for routine light microscopy. Five fish from each of the groups were processed for histology. Formalin fixed tissue was dehydrated through a series of ethanols and embedded in paraffin. 5 μm sections were cut from each paraffin block of tissue, stained with hematoxylin and eosin, and examined with a light microscope for the presence of diseases, and *Loma salmonae* xenomas were counted from all the tissue sections.

Figure 5.1. **A.** Schematic representation of experiment 1. One group of fish (n=60) is infected with *Loma salmonae* (IF) and fish are randomly distributed into two groups (n=30); naïve fish (NF) are distributed into two groups (n=30). One group of naïve fish and one group of infected fish are treated with dex (D). **B.** Schematic representation of experiment 2. Two groups of fish (n=96) are infected with *Loma salmonae* (IF) and given 12 weeks to clear the infections. The *Loma* cleared fish (CF) are distributed into 4 groups (n=12), which include a group which is treated with dex (D), a group left untreated, a group treated with dex and simultaneously rechallenged with *Loma* (D, I), and a group which is rechallenged with *Loma* without any treatment (I). Two groups of naïve fish (n=12) are infected with Loma (I) and one group is treated with dex (D). Groups are run in duplicate totalling 12 tanks.


A
IF
n=60
NF
n=60
IF
+(D)
n=30
IF
n=30
NF
+(D)
n=30
NF
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B
IF
n=48
IF
n=48
NF
n=48
clear infection
12 weeks
CF
n=48
CF
n=48
NF
n=48
CF
+(D)
n=12
CF
n=12
CF+
(D)(I)
n=12
CF
+(I)
n=12
NF+
(D)(I)
n=12
NF
+(I)
n=12
in duplicate

5.3.8 Statistics

In experiment 1, a Mann-Whitney test was run to compare *Loma* infected fish with and without dex treatment, due to a non-normal distribution of xenoma counts in the untreated group. In experiment 2, an ANOVA with tank random effects was used to compare xenoma counts in *Loma* infected fish with and without dex treatment. The data was cubic root transformed prior to analysis to meet the model's assumptions of normality. The analysis was carried out using the SAS version 9.1 software. The significance level was set at 0.05.

5.4 RESULTS

5.4.1 Experiment 1. Effects of dex on the innate immune response

Daily feeding of dex in the feed at concentrations of 300 mg/kg at 1% body weight resulted in mortalities beginning at 20 days post treatment and continued throughout the duration of the trial; a total of 12 mortalities were observed in the group fed infective gill material with dex treatment and 9 mortalities were observed in the *Loma*-naive group with dex treatment (Table 5.1). Necropsies on fish mortalities in both groups revealed consistently that gills contained lamellar fusion, while no gross lesions were observed in other organs. At the termination of the trial wet mounts of the gills of the remaining fish revealed that *Loma salmonae* infections were observed in the two groups of fish that were fed infective gill material, while absent in the *Loma*-naive groups. Xenoma counts on wet mounts done in 30 fish in the *Loma*-infected/non-treated group and 18 fish in the *Loma*-infected/dex treated

group had respective means (± SE) of 30 (± 5.9) and 169 (± 29) xenomas per hemibranch (Table 5.1). The difference between the groups was statistically different ($P<0.001$).

Histopathology revealed no lesions or pathogens in the *Loma*-naive/non-treated group. The *Loma*-naive/dex treated group had one fish with lesions in the kidney and a Gram stain revealed intracellular Gram-positive bacteria. Two fish within this group had lamellar hyperplasia and inflammation in the gills, but *Ichthyobodo* organisms were not observed, as in other groups which will be discussed in the following sections. In the *Loma*-infected/non-treated group all gills examined contained xenomas. No xenomas were observed in the head kidney, trunk kidney, liver, or spleen, but three fish examined contained xenomas in the ventricle of the heart. The *Loma*-infected/dex-treated group had large numbers of xenomas in the gills and xenomas were frequently observed in the other organs (Table 5.2). Of 43 xenomas found in the heart, 17 were in bulbus arteriosus, 16 in ventricle, and 10 in atrium.

5.4.2 HRLM and TEM of gills

The fish infected with *Loma* and left untreated contained 129 total xenomas in 30 HRLM sections and the xenoma area ranged from 225 μm^2 to 3,910 μm^2. The majority of these xenomas were over 1,000 μm^2 (the size variations of xenomas in treated fish vs. untreated fish can be seen in Fig. 5.2) and contained the typical developmental parasite stages (Fig. 5.3.A). There was no inflammatory response

Group	Mortality	Mean xenoma count (±SE)
LI+dex	12/30	169±29
LI	0/30	30±5.9
NF+dex	9/30	0
NF	0/30	0

Table 5.1. Experiment 1. Mortality and mean xenoma counts with standard error (SE) per wet mount microscope field of *Loma* infected fish (LI) and naïve fish (NF) with dex treatment and without treatment.

Group	Heart	Kidney	Spleen	Liver
LI+dex	43	19	8	4
LI	3	0	0	0

Table 5.2. Experiment 1. Total xenoma counts in organs other than gill of *Loma* infected fish (LI) treated with dex daily at 300 mg/kg or left untreated. The counts represent total xenomas counted from 5 μm sections from five fish for each group.

around intact xenomas although a few xenomas had ruptured and caused focal areas of inflammation, consisting of neutrophils and macrophages; only focal areas of xenoma rupture had lamellar fusion. No xenomas appeared to be undergoing degeneration and no other pathogens were observed.

The group of fish receiving the dex treatment and infected with *L. salmonae* had severe pathology of the gills, which consisted of a large number of intact and ruptured xenomas causing an inflammatory response at 6 weeks post infection. The total number of *Loma* xenomas counted in 30 HRLM sections was 763. The xenomas ranged in sizes from 40 μm^2 to 2,350 μm^2 in single section plane and the majority of xenomas were small (0-1,000 μm^2) (refer to Fig. 5.2.). Many xenomas appeared dark due to hypercondensation of cytoplasm and some spores had extruded their contents (Fig. 5.3.B). Transmission electron microscopy revealed that the early parasite stages (meronts and sporonts) were degenerating within these xenomas and the amorphous material was often present in droplets (Fig 5.4.C). Additionally, 26 HRLM sections contained a heavy accumulation of lipid within the epithelial cells of the lamellae (Fig. 5.4.D). All sections observed in the dex treated/*Loma* infected group contained moderate to heavy infections with the ectoflagellate, *Ichthyobodo necator* (Fig. 5.4.B) and rickettsia-like organisms were seen within macrophages (Fig. 5.5.). Lamellar fusion and inflammation was observed in all the sections and the lamellae appeared short due to cellular infiltration of the interlamellar zone. Uninfected control fish treated with dex did not appear to develop infections with *Loma*, *Ichthyobodo*, or the rickettsia-like organisms, but there was significant

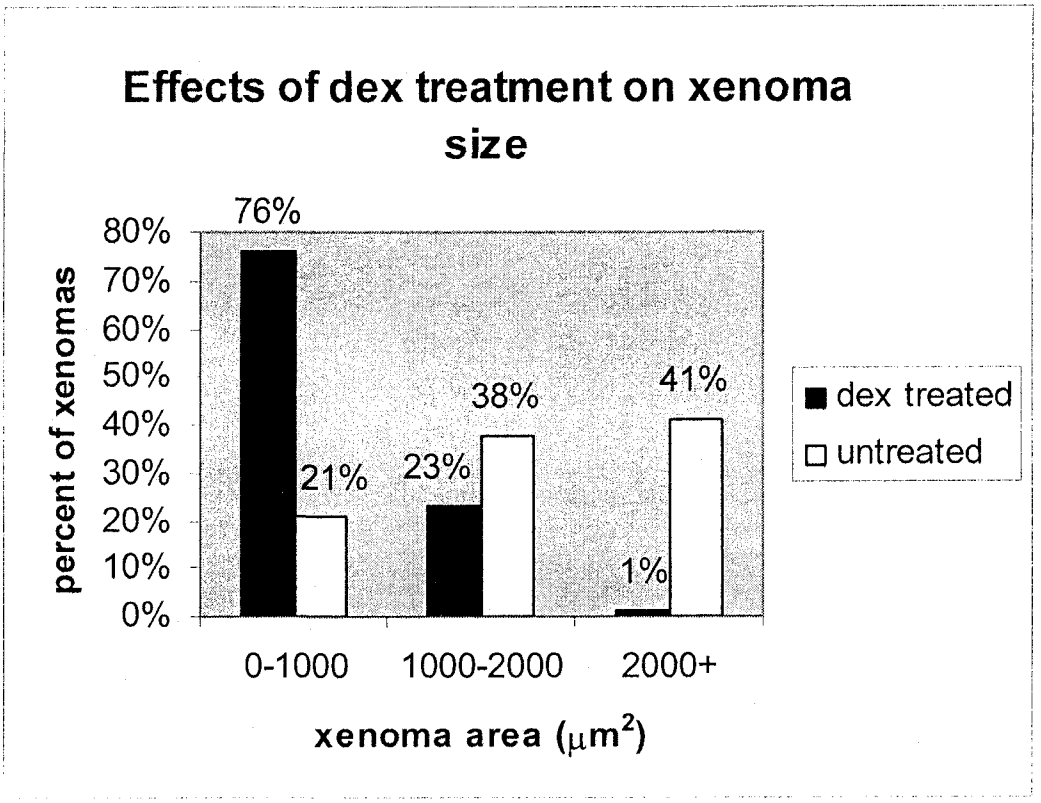


Figure 5.2. Xenoma sizes in sections using HRLM from fish treated with dexamethasone vs. untreated control fish.

inflammation in both the gill filament and in the lamellae. The inflammation consisted of infiltration with neutrophils, macrophages, and lymphocytes. Surface area of lamellae was lost due to lamellar fusion and infiltration with inflammatory cells and as a result of the inflammatory infiltrate the filament was frequently swollen. Tissue was carefully examined by HRLM and TEM, but no pathogens were observed.

5.4.3 Experiment 2. Effects of dex on the generated adaptive immune response

A total of 5 mortalities occurred throughout the trial (Table 5.3). Post mortem examination of the fish revealed that the 4 dead fish from the dex treated groups had *Ichthyobodo* organisms in the gills and the mortality from the *Loma*-rechallenged/non-treated group had a severe intestinal flagellate, likely representing a *Spironucleus* infection. No xenomas were observed in the gills of *Loma* cleared/non-treated, *Loma* cleared/dex treated, *Loma* rechallenged/non-treated, and *Loma* rechallenged/dex treated groups. Xenomas were observed in both duplicates of the two groups of fish infected a single time with *Loma* (*Loma* infected/non-treated and *Loma* infected/dex treated). Mean xenoma counts on the gills of the *Loma* infected/non-treated fish were lower than in the *Loma* infected/dex treated fish, but there was high variation between the duplicate tanks (Table 5.3). When accounting for tank variation, there was no significant difference between the dex treated group and non-treated group ($P=0.11$).

Histopathology demonstrated that organs other than gill did not contain xenomas in all groups except for the *Loma* infected/dex-treated and the *Loma*

Figure 5.3. Experiment 1: *L. salmonae* xenomas in the gills of rainbow trout. **A.** A HRLM of an untreated control fish with a typical xenoma containing various parasite stages including meronts (M), sporoblasts (sb), and mature spores (sp). The boundaries of the xenoma (arrowheads) are seen and there is little host response to the xenoma. (Bar= 10 μm) **B.** A HRLM of a xenoma (arrowheads) in dex treated fish with a darkly stained cytoplasm containing many empty spores (es); no developmental stages of the parasite are observed. (Bar= 10 μm) **C.** A TEM of a degenerating xenoma in the gills of dex treated fish. The early parasite stages (*) have hypercondensed and vacuolated cytoplasm and sections of sporulated polar tube (pt) can be seen within the xenoma cytoplasm (Bar= 3 μm)

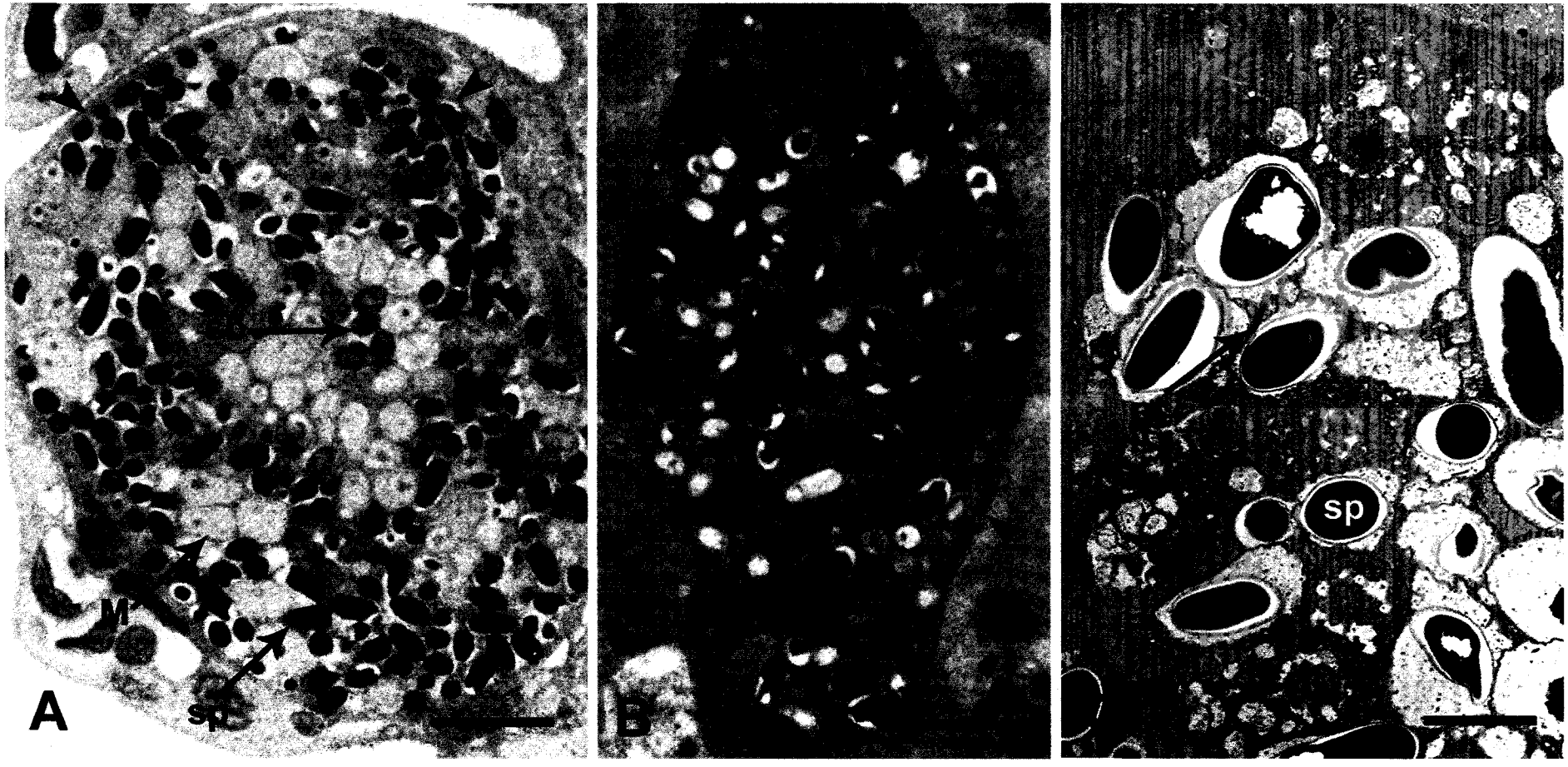

A
M
sp
B
sp

Figure 5.4. Experiment 1: Gills of dex treated rainbow trout. **A.** A HRLM of a xenoma (arrowheads) containing amorphous material, phagocytes (ph), and mature spores (sp). (Bar= 10 μm) **B.** A HRLM of a degenerated xenoma containing spores (sp) and red blood cells (rbc) in a gill lamella. The amorphous material (*) extends to nearby pillar cells and pillar channels. *Ichthyobodo* organisms (Ib) are observed around the lamellar epithelium. (Bar= 10 μm) **C.** TEM of a degenerated xenoma containing amorphous material which is arranged as lipid droplets (*), spores (sp), empty spores (es), and a macrophage with degenerated spores (arrowheads). Similar lipid material is seen within degenerated spores. (Bar= 3 μm) **D.** A HRLM of lamellae which contain lipid inclusions within the epithelium (arrowheads). (Bar= 20 μm)

