Loma salmonae in Chinook salmon (*Oncorhynchus tshawytscha)*: improving detection, preventing infection, and increasing our understanding of the host response to a microsporidian parasite

by

Catherine Ann Thomson
B.Sc., Malaspina University-College, 2002

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in the Department of Biology

Supervisory Committee

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Abstract

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Loma salmonae is a microsporidian parasite that infects economically important Chinook salmon (*Oncorhynchus tshawytscha*) farmed in British Columbia, Canada. Here a variety of research efforts aimed at improving early detection and diagnostic tools, developing preventative strategies, and increasing understanding of the parasite/host interactions are presented. First, the development of chicken-derived polyclonal antibodies (IgY) specific for *L. salmonae* is described. These antibodies have proven useful for immunohistochemical detection of parasites very early in the infection process. Next, the immune-modulating effects of intra-peritoneal β-glucan inoculation of Chinook salmon are presented. Intensity of *L. salmonae* infection was significantly reduced in fish inoculated with β-glucan 3 weeks prior to parasite exposure, although prevalence was not reduced in these fish. Gene expression analysis of head kidney from glucan-inoculated fish measured at 1, 2 and 3 weeks post-inoculation (PI) revealed that the majority of differential expression occurred at 1 week. Pathways related to antioxidant defence, innate immune responses, antigen presentation, as well as oxidative metabolism were up-regulated in glucan-inoculated fish at 1 week PI. Finally, temporal gene-expression

analysis on gill and kidney tissue from Chinook salmon infected with *L. salmonae* is described. Analysis at 4 weeks post-exposure (PE) in the gill revealed an early up-regulation of gas transport, whereas numerous pathways including oxidative metabolism, antioxidant defences, monooxygenases and immune receptors were down-regulated in the gill at the same time point. Similarly, oxidative metabolism, antioxidant defences, and monooxygenases were down-regulated in the kidney at 4 weeks PE. However, there is evidence for a developing immune response over time. Antigen processing and presentation pathways were up-regulated in the kidney at 4 weeks and in both tissues at 8 weeks PE. In addition a number of immune receptors and genes involved with innate immune functions were also up-regulated at 4 and 8 weeks PE in the kidney.

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List of Acronyms and Abbreviations

γC	- Cytokine receptor gamma chain
AAT	- Alanine-aminotransferase
Ab	- Antibody
ADCC	- Antibody dependent cell-mediated cytotoxicity
Ag	- Antigen
AP	- Alkaline phosphatase
APC	- Antigen presenting cell
BSA V	- Bovine serum albumin fraction V
CTL	- Cytotoxic T-lymphocytes
FASL	- FAS ligand
FcR	- Fragment crystallisable (Fc) receptor
FITC	- Fluorescein-isothiocyanate
G-CSF	- Granulocyte colony stimulating factor
GGT	- Gamma glutamyltranspeptidase
GPX	- Glutathione peroxidase
G-R	- Reduced glutathione
G-Ox	- Oxidized glutathione
GST	- Glutathione-S-transferase
HBSS	- Hank's buffered saline solution
H_2O_2	- Hydrogen peroxide
IFN	- Interferon
Ig	- Immunoglobulin
IgSF	- Immunoglobulin super-family
IL	- Interleukin
IP	- Intra-peritoneal
ITAM	- Immunoreceptor tyrosine-based activation motif
ITIM	- Immunoreceptor tyrosine-based inhibition motif
JAK	- Janus associated kinase
KIR	- Killer immunoglobulin-like receptors
mAb	- Monoclonal antibody
MAC	- Membrane attack complex
MBL	- Mannose-binding lectin
METC	- Mitochondrial electron transport chain
MHC	- Major histocompatibility complex (I and II)
MRP	- Multidrug resistance transporter
NBT	- Nitro blue tetrazolium
NCC	- Nonspecific cytotoxic cells
NCCRP-1	- NCC receptor protein – 1
NITR	- Novel immune-type receptors
NK	- Natural killer cells
NKTag	- NK target antigen
NO	- Nitric oxide
$NO^{\cdot}$	- Nitric oxide radical
NO_2	- Nitrite

NO_3	- Nitrate
NOS	- Nitric oxide synthase
O_2^-	- Superoxide anion
OH^-	- Hydroxyl radical
pAb	- Polyclonal antibody
PBS	- Phosphate buffered saline
PCR	- Polymerase chain reaction
PE	- Post-exposure
PI	- Post-inoculation
PIm	- Post-immunization
PMT	- Photo multiplier tube
qRT-PCR	- Quantitative reverse-transcriptase polymerase chain reaction
RBT	- Rainbow trout
RNS	- Reactive nitrogen species
ROS	- Reactive oxygen species
SDS	- Sodium dodecyl sulphate
SDS-PAGE	- Sodium dodecylsulphate - polyacrylamide gel electrophoresis
SOD	- Superoxide dismutase
SSC	- Buffer made from Sodium chloride and sodium citrate
STAT	- Signal Transduction and Activators of Transcription
TCR	- T-cell receptor
T_H cells	- T- helper cells (lymphocytes)
T_C cells	- T - cytotoxic cells (lymphocytes)
TNF	- Tumour necrosis factor

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Dedication

I dedicate this to my wonderful husband and children. Without you nothing would work. I love you more than I can say. Thank you especially to Andy for believing in me when I did not believe in myself, and for your incredible patience throughout this long process.

“I would thank you from the bottom of my heart, but for you my heart has no bottom.”
~Author Unknown

Introduction

Microsporidians are obligate intracellular parasites with a wide host range, infecting many vertebrate species, including teleosts. *Loma salmonae* is a microsporidian with a direct life cycle that infects members of the *Oncorhynchus* genus, including wild and cultured species. *L. salmonae* has been described in wild Pacific salmonids, including coho salmon (*O. kisutch*) (Kent et al. 1989), sockeye salmon (*O nerka*) (Shaw et al. 2000), and Chinook salmon (*O. tshawytscha*) (Shaw et al. 2000). Chinook salmon are of particular interest due to their importance as a farmed species in British Columbia, Canada (Speare et al. 1998). Infected fish develop microsporidial gill disease (MGD) characterized by the formation of spore-filled xenomas in the gill that eventually rupture, provoking a strong inflammatory reaction that may result in severe branchitis and asphyxiation of the host (Kent et al. 1995, Speare et al. 1998). Infection of a new host begins with ingestion of a spore from the surrounding aqueous environment. When the spore reaches the host's gut it makes contact with an epithelial cell, triggering biochemical changes that cause the spore to evert its polar tube and inject its sporoplasm directly into the adjacent host cell. The parasite migrates, presumably through the vascular system to the heart and, eventually, the gill, where most xenomas form (Sanchez et al. 2001a). It is thought that transport between the initial site of infection and the ultimate site of xenoma development may occur within a phagocytic host cell such as a macrophage (Rodriguez-Tovar et al. 2002).

Although there is little data available for wild salmon, the impact of *L. salmonae* infection to fish farmers is large, and includes both direct (MGD), as well as indirect

effects (increased susceptibility to other diseases). Mortalities due to MGD can reach upwards of 30% in some years, and the economic impact of these losses is maximized because most mortality occurs in fish nearing market-size (Kent et al. 1989). As such, research efforts are underway to improve early detection and diagnostic tools, develop preventative strategies, and increase understanding of the parasite/host interactions in the hope of preventing infection or lessening its impact.

Current detection and diagnosis of *L. salmonae* is either by direct examination of gill or gill clips, polymerase chain reaction (PCR) or microscopic examination of histological preparations of gill tissue. These techniques are valuable, although each has its own limitations. For example, estimating infection severity in histological preparations is both time-consuming and subjective. A more sensitive, specific and quantitative alternative to traditional stains would allow earlier diagnosis and more accurate assessment of the number of infectious organisms, aiding in timely management decisions.

In order to lessen the impact of a potential pathogen, strategies include preventing contact between the parasite and host, or preventing the development of disease in exposed fish, specifically through administering a vaccine or more generally through administration of an immune-stimulant. Unfortunately, preventing *L. salmonae* exposure is not practical for the aquaculture industry in British Columbia, since salmon are held in aquatic net pens, where free floating spores may be present in the environment. Disease prevention is a more viable alternative, as there have been promising findings in the area of immune-stimulation (Guselle et al. 2006, Rodriguez et al. 2009). The study of immunostimulants has expanded since the discovery of broadly specific receptors such as

Toll-like receptors (TLR) and pattern recognition receptors (PRR) on innate immune cells that bind to repeated molecular moieties displayed by pathogens, resulting in the activation of immune cells (Dalmo & Bogwald 2008). B-glucans are composed of repeating units of glucose that have been identified as potential immune modulators in a variety of species (Dalmo & Bogwald 2008). B-glucans show promise for mitigating *L. salmonae* effects in salmonids, with varying results based on the timing and nature of administration (Guselle et al. 2007, Dalmo & Bogwald 2008). Intra-peritoneal (IP) administration of β-glucan in rainbow trout (RBT) reduced the prevalence and intensity of *L. salmonae* infection (Guselle et al. 2006).

In addition to potential prevention strategies, it is important to further develop an understanding of parasite/host interactions. Microsporidian species infect a wide host range that includes a variety of commercially important fish and animals, as well as humans. For example there has been a dramatic increase in the prevalence of the intestinal parasite *Encephalitozoon cuniculi* in immuno-compromised AIDs patients (Didier et al. 1996). Although the immune systems and responses of fish vary from those of mammals, it is possible that a better understanding of the host/parasite relationship between microsporidians and Chinook salmon will not only aid in the fight against *L. salmonae*, but against other microsporidian species as well.

With these issues in mind research was undertaken to increase understanding of *L. salmonae* infection in Chinook salmon, both by improving detection techniques, and by increasing understanding of fish defence responses and host/parasite interactions. The following work is divided into 5 sections. Chapter 1 contains a review of current understanding of immune and defence responses of fish, specifically as they pertain to

interactions with intracellular parasites. Chapter 2 details the development of *L. salmonae*-specific polyclonal IgY from chickens that allows highly specific histological localization and visualization of developing xenomas very early in the infection process. In chapter 3 the efficacy of β-glucan as an immune-stimulant was assessed via measurements of prevalence and intensity of *L. salmonae* infection in glucan-inoculated Chinook salmon. In addition, chapter 3 includes a summary of differential gene expression analysis in glucan-inoculated fish. Chapter 4 consists of a summary of temporal gene expression analysis in *L. salmonae*-infected Chinook salmon not treated with β-glucan. In addition, variations in observable responses to *L. salmonae* infection are highlighted through observations of histological sections of gills from infected fish. The results from all chapters are summarized in chapter 5 and discussed in the context of current research.

Chapter 1

Immune and defence responses of fish to intracellular parasites

Due to the growing importance of finfish aquaculture, researchers are actively attempting to understand diseases and parasitic infections that negatively impact the industry. Vertebrates have evolved complex protective systems that include immune functions as well as a variety of additional mechanisms that work together to protect the organism. Although the majority of information detailing these systems has come from mammalian studies, research is continuing to identify comparable defence systems in fish.

Defence responses to parasites pose particular challenges, since these organisms have evolved complex and highly effective mechanisms by which they are able to evade and manipulate their host's defence systems. Intracellular parasites take advantage of the immune-privileged state that exists inside individual host cells. While inside a cell, these parasites are shielded from direct attack by much of the host defence repertoire. This shielding can be so effective that, in some cases, intracellular pathogens are able to completely transform the morphology of their host cell without triggering an effective immune response (Kent et al. 1989).

Intracellular parasites, such as the microsporidians, have specialized apparatus that allow them to penetrate the host cell membrane and inject their infective agent (sporoplasm) directly into the cytosol (Franzen 2005). The microsporidian parasite *Loma salmonae* enters its fish host by such a mechanism, triggered when a mature spore is

ingested by the host and comes into contact with a gut epithelial cell (Sanchez et al. 2001a).

Although the specific patterns of immune response are unique to each parasite-/host-species pair, the majority of studies indicate that cell-mediated immunity is the primary mechanism by which mammals develop an effective response to intracellular parasites. However, teleosts may rely more heavily on innate immune mechanisms than mammals do, and if that is the case such differences may be reflected in their responses to intracellular parasites. As research of teleost immunity continues it is apparent that, like other vertebrates, fish possess highly specialized cells in both the innate and adaptive branches of the immune system that are capable of attacking and destroying infected cells in order to eliminate pathogens within them.

1-1. Defence Responses to Intracellular Pathogens

1-1a. Cells of the Innate System

Innate effector cells make up an important part of the early response to pathogens. These broadly specific cells recognize repeating molecular patterns on pathogenic organisms and infected cells. Innate cells of the mammalian system include monocyte/macrophages, granulocytic neutrophils, and natural killer (NK) cells, whereas fish appear to possess an additional class of cells termed non-specific cytotoxic cells (NCC). Monocyte/macrophages and neutrophils are phagocytic cells, capable of engulfing pathogens and destroying them, as well as contributing to additional immune functions through the release of cytokines. NK cells and NCC cells have cytotoxic effects, inducing target-cell death through apoptosis (Shen et al. 2002).

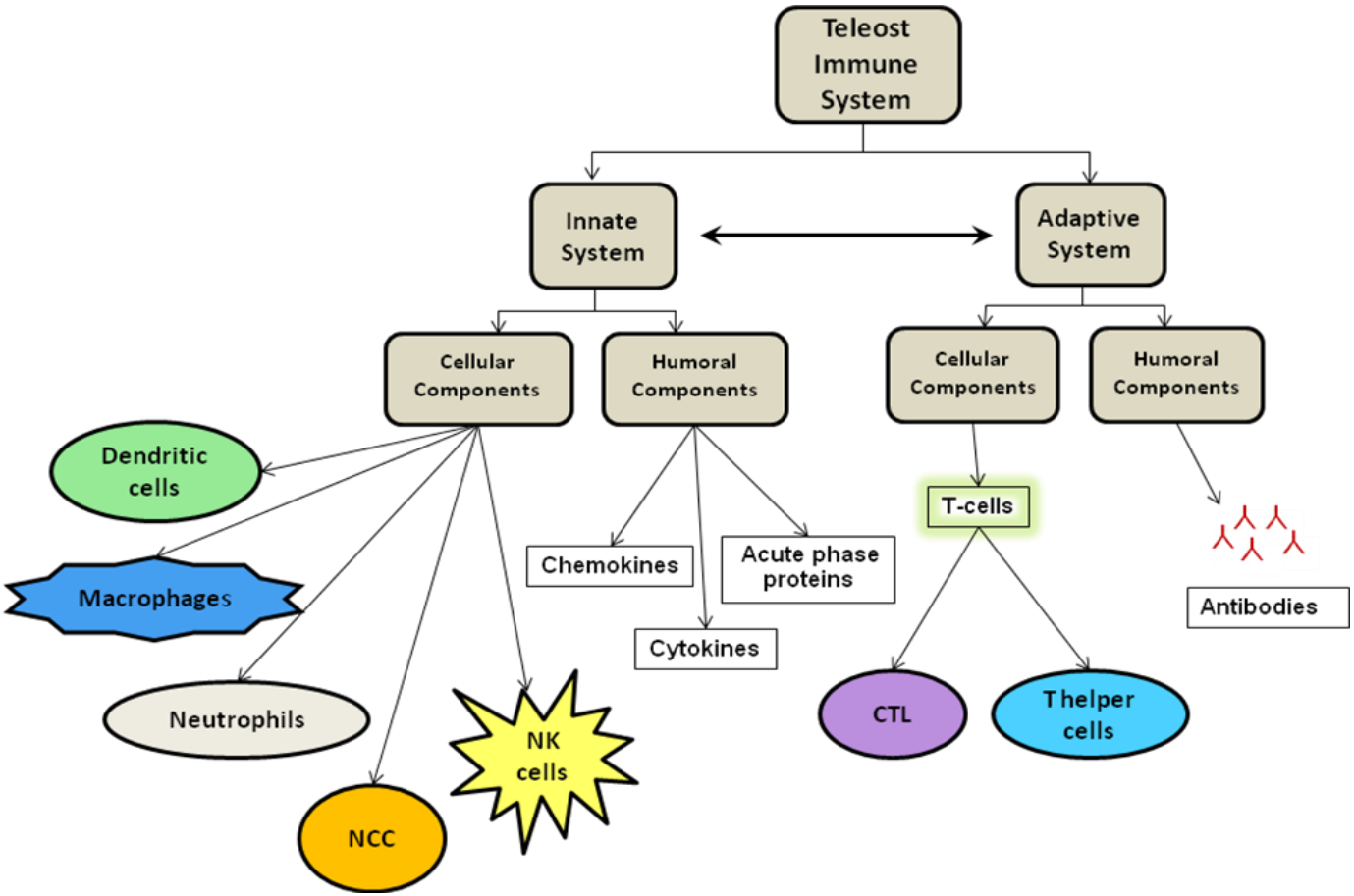

Figure 1.1. An overview of the teleost immune system. Like the mammalian system, it is divided into innate and adaptive factors that interact in order to mount effective responses. Individual components are described in the following sections.

1-1a.1. Monocytes/Macrophages

Monocytes are cells of the myeloid lineage, originating in the bone marrow of mammals and the hematopoietic kidney tissue of fish (Zapata et al. 2006). Monocytes are released to circulate through the bloodstream before migrating into tissues and differentiating into macrophages. Macrophages, as their name implies, are large phagocytic cells that make up one of the key components of the innate surveillance system. These phagocytes are broadly-specific cells, incapable of immunological memory, that recognize phagocytic targets through their expression of a variety of receptors for such ligands as toll-like receptor (TLR), antibody (FcR) and complement. Macrophages also act as antigen presenting cells (APC), and express MHC II on their surface. Under the influence of cytokines such as interferon-γ (IFN-γ), macrophages become activated upon phagocytosis of particulate antigen. Once activated, macrophages upregulate their expression of MHC II, making them more efficient APC.

Phagocytic cells such as macrophages and neutrophils produce antimicrobial and cytotoxic substances in order to facilitate the killing of ingested microorganisms. These substances are generated via mechanisms that are classified as oxygen-dependent or oxygen-independent, depending on their requirement for oxygen.

Oxygen-dependent reactions result in the formation of reactive oxygen species (ROS), or reactive nitrogen species (RNS), which are highly toxic to engulfed microorganisms (Goldsby et al. 2000). The mechanism by which ROS are generated by specialized immune cells is called the respiratory burst, beginning with the reduction of oxygen to superoxide anion (O_2^-), a reaction catalyzed by the enzyme NADPH oxidase. Much of the O_2^- generated is subsequently converted to other ROS, including hydrogen

peroxide (H_2O_2) and hydroxyl radicals (OH^-), either spontaneously, or by the enzyme superoxide dismutase (SOD) (Neumann et al. 2001).

Phagocytic cells of fish are capable of generating ROS by means of the respiratory burst. Respiratory burst activity has been well described in fish functional and biochemical studies, and *in vitro* assays to measure ROS are commonly used to evaluate the phagocytic activity of teleost immune cells (Secombes 1996, Neumann et al. 2001).

In addition to the oxygen-dependent cytotoxic ROS, phagocytic cells of both mammals and fish possess a variety of oxygen-independent, pre-formed antimicrobial substances to facilitate the killing and breakdown of ingested microorganisms. Macrophages and neutrophils contain degradative enzymes (proteases, nucleases, phosphatases, lipases, etc.) as well as antimicrobial peptides, stored within granules and lysosomes (Secombes 1996, Stafford et al. 2002). Upon ingestion of foreign particles the granules fuse with the phagosome, releasing their contents into the resulting phagolysosome and destroying the pathogen (Secombes 1996).

Some intracellular parasites have evolved to make use of their host cell's innate phagocytic defences in order to gain access into the cell. Once inside, the parasites are able to survive host killing by a variety of mechanisms. For example, macrophages of turbot and rats exhibit reduced levels of ROS after ingestion of viable microsporidian spores compared with killed spores (Leiro et al. 2000, Leiro et al. 2001). Similarly, Kim et al. (1998) found that O_2- production was inhibited in macrophages of Ayu after ingestion of spores from the microsporidian *Glugea plegogloss*, suggesting a mechanism of parasite survival. (Kim et al. 1998).

1-1a.2. Neutrophilic Granulocytes

Neutrophilic granulocytes are phagocytic cells of the myeloid lineage. Granulocytes are so named for the presence of large granules in their cytoplasm that contain a variety of lytic enzymes and bactericidal agents. Neutrophils are highly mobile cells that are recruited in large numbers to sites of injury as part of an inflammatory response. Like macrophages, neutrophils recognize targets such as antibody and complement. Neutrophils are also known to generate ROS from respiratory burst (Secombes 1996). In addition to their phagocytic/killing role, acidophilic granulocytes in fish (functionally equivalent to neutrophilic granulocytes in mammals) express MHC II, suggesting that they may also play a role as APC in teleosts (Cuesta et al. 2006).

1-1a.3. Non-specific Cytotoxic Cells (NCC)

Non-specific cytotoxic cells are an additional class of innate effector cells first discovered in channel catfish that appear to be unique to teleosts. Since their discovery NCC have been described in other fish species including rainbow trout, (Evans et al. 1984, Greenlee et al. 1991), and are known to kill xenogeneic targets in vivo, including protozoan parasites of fish (Graves et al. 1985). NCC were originally characterized as possible "evolutionary precursors" to NK cells in mammals, based on their purportedly similar cytotoxic properties, as well as the lack of evidence at the time for a true NK cell population in fish (Evans et al. 1984). Since then, accumulating evidence has called this idea into question. The small, agranular NCCs are morphologically distinct from mammalian NK cells, which are larger and contain numerous cytotoxic granules. In addition, there is accumulating evidence for NK-like cells distinct from NCC (see below).

Whatever their possible evolutionary relationship to NK cells, NCC remain among the best studied of cytotoxic cells in teleosts.

The mechanism(s) by which teleost NCC kill their target cells is still being elucidated. Mammalian cytotoxic cells (including NK cells and CTL) are known to kill via two apoptotic mechanisms. The first involves interaction between cell surface molecules (such as tumor necrosis factor [TNF] family members FAS/FASL) on the cytotoxic and target cells, triggering death domains located on the target cell, and initiating a calcium independent pathway resulting in apoptosis. The second killing mechanism displayed by mammalian cytotoxic cells involves a perforin/granzyme-mediated lytic pathway that also ends in apoptosis of the target cell (Goldsby et al. 2000, Muller et al. 2003). It appears that mammals and teleost cytotoxic cells utilize similar killing mechanisms, with studies providing evidence for the existence of both apoptotic mechanisms in fish (Jaso-Friedmann et al. 2000, Bishop et al. 2002, Praveen et al. 2004, Praveen et al. 2006).

1-1a.4. Natural Killer Cells

NK cells are believed to play an important role in immune surveillance, being able to respond to foreign pathogens and altered self cells during the lag time required while adaptive defences are being activated. NK cells form a subset of lymphocytes that are characterized by their lack of recombined receptor genes (TCR^-, Ig^-) (Fischer et al. 2006).

Accumulating evidence points to the existence of true NK-like cells in teleosts. NK-like cell lines have been successfully derived from channel catfish peripheral blood

leukocytes, with particular subsets displaying unique target specificities as well as gene profiles (Shen et al. 2002).

Mammalian NK cells become activated via a mechanism that can be described as a "missing self" model. NK display both inhibitory and activating receptors on their surface, and it is the overall balance of these signals that determines whether a particular NK will become activated. Inhibitory receptors recognize determinants on self-MHC (MHC I), independent from presented antigen. If MHC I molecules are appropriately expressed on a potential target cell, then the NK cell will receive sufficient inhibitory signals to prevent it from initiating target cell lysis. Some tumour and virally-infected cells express decreased levels of MHC I, leading to a reduction in inhibitory signals, and allowing activation of the NK (Shen et al. 2002, Yoder et al. 2004).

Two structurally distinct families of activation receptors have been identified on mammalian NK cells: the first, a group of IG superfamily (IgSF) members termed killer immunoglobulin-like receptors (KIR), which interact with MHC I-like molecules; and the second, a group of C-type lectin receptors (eg. NKG2/CD94), that includes both activating and inhibitory forms. Similar cytotoxic cell receptors appear to exist in teleosts. Studies have allowed the identification of genes encoding IgSF receptors called novel immune-type receptors (NITR) that are putative orthologues of KIR (Litman et al. 2003). In addition, IgSF immune receptors distinct from NITR have recently been described for carp (Stet et al. 2005). There is also genomic evidence for the existence of teleost C-type lectin receptors specific for MHC I-like molecules that are proposed to be orthologous to mammalian NKG2/CD94 (Sato et al. 2003).