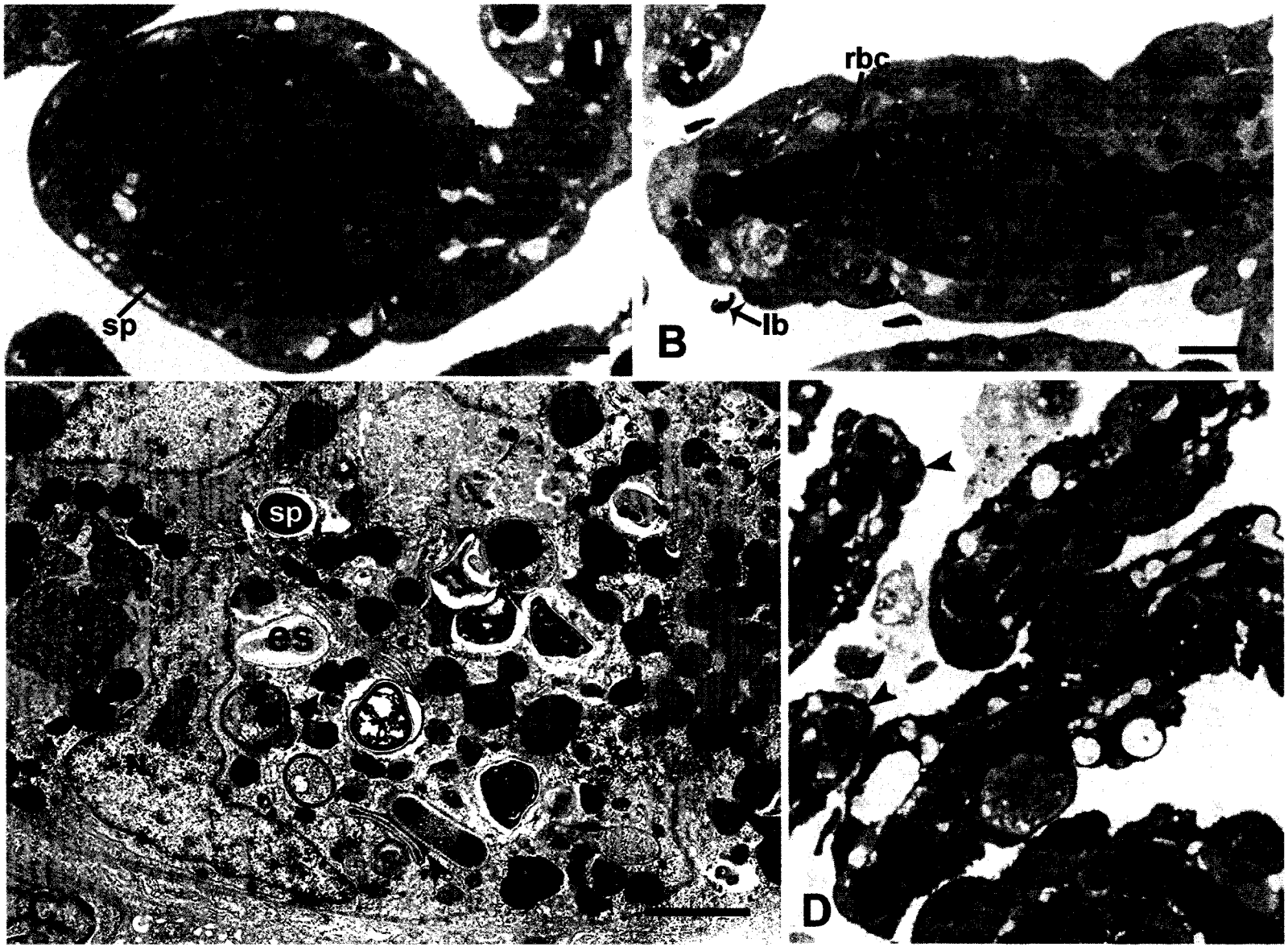
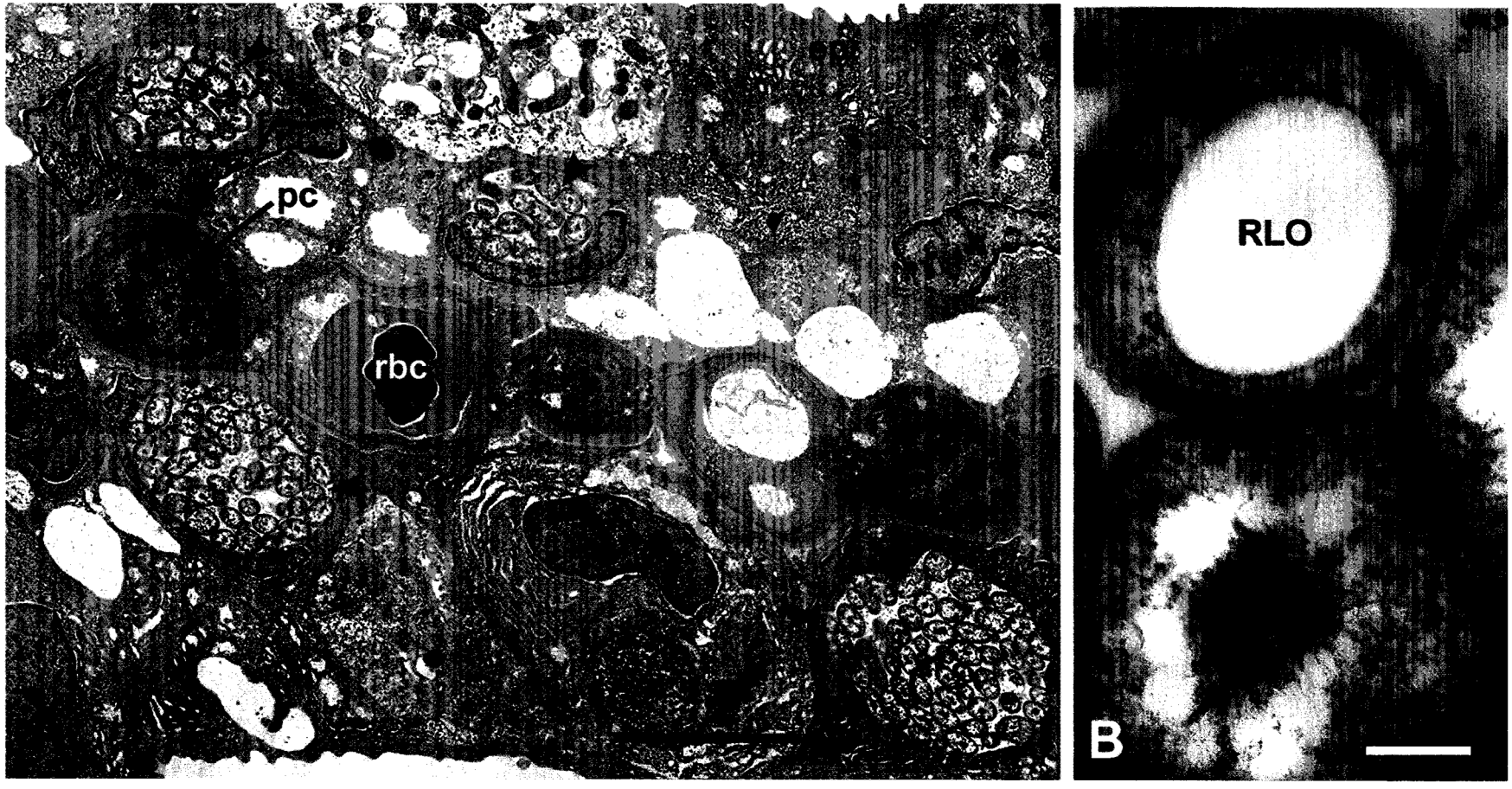

sp
rbc
lb
B
sp
es
D

Figure 5.5. Experiment 1: TEM of rickettsia-like organisms (RLOs) within macrophages in the gills of rainbow trout. **A.** A gill lamella with pillar cells (pc) and red blood cells (rbc) within pillar channels. Between the epithelium (ep) and the lamella are macrophages containing RLOs (arrowheads). (Bar= 5 μm) **B.** RLOs containing a double membrane wall (arrowheads). (Bar= 100 nm)


pc
rbc
RLO
B

infected/non-treated groups. In the *Loma* infected/non-treated group xenomas were rarely observed in the heart, while the *Loma* infected/dex treated group more commonly had xenomas in the heart and other organs (Table 5.4).

5.5 DISCUSSION

Dexamethasone (dex) treatment had an apparent effect on the immune response of rainbow trout when concurrently challenged with the microsporidian, *L. salmonae*. It was evident that daily feeding of fish at 1% body weight with dex at a concentration of 300 mg/kg of feed caused about 5-fold higher infections with the parasite in the gills and the parasite was more frequently found in the other organs, particularly in the heart and trunk kidney, but also in the spleen and in small numbers in the liver. The dex may diminish some aspects of the innate immune response or alternatively it could be influencing the development of adaptive immunity. Dex is known to suppress the innate immune responses to intracellular pathogens. This is supported by studies demonstrating inhibition of cytotoxic activity of human and bovine natural killer cells by dex (Bray et al. 1983; O'Brien and Duffus 1990) and inhibition of important type 1 immune response cytokines, IL-2 and IL-12, while production of cytokines which increase susceptibility to intracellular pathogens, such as IL-4 are enhanced (Schaffner et al. 1983, Daynes and Araneo 1989; Hennebold et al. 1997). It is difficult to explain what aspects of the innate immune responses are affected by dex to cause heavier infections with *L. salmonae* because the early stages

Group	Mortality	Mean xenoma count (±SE)
LCR+dex	2/24	0/0
LCR	1/24	0/0
LC+dex	2/24	0/0
LC	0/24	0/0
LNI+dex	0/24	300±63/922±222
LNI	0/24	53±27/140±76

Table 5.3. Experiment 2. Mortality and mean xenoma counts with standard error (SE) per gill arch in both duplicates (a/b). Loma cleared and rechallenged (LCR), Loma cleared (LC), and Loma naïve and infected (LNI); each group is either left untreated or treated with dex.

Group	Gills	Heart	Kidney	Liver	Spleen
LI+dex	209/751	8/14	2/5	0/0	0/8
LI	52/78	1/2	0/0	0/0	0/0

Table 5.4. Experiment 2. Xenoma counts in organs of *Loma* infected fish (LI) either treated twice weekly with dex at 60 mg/kg or left untreated in both duplicates (a/b). The counts represent total xenomas counted from 5 μm sections from five fish from each group.

of infection are not fully understood. It is unclear how the parasite migrates from the GI tract to the heart and how it is transported from the heart to the gills, although it is thought to use a white blood cell as a vehicle (Rodriguez-Tovar et al. 2002).

Chinook salmon are known to be more susceptible to *L. salmonae* than rainbow trout indicating that rainbow trout have a degree of resistance to the parasite that Chinook salmon do not have (Ramsay et al. 2002). Additionally, Chinook salmon commonly develop infections within the branchial filament arteries, while rainbow trout do not (Ramsay et al. 2002). Infections in other internal organs are commonly seen in Chinook salmon, while rainbow trout rarely develop xenomas outside the gills when left untreated with dex. It is likely that the resistance to the parasite in rainbow trout was diminished with dex treatment, causing similar infections in the gills and organs as seen in Chinook salmon. These results show that dex treated rainbow trout infected with *L. salmonae* provide a more accurate model of MGDS as seen in Chinook salmon.

A similar trend suggesting that dex caused increased infections with *L. salmonae* was repeated in experiment 2 demonstrating that dex treatment increased the number of xenomas in the gills and other organs of fish that were exposed to the parasite a single time. Mean (± SE) xenoma counts for the duplicate dex treated groups were 922 (±222) and 300 (± 63), while the non-treated group the means were 140 (± 76) and 53 (± 27). Although the difference between the four groups was not significant when analyzed with an ANOVA with tank random effects, there was an indication that dex treatment caused heavier infections. One possible explanation of

the substantial tank variation in experiment 2 was that in experiment 1 all fish were infected within the same tank and then randomly distributed into groups thus avoiding possible tank effects for the infection procedure itself, whereas in experiment 2 fish were infected in separate tanks. Accounting for tank effects in the analysis resulted in a low power for the test, making it more difficult to determine a significant difference.

All fish in this study demonstrated a very efficient protective immune response to *Loma salmonae*. Fish that cleared an infection never developed any xenomas in the gills or other tissues after exposure to the parasite. Even fish that were rechallenged with the parasite and concurrently fed with dex at 2% body weight twice weekly with a concentration of 300 mg/kg of feed for 3 weeks did not develop xenomas. Based on this study it appears that when fish have generated an adaptive immune response to *Loma*, dex has no effect on the activation of that response to block the infection from proceeding to the gills and other organs. The host immune response to *L. salmonae* has been shown to be a cellular response (Rodriguez-Tovar et al. 2006) and thus the inability of dex to disrupt the adaptive immune response to *Loma* suggests that once the T cell response is primed by the parasite, it is not affected by this steroid. Contrary to our findings with *L. salmonae*, it was shown that corticosteroid treatment in carp, *Cyprinus carpio*, suppressed the adaptive immune response to *Ichthyophthirius multifiliis* during rechallenge to the parasite (Houghton and Mathews 1990). Houghton and Mathews (1990) demonstrated that the humoral response was unaffected by corticosteroid treatment, therefore it was suggested that

the treatment affected the cellular immune response. However, the suppressed adaptive immune response was caused by treatment with triamcinolone and cortisol, and their mode of action may differ from that of dex. Additionally, *I. multifiliis* is a different disease model and the generation of the adaptive immune response likely differs from that of *L. salmonae*. The anti-inflammatory effects of dex treatment in mammals have been shown to be upstream of the presentation of processed antigen to T cell receptors (Holt and Thomas 1997). In humans, the cytotoxic activity of cytotoxic T cells is unaffected by dex treatment and it has been concluded that dex inhibits the activation of naïve T cells, but is unable to block proliferation of activated T cells (Strauss et al. 2002, Brandl et al. 2007). Dendritic cells previously treated with dex failed to mature when exposed to inflammatory stimuli, but cells exposed to inflammatory stimuli prior to dex treatment were not affected by the treatment (Matyszak et al. 2000). Based on the conclusions of this study, dex seems to have similar affects in fish as in mammals, demonstrating an inhibitory effect on the innate immune responses, but not on an acquired adaptive response to an intracellular pathogen.