1-1b. Cell-Based Responses to Intracellular Pathogens - Cytotoxic and Accessory Cells of the Adaptive System

Functional studies have long indicated the existence of Ig(+) B-cells and Ig(-) T-cell-like lymphocyte subpopulations in teleosts, based on differential reactivity with B- and T-cell mitogens, acute allograft rejection, and mixed lymphocyte reactions (Miller et al. 1998). In addition, an increasing number of teleost Ig heavy (IgH) and light (IgL) chain isotypes have been recognized, allowing teleost B-lymphocyte subsets to be identified (Pilstrom 2002, Danilova et al. 2005, Hansen et al. 2005). The characterization of T-cells has been more difficult due to a lack of definitive T-cell-specific cell-surface markers. As a result, presumptive T-cell subsets have traditionally been characterized as Ig(-) T-like cells. However, the genes for all four T-cell receptor (TCR)-chains (α, β, γ, and δ) as well as CD4 and CD8 homologues have now been identified in teleosts (Nam et al. 2003, Moore et al. 2005, Moore et al. 2009).

1-1b.1. T Lymphocytes

T lymphocytes develop in the thymus, and are characterized by their expression of recombined T-cell receptors (TCR) (αβ-TCR or γδ-TCR), plus accessory molecules such as CD28 (Goldsby et al. 2000, Martins et al. 2004). αβ T-cells are generally divided into two functional subpopulations based on their expression of the molecular markers CD4, expressed on T helper (T_H) cells and/or regulatory T (T_{Reg}) cells, or CD8, expressed on cytotoxic T lymphocytes (CTL or T_C). $CD8^+$ T_C cells represent the antigen-specific cytotoxic effector cells of the adaptive immune system, and kill their targets via apoptotic mechanisms similar to NK cells: 1) an exocytotic perforin/granzyme mediated pathway, and 2) activation of death domains (eg.FAS/FASL). The role of $CD4^+$ T_H cells, as their

name implies, is to provide accessory "help", in the form of cytokine secretion to aid in the immune response.

Unlike B-cells, which become activated upon binding to soluble antigen via their membrane-bound Ig, T cell activation is said to be MHC-restricted, meaning that T-cells will only bind to antigen that is displayed in the context of a major histocompatibility molecule (MHC). The TCR of $CD8^+$ T_C cells bind antigen displayed by MHC class I (MHC I) present on "self" cells, which include all of the cells of the body except erythrocytes, in mammals, and including erythrocytes, in fish. $CD4^+$ T_H cells, on the other hand, bind specifically to antigen displayed by MHC II on the surface of antigen presenting cells (APC), which include macrophages, activated B-cells, and dendritic cells (Goldsby et al. 2000). Activation of antigen-specific T-cells stimulates clonal proliferation and differentiation into effector and memory T-cell subsets. Memory T-cells allow accelerated response to subsequent antigenic challenge (Schepers et al. 2005).

The majority of T-lymphocyte information has been derived from studies on αβ T-cells. Another group of T-cells, which are relatively poorly understood, are the γδ T-cells, whose TCR is made up of a γ-, and a δ-chain. Unlike αβT-cells, which recognize specific antigen in an MHC-restricted manner, γδ T-cells act as innate effectors, recognizing intact proteins and organic molecules that are not presented in the context of class I- or class II-MHC, and which are not presented by APC. In mammals, γδ T-cells migrate from the thymus and primarily populate epithelial tissues, where they have been suggested to be important in early immunity against invading pathogens. γδ T-cells may also have additional roles, amplifying dendritic cell functions as well possibly acting as APC (Casetti & Martino 2008).

Yet another group of poorly understood T-cells are the mammalian NKT cells. NKT-cells are αβ T-cells that derive their name from their expression of cell-surface markers characteristic of natural killer cells. NKT cells differ from conventional αβ T-cells by the limited diversity of their TCR. The TCR of NKT-cells are composed of an invariant α-chain combined with a β-chain of limited diversity. Rather than recognizing specific antigenic peptides complexed with MHC, NKT-cells recognize glycolipid antigens displayed by MHC I-like cell surface molecules termed CD1d. Activated NKT-cells can play an important role in determining the direction of an immune response through their ability to produce both T_H1 (IFN-γ) and T_H2 (IL-4) type cytokines. NKT-cells are important for mammalian defence against intracellular parasites (Ishikawa et al. 2000), and may represent a link between the innate and adaptive immune systems (Yu & Porcelli 2005).

As mentioned, there is now conclusive evidence for true T cell populations in fish (Levraud & Boudinot 2009). Researchers are continuing to identify T-cell associated molecules, including TCR, as well as CD4 and CD8 homologues (Moore et al. 2005, Moore et al. 2009). The genes for all four TCR chains (α, β, γ, and δ) have been identified in numerous teleost species, including Atlantic salmon (Yazawa et al. 2008a, Yazawa et al. 2008b). MHC genes have also been characterized for fish and, similar to mammals, MHC diversity in teleosts influences disease resistance (Kurtz et al. 2004). MHC I has been characterized in several teleost species, including carp, zebrafish, and Atlantic salmon (Grimholt et al. 1993, Okamura et al. 1993, Takeuchi et al. 1995), and descriptions show functional homology to mammals (Nakanishi et al. 1999).

1-1b.2. TH Cells

T_H cells do not kill target cells or pathogens directly, but play a key role in the development of an immune response by recruiting innate cells such as macrophages to the site, and activating effector cells of both the innate and adaptive immune systems. Activated T_H cells are also largely responsible for determining the direction of the adaptive response toward a predominantly humoral (B-cell)- or cell-mediated (T-cell)-based immune response. In mammals, naïve CD4(+)T_H cells become activated by binding through their TCR to specific antigen displayed in the context of MHC II on antigen presenting cells (APC), including dendritic cells, B-cells, and activated macrophages. The identity of APCs in fish is still under investigation. Macrophages and B-cells have been identified as APCs in teleosts, with putative APC roles suggested for neutrophilic granulocytes and thrombocytes in fish (Secombes 1996, Kollner et al. 2004, Chaves-Polo et al. 2005, Cuesta et al. 2006). In addition, dendritic-like cells similar to human Langerhans cells have recently been identified in the spleen and head kidney of salmonids (Lovy et al. 2009). Under the influence of the particular cytokines released by innate immune cells into the surrounding mileau, ligation of the TCR induces T_H cells (via TCR/CD3 signal transduction) to proliferate and begin secreting cytokines (Schepers et al. 2005).

The particular array of cytokines secreted by T_H can be divided into a Type 1 or Type 2 profile, similar to NK cells. The T_H1 (Type1) cytokine response is characterized mainly by the production of IFN-γ and interleukin-2 (IL-2). Cytokines secreted by T_H1 cells are important for the development of adaptive cell-mediated responses. For example, IFN-γ activates monocytes/macrophages, (Sinigaglia et al. 1999). The T_H2

subset, on the other hand, secretes Type 2 cytokines such as IL-4, IL-5, and IL-10, which are known to promote B-cell activation.

1-1b.3. Regulatory T Lymphocytes

In addition to the well characterized T_C and T_H cell subsets, an additional subset of lymphocytes termed regulatory T (T_{reg}) cells have gained prominence for their important role in regulating the immune response. T_{reg} are classified as either natural T regulatory cells (nT_{reg}) or adaptive T regulatory cells. nT_{reg} are self antigen specific cells that are characterized by their expression of $CD4^+$ along with CD25 and Foxp3. Adaptive T_{reg} cells may be mature T cells that become activated without optimal antigen exposure, or mature T cells that are activated in the presence of inhibitory cytokines (Nandakumar et al. 2009). T_{reg} cells act to inhibit immune responses and protect against injury to tissues caused by inflammation in mammals (Bettelli et al. 2006). At present evidence for teleost-specific T_{reg} is lacking.

1-1b.3. Cytotoxic T Lymphocytes

Activation of naïve CD8(+)T_C cells occurs when TCR interacts with specific antigen displayed in the context of MHC I on altered self cells such as tumor, virus-, or parasite-infected cells. Activation of T_C requires strong co-stimulatory signals, which may be provided by dendritic cells presenting Ag/MHC I, aided by cytokine stimulation from activated T_H1 cells (Gaffen & Liu 2004).

As mentioned, it appears that cytotoxic cells (including NCC, NK and CTL) of both mammals and teleosts utilize similar killing mechanisms (Praveen et al. 2004). Mammalian CTL are known to kill their targets via one of two apoptotic mechanisms,

namely the FAS/FASL pathway, or the perforin/granzyme pathway, both of which initiate apoptosis of the target cell (Berke 1995, Goldsby et al. 2000).

1-1c. Humoral Factors

1-1c.1. Antibodies

Antibodies have numerous important immune functions, including opsonization, activation of complement, and antibody dependent cell-mediated cytotoxicity (ADCC) (Goldsby et al. 2000). For years it was believed that teleost fish display limited diversity of antibody isoforms compared with those of mammals. The predominant serum Ig in teleosts is a tetrameric homolog of mammalian Igμ, and an additional serum antibody isoform is a homolog of mammalian Igδ (Bengten et al. 2006). However, in addition to the previously identified teleost Igμ and Igδ heavy chain isotypes, the growth of sequence data has contributed to the discovery of additional heavy-chain isotypes, including Igτ, identified in zebrafish (Danilova et al. 2005), and Igζ in rainbow trout (Hansen et al. 2005), neither of which has yet been well characterized. A variety of light chain isotypes in different teleost species that may also contribute to antibody diversity (Pilstrom 2002).

1-1c.2. Complement

The complement system consists of more than 35 soluble blood proteins that play an important role in innate defence. Activation of complement triggers an enzymatic cascade capable of direct lysis of pathogens, opsonization, solubilization of immune complexes, and respiratory burst (Goldsby et al. 2000). The complement cascade can be activated by any of three distinct pathways - the classical, the alternative, or the lectin pathway - consisting of different sets of proteins. The classical pathway is triggered by the binding of complement proteins to antibodies complexed with soluble or cell-surface

antigens. The antibody-independent alternative pathway is activated when complement proteins bind to a variety of cell-surface moieties displayed by viruses, bacteria, and some tumor cells, among others. The lectin pathway is initiated by the binding of lectins, such as mannose-binding lectin (MBL), to sugars on carbohydrates or glycoproteins that are displayed by microorganisms. However they are activated, the three pathways converge at the critical C3 component. Each pathway activates a C3 convertase enzyme, which splits C3 into C3a and C3b. C3b is a potent opsonin, which is able to bind to the activating surface (bacteria, fungi, viruses, parasites) and also to complement receptors on phagocytic cells, promoting phagocytosis, respiratory burst, and antigen uptake by APCs. C3b is also a component of the next enzyme in the cascade, the C5 convertase, which breaks C5 into C5a, and C5b. C5b combines with additional proteins C6, C7, C8, and C9 to form the membrane attack complex (MAC), which is able to directly lyse target cells by forming pores in their membrane. C5a and C3a are anaphylatoxins, which are able to induce inflammation and elicit both innate and adaptive responses (Boshra et al. 2006).

The complement system of fish is similar, although apparently more complex, in both form and function to that of mammals (Sunyer et al. 2005). The complement cascade can be activated by any of the three pathways (classical, alternative, or lectin) converging, as in mammals, at C3. Unlike the single isoforms of key proteins found in mammals, however, multiple isoforms of C3 and C7 have been documented in fish (Sunyer et al. 1996, Sunyer et al. 1997, Papanastasiou & Zarkadis 2005). In addition, the cleavage of teleost C3 generates multiple anaphylatoxin derivatives corresponding to the C3 isoforms. Serum fractions containing anaphylatoxins isolated from fish are able to

dramatically enhance phagocytosis by head kidney and peripheral blood leukocytes in rainbow trout (Li et al. 2004). The complement system of fish is also active at low temperatures compared to mammals, allowing a strong innate response at temperatures prohibitive for components of the adaptive system (Sunyer & Lambris 1998). The broadened set of complement proteins of fish, as well as their extended activation range compared with the mammalian complement system provide evidence for the relative importance of complement in teleost immune function.

1-1d. Antioxidant defences

Antioxidant defences are generated in cells in order to combat ROS produced during oxidative stress, infections and inflammatory processes. ROS can be generated by several mechanisms, and the products of these reactions are highly reactive and capable of damaging cellular macromolecules. As discussed above, ROS are produced by specialized enzyme complexes in phagocytic immune cells as a killing mechanism aimed at eliminating pathogens. In addition, ROS can be generated as byproducts of metabolism in cells, or by enzymes such as cyclooxygenases (COX) or Cytochrome P450 monooxygenases. Whatever their origin, ROS must be quickly eliminated in order to protect cellular integrity. As a first line of defence, cells employ numerous enzymatic and non-enzymatic anti-oxidant mechanisms to eliminate ROS, but even with rapid detoxification, some cellular damage may occur. A second line of anti-oxidant enzymes act to minimize this damage by preventing free radicals from propagating redox chain reactions. Once they have been detoxified, the metabolites are removed from the cell via energy dependent efflux pumps (Figure 1-2).