Dex treatment during infection with *Loma* appeared to affect the development of the parasite in the gills. The xenomas in dex treated fish were generally much smaller than the xenomas in the control infected fish, and the xenomas in treated fish had morphology suggestive of a degenerative condition. Two types of degenerative xenomas were observed; one type was characterized by a darkly stained host cell cytoplasm, hypercondensed cytoplasm along with vacuolation of early parasite

stages, and germination of spores within xenomas. In this type of degenerative xenoma (Fig. 5.3) the xenoma plasma membrane appeared intact and no cells were seen infiltrated within the xenoma. A second type of degeneration was observed as a severe accumulation of amorphous material and lipid within the xenoma. Among this material were phagocytic cells degenerating spores and early parasite stages were not observed (Fig. 5.4). These appeared to be xenomas that have been broken down, possibly caused by a breach in the xenoma wall by phagocytes and degeneration of the parasites within the xenoma. The accumulation of amorphous material and lipid is likely a result of degeneration of the early and late parasite stages. Although phagocytosis in fish leukocytes have been shown to be inhibited by dex treatment (Leiro et al. 2000, Law et al. 2001), a study on human monocytes shows that dex promotes phagocytosis of particles (Van der Goes et al. 2000). In this study, phagocytic cells in fish with dex treatment were commonly observed to contain degenerated parasites and macrophages were seen infiltrated within xenomas. In the infected control fish, no xenomas were infiltrated with inflammatory cells and no degenerative conditions were observed in the xenomas. These findings suggest that dex treatment delayed the growth of xenomas or inhibited further development of xenomas. A possible theory is that the treatment had an affect on the host cells that harbor the parasite and produced unfavorable conditions for parasite development. Dex is a known inducer of apoptosis of various cell types, such as thymic cells (Zavitsanou et al. 2007), eosinophils (Druilhe et al. 2003), and human bone marrow derived mesenchymal cells (Oshina et al. 2007). It is possible that dex shortened the

life of the host cells that harbour the parasite by inducing apoptosis of the cells. *Loma salmonae* is known to rely on host cells for development, and the life cycle is thought to develop within a single host cell in the gills for approximately 4 weeks (Rodriguez-Tovar et al. 2002, 2003). If apoptosis was induced in the xenomas then the parasite would fail to develop and phagocytic cells would likely infiltrate the xenoma to clear the parasites from the tissue. This theory is supported by the findings of smaller xenomas in treated fish, the presence of degenerative xenomas which appear unbreached, and xenomas which contain degenerated parasites infiltrated with host inflammatory cells.

Dex treatment in fish also caused an increased susceptibility to other intracellular infections including RLOs and a bacterial infection consistent with *Renibacterium salmoninarum* in macrophages. High doses of dex as used in experiment 1 resulted in mortalities of fish which were thought to be caused predominantly by *R. salmoninarum*. The ectoparasite, *Ichthyobodo* was found in all fish treated with dex and infected with *L. salmonae*, while fish that were left uninfected with *Loma* and treated with dex did not develop infections with this parasite, suggesting that the infection was likely introduced with the *Loma* infective gill material that was fed to fish. The untreated infected fish remained uninfected by *Ichthyobodo*, so it is likely that dex given orally increased the susceptibility of the gills to this ectoparasite. Previous studies showed that dex treated fish become more susceptible to infections with other ectoparasites, such as *Gyrodactylus derjavini* (Lindenstrom and Buchmann 1998; Nielsen and Buchmann 2003). The increased

ectoparasitic infestations in dex treated fish could be explained by dex inhibiting some innate immune factors in the mucus of fish. Increased infections of *G. derjavini* caused by dex treatment in Atlantic salmon were thought to be caused by dex inhibiting the mucous cell discharge (Olafsdottir and Buchmann 2004).

In this study the use of per os treatment of dex as a model of stress related immunosuppression had severe affects on the innate immune responses of fish to *L. salmonae*, although it had no affect on infections after the adaptive immune response was generated. It was previously hypothesized that stress in a farm environment could override the adaptive immune response generated by Chinook salmon, which could partially explain why fish in their second year in the ocean are affected by MGD. The results in this study show that dex related immunosuppression does not have any affects on the adaptive immune response and in our model stress cannot override the adaptive immune response. Although it is possible that the induced protective response in Chinook salmon responds differently than rainbow trout when the fish are treated with dex, this study suggests that protective immunity may not be efficiently generated in netpen reared Chinook salmon or that there are other factors involved in the generation of disease in second year fish in the netpens.

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6 A HISTOPATHOLOGICAL STUDY ON THE EFFECTS OF INDOMETHACIN IN RAINBOW TROUT[7] AND ITS USE AS AN ANTIINFLAMMATORY AGENT IN MICROSPORIDIAL GILL DISEASE

6.1 ABSTRACT

The purpose of this study was to (1) determine adequate dose ranges and test for side effects associated with chronic treatment of indomethacin in fish and (2) determine if indomethacin treatment effects xenoma clearance and healing in the gills of fish experimentally infected with MGDS. Rainbow trout *Oncorhynchus mykiss* were orally treated with indomethacin at various nominal concentrations including, a negative control (0 mg/kg), low (5 mg/kg), medium (10 mg/kg), and high doses (15 mg/kg) daily for 30 days. A dose associated mortality was observed where a 3%, 13%, and 33% total mortality occurred respectively in the indomethacin dose groups. No lesions were observed grossly or with histopathology in the control and low dose treatment groups. Gross lesions were observed in the medium and high dose groups that included skin ulcers, abdominal distension, and necrosis of abdominal wall muscle. Histopathology of fish in the medium and high dosage groups displayed severe granulomatous peritonitis with a large number of foreign body type giant cells present around proteinaceous and plant material. The inflammatory response spread from the peritoneum through the somatic muscle to the epidermis, causing lesions within all layers of the skin. A large number of bacteria were noted within the peritonitis, which were observed intracellularly and in large aggregates

[7] Lovy J, Speare DJ, Wright GM. Pathological effects caused by chronic treatment of indomethacin in rainbow trout. J Aquat Anim Health 2007; 19: 94-98.

extracellularly. Perforations occurred within the anterior intestine, and the thick muscularis layer was replaced with inflammatory tissue. This work shows that chronic indomethacin treatment in rainbow trout produces similar gastrointestinal side effects as are seen in mammals. A chronic exposure model of MGDS consisting of fish exposed daily to the parasite, *Loma salmonae* for 3 weeks was developed and utilized to see the effects of indomethacin treatment during the rupture of parasitic xenomas. Two samples for high resolution light microscopy were taken in the chronic exposure model, one at 8 weeks PE and another at 11 weeks PE. In gills sampled at 8 weeks PE xenomas were observed intact and in various stages of degradation with an associated inflammatory response. Xenoma rupture was associated with fusion of secondary lamellae, an inflammatory infiltrate, and necrosis of secondary lamellae. Areas of fusion contained goblet cells which were arranged peripherally to the lesion. By 11 weeks PE all the xenomas were cleared from the lamellae and rarely large xenomas remained in the gill filament. Eosinophilic granular cells (EGC), which were occasionally in mitosis, were seen in the gill filament tips and some lamellar fusion was still present, which had evidence of lamellar regeneration. Indomethacin treatment did not delay the clearance of xenomas and had no effect on the regeneration of the lamellae. In fish sampled at week 8 PE, indomethacin treated fish had similar inflammatory lesions consisting of neutrophils and macrophages as untreated fish. Indomethacin treated fish at 11 weeks PE had a more severe EGC response in the primary gill filament adjacent to

lamellar fusion. This EGC infiltrate in the gill filament appeared to be at least in part the result of local proliferation of EGC's within the gill.

6.2 INTRODUCTION

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) that suppresses production of prostaglandins through inhibition of cyclooxygenase (COX) on arachidonic acid. The drug suppresses the action of both the constitutive COX-1 and inducible COX-2; suppression of the former is thought to result in serious side effects such as increased intestinal permeability and inflammation, which can lead to intestinal perforations. Specific COX-2 inhibitors have been developed that decrease risk of gastrointestinal toxicity, but can still result in toxicity to the lower intestinal tract of mammals (Thiefin and Beaugerie 2005).

It appears that NSAIDs in fish, as in higher vertebrates, may cause suppression of prostaglandins by inhibiting the COX pathway. Tilapia *Oreochromis mossambicus* treated with acetylsalicylic acid were shown to exhibit reduced levels of prostaglandins in their plasma (Van Anholt et al. 2003) and rainbow trout *Oncorhynchus mykiss* thrombocyte aggregation was inhibited by treatment with indomethacin (Hill et al. 1999), although the mechanism of inhibition is unknown. Activated fish macrophages express COX-2 and it has been reported that release of prostaglandins by fish macrophages modulate inflammatory responses. In a macrophage-like cell line of Atlantic salmon *Salmo salar* there is a 30-fold increase in the expression of the proinflammatory cytokine IL-1β upon incubation with LPS

and prostaglandin E_2 (Fast et al. 2005). Indomethacin ablated generation of prostaglandin E_2 in the fish macrophage cell line (Zou et al. 1999).

Several fish diseases result in an inflammatory response that may be more detrimental to the host than curative. An example is proliferative gill disease, a serious disease in farmed channel catfish *Ictalurus punctatus* associated with gill inflammation and hyperplasia in response to a myxosporean parasite. Fish with proliferative gill disease which were treated with indomethacin developed less severe lesions as demonstrated by histopathology (Davis 1994). Microsporidial Gill Disease of salmonids (MGDS) causes serious gill pathology attributed to the rupture of parasitic xenomas and the associated granulomatous branchitis. The disease is associated with a heavy infiltration of inflammatory cells into the branchial filaments and lamellae with a vasculitis causing thrombosis of arterioles and arteries (see chapter 3).

Experimentally induced MGDS in rainbow trout, *Oncorhynchus mykiss* provides a model in which the infection can be followed from the parasite arrival in the gills, 2 weeks PE, to the xenoma rupture/inflammation phase, 7-8 weeks PE (Rodriguez-Tovar et al. 2002, 2003). Differences exist between clinically infected Chinook salmon with MGDS and experimentally induced MGDS in rainbow trout. Chinook salmon are more susceptible to the parasite and carry heavier infections than seen in rainbow trout (Ramsay et al. 2002). Netpen reared Chinook salmon contain infected host cells that contain only early parasite stages (meronts) within host cells and fully matured xenomas, which have ruptured and elicited an inflammatory

response (Chapter 3). According to a single dose exposure model for rainbow trout the parasite first arrives in the gills as meronts infecting host cells at 2 weeks PE and at 7 weeks PE xenomas rupture and inflammation is observed (Rodriguez-Tovar et al. 2002, 2003). Xenomas are known to persist in the gills of laboratory infected Chinook salmon for up to 21 weeks PE (Kent et al. 1999). This shows that clinically infected netpen reared Chinook salmon carry infections that vary in age from at least 2 weeks PE up to 21 weeks PE, suggesting that an adaptive immune response is not efficiently generated as reported in rainbow trout (Rodriguez-Tovar et al. 2006).

With Chinook salmon carrying various stages of xenomas, it is likely that disease in these fish is more chronic than in the rainbow trout single dose experimental model, which would clear the xenomas in a single wave. In this study a chronic infection model is examined, which consists of feeding rainbow trout infective gill material over a period of three weeks. This was chosen because it has previously been shown that rainbow trout induce an adaptive immune response to the parasite starting at 3 weeks post exposure (Rodriguez-Tovar et al. 2006). It is hypothesized that this model will extend the xenoma rupture period and the inflammatory response will be more representative to that which is observed in clinically infected Chinook salmon. With evidence showing that indomethacin has similar inhibitory action on prostaglandin generation in both fish and mammals, there is the potential to administer this drug to fish which exhibit inflammatory diseases. Therapeutics for fish in aquaculture may be applied in various forms, but the most efficient method for salmonids in netpens is likely through the feed. The intent of

this study is to determine oral doses that are acceptable for rainbow trout and to determine if side effects of the drug are present after chronic treatment at various doses. Additionally the chronic exposure model of MGDS is utilized to examine the effects of indomethacin on the clearance of xenomas and the healing of gills after xenoma rupture.

6.3 MATERIALS AND METHODS

6.3.1 In feed treatment

Three mm extruded pellets (Corey Aquafeeds; Fredericton, New Brunswick, Canada) were coated with indomethacin (Novopharm; Toronto, Canada), which was mixed into gelatin (Oxoid; Basingstoke, Hampshire, England). For every kg of feed, 4 g of gelatin was dissolved into 70 ml of boiled distilled water and allowed to cool to about 25°C. Appropriate amounts of indomethacin were slowly added to the cooled gelatin mixture with constant mixing until the indomethacin appeared evenly dispersed throughout the mixture. The mixture of gelatin with indomethacin was slowly poured onto the feed with constant mixing using a rubber policeman to evenly coat the feed. Feed was immediately spread over a tray to dry at room temperature for one hour. The feed was stored at 4°C until use. Appropriate feed treatment was given to the fish groups by feeding fish their respective treated feed at 2% body weight per day. Assuming a 2% weight gain per day, the feeding regime was adjusted weekly. Mortalities were monitored throughout the treatment duration and amounts of feed given to groups were adjusted based on loss of biomass.

6.3.2 Experiment 1: Dosage effects of indomethacin

6.3.2.1 Experimental design

Rainbow trout were acquired from the Cardigan Fish Hatchery on Prince Edward Island and maintained at 10° ±1°C until one week prior to the experiment. Fish were approximately six months old and weighed 15-25 g each. The fish were separated into four 70 L circular tanks maintained at approximately 15°C, with 30 fish per tank. The mean (± SE) biomass of each tank was approximately 560 g (± 15). After allowing a one week period for fish to acclimate to the new tanks they were treated with indomethacin at various nominal concentrations, of which one group served as an untreated control. The treatment concentrations were applied at a control, low, medium, and high dose, which were 0 mg/kg, 5 mg/kg of fish, 10 mg/kg of fish, and 15 mg/kg of fish, respectively. The fish were given the appropriate nominal concentrations daily for a period of 30 days.

6.3.2.2 Sampling procedure

Mortalities were monitored throughout the duration of the study and upon termination of the experiment (30 days) the remaining fish were sampled. All fish were examined grossly for external lesions and five fish from each group were fixed in 10% neutral buffered formalin for histopathological examination. Incisions were made in abdomens of fish to allow better fixative penetration, and five fish from each group were transferred to jars containing 1 L of fixative. After routine processing the tissue was embedded in paraffin wax and 5 μm sections were cut , stained with hematoxylin and eosin, and examined with light microscopy. Two transverse

sections were cut from each of the fish in all treatment groups, one section from the anterior portion of the body cavity through the liver and one from the area of the stomach and pyloric caeca.

6.3.3 Experiment 2. Effects of indomethacin on fish with MGDS

6.3.3.1 Experimental design

120 rainbow trout (weight 15-25 g each) were randomly distributed into four 70 L tanks, so each tank would contain 30 fish, and maintained at 15° ± 1°C. After allowing one week for acclimation, two of the tanks of fish were given a chronic infection with *L. salmonae*, which consisted of feeding fresh infective gill material daily for 21 days. Five weeks post the initial infection day, one tank of infected fish and one tank of naïve fish were treated daily with indomethacin at a nominal concentration of 15 mg/kg for the duration of the trial. Two samples were taken from each of the tanks, one to represent the xenoma rupture period, 8 weeks post initial infection, and one to represent the clearance/healing period, 11 weeks post initial infection. Mortalities in the tanks throughout the trial were monitored and recorded.

6.3.3.2 Sampling procedure

Each sample consisted of 10 fish from each of the 4 groups; the gills of all the fish were fixed in formalin for standard histopathology and one gill arch from all fish from each group was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for HRLM and TEM. Because of mortality in the *Loma* infected and indomethacin treated group the week 11 sample was represented by 8 fish. Samples for HRLM were processed as previously described (2.3.4). Three pieces of gill tissue from each

fish were individually embedded into BEEM capsules; in the week 11 sample of *Loma* infected and indomethacin treated fish, 4 pieces of gill tissue were taken from each fish. Semi-thin sections (0.5 μm) were cut from a total of 120 blocks (30 blocks from each of the two samples of infected fish treated with indomethacin and 30 blocks from each of the two samples of infected fish left untreated), stained with toluidine blue, and examined with light microscopy. Areas of interest were re-trimmed and ultra thin (90 nm) sections were cut, retrieved onto copper super grids (200 mesh), and stained with uranyl acetate and Sato's lead stain. Grids were viewed and photographed with a Hitachi 7500 transmission electron microscope.

6.3.3.3 Source of infective gill material for chronic infection:

The innoculum originated from gills of infected Chinook salmon from British Columbia and the parasite was maintained in the laboratory by serial passage through rainbow trout. Two 70 L tanks each containing 50 rainbow trout were maintained at 15° ±1°C and infected with *Loma salmonae* by feeding infective gill material from previously infected rainbow trout. From 5 weeks PE (35 days) through 8 weeks PE (55 days), 5 fish were euthanized daily with an overdose of benzocaine and the gills were dissected and fed to fish in the chronic infection tanks.

6.4 RESULTS

6.4.1 Experiment 1. Dosage effects of indomethacin

6.4.1.1 Drug palatability and Mortality

Feed palatability was good and fish continued feeding on the treated feed throughout the duration of the study with no feed remaining in any of the tanks. Mortalities were encountered in this study prior to termination of the experiment. There appeared to be a treatment associated mortality, where the low, medium, and high dose groups experienced a 3.33%, 13.33%, and 33.33% respective total mortality (Figure 6.1.). Most mortalities occurred after 12 days of treatment and prior to death fish appeared inactive and were localized near the sides of the tank near the water surface.

6.4.1.2 Gross Lesions

Fish from the control and low dose treatment groups had no external lesions. Fish from the medium and high treatment groups had large irregularly shaped ulcers that were located posterior to the pectoral fin on the lateral body surface. These large ulcers had central areas that were red with black margins, and the area on the skin peripheral to the lesion was pale (Fig. 6.2.A). Also observed were circular skin ulcers located on the ventral abdominal surface, which were variably dark red and black (Fig. 6.2.B). External lesions were observed in 30.77% and 25% of fish in the medium and high dose groups respectively. Fish that had external lesions also had abdominal distension. The stomach was swollen and full of fluid and abdominal

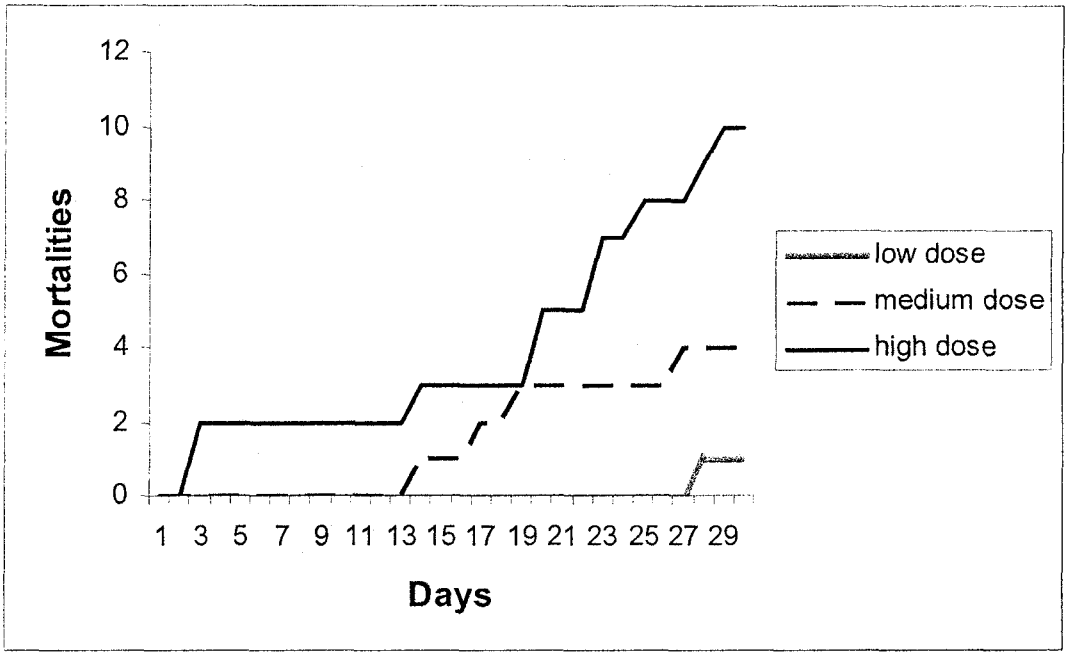
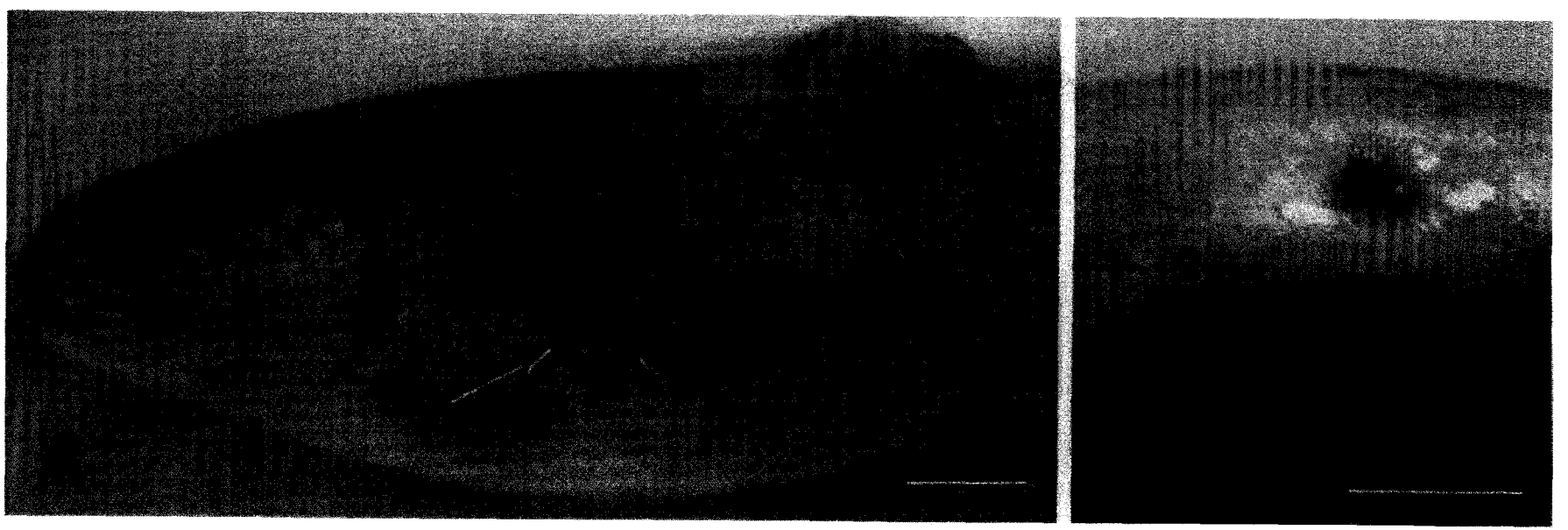


Figure 6.1. Mortalities of rainbow trout given various doses of indomethacin

Figure 6.2. Experiment 1: Rainbow trout with skin ulcers after daily treatment with a medium dose (10 mg/kg) of indomethacin for 30 days. **A.** Large irregular ulceration (arrow) located laterally. **B.** A circular ulcer in the ventral abdominal area. (Bar= 1 cm)



muscle was excessively soft. There was no marked difference in severity of the lesions between the medium and high dose treatment groups.

6.4.1.3 Histopathology

No lesions were observed in any fish within the control group or the low dose group. Fish from the medium and high dose group had a number of pathological alterations. A common finding was the presence of granulomatous peritonitis, which caused adhesions among the internal organs. Adhesions were observed between liver, gastro-intestinal (GI) tract, spleen, pancreas and frequently adhesions extended to the body wall (Fig.6.3.A). Abundant eosinophilic material, likely protein from feed contents, was found throughout the inflammatory infiltrate (Fig. 6.3.A). Many macrophages and fibroblasts were present in the lesion and a consistent finding was a large number of foreign body type giant cells seen within the infiltrate. The giant cells often contained eosinophilic material and in some cases contained cellular material containing cell walls, which was presumably plant material. The inflammation extended from the peritoneal cavity through the somatic muscle of the body wall (Fig. 6.3.B). The muscle and adipose tissue had degenerative areas, in which the tissue was replaced with inflammatory cells (Fig. 6.3.B). The inflammation extended from the somatic muscle through the dermis and into the epidermis. This inflammation was associated with a loss of the adipose tissue of the hypodermis, loss of the adjacent iridophore layer, disorganization of the stratum compactum of the dermis, loss of scales and scale pockets in affected areas, and thickening of the epidermis with a reduction of mucous cells (Compare normal skin,

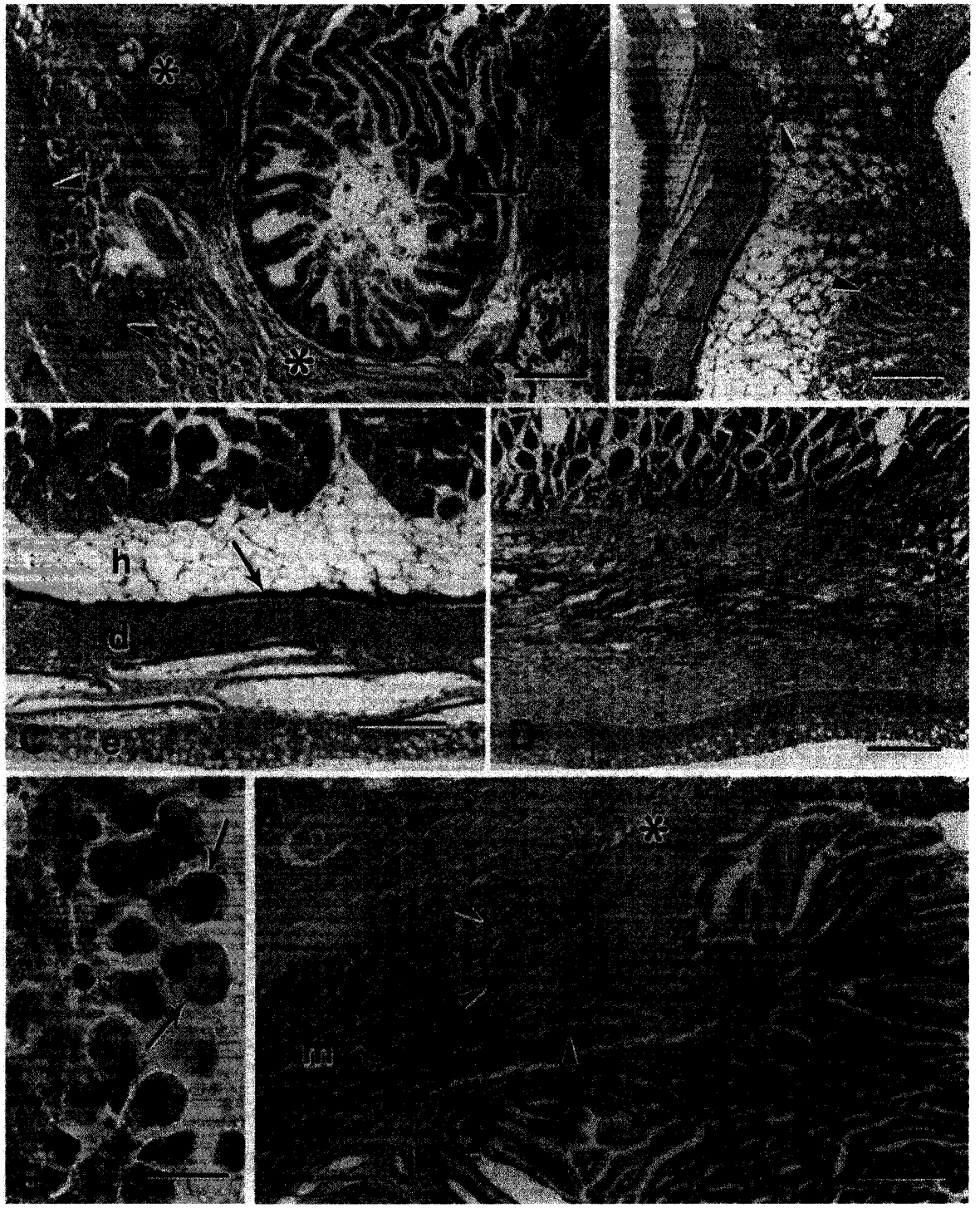
Fig. 6.3.C with affected area, Fig. 6.3.D). Another finding within the peritonitis was the presence of bacteria. The bacteria were extracellular within the inflammation, in which large aggregates were formed, and intracellular, in which cells were engorged with the bacteria (Fig. 6.3.E). Fish with peritoneal granulomatous inflammation also had lesions within the gastrointestinal (GI) tract. In particular, areas of necrosis and severe inflammation were evident in the muscular tunic of the anterior intestine. The damage extended into the submucosa, where inflammatory cells covered an ulcerated area (Fig. 6.3.F).