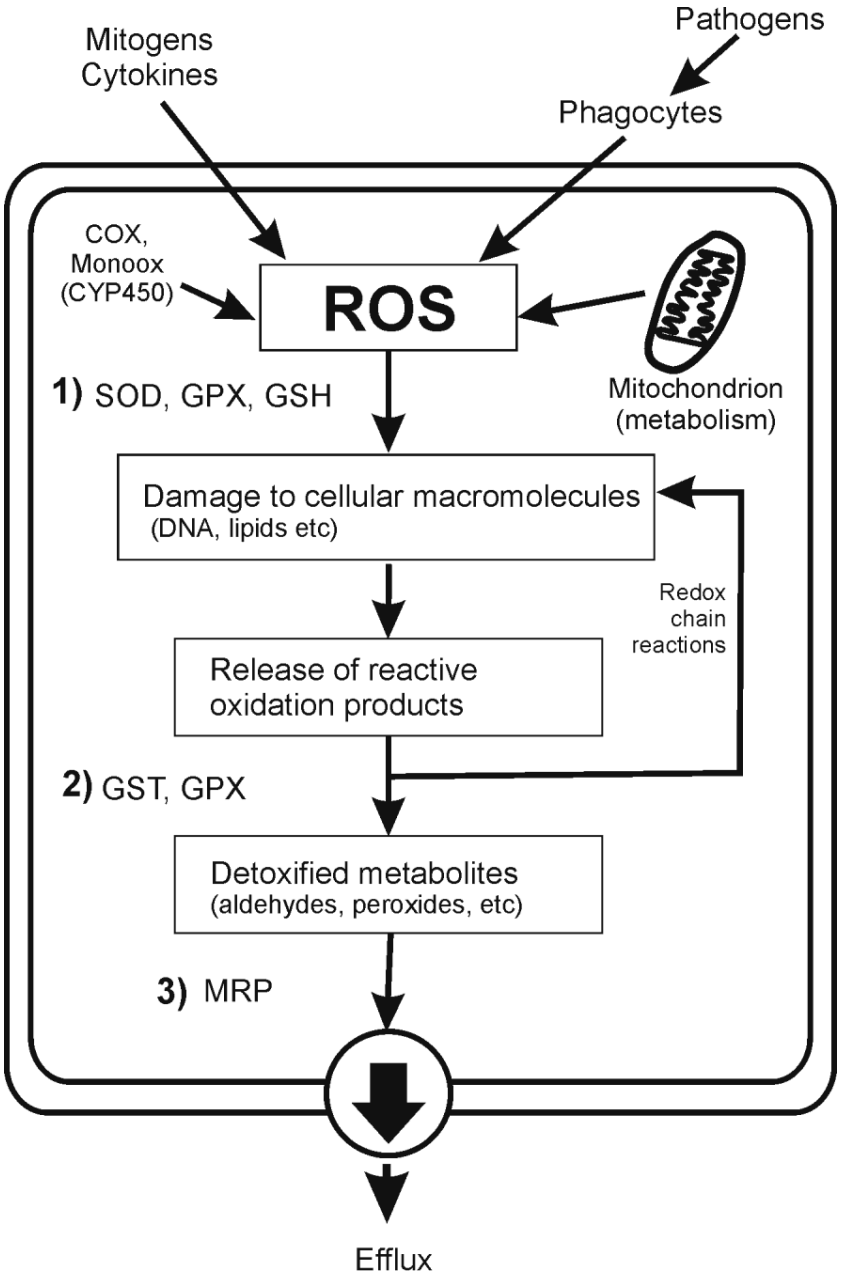

Figure 1-2. Antioxidant mechanisms.

Multiple levels of defence against reactive oxygen species (ROS). 1) Enzymes of the first layer include superoxide dismutase (SOD), glutathione peroxidases (GPX), reduced glutathione (G-R) and others that act to eliminate the superoxide anion and hydrogen peroxide. Second layer enzymes such as glutathione-S-transferases (GST) and GPX detoxify the resulting reactive oxygen products. 3) The resulting metabolites are eliminated from the cell by energy dependent efflux pumps such as the multidrug resistance protein (MRP). Adapted from (Hayes & McLellan 1999).

A high degree of similarity exists between the immune components of mammals and fish. The rapidly expanding sequence data becoming available for several different, and evolutionarily distant, teleost species supports this conclusion. However, as more immune-related genes and gene products are identified in fish, it is also apparent that many differences have evolved. Some components of the adaptive immune system show reduced activity at low temperatures (Manning & Nakanishi 1996). It is generally believed that, for this reason, poikilothermic vertebrates rely more heavily on innate defence mechanisms than do their mammalian relatives. Evidence of expanded repertoires of such innate components as the complement proteins would tend to support this idea. It is not clear, as yet, what effect such differences may have on the comparative immune response of fish and mammals to intracellular parasites.

1-2. Immune Responses to Intracellular Parasites

As outlined above, the vertebrate immune system has evolved into a highly complex, interconnected system capable of mounting defences against intracellular parasites. Once again, most investigations of immune response to intracellular parasites have focussed on mammals; however, because of the growing importance of finfish aquaculture there is increasing interest in understanding the defence mechanisms in teleosts. This section will review some of what is known about the immune response to intracellular parasites in mammals and identify areas where similar mechanisms may be at work in the immune systems of fish.

1-2a. Immune Recognition of Infected Cells and Parasite Strategies for Avoidance:

In order for infected host cells to be targeted for attack by immune cells, they must first be recognized as targets, or “altered self” cells. Generation of an effective CTL

response requires the participation of both T_C and T_H cells. CTL recognize cells displaying foreign Ag in the context of self-MHC I as targets for cytolysis. Appropriate antigenic peptides for display by MHC I are generated via the endogenous processing pathway (Figure 1-3). Foreign proteins are degraded in the cytosol by a proteasome complex, generating peptides that are translocated into the RER, where they can be complexed with the α- and β2-microglobulin- chains of MHC I. Ag/MHC I complexes are transported through the Golgi to the cell surface where, if present in high enough concentration, they may be recognized by antigen-specific CD8(+) T_C-cells.

Displays of antigen/MHC I mark altered-self cells for lysis, but in order for CTL to respond, they must receive sufficient co-stimulatory signals to become activated, which usually involves T_H-cell activation toward a T_H1 response. T_H cells become activated by binding to Ag displayed in the context of MHC II by APC. Antigen presentation by MHC II requires antigen processing through the exogenous pathway, which involves internalization via phagocytosis, followed by degradation of the pathogen into antigenic peptides within endocytic vesicles (Figure 1-3). MHC II components are transported from the RER, through the golgi and into endocytic compartments, where they complex with appropriate peptides. The Ag/MHC II complexes are then transported to the cell surface for display to T_H cells.

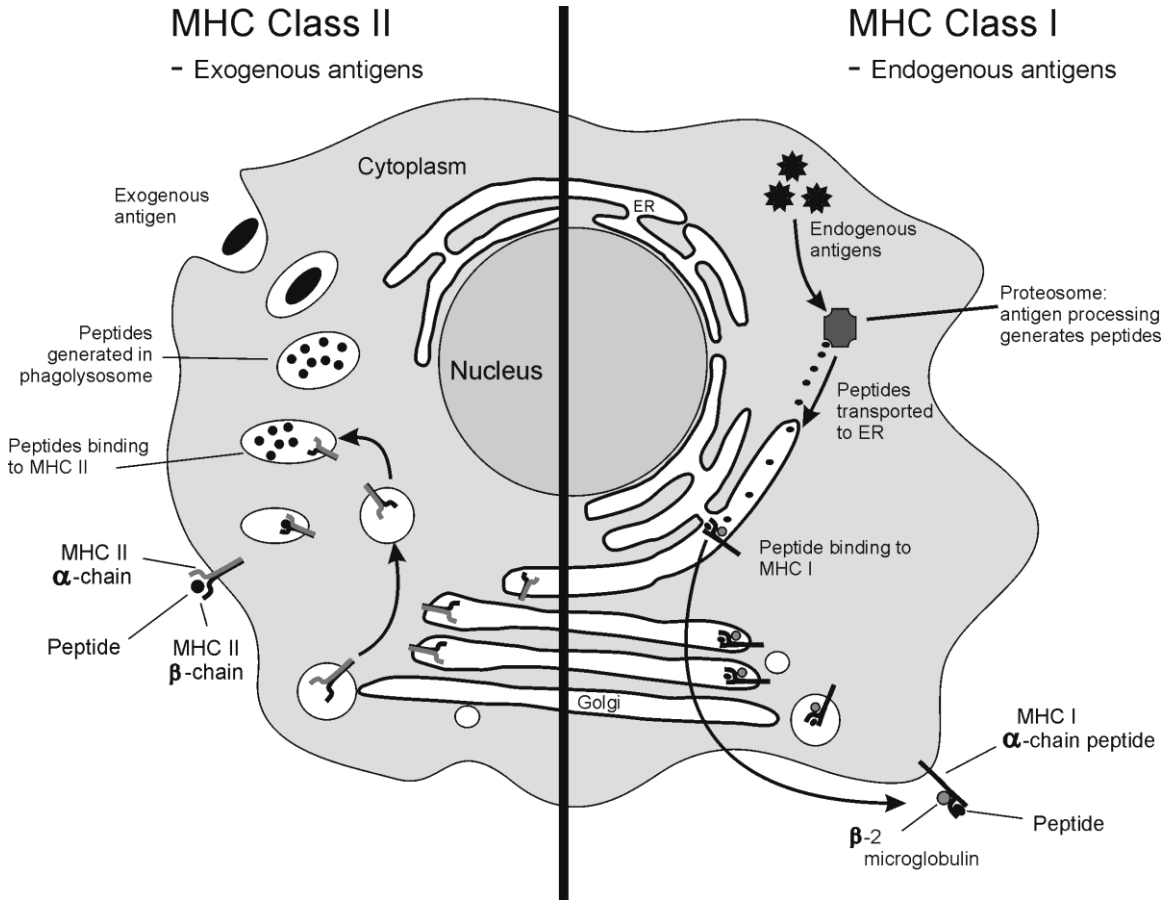

Figure 1-3. Antigen processing and presentation pathways.

MHC Class II - External antigens are processed through the exogenous pathway to be presented in the context of MHC II. Peptides are generated as the pathogen is broken down within increasingly acidic endocytic vacuoles and complexed with MHC II α and β chains. **MHC Class I** - Internal antigens are processed through the endogenous pathway to be presented in the context of MHC I. Antigenic peptides are generated by the proteasome and complexed with MHC I α-chain and β-2 microglobulin. Adapted from: (Parham 2005).

A strong adaptive cytotoxic response requires the breakdown and processing of intracellular parasites via both endogenous and exogenous pathways. However, if a parasite is able to prevent or withstand degradation in the phagosome, they may thereby prevent effective display of antigen to T_H cells. If the parasite escapes from the phagosome, they also avoid exogenous processing and therefore will not trigger a T_H response. Although escape to the cytosol may allow pathogens to be degraded and processed for MHC I presentation to T_C-cells, without a strong T_H1 response, CTLs may not become activated. *L. salmonae* is believed to be transported through the host vascular system within some type of phagocytic cell, but it is not known how the parasite survives or evades the killing mechanisms generated within these cells.

Targeting of infected cells for cell-mediated lysis may also occur through some alteration to cell surface molecules involved in signaling, such as reduced MHC I expression on altered self cells, or the increased expression of cell-surface molecules on infected or cytotoxic cells. For example, activated T-cells up-regulate FASL, making them more efficient killers of cells displaying FAS on their surface (Dockrell 2003).

Clearly, one of the key factors involved in the development of an effective immune response to intracellular parasites is immune recognition. At what point and by what means do components of the immune system (both innate and adaptive), become alerted to the presence of the parasite within infected cells? The success of some intracellular parasites may be based on their ability to delay the host immune response long enough for them to complete development. *L. salmonae* is able to develop within cells of the gill in Chinook salmon while apparently provoking limited responses. Eventually, the integrity of the severely hypertrophied host cells is compromised, releasing mature spores

into the water and allowing them to be passed to a new host. It is unclear whether xenoma rupture is host-cell or parasite driven. At this point an inflammatory response develops in the gill, with an influx of phagocytic cells (macrophages, neutrophils) that appear to ingest the released spores. The infection is cleared, and recovered fish are resistant to re-infection, indicating the development of immunological memory (Kent et al. 1999). The mechanism of immunity is believed to be cell-mediated, since passive transfer of serum from infected fish does not confer immunity to naïve fish (Sanchez et al. 2001a). In addition, spores have been observed in head kidney phagocytes several months after resolution of disease, where they were speculated to act as a continual immune stimulant, reinforcing memory responses (Kent et al. 1999).