6.4.2 Experiment 2. Effects of indomethacin in fish with MGDS

6.4.2.1 Treatment associated mortality:

Mortalities occurred in both *Loma* infected fish and control fish that were treated with indomethacin. The mortality started at 3 days post treatment in both groups and was chronic throughout the trial. Up until the first sample period (3 weeks of indomethacin treatment) mortalities were observed in 8/30 fish in the control treated fish and 6/30 mortalities were observed in *Loma* infected/treated fish. The time between the 8 week sampling (after removing 10 fish from each group) and the 11 week sampling mortalities occurred in 4/12 fish and 2/14 fish in the control treated fish and *Loma* infected/treated fish respectfully. No mortalities were observed in either of the *Loma* infected or control groups that were not given indomethacin.

Figure 6.3. Light micrographs of lesions associated with chronic treatment of medium and high doses of indomethacin; **A.** Granulomatous peritonitis (*) causing adhesion of pancreas and GI tract to the body wall. Inflammation extends to the muscle (arrowheads) of the body wall. The peritonitis contains eosinophilic material (arrow). (Bar= 250 μm) **B.** Ventral body wall showing inflammation originating from the peritoneal cavity (right) extending to the skin (left). Inflammation is seen throughout the adipose and muscle tissue (arrowheads). (Bar= 250 μm) **C.** Area of normal skin showing the epidermis (e), dermis (d), iridophore layer (arrow), and hypodermis (h). (Bar= 250 μm) **D.** Skin lesion showing inflammation extending from the muscle to the epidermis. Note the loss of the iridophore layer and hypodermis and that the dermal collagen layers have lost their densely packed arrangement. (Bar= 250 μm) **E.** Bacteria engorged cells within the peritoneal cavity (arrows) and aggregates of extracellular bacteria (left). (Bar = 15 μm) **F.** Inflammatory tissue (*) replacing the muscularis and submucosa of the anterior intestine. Arrowheads show boundaries of the inflammation and the intact muscularis layer. (Bar= 250 μm)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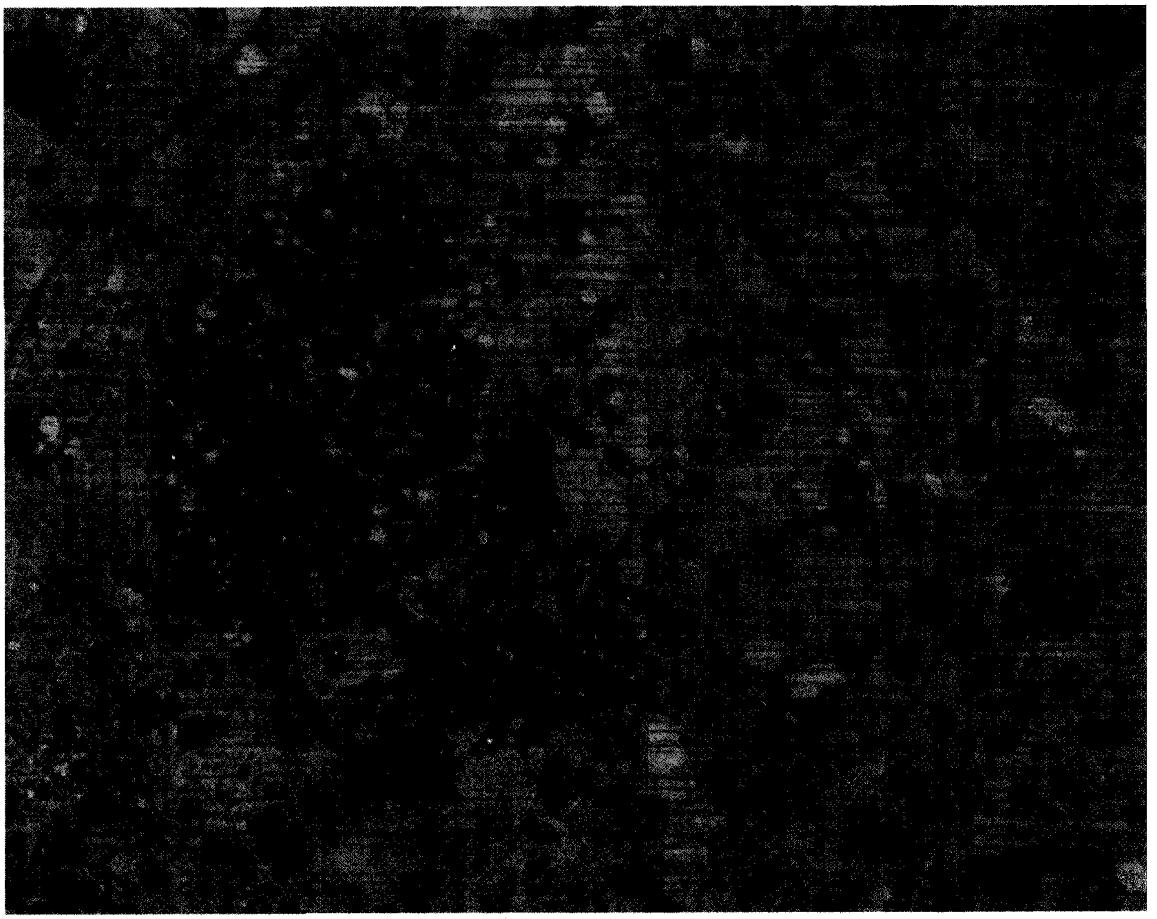
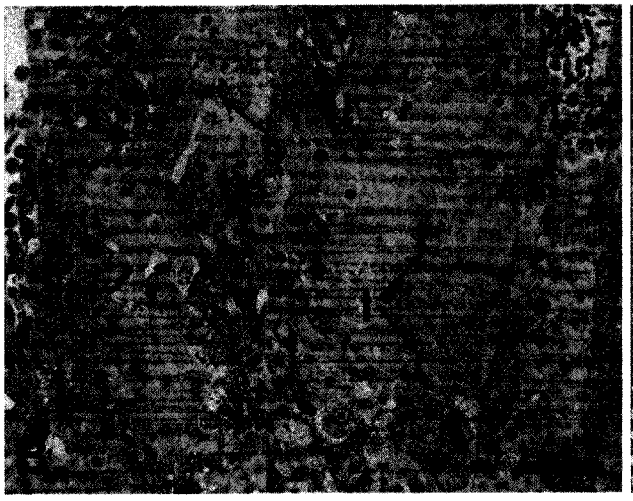
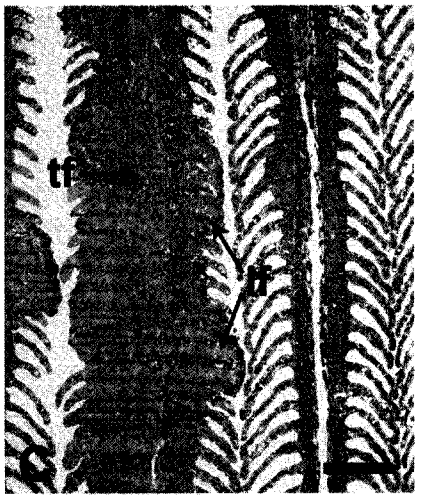
6.4.2.2 Chronic exposure Loma model:

Week 8 sample of *Loma* infected fish untreated: Xenomas were observed in the gills of fish in various stages of development and degeneration. Many xenomas appeared intact, while it was common to see xenomas in various stages of degradation. Xenomas were observed in both the lamellae and in the filament associated with the filament arteries. In some, the xenoma membrane was disrupted and neutrophils and macrophages were invading within the xenoma (Fig. 6.4.A). The inflammatory infiltrate was surrounding the xenoma within the gill filament and within the xenoma, intact and empty spores were observed (Fig. 6.4.A). Degraded xenomas were also observed within the lamellae (Fig. 6.4.B), which were associated with an inflammatory infiltrate and lamellar fusion. Within the fusion areas, individual spores were within the tissue and the lamellae were infiltrated with inflammatory cells. The lamellar structure was frequently lost or appeared to undergo degeneration in severely affected tissue (Fig. 6.4.B). Lesions typically involved both the gill filament and the lamellae (Fig. 6.4.C). In addition to lesions containing intact parasitic spores, fusion of lamellae was observed without the presence of spores. These, likely older lesions, contained a peripheral arrangement of goblet cells arranged within the epithelial layer of the affected areas (Figs. 6.5.A and B). The goblet cells were strongly associated with the lesions, while they were seen only in small numbers within unaffected lamellar epithelium. The structure of the lamellae was lost (Fig. 6.5.B) and the fusion areas contained undifferentiated cells and inflammatory cells.

Figure 6.4. Xenoma rupture in the chronic exposure MGD model in *Oncorhynchus mykiss*. **A.** A xenoma, within the gill filament, with the xenoma membrane (XM) disrupted on one side and host cells (arrowheads) infiltrating the xenoma. Within the xenoma both intact spores and empty spores (ES) are seen, and outside the xenoma neutrophils (N) are abundant. (Bar= 20 μm) **B.** A xenoma which has ruptured in the gill lamellae. Spores (s) are observed within the tissue and lamellae (l) have lost their structure and some are missing. The gill filament (f) adjacent to the lesion is infiltrated with inflammatory cells. (Bar= 50 μm) **C.** A lesion associated with a ruptured xenoma. There is lamellar fusion (lf) and a thickened gill filament (tf) which is infiltrated with inflammatory cells. (Bar= 100 μm)



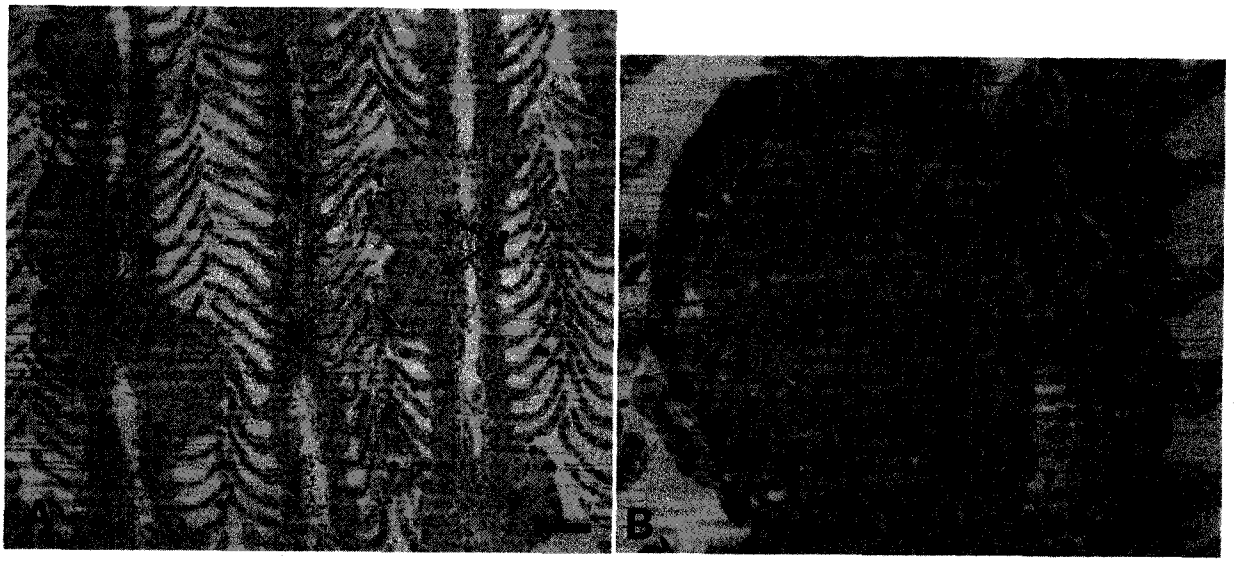



tf

Week 11 sample of *Loma* infected fish untreated: The majority of xenomas were cleared from the tissue and large xenomas were rarely observed within the primary gill filament, while all the xenomas were cleared from the lamellae. The large xenomas persisting within the filament had endothelial cells separating the xenoma membrane from the blood vessel lumen (Figs. 6.6.A and B). While all of the xenomas were cleared from the lamellae, small lesions were still present and eosinophilic granular cells (EGCs) were commonly within the filament. The EGCs were most frequently arranged around the filament cartilage in the tips of the gill filaments, but not associated with lesions. Mitotic EGCs were observed throughout the filament. Areas of lamellar fusion and degeneration contained signs of lamellar regeneration. The basement membrane of regenerating lamellae first appeared as a bulge in the basement membrane that separated the connective tissue of the gill filament from the lamellar zone. Within this early lamellar bud mitotic cells and early blood vessels were observed (Fig. 6.7.A). Undifferentiated cells accumulated at the base of regenerating lamellae, which originated from the filament, and blood vessels continued to form throughout the developing lamellae (Fig. 6.7.B). Within the base of regenerating lamellae and the connective tissue of the filament the accumulation of undifferentiated cells had a stacked appearance in the filament and some were arranged peripherally within the basement membrane of the newly forming lamellae (Fig. 6.7.C). At that stage early pillar cells were observed bridging the two sides of the basement membrane of the lamellae (Fig. 6.7.C). Lamellae were

Figure 6.5. Lesions associated with xenoma rupture. **A.** Lamellar fusion (lf) with a peripheral arrangement of goblet cells (gc) within the fused lamellae. (Bar= 100 μm) **B.** Lamellar fusion area with goblet cells with a peripheral arrangement in the lesion. Within the lesion lamellar structure is no longer present; normal lamellae (l) are seen on the opposite side of the lesion. (Bar= 20 μm)

Figure 6.6. Large xenomas in the gill filament of fish sampled 11 weeks PE. **A.** Indomethacin treated fish with a xenoma within the gill filament; the xenoma is beneath the basement membrane (arrowheads) that separates the lamellae from the gill filament. The xenoma is bulging into the blood vessel (v). (Bar= 20 μm) **B.** A xenoma in an untreated fish. The xenoma is compressing the blood vessel lumen (v) and intact endothelial cells are separating the xenoma from the blood vessel lumen. (Bar= 10 μm)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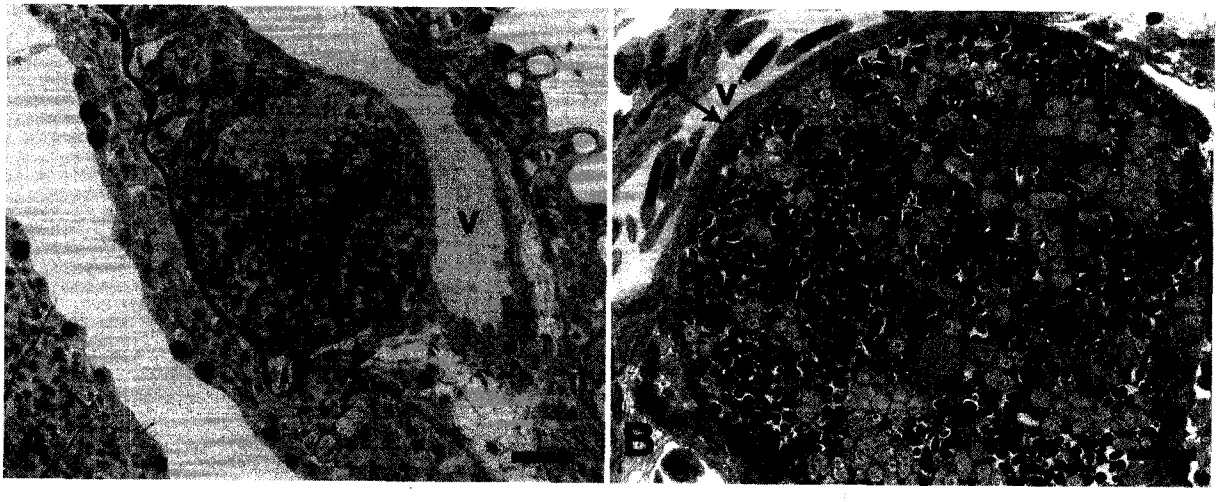
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observed containing blood vessels with endothelial cells, likely pillar cells, which have not yet formed the typical pillar formation within the lamellae (Fig. 6.7.D).