1-2b. Immune Responses to Intracellular Parasites: The TH1 Paradigm and Cell-mediated Responses

Mammalian responses to parasitic infection have been extensively studied for a variety of protozoan species, and much of the current understanding of immune function comes from work on such species as *Leishmania major*, *Toxoplasma gondii* and others. An effective mammalian immune response appears to involve the cooperation of multiple components of the innate and adaptive immune systems. Studies of *L. major* infection in mice have provided a paradigm, demonstrating that intracellular parasites provoke a predominantly T_H1-driven, cell-mediated host response (Gumy et al. 2004). A T_H1 response is triggered when phagocytic cells engulf free parasites or infected host cells. Macrophages, dendritic cells, and neutrophils release the cytokines IL-12 and TNF-α, which stimulate nearby NK cells to release IFN-γ. Macrophages and dendritic cells also function as APCs to T_H cell progenitors, triggering their activation and proliferation, and

(under the influence of secreted cytokines) promoting a T_H1 response. Activated T_H1 cells secrete the cytokines IFN-γ and IL-2, which help to activate CTL. Effector cells from both the innate and adaptive systems, stimulated by cytokines, target free parasites and infected cells for killing through apoptotic mechanisms (Gumy et al. 2004).

NK cells play important roles in cell-mediated immunity against intracellular parasites in mammals. NK cells secrete IFN-γ, which activates monocytes/macrophages and promotes phagocytosis (Sinigaglia et al. 1999, Sague et al. 2004). NK cells may also be involved in promoting $CD8^+$ T_C-cell immunity. In mice lacking $CD4^+$ T_H cells, NK cells secrete IL-12, critical for priming of CTL, and showed an extended response that resulted in the priming of parasite-specific $CD8^+$ T-cells (Combe et al. 2005).

As outlined above, the panel of cytokines released by T_H cells can be characterized as fitting either a T_H1 or T_H2 profile. T_H1 cytokines (IFN-γ, IL-2, etc.) are associated with bias toward a predominantly cell-mediated response associated with the fight against intracellular parasites (Alexander & Bryson 2005). Strong T_H1 responses have been described in response to *Leishmania major* (Rogers & Titus 2004). However, the expression of a T_H1 cytokine profile may vary amongst individual hosts in response to the same parasite, offering a possible explanation for the differential susceptibility of individuals of the same host-species (Cardoni et al. 1999).

1-2c. Immune Responses to Intracellular Parasites: Humoral Responses

The majority of studies point to T_H1 driven cell-mediated mechanisms as the primary response to intracellular infection. However, there is evidence of limited humoral response to some intracellular parasites of fish. Results of ELISA studies showed a specific humoral response of the grouper *Epinephelus akaara* to spores of the

microsporidium *Glugea epinephelusis* following natural infection. However, antibody titers did not correlate with the intensities of infection in these fish (Zhang et al. 2005). Further evidence for antibody responses to intracellular parasites comes from studies of the microsporidian *L. salmonae*, in which passive serum transfer from infected to naïve fish delayed, but did not prevent, the progression of infection (Sanchez et al. 2001a). There have also been indications of parasite-induced immuno-suppression of humoral responses in fish. For example, the infection with the microsporidian *Glugea stephani* resulted in reduced overall serum Igμ levels in winter flounder and summer flounder (Laudan et al. 1986, 1989). At present the role that antibodies play in the immune responses of teleost fish to infection by intracellular parasites is not well understood.

Although there is functional evidence that fish are capable of cell-mediated responses, it remains unclear if fish mount T_H1-driven cell-mediated defences against intracellular parasites. However, some fish are capable of developing immunity to re-infection by certain parasites, implying the development of immunological memory associated with adaptive responses. Humoral responses, where present, are not protective, suggesting the importance of cell-mediated responses. Fish are known to possess many of the key components involved in the mammalian T_H1 response. As any adaptive response depends on the participation of numerous innate factors, it is likely that the development of resistance to reinfection by intracellular parasites involves the participation of both innate and adaptive systems. Although *L. salmonae* continues to be studied, the full picture of host response to this parasite is still elusive. However, what is known about host responses to *L. salmonae* suggests that an effective response is cell-

mediated, and involves both innate and adaptive factors. In addition, a possible role for humoral factors has been suggested.

In order for an effective immune response to develop, components of the host immune system must recognize the parasite or parasite-infected host cells as non-self, or "danger". *L. salmonae* is thought to migrate within a host phagocytic cell through the vascular system to the gill, where xenomas develop and grow for several weeks before they begin to break down. During this time the parasite maintains its intra-cellular existence, likely preventing strong immune recognition and activation. Once xenomas are fully developed in the gill it is in the parasite's best interest for xenomas to be disrupted in order for mature spores to be disseminated. It is not known whether the breakdown of the xenoma structure is parasite or host driven, but however it is initiated, the dissolution of xenomas likely represents an optimal opportunity for immune recognition.

Once recognition has occurred, immune cells accumulate in the gill, and this stage is characterized by phagocytic cells engulfing freed spores. Innate phagocytic cells likely play a key role in clearing the infection, since *L. salmonae* spores have been observed within both neutrophils and macrophages in gills of naturally infected Chinook salmon. However, spore degradation was not observed within neutrophils, whereas macrophages appeared to be actively degrading engulfed spores (Lovy et al. 2007). Humoral factors such as antibodies may also play a role in the phagocytic uptake of *L. salmonae.* Passive transfer of sera from *L. salmonae*-exposed RBT to naive RBT, followed by parasite exposure, was shown to delay but not prevent xenoma formation, suggesting increased

opsonisation by *L. salmonae* –specific antibody, resulting in improved phagocytic uptake by macrophages (Sanchez et al. 2001b).

Recovered fish develop strong resistance to reinfection that may continue for upwards of a year, indicating the development of immunological memory. *L. salmonae* spores have been documented within head kidney of Chinook salmon at 22 weeks PE, after xenomas were fully cleared from the gill (Kent et al. 1999). These spores may have been transported to the reticulo-endothelial system of the kidney by phagocytic cells clearing spores from ruptured xenomas in the gill. Within the kidney spores may provide a continual immune stimulus, promoting and prolonging a memory response. Although cell-mediated factors have been implicated in response to *L. salmonae* infection, more work is required to identify the particular cells and pathways involved in mounting an effective response and to determine the patterns of interaction in order to develop an increased understanding of this complex protective system.

Chapter 2

Chicken-derived IgY recognizes developing and mature stages of *Loma salmonae* (Microsporidia) in Pacific salmon, *Oncorhynchus* spp.

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Introduction

Loma salmonae (Microsporidia) is the causative agent of microsporidial gill disease (MGD) in several members of the Oncorhynchus genus, particularly chinook salmon (*O. tshawytscha*) and coho salmon (*O. kisutch*) that are farmed in British Columbia, Canada (Speare et al. 1998). Infections are also known to occur in wild Pacific salmonids such as pink salmon (*O. gorbuscha*) and sockeye salmon (*O. nerka*), although the parasite has not been well studied in wild species (Shaw et al. 2000). Infection results in the formation of spore-filled xenomas in the gill that eventually rupture, provoking a strong inflammatory reaction that may result in severe branchitis and asphyxiation of the host (Kent et al. 1995, Speare et al. 1998). Spores, containing the sporoplasm and a polar tube, are released from ruptured xenomas into the water and ingested by a new host. The infective sporoplasm is injected through the everted polar tube into a cell associated with the gut epithelium. The parasite migrates to the heart and, eventually, to the gill, where most xenomas form (Sanchez et al. 2001a). Xenoma maturation is temperature-dependent and takes approximately 6 to 8 weeks in chinook salmon at 14 ºC. Current detection and diagnosis of *L. salmonae* is performed either by direct examination of gill or gill clips, polymerase chain reaction (PCR) or microscopic

examination of histological preparations of gill tissue. *L. salmonae* DNA can be detected by PCR in rainbow trout (*O. mykiss*) gill tissue as early as 2 weeks post -exposure (PE) (Sanchez et al. 2000). Although PCR analysis is very sensitive, it requires specialized equipment and does not provide a quantitative estimate of the severity of infection (Docker et al. 1997). Histology, utilizing staining techniques such as Giemsa or haemotoxylin and eosin (H&E), is less sensitive than PCR (Sanchez et al. 2000, Rodriguez-Tovar et al. 2002). Detection and quantification of infection by histology is possible approximately 6 – 8 weeks PE in Chinook salmon, when xenomas are nearly mature. At earlier times, the developing xenomas are small, morphologically indistinct and easily overlooked in histological preparations. Estimating infection severity in histological preparations is both time-consuming and subjective. A more sensitive, specific and quantitative alternative to traditional stains would result in earlier diagnosis and more accurate assessment of infection severity, leading to more timely management decisions.

Antibody-based (serological) diagnostic tools including immunofluorescence, agglutination and enzyme immunoassays are used to detect bacterial, virus and parasite pathogens of finfish. Antibody (Ab) preparations can also be utilized to stain histological sections, providing highly sensitive and specific recognition of the target organism. Monoclonal Abs bind to a single epitope, making them highly specific, but they are both costly and invasive, as their production generally involves raising mice in very expensive animal care approved facilities, followed by euthanization of the mice in order to harvest hybridomas (Ziegelbauer & Light 2008). In addition, Abs from particular clones may bind to epitopes that are displayed non-continuously during parasite development (Young

& Jones 2005). Polyclonal antibodies (pAb), on the other hand, are generally cheaper and easier to produce, since they can be generated in live animals and then harvested by collecting serum repeatedly from the live animal. pAb bind specifically to multiple epitopes that may be displayed across a wider range of developmental stages. This report describes the application of chicken yolk polyclonal antibodies (IgY) in immunofluorescent and immunohistochemical assays to detect *L. salmonae* in salmon tissues.

2-1. Materials and Methods

2-1a. Fish husbandry:

Chinook salmon smolts were obtained from Sea Springs Hatchery (Chemainus BC), and maintained in ambient seawater (10°C – 15°C) in 1000 L tanks for the duration of the study. Pink salmon fry were obtained from Quinsam Hatchery (Fisheries and Oceans Canada) and maintained on normal freshwater until transfer to ambient seawater. Fish were fed a commercial diet daily.

2-1b. Fish infection and sampling:

Chinook salmon (average wt 23 g.) with no history or symptoms of *L. salmonae* infection, were exposed to *L. salmonae* by gastric intubation with an inoculum of 1×10^6 spores in macerated gill tissue and held in ambient seawater in a 1000 L flowthrough tank. Gill, spleen and heart were collected from 15 exposed fish at 4 and 8 weeks PE immediately following killing by an overdose of tricaine methane sulphonate (TMS). Tissues were fixed for 24 h in neutral buffered 10% formalin and processed for histology. Thin (5 μm) sections were mounted on silane-coated slides (Sigma) in order to promote strong adherence. Similarly, gill tissue was collected and processed from 15 pink salmon

intubated 75 days earlier with an inoculum containing 1 x 10^6 spores collected from Chinook gills. Gill and heart tissues obtained from adult sockeye salmon that had returned to spawn in Cultus Lake, B.C., were fixed and processed for histology as described above.

2-1c. Spore purification:

Loma salmonae spores were purified according to the methods of Shaw (Shaw et al. 1998). Briefly, complete gill arches were removed from heavily infected Chinook or pink salmon. Infections were confirmed through microscopic examination of gill squashes. Gills were scraped in order to detach soft tissue from cartilage and the resulting tissue slurry was homogenized for 30 seconds using a Polytron tissue homogenizer. Spores were collected at this stage in the protocol for use in inocula to infect salmon. For further purification, homogenates were flushed through a 50 μm Nitex filter backed by a wire mesh screen with Hank's balanced salt solution (Sigma) supplemented with 1% (v/v) of an antibiotic and antimycotic solution (Gibco) (HBSS/Ab). The spores were washed three times by suspending them in HBSS/Ab followed by cold (4ºC) centrifugation at 1000 x g for 15 minutes. Washed spores were re-suspended in 40 ml HBSS/Ab. Ten ml aliquots of the rinsed spores were then combined with 15 ml ddH2O and 25 ml 54% Percoll, centrifuged as before, and the supernatant discarded. The spores were resuspended in HBSS/Ab and layered over a Percoll gradient (3ml 100%, 2 ml 54%) and cold-centrifuged as above. Spores concentrated at the interface were collected and washed with ddH2O, centrifuged again, and the supernatant discarded. The purified spores were resuspended in phosphate

buffered saline, pH 7.4 (PBS) (Sigma), enumerated with a haemocytometer, and stored at -20ºC.

2-1d. Immunization of chickens:

Three adult Barred Plymouth Rock hens were immunized by intramuscular injection with $1x10^6$ purified *L. salmonae* spores. For the primary inoculations, spores were emulsified in one-quarter volume Freund's incomplete adjuvant (Sigma) and the 1 ml inoculum was divided among four locations in the pectoral muscle of each bird. Booster inoculations with $1x10^6$ spores were administered without adjuvant by sub-cutaneous injections at 28, 35, and 42 days post-immunization (PI). Eggs were collected daily from 28 to 65 days PI and stored at 4°C until extraction.

2-1e. Extraction and characterization of polyclonal IgY from eggs:

IgY was extracted from eggs using a commercially available kit (Eggspress; Gallus Immunotech). Purified IgY was resuspended in PBS plus 0.02% sodium azide, and stored at -20°C. Protein concentrations of the IgY extracts were measured using the BCA Protein Assay kit (Pierce). Purity of the yolk protein extracts was determined by SDS-PAGE analysis and Western blotting. Yolk proteins were boiled in sodium dodecyl sulphate and separated on a 10% polyacrylamide gel (Novex) before being transferred to a nitrocellulose membrane according to standard Hoefer protocols (Hoefer 1994). Blots were stained for total protein with Ponceau-S. In order to distinguish IgY bands, blotted proteins were probed in duplicate lanes with either alkaline phosphatase (AP) conjugated rabbit anti-chicken-IgY or AP-conjugated goat anti-rabbit-IgG (Jackson Immunolaboratories), followed by Blue-Phos substrate (KPL Laboratories).

2-1f. Immunofluorescence:

Purified spores (7.5 x 10^4 in 100 µl PBS) were aliquoted onto silane coated slides, which were air-dried and fixed in acetone for 1 minute. For non-lethal detection of spores, three infected pink salmon were sedated by immersion in TMS (40 mg/L) and a small sample was clipped from the first left gill arch. The gill clips were blotted onto silane-coated slides, dried, and then fixed in acetone for 1 minute. Infection was confirmed by microscopic examination of the gill clips. The fixed preparations were rehydrated 5 minutes in PBS and incubated with 100 µl anti- *L. salmonae* IgY diluted 1:1000 in PBS plus 2% Tween-20 (PBS-T) in a humidified chamber for 1 h. Slides were rinsed briefly with PBS, followed by 3-5 minute washes in PBS, before being incubated with 100 µl fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-IgY diluted 1:50 in PBS-T. Slides were rinsed and washed as above, Slow-Fade™ Equilibration buffer and fluorescence-fading inhibitor (Molecular Probes) was added and a coverslip applied prior to observation with a Zeiss epi-fluorescence microscope.

2-1g. Immunohistochemistry:

Thin (5µm) tissue sections were deparaffinized and rinsed for one minute in running tap water. Tissue sections were incubated with 80 µl anti- *L. salmonae* antibody diluted 1:1000 in PBS-T in a humidified chamber for 1 h. Slides were briefly rinsed and then washed in PBS-T (3 x 5 min.) followed by incubation for 45 min with alkaline-phosphatase (AP)-conjugated rabbit anti-chicken-IgG (100 µl) diluted 1:50 in PBS-T. Slides were washed as above and then incubated for 30 minutes with Blue-Phos substrate solution (100µl) (KPL). Slides were immersed in water in order to stop the reaction,

counterstained with a five-second immersion in eosin, dehydrated in 100% isopropanol (3 x 30 sec.) and xylene (3 x 30 sec), and cover-slipped with Permount.

In addition to assays of tissues from Chinook and pink salmon, the specificity of the IgY was assessed by immunohistochemical analysis of *L. salmonae* in sockeye salmon (*O. nerka), Loma branchialis* in Atlantic cod (*Gadus morhua*), *Pseudoloma neurophilia* in zebrafish (*Danio rerio*), *Glugea anomala* in 3-spined stickleback (*Gasterosteus aculeatus*), *Kudoa thyrsites* in Atlantic salmon (*Salmo salar*), and *Parvicapsula minibicornis* in sockeye salmon (*O. nerka*).

2-2. Results

2-2a. Extraction of IgY:

Fifty-three eggs were collected from three immunized hens over a period of 35 days, beginning 28 days post initial immunization (PI). Protein levels of extracted IgY ranged from 1.6 mg/ml to 8.3 mg/ml, with an average of 5.25 mg/ml. Between 24 mg and 125 mg protein were recovered per egg for an average yield of 79 mg of protein. Mean protein concentrations (mg/ml) in yolk extracts were 5.35 ± 0.35., 5.62 ± 1.83., 6.27 ± 1.78 and 7.04 ± 0.69 at 32, 45, 55 and 60 days PI. Indirect immunofluorescence (IFAT) using these extracts on fixed, purified spores showed that the minimal IgY concentration at which fluorescence was detected decreased with time following immunization (Table 2-1).

2-2b. Western Blots:

Proteins extracted from eggs were separated by SDS-PAGE and resolved into four bands that stained intensely with Ponceau-S as well as several faintly stained bands (Figure 2-1). Four immunoreactive bands were observed when the separated proteins

were probed with the rabbit-anti-IgY, whereas duplicate lanes probed with goat anti-rabbit-IgG showed no reactivity (Figure 2-1).

2-2c. Immunofluorescence:

Fixed spores showed strong, specific fluorescence with very low signal-to-noise ratio (Figure 2-2). In some spores the outline of the polar capsule could be discerned. Blots made from non-lethal gill clips of infected pink salmon showed the presence of immuno-reactive spores for all three fish.

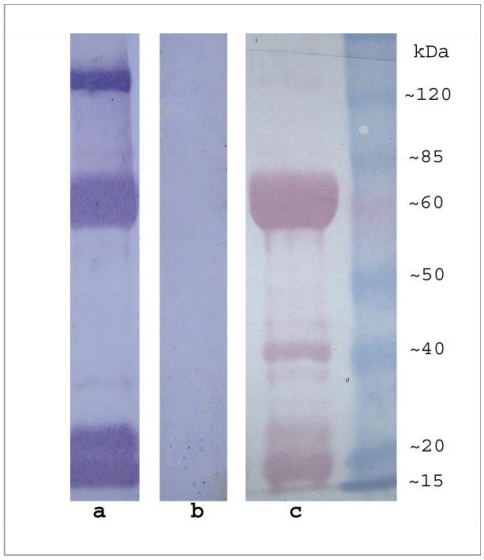

Figure 2-1. Western blot of chicken egg proteins separated by SDS-PAGE. Proteins in lane (a) were probed with AP-conjugated rabbit-anti-IgY and represent (from the top) non-reduced, native IgY (> 120 kDa), IgY heavy chain (65 kDa), and IgY light chain (~18 kDa). Proteins in lane (c) were stained with Ponceau-S, and represent all proteins. Differing levels of protein reduction resulted in a very faint band corresponding to native IgY (> 120 kDa) in lane c, whereas a strong band is visible in lane (a). Bands in lane (b) were probed with AP-conjugated goat-anti-rabbit-IgG.

Figure 2-2. *Loma salmonae* spores of Chinook salmon (*Oncorhynchus tshawytscha*) stained with IgY. Acetone-fixed *L. salmonae* spores stained with IgY followed by FITC-conjugated rabbit-antiIgY (IgG). Scale bar represents 5 µm.

2-2d. Immunohistochemistry:

L. salmonae stained strongly in immunohistochemical preparations of Chinook, pink, and sockeye salmon tissues. Mature xenomas in gill, heart, and spleen tissues from Chinook and pink salmon (Figure 2-3a), and in heart tissue from sockeye salmon (not shown) reacted strongly with IgY. Very early development was also observed, beginning at 4 weeks post-exposure (PE). In Chinook salmon gill at 4 weeks PE, very small (~ 5 µm) pre-xenomas were clearly visible (Figures 2-3b, 2-3c). Similar early development was observed in Chinook heart tissue (not shown). *L. salmonae* could not be detected in adjacent thin sections when stained with H&E. Immunoreactive spores were clearly visible towards the periphery of mature xenomas, whereas a non-reactive zone was also noted at the centre of many xenomas (Figure 2-3d). Xenomas of *Loma branchialis* in Atlantic cod (*G. morhua*) reacted strongly with the IgY (Figure 2-4). In contrast, the antibodies showed no reactivity with the microsporidian parasites *Glugea anomala* or

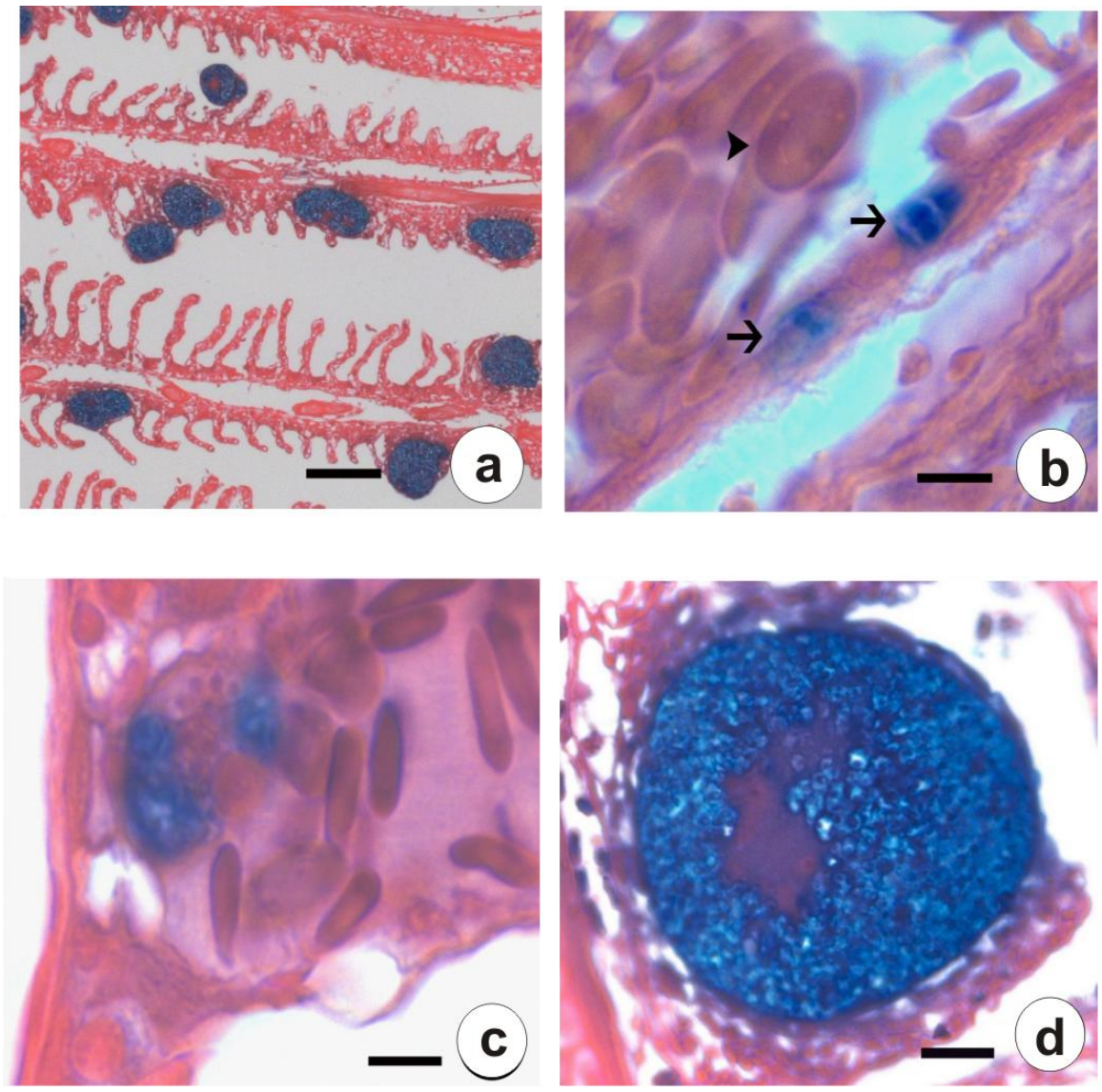

Figure 2-3. *Loma salmonae* of Pacific salmon stained with IgY.

a) Mature xenomas in pink salmon gill stained with IgY followed by AP-conjugated rabbit anti-IgY. Scale bar represents 100 µm. (**b & c)** Early *L. salmonae* development in Chinook salmon gill at 4 weeks post-exposure. **b)** Two meronts within the epithelium of the primary lamella (arrows). The small size of the meront is illustrated by comparison with a nearby red blood cell (arrowhead). Scale bar represents 5 µm. **c)** Meronts within a developing xenoma. Scale bar represents 5 µm. **d)** Mature xenoma from heavily infected pink salmon gill. Scale bar represents 20 µm.