6.4.2.3 Indomethacin treatment on *Loma*-infected fish

Week 8 sample: The gills of fish treated with indomethacin had xenomas which were intact and in various stages of degeneration. The pathology was similar to what was described for fish without indomethacin treatment. Lamellar fusion was characteristic and inflammatory cells were observed infiltrated within the filament and the lamellae (Figs. 6.8.A and B). Xenomas that ruptured within the lamellae caused necrosis and loss of structure of the lamellae (Fig. 6.8.B).

Week 11 sample: The majority of xenomas were cleared and only rarely xenomas were found within the filament (Figs. 6.6.A and B). Similarly to the untreated fish all the xenomas were cleared from the lamellae. Mitotic cells within the lamellar fusion areas and regeneration of lamellae were observed in these fish. There was a pronounced EGC reaction in the filament adjacent to areas of lamellar fusion and lamellar regeneration (Figs. 6.9.A and B). The EGCs were heavily infiltrated within the filaments and caused swelling of the filament (Fig.6.9.A). The pronounced infiltrate within the filaments contained mitotic EGCs (Figs. 6.9.C-F). Heavy infiltration of EGCs adjacent to lesions was observed in 11/30 sections.

Figure 6.7. Lamellar regeneration in the chronic exposure model of MGD in untreated fish. **A.** The basement membrane (arrowheads) bulging out towards the lamellar surface with a mitotic cell (mc) and a blood vessel (v) within the basement membrane bulge. **B.** Two regenerating lamellae; there is an aggregation of nondifferentiated cells at the base of the lamellae within the basement membrane (arrowheads) and blood vessels (v) are formed throughout the lamellae, goblet cells (gc). **C.** A regenerating lamella with an aggregate of nondifferentiated cells (nc) which appear stacked within the gill filament and within the lamella some of the nondifferentiated cells are arranged along the inside of the basement membrane forming a circular pattern. A newly formed pillar cell (pc) within the blood vessel (v) is not yet fully attached to both sides of the basement membrane of the lamella. **D.** Two regenerating lamellae. The one on the left has fully developed pillar cells (pc) and the lamella on the right has a single blood vessel with endothelial cells (en) without developed pillar cells. (Bar= 10 μm)

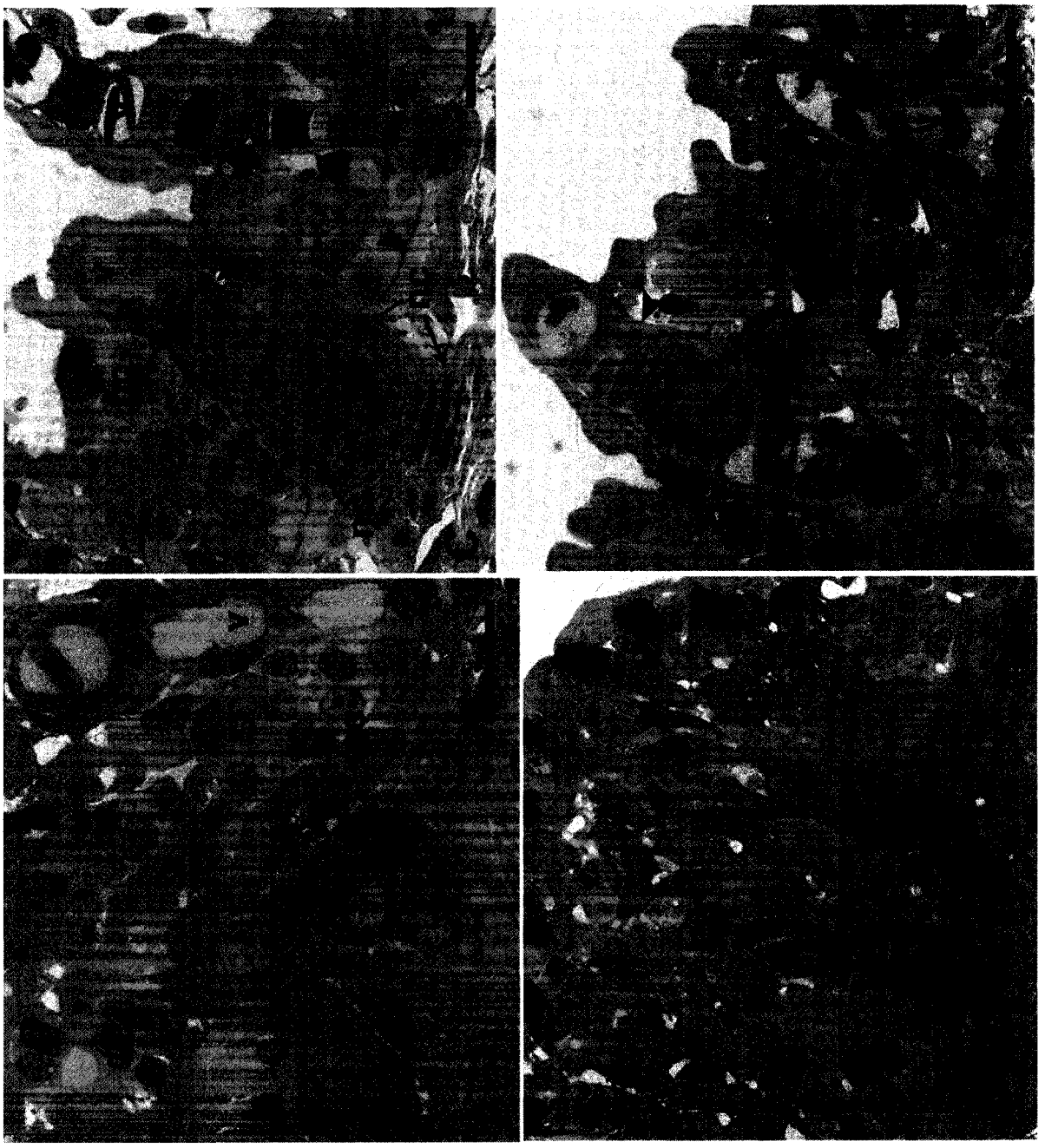
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Figure 6.8. The gills of indomethacin treated fish sampled 8 weeks PE to *L. salmonae*. **A.** Lamellar fusion (lf) and intact xenomas (xe) are observed throughout the gills. (Bar= 200 μm) **B.** Lamellar fusion and lamellar necrosis is associated with individual spores (s) within the tissue of the lamellae. Below the intact lamella (l) the other lamellae are necrotic or missing. The gill filament adjacent to the lesion is infiltrated with inflammatory cells. (Bar= 20 μm)

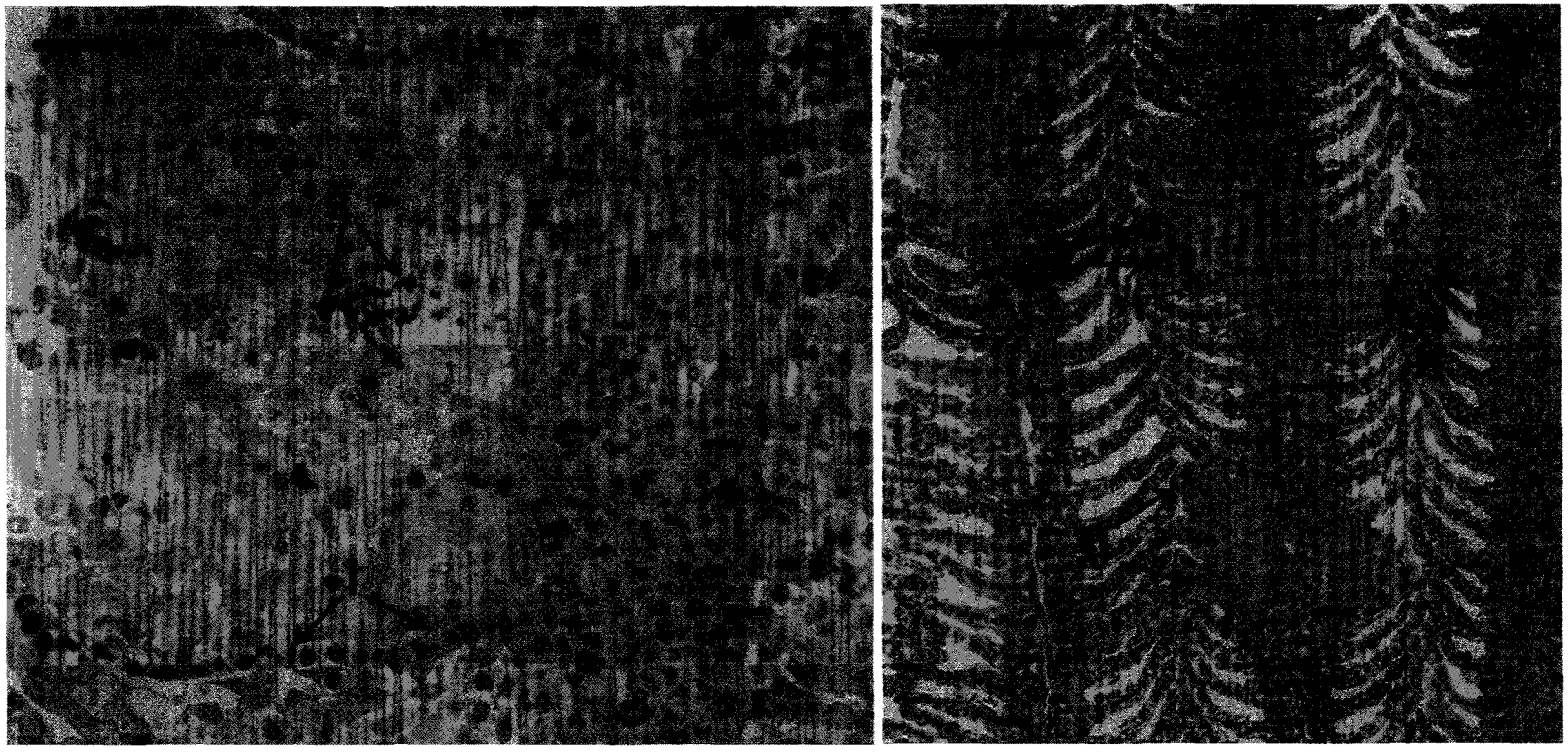
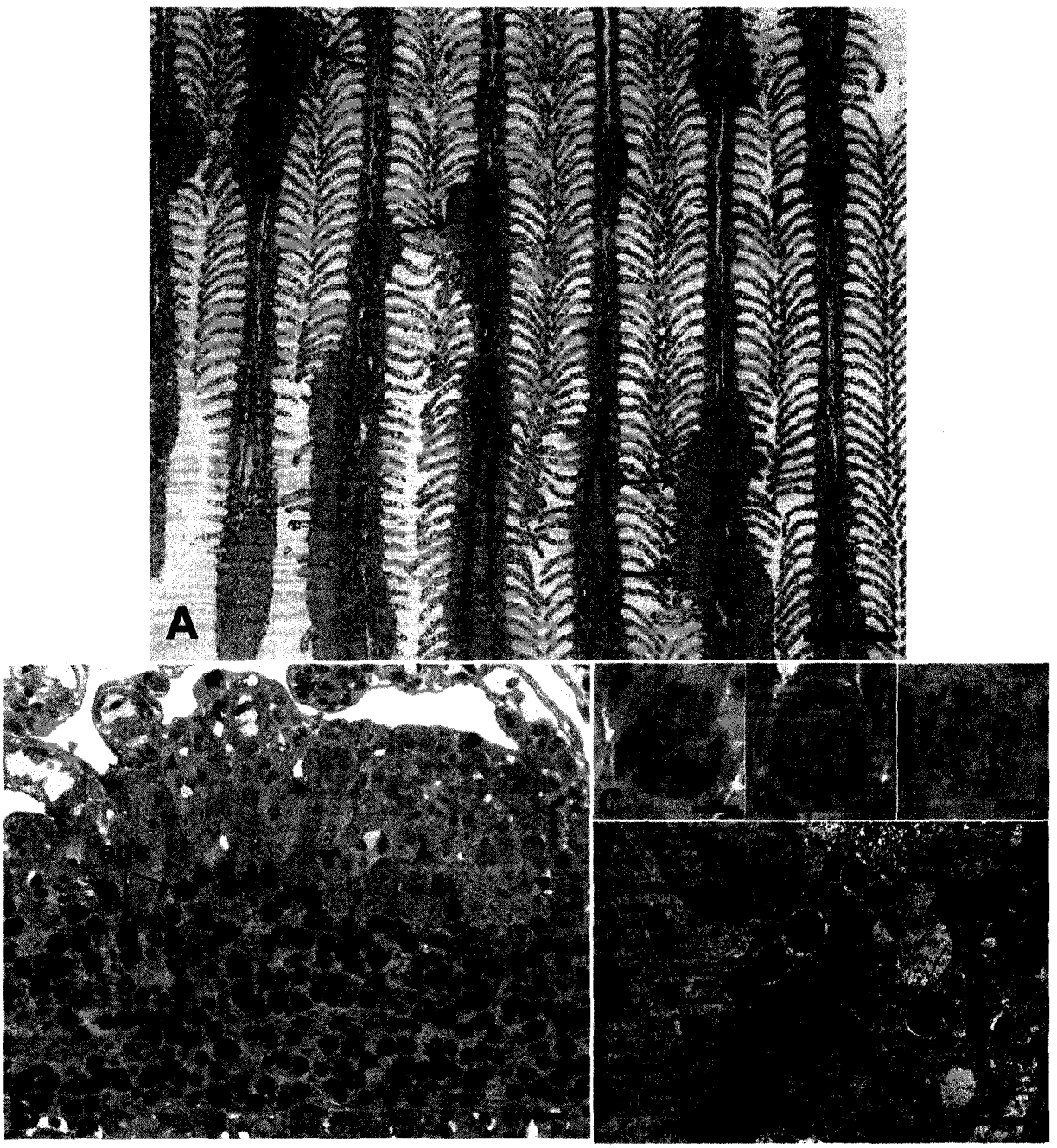