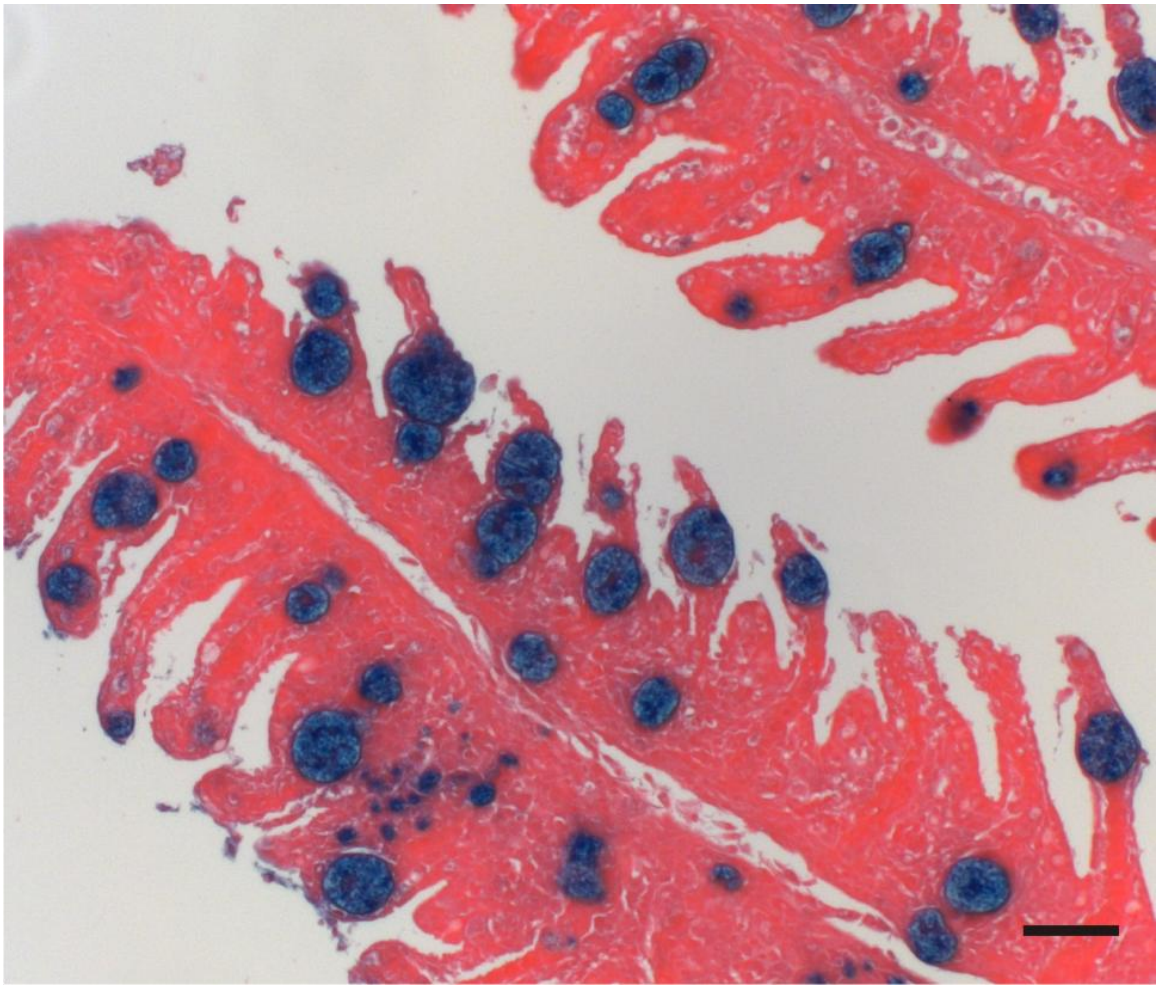

Figure 2-4. *Loma branchialis* in Atlantic cod (*G. morhua*) gill stained with IgY and AP-anti-IgY. Scale bar represents 50 μm.

Pseudoloma neurophilia. Similarly, the IgY showed no reaction with myxosporean parasites *Kudoa thyrsites* or *Parvicapsula minibicornis* (not shown).

2-3. Discussion

Antibodies are multipurpose tools with widespread utility in many biological applications. Polyclonal antibody preparations have traditionally been generated via the injection of a purified antigen into a mammal (e.g. rabbit), followed by periodic bleedings

and preparation of antisera. However, recent concerns over animal welfare make less invasive alternatives increasingly desirable. The immunoglobulin laid down in the yolk of avian eggs (IgY) is an alternative to serum Ig because of its specificity, high yield and the relative non-invasiveness of collection (Schade et al. 2005).

The IgY generated in this work recognized *Loma* spp. in histological sections of fish tissues. In a previous study of rainbow trout gill, Rodriguez-Tovar et al. found that the smallest xenomas detected by standard histology utilizing H & E staining had a mean diameter of 21 μm (Rodriguez-Tovar et al. 2004). In contrast, the IgY preparation identified 5 μm pre-xenoma-like structures in chinook gill. In addition, the strong contrast provided between the Blue-Phos product and the eosin-counterstained background enhanced the identification and enumeration of parasite stages. Similar structures were not recognized in adjacent sections stained with H & E. The absence of immunoreactivity at the centre of mature xenomas probably reflected degenerate host cell residue at this location. The relative specificity of the IgY preparation was demonstrated by its reactivity with *Loma* species but not with more phylogenetically distant microsporidians such as *Pseudoloma neurophilia* or *Glugea anomala*. The IgY also failed to react with myxosporean parasites of fish.

The polyclonal antibodies developed here showed the ability to bind strongly to early, pre-xenoma stages of *L. salmonae* in gill tissue. Previously, Speare et al. (1998), utilized a spore-wall specific monoclonal antibody (MAb), clone 4HB, in immunohistochemical detection of *L. salmonae* in rainbow trout (RBT) (Speare et al. 1998). In that work, spore wall antigens were detected as early as 3 weeks PE in RBT gill tissue. It is difficult to directly compare the results from this pAb preparation vs. the

mAb, both because this study did not include a 3 week PE sample date, and also because *L. salmonae* development proceeds more quickly in RBT than in Chinook salmon. Further work will be necessary to determine the lowest temporal detection limit for this pAb in Chinook tissues. It is possible that pAb preparations may be generally preferable to mAbs for early detection of the parasite, due to their reactivity with multiple epitopes that may be expressed during a broader range of developmental stages. In previous work with *K. thyrsites*, epitopes recognized by mAbs generated against mature spores were not present at earlier developmental stages of the parasite (Young & Jones 2005).

Fixed purified *L. salmonae* spores as well as spores transferred to slides from non-lethal gill clips showed strong reactivity with the IgY. Additional work must be done to determine whether spores can be directly transferred from intact gills of anesthetized fish onto slides, which could allow for the development of a relatively non-invasive detection method for *L. salmonae*.

High quantities of protein were extracted from individual eggs, and western blots confirmed that the majority of extracted protein was IgY (Figure 2-1). These results were consistent with earlier reports of yields in the range of 3 to 7 mg/ml (Carlander et al. 2001). The yield of IgY varies between strains of birds, as well as between individual birds within a strain (Carlander et al. 2001). Approximately 1500 mg of total IgY, in which specific IgY comprised 2% to 10%, was obtained from one hen over a laying cycle (Carlander et al. 2001). In comparison, a typical rabbit yields approximately 200 mg of IgG in the same time period, with approximately 5% representing specific Ig (Schade et al. 1994). The size of immunoreactive proteins in yolk extracts visualized here agreed closely with the expected molecular weights for native IgY (165 kDa), IgY heavy chain

(65 kDa) and light chain (~18 kDa). The ~22 kDa immunoreactive band was not identified but may represent glycosylation heterogeneity among IgY light chains (Tachibana et al. 1992). In the present study, the immunized hens were kept in the same cage, thus eliminating the possibility of tracking individual protein levels and IgY specificities. While the mean protein yield did not change significantly over time, specific antibody titre appeared to increase following immunization, demonstrated by both the quantitative and qualitative changes in immunofluorescence observed using extracts from eggs collected at 35, 42, and 60 days PI. Extracts collected 60-days post-immunization showed bright, apple green fluorescence that appeared to completely cover the spore surface, whereas extracts from earlier eggs showed much less fluorescence at lower concentrations of IgY extracts, as well as patchy, pinpoint fluorescent spots. It is unknown whether this qualitative change represents increased IgY transfer to yolk over time, and therefore an increased IgY concentration, or also an increase in affinity of IgY for particular spore epitopes, given a secondary response following booster inoculations. A classical anamnestic immune response, characterized by an increased antibody response to a second antigen dose, has been described in chickens, although this response may not be consistent in all individuals (Patterson et al. 1962, Woolley & Landon 1995). Affinity maturation has not been well described for IgY of chickens, although somatic hypermutation is known to occur (Arakawa & Buerstedde 2004). The present results demonstrate the value of IgY as a specific, abundant and readily available reagent for the detection and diagnosis of other fish pathogens.

2-4. Conclusions

IgY obtained from avian egg yolk has gained recent prominence as concerns over animal welfare grow. IgY is a practical and useful alternative to traditional techniques for the detection and quantification of *Loma salmonae* in Pacific salmon. The advantages of IgY technology include the relatively low cost and non-invasiveness of collection and Ab extraction, as well as extremely high yield of IgY. The relatively specific IgY generated in this study allows simple and early detection of extremely small pre-xenoma stages within tissues. In addition, the polyclonal nature of the Abs generated here may allow detection over a wide range of developmental stages of the parasite. Further studies will determine the applicability of this reagent to enzyme immunoassays such as ELISA for detection of *L. salmonae* infection in Pacific salmonids. In addition, researchers may be prompted to utilize this approach for the diagnosis of other pathogens.

Table 2-1. Indirect fluorescence antibody tests.

Tests were conducted using IgY extracts from eggs collected 32, 45, 55 and 60 days post-immunization (PI) on purified *Loma salmonae* spores. Strong fluorescence is indicated by (+). The lack of observable fluorescence is indicated by (-), whereas (+/-) indicates very faint fluorescence. (n = 3). Detection threshold (minimum concentration of protein at which strong fluorescence was detected) decreases with time.

Days PI	**Protein concentration in IgY extract (µg/ml)**					
	10	**5**	**2.5**	**1.25**	**0.675**	**0.3375**
32	+	+	+	+/-	+/-	_
45	+	+	+	+	+/-	_
55	+	+	+	+	+	+/-
60	+	+	+	+	+	+

Chapter 3

Preventing *Loma salmonae* infection in Chinook salmon: Analysis of β-glucan as an immune-modulant, including gene expression analysis

Introduction

Loma salmonae (Microsporidia) is the causative agent of microsporidial gill disease (MGD), infecting members of the *Oncorhynchus* genus, particularly Chinook salmon (*O. tshawytscha*), (Speare et al. 1998). Infected fish form spore-filled xenomas in the gill that eventually rupture, provoking a strong inflammatory reaction that may result in severe branchitis and asphyxiation of the host (Kent et al. 1995, Speare et al. 1998). *Loma salmonae* is transmitted directly when a potential host ingests spores from the surrounding water. Once a spore makes contact with cells of the gut it triggers eversion of the spore's polar tube, which penetrates the host cell membrane and allows the infective sporoplasm to pass directly into the cell. The parasite migrates to the heart and, eventually, the gill, where the majority of xenomas form (Sanchez et al. 2001a). It is believed that transport between the initial site of infection and the ultimate site of xenoma development may occur within a phagocytic host cell such as a macrophage (Rodriguez-Tovar et al. 2002).

The impact of *L. salmonae* infection to fish farmers is large, and includes both the direct impact of MGD, as well as indirect effects such as reduced growth rate and increased susceptibility to other diseases (Ramsay et al. 2004). Mortalities from MGD can reach upwards of 30% in some years, and the economic impact of these losses is maximized due to the fact that most mortality occurs in fish nearing market-size (Kent et

al. 1989). As such, research efforts are underway to develop therapies with the potential to reduce the impact of *L. salmonae* infection.

Glucans have been widely studied as potential immune modulators in a variety of species, and increase vertebrate resistance to pathogens in some cases. Glucans are carbohydrate polymers composed of glucose subunits linked through β-1,3 and β-1,6 glycosidic linkages, and are key components of the cell walls of yeast, fungi and bacteria. Many studies involve administration of glucan as a dietary additive, although the efficacy of dietary dosage is unclear, with varied reports of innate immune enhancement. For example, innate immune parameters such as complement activity were enhanced in sea bass in response to dietary glucan under certain conditions, whereas no significant effect was measured under others (Bagni et al. 2005). The low pH of the digestive system may result in hydrolysis of the branched chains of the glucans, resulting in severe reductions to their effectiveness as immune-stimulants when administered by this method (Kudrenko et al. 2009).