Figure 6.9. Eosinophilic granule cell (EGC) response in *Loma*-infected, indomethacin treated fish at 11 weeks PE. **A.** EGC infiltration within the gill filament is frequently adjacent to areas with lamellar fusion (arrows). (Bar= 300 μm). **B.** A lesion with lamellar fusion and lamellar regeneration (arrowheads) adjacent to a severe accumulation of EGCs within the gill filament. (Bar= 20 μm) **C, D, E.** Various examples of EGCs in mitosis as seen with HRLM. (Bar= 2 μm) **F.** A transmission electron micrograph of a EGC with intact granules and a mitotic nucleus. (Bar= 2 μm)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6.5 DISCUSSION

6.5.1 Experiment 1. Dosage effects of indomethacin

The work herein shows that there is a detrimental effect to fish chronically fed medium and high concentrations of indomethacin. Mortalities occurred in all the treated groups in a dose responsive manner and no mortalities occurred in the control group. Mortalities were most prevalent in the high dose treatment group (33.33%), followed by the medium dose group (13.33%), and lastly the low dose group (3.33%). An important finding is that fish treated over the study period with 10 and 15 mg/kg daily had severe peritonitis and ulcers in the anterior intestine. The abundant proteinaceous and plant material that was seen within the peritonitis likely originated from the feed. It is probable that ulcers within the GI tract perforated and the feed within leaked out into the peritoneal cavity. The bacteria inhabiting the peritoneum may also have originated from the GI tract as a result of the perforation. The inflammation and bacteria likely damaged the muscle of the body wall, leading to a transmural ulcer that extended from the peritoneum to the epidermis. This is consistent with the external finding of necrosis in the muscle of the peritoneal cavity and the gross skin lesions which were described. Granulomatous peritoneal inflammation frequently occurs in Atlantic salmon, *Salmo salar* when given vaccines administered through the peritoneum. The inflammatory response occurs around the oil adjuvant and characteristically consists of macrophages, foreign body type giant cells, and proliferating fibroblasts (Ferguson 2006). This reaction is similar to the peritonitis seen in this study.

The toxic side effects of indomethacin have been thoroughly investigated for mammals and damage to the gastrointestinal tract is widely recognized (Brodie et al. 1970, Yamada et al. 1993, Evans and Whittle 2003, Thiefin and Beaugerie 2005). It is reported that 100,000 humans that use NSAIDs are treated for adverse GI effects and that 15% of them die of their complications. It is also thought that small animals such as cats and dogs are more sensitive to the consequences of NSAIDs than are humans (Bergh and Budsberg 2005). The early pathogenesis of gastrointestinal toxicity is reported to involve an increase in intestinal permeability, where the mucosa becomes vulnerable to injury from the bile salts and intra-intestinal bacteria. The bacterial injury leads to inflammation and intestinal ulcers, which can contribute to perforation and bleeding (Evans and Whittle 2003, Thiefin and Beaugerie 2005). Bacterial translocation, which occurs when bacteria cross the intestinal membrane into extra-intestinal sites, can occur and it has been demonstrated that treatment with antibacterial compounds during treatment with indomethacin leads to less severe intestinal injury and reduced bacterial translocation (Evans and Whittle 2003). This is the first study to examine the toxic side effects of indomethacin in fish, therefore the pathogenesis is unknown. The intestinal perforation and inflammation in the muscularis of the anterior intestine seen in this study with rainbow trout is consistent with the indomethacin-induced lesions of mammals. Bacterial translocation from the GI tract to the peritoneum was also seen in the present study.

It was interesting to observe similar gastrointestinal toxicity in rainbow trout treated with indomethacin to that described for mammals. Due to variable feeding

responses among fish within a group it is likely that some fish may receive higher doses than others; therefore, injection may be a better method if precise dosing is required. According to this study, chronic per os treatment of indomethacin daily at concentrations of 10 mg/kg or more resulted in severe toxicity. These doses may be tolerated with short duration treatments; the first mortality in the 10 mg/kg group was observed 12 days after the start of treatment. It appears that 5 mg/kg daily treatments are tolerated for prolonged periods, as only one mortality occurred in this group after 27 days of treatment. This study suggests that if indomethacin will be utilized as a therapeutic for inflammatory diseases in fish, then similar precautions used for mammalian treatment regimes must be taken.

6.5.2 Experiment 2. Effects of indomethacin on fish with MGDS

The chronic exposure model of *L. salmonae* in rainbow trout caused moderate infections and xenomas were encountered in various stages of development and degeneration in the week 8 sample. Considering the week 8 sample represents infections which are 6-8 weeks old, the tissue was ideal for observing xenomas during their rupture phase and a significant inflammatory reaction. The tissue in the week 11 sample, which represents infections that are 9-11 weeks old, was almost completely cleared of the parasite and the tissue was ideal for examining the clearance of the parasite and the recovery/healing stage of the gills. The chronic exposure model in this study was still not representative of the gills of clinically infected Chinook salmon because the parasite was still only present within a three week infection period, but the duration of xenoma rupture and inflammation may

have been extended compared to the single dose exposure method. Considering that the majority of xenomas were ruptured by week 11 PE, the chronic exposure model is more similar to the single dose exposure model in rainbow trout rather than that which is observed in clinically infected Chinook salmon.

The gills in the week 11 sample contained several occurrences of lamellar regeneration and fully recovered lamellae with pillar cells. Teleost fish are known to fully regenerate various tissues without scarring and fibrosis which is common in the repair of many mammalian tissues (Poss et al. 2003). Gill lamellae have been reported to regenerate following damage caused by hydrogen peroxide exposure (Speare et al. 1999) and full recovery by regeneration was reported 14 days after exposure to chloroanilline (Burkhardt-Holm et al. 1999). In this study lamellar regeneration was first observed as a small group of undifferentiated cells that aggregated under the basement membrane of the regenerating lamellae; some of these cells were in mitosis, so it is possible that these cells migrate to the area from the gill filament and once there they proliferate. The aggregate of undifferentiated cells at the base of the regenerating lamellae is similar to blastema formation, which is typical for epimorphic regeneration in the teleost caudal fin (Santos-Ruiz et al. 2005, Poss et al. 2003, Nakatani et al. 2007). The populations of cells at the base of the lamellae likely migrate up the lamellae and mature into pillar cells, which are modified endothelial cells that provide structural support for the lamellae.

High doses of indomethacin treatment in the chronic infection model of MGDS did not delay the rupture or clearance of xenomas from the gills. It has been

suggested by the accumulation of inflammatory cells around mature xenomas at week 6 PE that inflammatory cells could initiate the breakdown of xenomas (Rodriguez-Tovar et al. 2003) and it was believed that indomethacin would delay the breakdown and clearance of the parasite. Contrary to this hypothesis, indomethacin treatment did not prevent accumulation of inflammatory cells around xenomas and inflammatory lesions caused by ruptured xenomas were similar in the indomethacin treated and untreated fish. There also did not appear to be an effect of indomethacin on the regenerative capacity of the gill, since both the treated and untreated groups showed evidence of lamellar regeneration. A previous report showed that aspirin and indomethacin treatment inhibited the regeneration of the caudal fin in tilapia, *Tilapia rendalli* and carp, *Cyprinus carpio*; the inhibition was attributed to the disruption of the collagenous scaffolding in the regenerating dermoskeleton (Bechara et al. 2000). It is possible that indomethacin had no effect on regenerating gill lamellae as seen in this study because it would be expected that there would be less collagen required for gill lamellar regeneration compared to the regeneration of a caudal fin.

An interesting finding was the severe infiltration of EGC's within the gill filament localized near lesions caused by xenoma rupture in indomethacin treated fish. Although fish in the untreated group contained EGC's in the filament, they were arranged around the filament tips and not associated with areas of lamellar fusion as seen in the indomethacin treated group. There is considerable evidence that EGCs of fish are homologous to mast cells in mammals (Reite and Evensen 2006). In the indomethacin treated group the EGCs were most commonly accumulated

adjacent to areas of lamellar fusion, but they did not typically infiltrate the lamellae. The accumulation of EGCs in the gill filament caused swelling of the tissue. In mammals mast cells are known to accumulate during some forms of chronic inflammation (Hay et al. 1988) and are an important cell type during wound healing in the skin of humans (Trautmann et al. 2000). Transforming growth factor-beta is also known to be a strong chemoattractant for mast cells in mice (Gruber et al. 1994), supporting the idea of mast cells being involved in wound healing. In the present study mitotic EGCs were commonly observed in the gill filament, which coincides with a previous report of mitotic EGCs in the gill filament of healthy rainbow trout (Flano et al. 1997). The common finding of mitotic EGCs within the lesions suggests that the accumulation of these cells is at least in part a result of local proliferation of the cells. Contrary to this, the mast cell accumulations around the fibrotic border of wounds in human skin are attributed to recruitment from the peripheral blood rather than local proliferation of mast cells (Trautmann et al. 2000). The reasons for seeing a more pronounced EGC response adjacent to lesions in indomethacin treated fish than in untreated fish is unknown, but it may be related to effects that indomethacin may have on the release of mediators by the EGCs. If some functional EGC mediators that are normally released are inhibited then it may interfere with the negative feedback loop and recruitment of EGCs may continue due to a lack of an inhibitory signal. Anti-inflammatory treatments have been shown to have effects on mast cells in mammals. Some NSAIDs can directly inhibit mast cell degranulation by inhibiting the lipoxygenase pathway of arachidonic acid metabolism, but

indomethacin, a COX inhibitor is not reported to do so (Chi et al. 1982). A study conducted by Grupe and Ziska (1994) showed that COX inhibitors under certain conditions could inhibit histamine release from mast cells. High doses of acetylsalicylic acid inhibited mast cell degranulation and the release of TNF-alpha and IL-6, although this is thought to be independent of COX activity and prostaglandin synthesis inhibition (Mortaz et al. 2005).

The chronic exposure model was an adequate model to study the effects of indomethacin on the inflammatory response in rainbow trout gills with MGDS. The work herein suggests that indomethacin does not reduce the inflammatory response in *Loma* infected tissues and in fact seems to cause a more pronounced EGC reaction. The rainbow trout model did not have branchial vasculitis to the same degree as clinically affected Chinook salmon, and thrombosis was not common, therefore the effects of indomethacin on thrombosis could not be determined. There is evidence that fish leukocytes produce prostaglandins through the COX pathway and that indomethacin has an inhibitory effect on this pathway (Hill et al. 1999, Zou et al. 1999, Van Anholt et al. 2003, Fast et al. 2005), but this study failed to show that indomethacin can cause reduced inflammatory lesions as seen with histopathology even at doses high enough to cause treatment related mortality. It is possible that although no difference was observed histologically, there were differences in the cytokines expressed by the leukocytes within the lesions. The dose of indomethacin used was very high and caused mortalities in all the treatment groups. It is possible that if a lower and more therapeutic dose was used then different results could have

been obtained. The combination of the adverse reactions induced by indomethacin in fish and the failure to reduce inflammatory lesions in the MGDS model in rainbow trout suggests that this drug cannot be supported for use as a treatment for fish with MGDS.

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7 GENERAL DISCUSSION

7.1. CONCLUSIONS AND DISCUSSION

Microsporidial gill disease of salmonids (MGDS) causes seasonal economic losses in the Chinook salmon aquaculture industry (Kent 1999). The disease can result in 10% cumulative mortality in fish that are in their second year in the ocean netpens (Chapter 3). To the farmer these represent significant economic losses because the fish that succumb to MGDS are ones that have the most significant investment in them, in terms of maintenance and rearing for the two years in saltwater. Although avoidance of the parasite would be the most efficient management technique to reduce prevalence of MGDS in farms, it remains difficult because the parasite is indigenous throughout the region where Chinook salmon are farmed (Kent 2000). Treatments against *Loma salmonae* have proven to be difficult due to their intracellular localization. Through the thesis, host-pathogen relationships were examined in order to better understand the pathogenesis of MGDS. It is believed that by understanding both how the parasite utilizes the host cell for development and how the host inflammatory response may contribute to the disease, therapeutic manipulations may be developed to either inhibit parasite development or reduce gill inflammation associated with disease.

Anti-parasitic compounds against *L. salmonae* would be useful for aquaculture by minimizing the numbers of parasites or delaying the development of xenomas in the gills of affected fish. The efficacy of several therapeutic agents aimed at reducing levels of *L. salmonae* infections in the gills were assessed by Speare et al. (1998a, 1999a) and Becker et al. (2002) who found that quinine

hydrochloride, fumagillin, and albendazole delayed xenoma development (Speare et al. 1998a, 1999a) and monensin reduced the numbers of xenomas in the gills (Becker et al. 2002). In studying the parasite development within xenomas with TEM, a theory was proposed suggesting *L. salmonae* uses the intracellular process of autophagy to aid in parasite development (Chapter 2). It was suggested that the parasite derived its outer membrane and formed its parasitophorous vacuole from the host endoplasmic reticulum, which surrounded the parasite during autophagy. If in fact the parasite development is aided by autophagy then it may be possible to inhibit its development within the host cell by applying drugs to affect the autophagic process. In farmed salmonids, in order to be effective, anti-parasitic compounds would likely have to be administered during the early stages of infection. Becker et al. (2002) showed that monensin treatment most efficiently reduced numbers of parasites in the gills when applied during or one week prior to exposure to the parasite. The early administration of these compounds would require farmers start treatment prior to the presence of clinical signs of MGDS on the farm. If MGDS is a seasonal problem at farms, then it is possible for farmers to predict the time course for disease outbreaks based on a temperature model used to predict xenoma development in the gills (Speare et al. 1999b). Drugs that inhibit parasite development have the obvious benefits of reducing parasites in the gills and thus reducing disease intensity, but drugs that delay the formation of xenomas may have benefits in aquaculture as well. Typically, outbreaks occur in late summer and if the development of the parasite is delayed then disease outbreaks may be pushed to the fall when water temperatures decline and more dissolved oxygen is present in the

water (Speare et al. 1999a). With the hypothesis of *L. salmonae* utilizing the intracellular process of autophagy, new therapeutic drugs can be assessed targeting this cellular process in attempts to reduce infection intensity in the gills of fish.

Targeting the inflammatory response during the rupture of parasitic xenomas with anti-inflammatory compounds is a promising treatment approach for MGDS. Treating the inflammatory response has the benefit of being able to treat fish during a later stage of infection when they would be showing clinical signs of disease. In chapter 3 the ultrastructural pathology in the gills of netpen reared Chinook salmon with MGDS showed that disease in fish was associated with an extensive cellular infiltration of neutrophils and macrophages, and numerous vascular changes including thrombosis within arteries and arterioles and neovascularization during chronic inflammation. The common finding of vascular thrombosis and inflammatory cell infiltration, which affected the architecture of the gills of netpen reared Chinook salmon, suggested the possible use of indomethacin as an anti-inflammatory compound. Indomethacin treatment is known to inhibit aggregation of rainbow trout thrombocytes (Hill et al. 1999) and ablate generation of prostaglandins, which are known modulators of inflammatory responses (Zou et al. 1999). When indomethacin was tested in an experimental model of chronically infected rainbow trout with *L. salmonae* there was no delay in the clearance of the parasite or in the healing of the tissue and treatment did not seem to have an anti-inflammatory effect (Chapter 6). It is possible that infecting rainbow trout with *L. salmonae* is not an adequate experimental model of MGDS in Chinook salmon. Rainbow trout serve as a great experimental model of parasite development in salmonid hosts (Speare et al.

1998b), but they do not represent the same degree of inflammation as seen in *L. salmonae* infected Chinook salmon. Chinook salmon are known to carry heavier infections than rainbow trout and the xenomas in Chinook salmon more frequently develop within the branchial filament artery (Ramsay et al. 2002). This explains the difference in vascular pathology between the two species. Thrombosis not commonly observed in the rainbow trout model of MGDS, is common in netpen reared Chinook salmon. The cellular responses of Chinook salmon and rainbow trout are similar, but lesions are less dramatic in rainbow trout because of the lighter infections. Indomethacin treatment has no effect on the clearance of *L. salmonae* or the associated inflammatory response in rainbow trout. However, its effect on thrombosis in Chinook salmon is not known.

An experimental model of MGDS in rainbow trout that has infections similar in intensity to Chinook salmon would provide a better model of gill inflammation. It was previously suggested that rainbow trout develop lighter infections because they have a degree of innate immunity that is lacking in Chinook salmon (Ramsay et al. 2002). Dexamethasone (dex) treatment in rainbow trout resulted in 5-fold heavier infections with *L. salmonae* than in control infected fish, as demonstrated in chapter 5. It is likely that dex reduces the innate immune response to the parasite and infections become more comparable to those seen in Chinook salmon. In addition to gills, other organs of rainbow trout were more heavily infected with the parasite (Chapter 5), again reflecting a similarity to Chinook salmon, as which commonly have systemic infections with the parasite (Hauck 1984). The pathology of the gills in dex treated rainbow trout was not characterized to date, but with increased infections

it may more accurately represent the severity of the inflammatory response seen in Chinook salmon. At high doses, dex also appears to have an affect on xenoma development as seen by reduced size and some degenerative changes (Chapter 5). It is likely that, for the purpose of an inflammatory model, dex can be given to fish only during the first week of infection. This model may be of use in future studies using *L. salmonae* as a model of gill inflammation.

Management strategies could help to control disease caused by *L. salmonae* if farm factors such as stress contribute to the pathogenesis of disease. It is a strange phenomenon that netpen reared Chinook salmon die from MGDS during their second year in the ocean when they are likely exposed to the parasite during their first year in the ocean. It was reported by Kent et al. (1999) that in experimental infections of *L. salmonae* in Chinook salmon a strong protective response is generated against the parasite. This would suggest that fish in their second year should have a protective immune response to the parasite. It was thought that even if fish had developed immunity to the parasite then stress caused by farm factors could still cause infection and MGDS. This was tested in chapter 5, and it was concluded that once rainbow trout generated an adaptive immune response to the parasite then dex-induced immunosuppression did not result in infections. This would suggest that stress alone cannot override the protective cellular immune response and other factors are involved in disease of fish in their second year. Considering *L. salmonae* is endemic, it is possible that fish in the summer of their first year in the ocean are exposed to light concentrations of the parasite leading to only light infections in some fish. If the pathogen persists through the winter then the following summer there will be

heavier concentrations of the parasite. There may be a small population of fish within the netpens that were never exposed to the parasite during the first year and these may be the fish most affected by the parasite in the second year. A 10% cumulative mortality was reported in fish (Chapter 3) and these may represent fish that did not generate an adaptive immune response because they were never infected with the parasite. Although this hypothesis may be possible it is not likely based on results from chapter 3, which demonstrate the presence of early parasite stages, fully developed xenomas and ruptured xenomas with an associated chronic inflammatory response within individual fish. This suggests that netpen reared Chinook salmon do not generate an adaptive immune response to the parasite as seen in a single dose exposure model of *L. salmonae* in rainbow trout (Chapter 5).

It is not understood why farmed Chinook salmon are unable to generate an adaptive immune response, but it may be partially related to the differences in the exposure methods to the parasite. It is likely that farmed fish are chronically exposed to high concentrations of the parasite, while in the experimental model fish are exposed to a single dose. If Chinook salmon are chronically infected with the parasite then their immune responses to the parasite may be different from fish given single dose infections in the laboratory. Chronic infections have been reported to cause T cell dysfunctions and anergy in mammals and these similar mechanisms may contribute to the prevalence of disease in Chinook salmon in their second growing season. During chronic viral infections in mammals, exhaustion of effector functions occurs in T cells; the virus specific $CD8^+$ T cells still remain, but they are unable to produce effector cytokines such as IL-2, TNF, and during late stages of exhaustion

IFN-γ (Shin and Wherry 2007). In addition to the effector funtions being affected, the T cells themselves may become dysfunctional and have low expression of cytokine receptors, lack antigen-independent homeostatic self-renewal, overexpress inhibitory receptors, display altered expression of genes involving chemotaxis, adhesion, and migration, and express a distinct set of transcription factors (Shin and Wherry 2007, Wherry et al. 2007). Chagas disease is an inflammatory disease caused by chronic infection with the protozoan parasite *Trypanosoma cruzi*. The disease causes inflammation in the muscle tissue, where the parasite persists; it has been demonstrated that the $CD8^+$ T cells in the chronically affected areas have limited cytotoxic and IFN-γ-producing activity when compared to splenic T cells (Leavey and Tarleton 2003). With evidence that viral and parasitic pathogens chronically infecting tissues cause dysfunctions and anergy in T cells it is possible that these phenomena occur in *L. salmonae* infected Chinook salmon.

Considering the difficulty in treating this intracellular parasite the best method is prevention by limiting exposure to the parasite. Prophylactic treatment with a vaccine against the parasite may be an efficient method for avoiding infections with the parasite in the gills. Speare et al. (2007) demonstrated that a killed whole spore vaccine derived from the SV low-virulent parasite strain can induce protection in rainbow trout that results in an 85% reduction in xenomas in the gills compared to unvaccinated controls. The efficacy of this vaccine is yet to be tested in Chinook salmon and its general effectiveness needs to be demonstrated in farmed salmon, considering the results demonstrated in chapter 3 suggest that farmed Chinook salmon do not generate an efficient adaptive immune response. If the vaccine proves

successful in reducing infections in Chinook salmon then it may be successful in farms when applied to fish prior to their introduction into seawater netpens. Another promising treatment method is the use of anti-inflammatory treatments mixed into the feed. This method, instead of prophylactic treatment, could be applied to fish that are showing clinical signs of disease. It is apparent that disease is associated with a severe inflammatory response (Chapter 3) and treating this response may reduce clinical signs associated with the parasite and thus reduce mortalities.

MGDS was demonstrated to be a good disease model to study the inflammatory response of fish. The full characterization of pathology and host responses to the parasite during disease was described in chapter 3. These findings indicate that neutrophils are an important component of the inflammatory response to the parasite in both Chinook salmon and the rainbow trout experimental model. *L. salmonae* spores were frequently engulfed, but once internalized there was no evidence of subsequent fusion with lysosomes or degradation of the parasite. Empty spores, which only consisted of the spore wall, were also found within neutrophils and there were no signs of degeneration of the spore wall in these cells. Rodriguez-Tovar et al. (2002) suggested that infections may be carried to the gills through the blood by an infected white blood cell. It is possible that spores inhibit intracellular degradation within neutrophils, and these cells may be responsible for carrying the infections to the gills. Franzen (2005) also suggests the possibility for phagocytosis of spores to spread the infection to other cells. Autoinfection could occur within the individual fish if the germinated spores infect other host cells. It has been shown that autoinfection does not occur in rainbow trout, since infections are normally cleared

after 12 weeks PE and a second wave of infection is not seen. Autoinfection in rainbow trout may be prevented by the cellular protective response that is effectively generated by week 6 PE. On the other hand, in farmed Chinook salmon it is possible that autoinfection is occurring based on the presence of early parasite stages in the gills concurrently with ruptured xenomas (Chapter 3).

7.2 FUTURE DIRECTIONS

The work represented in this thesis has contributed to the understanding of the pathogenesis of *Loma salmonae* in the gills of salmonids and in doing so has generated numerous new questions that require more research for their verification. These include, *L. salmonae*'s ability to utilize the cellular process of autophagy to aid in parasite development, the presence of dendritic cells within both inflammatory lesions of MGDS affected fish and immune organs of healthy fish, and adaptive immunity, which may not be adequately generated in netpen reared Chinook salmon. In addition to verification of these questions, additional work should be directed towards identifying the innate immune mechanisms that affect susceptibility to *L. salmonae* and towards finding effective treatments for inflammation in fish.

There are at least two strategies that could be used to test the hypothesis generated in chapter 2, which suggests that *L. salmonae*, during development, utilizes the host cell's endoplasmic reticulum during autophagy to create a parasitophorous vacuole and a surface parasite membrane. One way is to use immunohistochemistry and electron microscopy. With this method it may be possible to find markers to the proteins found in the lumen of the host endoplasmic reticulum or to use a marker to

label the endoplasmic reticulum membranes. These markers could then be observed with TEM and it could be determined if the parasitophorous vacuole lumen contains proteins normally found in the lumen of the endoplasmic reticulum or if the parasitophorous vacuole membrane contains proteins that are found in the membranes of endoplasmic reticulum. A similar method was used to demonstrate that poliovirus-induced membranous structures contain both virus proteins and proteins associated with autophagy, thus demonstrating that autophagy is involved in the formation of the virus-induced membranous structures (Schlegel et al. 1996). Another way to verify this hypothesis is much more complicated and involves first finding an adequate cell culture method for *L. salmonae*. The cell culture can then be treated with wortmannin, a phosphatidylinositol 3-kinase inhibitor, a known inhibitor of autophagy (Blommaart et al. 1997), and the effects on parasite development can be observed with TEM. If the treatment has an effect on parasite development, this would provide evidence for the role of autophagy in parasite development. Work can then be directed towards using inhibitors of autophagy as a treatment method in reducing parasite numbers in the gills of infected salmonids.

The further characterization of dendritic-like cells that were described in chapter 4 is important in expanding the general knowledge of this cell type in the immunology of fish. The knowledge may be useful for vaccine development. First, a characterization of the cells would need to be conducted to determine that the cells biochemically resemble dendritic cells. This could be done by immunohistochemistry with known markers for antigen presenting cells. Dendritic cell-based vaccination strategies involve injection of dendritic cells that are pre

stimulated with a known antigen in order to induce a protective immune response to the specific antigen. This has been suggested to be a good strategy for prophylactic treatment against Leishmaniasis (Moll and Berberich 2001). If dendritic cells of fish could be isolated and put into cell culture then experiments on dendritic cell-based vaccines could be conducted. It is possible that this would be an efficient prophylactic treatment strategy for fish with MGDS because the protective response is cell mediated (Rodriguez-Tovar et al. 2006) and dendritic cells likely play an important role based on their presence within MGDS associated lesions. The results of the thesis also identified that the dendritic-like cells are present in the spleen of healthy fish, demonstrating that this organ could be used in immunohistochemical studies or in attempts to try to isolate this cell type.

The nature of the adaptive immune response in netpen reared Chinook salmon needs further investigation. Having an understanding for the reasons responsible for the apparent lack of protective immunity in farmed Chinook salmon would be important, because vaccination may be an appropriate control strategy against MGDS. Similar trials that were run on rainbow trout with dex treatment could be run on Chinook salmon to verify that the protective immune response is not affected by dex treatment. It would also be good to determine the extent of the protective immune response when fish are chronically challenged with a high concentration of the parasite, considering all the results demonstrating protective immunity examined during a single dose exposure model. The best way to identify if vaccination has potential in farmed salmon is to run vaccine trials in fish kept within netpens. This could include a cage within a netpen site that is given the vaccine, while fish in other

areas of the pens remain unvaccinated, thus serving as controls. This information would demonstrate if the vaccine works efficiently during a disease outbreak when there are high concentrations of the parasite in the area. The vaccine may be most effective in keeping the parasite numbers down in the first year, thus avoiding high numbers in the second year, so it would be important to demonstrate the effectiveness of the vaccine in fish before the parasite reaches the high concentration. This could involve running vaccine trials in a site where *L. salmonae* is known to be endemic and all the fish in the system would be vaccinated and allowed to grow for two years. The controls would be in a separate netpen system in a similar area, but with no vaccinated fish. After two years the prevalence, intensity of infections, and mortality could be compared between the two pens. The results of these trials would directly demonstrate the effectiveness of the vaccine in farmed Chinook salmon.

Further work on characterizing the cytokines involved with the inflammatory responses to *L. salmonae* would better characterize the nature of the response associated with MGDS. It is important to establish a method for measuring and comparing inflammatory responses between fish. This is important for testing anti-inflammatory compounds to assess their effectiveness. The current method to evaluate the inflammatory response is to use histopathology, but this method has flaws because only a 5 μm thick section is evaluated. It is difficult to compare lesions between fish when the lesions vary depending on their plane of section, for example similar lesions can appear mild if sectioned around the periphery or they can appear severe if sectioned through the center. This could be avoided if serial sections were cut for each fish, but this becomes laborious to cut and examine this many

sections. It would be helpful to have another approach to measure the inflammatory response and these may include measuring the expression of several cytokines in tissue, or to use a marker of inflammation and view the whole tissue with confocal microscopy. Establishing a consistent and accurate method to evaluate the degree of the inflammatory response to *L. salmonae* will be a critical first step in further identifying anti-inflammatory compounds for MGDS. Further characterization of the innate immune response to the parasite would help to understand the mechanisms involved in the innate resistance to the parasite that some fish have. Strains of Chinook salmon have demonstrated greater resistance than others (Shaw et al. 2000a) and Atlantic salmon and lake trout have been shown to be resistant to infections (Shaw et al. 2000b). First it would be important to show that resistance is attributed to innate immunity differences in the host and not parasite factors. This could be determined by suppressing the innate immune response of both Atlantic salmon and lake trout with dex and seeing if this increases their susceptibility to the parasite. If determined that the susceptibility differences are attributed to innate immunity then innate immune responses could be studied between resistant and non-resistant species.

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