Glucans administered through intra-peritoneal (IP) injection are also able to reduce the effects of pathogenic organisms *in vivo*. In previous studies with rainbow trout (RBT), IP injection of β-1,3/1,6-glucan prior to experimental exposure with *L. salmonae* resulted in reduced prevalence and intensity of the parasite (Guselle et al. 2006, 2007). However they are administered, the mechanism of β-glucan protection is not fully understood. It is believed that glucans induce innate immune responses through the recognition of their repeated molecular patterns by cell-surface receptors including Toll-like receptor 2 (TLR-2), dectin-1, and complement receptor 3 (CR3) on immune cells such as macrophages and neutrophils (Novak & Vetvicka 2008).

Although IP glucan inoculation reduced both the prevalence and intensity of *L. salmonae* infection in RBT, it is unknown whether β-1,3/1,6-glucan inoculation would have similar effects in Chinook salmon. Here the immune-modulating effects of β-glucan on Chinook salmon *in vivo* were investigated through several measures. In order to determine the protective effect of glucan exposure, the prevalence and intensity of *L. salmonae* were measured in Chinook salmon that were experimentally infected with the parasite 3 weeks post-glucan inoculation (PI). Glucan exposure significantly reduced the severity of infection, although prevalence of *L. salmonae* was not reduced. In addition, gene expression analysis was performed on head kidney from groups of fish inoculated with β-glucan at 1, 2 and 3 weeks PI. Gene expression was analysed through the use of a 16,006 feature salmon cDNA microarray developed by the Genome Research on Atlantic Salmon Project (GRASP: http://web.uvic.ca/cbr/grasp; B.F. Koop & W. Davidson), (von Schalburg et al. 2005), containing 13,421 Atlantic salmon features and 2,576 rainbow trout features. cDNA generated from a variety of Chinook salmon tissues has previously been successfully cross-hybridized to this array (Rise et al. 2004, von Schalburg et al. 2005). A subset of differentially-expressed genes identified in the microarray study was validated using quantitative reverse-transcriptase PCR (qRT-PCR).

3-1. Materials & Methods

3-1a. Fish Treatments and sampling:

Chinook salmon smolts (average wt. ~ 25 g) of unknown health status and with no exposure to glucan were obtained from Omega Pacific Hatchery, Port Alberni, BC, and held in ambient seawater (10°C - 13°C) for the duration of the study. Incoming water was UV- sterilized by passage through two 40 watt sterilizers connected in series (Pentair

Aquatics) at a rate of 12 L/min. Test fish (n = 100/group) were inoculated by intra-peritoneal (IP) injection with either 100 µg of β-glucan (Sigma) dissolved in 100 µl phosphate buffered saline (PBS) (glucan) or with PBS alone (control). Test fish (total n = 200) were divided into 8 tanks (4 tanks per group - 25 fish/tank). Samples of head kidney were harvested from 20 fish per group at 1, 2 and 3 weeks PI for microarray (n = 5) and qPCR analysis (n = 15). Three weeks post-inoculation (PI) the remaining fish in the glucan and control groups (n = 40 fish/group) were separated and half (n = 20) of each of the groups was intubated with 5 x 10^5 *L. salmonae* spores in homogenized gill tissue suspended in 1X Hank's buffered saline solution (HBSS), whereas the other half (n = 20) was intubated with HBSS alone. Gills from 8 fish per group were sampled at 8 weeks post-*L. salmonae*-exposure (PE) for PCR and immunohistochemistry. PCR analysis of control fish (*L. salmonae* unexposed) confirmed their *L. salmonae*-free status.

3-1b. DNA extractions and PCR:

DNA was extracted from ~25 mg of kidney tissue, using the Qiagen DNeasy tissue kit according to the manufacturer's instructions. 1.5 µl aliquots of extracted DNA (<50 µg/ml) were then used as template in PCR reactions to amplify a 272 base pair fragment of the *L. salmonae* 18S rRNA gene. Each PCR reaction included: 0.625 U Platinum Taq polymerase (Invitrogen), 1x PCR buffer (Invitrogen), 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer (LS1F: 3' - CTG GAT CAG ACC GAT TTA TAT A – 5', LS1R: 5'- ATG ACA TCT CAC ATA ATT GTG A– 3') (Docker et al. 1997), with the remaining reaction volume (25 µl) made up with filter-sterilized, deionized, double-distilled water (ddH_2O). PCR reactions were performed in a PTC – 200 thermocycler (MJ Research). The PCR consisted of denaturation at 94°C for 1 minute, followed by 45 cycles of 94°C

for 35 seconds, 53°C for 20 seconds, and 72°C for one minute, followed by a final extension of 72°C for 7 minutes. PCR products were visualised on a 1.5 % agarose gel containing 0.1 μg/ml SybrSafe (Invitrogen).

3-1c. Immunohistochemistry:

Thin (5μm) tissue sections were deparaffinized and rinsed for one minute in running tap water. Tissue sections were incubated with anti- *L. salmonae* polyclonal IgY diluted 1:1000 in phosphate-buffered saline plus 2% Tween-20 (PBS-T) in a humidified chamber for 1 hour (Young et al. 2007). Slides were briefly rinsed and then washed in PBS-T (3 x 5 min.) followed by incubation for 45 min with alkaline-phospatase (AP)-conjugated rabbit anti-chicken-IgG (100 μl) diluted 1:50 in PBS-T. Slides were washed as above and then incubated for 30 minutes with Blue-Phos substrate solution (100μl) (KPL). Slides were immersed in water in order to stop the reaction, counterstained with a five-second immersion in eosin, dehydrated in 100% isopropanol (3 x 30 sec.) and xylene (3 x 30 sec), and cover-slipped with Permount. The sections were observed with a Zeiss Axioskop Imager A1 microscope (Götingen, Germany) and photographed with an Axiocam Mrc5 camera.

3-1d. Microarray Analysis - RNA extraction, cDNA generation and labeling:

For microarray experiments total RNA was extracted using Trizol, according to a modified manufacturer's protocol (Sigma). Tissues were transferred from RNAlater into one ml Trizol with a 3 mm stainless steel ball, incubated at room temperature (RT) for 5 minutes, and then homogenized for 3 minutes at 20 Hz on a Retsch mixer mill (Qiagen). 100 μl of 1-bromo-3-chloropropane were added to the homogenate and the tube was shaken vigorously for 15 seconds. The mixture was incubated for 3 minutes at RT and

centrifuged at 12,000 x g for 8 minutes. The aqueous phase was transferred to a new tube containing 500 µl of 2-propanol, mixed thoroughly, and incubated for 5 minutes at RT before being centrifuged at 12,000 x g for 5 minutes. The isopropanol was removed and the pellet washed with 750 ml of ice-cold 75% ethanol, vortexed, and then centrifuged at 7,500 x g for 5 minutes. The ethanol was carefully removed and the pellets dried for 10 minutes before being dissolved in 50 µl of RNase-free water at 55°C for 10 minutes. The integrity of the RNA was determined by spectrophotometric analysis and gel electrophoresis. Extracted RNA was stored at – 80°C until used.

Five µg of total RNA were reverse-transcribed into cDNA using the Invitrogen Superscript Indirect labeling kit according to a modified manufacturer's protocol. Briefly, first-strand cDNA was synthesized and precipitated in ethanol overnight. The cDNA was centrifuged at 16,000 x g for 60 minutes at 4°C, washed in 70% EtOH, and air dried before being resuspended in 5 µl of coupling buffer. Cy 3 and Cy 5 monoreactive dye packs (Amersham Biosciences) (GE Healthcare Life Sciences) were each dissolved in 45 µl DMSO and 5 ul of dye were added to the samples and incubated for 2 hours in the dark at RT. 10 µl of sodium acetate were added to the labeled samples before they were purified on SNAP columns, pooled, and precipitated in EtOH overnight. Pellets were spun at 16,000 x g for 60 minutes at 4°C, washed once with EtOH, dried, and resuspended in 27 µl DEPC water at RT.

3-1e. Microarray hybridization:

Microarray slides were washed twice for 5 minutes in 0.2% sodium dodecyl sulphate (SDS), 5 times for 1 minute in ddH_2O, and dried by centrifugation in a 50 ml conical tube for 5 minutes at 514 x g at RT. Dried arrays were immersed in

prehybridization buffer (5XSSC [1X SSC =150 mM sodium chloride, 15 nM sodium citrate], 0.1% SDS, 3% bovine serum albumin [BSA] V) for 90 minutes @ 49°C, washed 3 x 20 sec. in ddH_2O, and then centrifuged dry for 5 minutes at 514 x g at RT. Dry arrays were held at 49°C until hybridized.

Cyanine-labeled samples were mixed with 2X formamide buffer (50% formamide, 8X SSC, 1% SDS, 4X Denhardt's), and 4 µl LNAdT blocker (Genisphere), incubated for 10 minutes @ 80°C, and held at 65°C until hybridized. The microarray slides were placed into Corning hybridization chambers and ddH_2O was added to the reservoirs in order to maintain humidity within the chambers. Pooled cyanine-labeled samples were aliquoted onto the slide and covered with a 22 x 60 mm hybrislip (Invitrogen). Slide chambers were quickly assembled and incubated in a 49°C water bath for 16 hours. The hybridized arrays were washed 1 x 10 minutes (49°C) and 2 x 5 minutes (RT), in 2X SSC/0.1% SDS, followed by 2 x 5 minutes 1X SSC (RT), and finally 4 x 5 minutes in 0.1% SSC (RT). Slides were immediately dried by centrifugation for 5 minutes at 514 x g and held in the dark until scanned.

The hybridized microarrays were scanned using a Perkin Elmer ScanArray Express (Perkin Elmer, Boston, MA), with adjustments to the photo multiplier tube (PMT) gain in order to optimize signal/noise ratio in each sample. The images were then quantified using Imagene 5.6.1 (Biodiscovery, El Segundo, CA) and the data were analyzed with GeneSpring 7.3.1 data analysis software (Agilent Technology). Raw data were imported into GeneSpring, dye-swaps were identified, and the pairs were normalized by Lowess normalization with 20% of data used for smoothing. The data set was filtered to eliminate absent or marginal signals and those with values below the

average of base/proportional values for control signals. The data from normalized dye swap pairs were exported and re-imported into GeneSpring without further normalizations in order to compensate for GeneSpring's treatment of dye-swap technical replicates as biological replicates.

T-tests were performed on data from each treatment group and genes were retained if they were significantly differently expressed between exposed versus control, (P-value of ≤ 0.05), with a fold-change ≥ 1.40 up- or down-regulated. Due to the small number of samples tested, a multiple test correction was not employed in the statistical analysis. Multiple test corrections reduce the likelihood of reporting a false positive (reporting a gene as significantly differentially regulated when it was not), however, employing a multiple test correction then increases the chance of failing to recognize a true positive (eliminating a truly differentially regulated gene from a gene list). Small sample sizes reduce the statistical strength of each result, reducing the resultant p-value, and likely preventing genes from passing stringent multiple test corrections. To compensate for the lack of a multiple test correction, differentially regulated genes were sorted according to gene ontology (GO terms) and grouped into major biological functions or pathways, with the idea that numerous genes from the same pathway are unlikely to all be false positives.

3-1f. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

Total RNA was extracted as above using Trizol according to the manufacturer's instructions (Invitrogen). Possible contaminating DNA was removed from extracted RNA by an RNeasy on-column DNase digestion according to the manufacturer's instructions (Qiagen). cDNA was reverse transcribed using AffinityScript 1st strand

cDNA kit according to the manufacturer's instructions. Briefly, 1 µg aliquots of RNA were added to a reaction mix consisting of 10 µl 2x First strand master mix, 3 µl Oligo (dT) 20 primer (0.1 µg/µl), 1 µl AffinityScript RT/ RNase block enzyme mixture, and RNase/DNase-free water to a total volume of 20 µl. The reaction mixture was incubated at 25°C for 5 minutes to allow primer annealing, followed by synthesis at 42°C for 15 minutes, and the resulting cDNA was immediately placed at 4°C. For qRT-PCR reactions, 1 µl aliquots of cDNA were added to qRT-PCR reaction mixture (12.5 µl 2x Brilliant Sybr green master mix, 0.625 µl each of forward and reverse primers (10 µM) (Table 3-1), 0.375 µl ROX reference dye, and 9.875 µl ddH_2O) in 96 well plates and amplified in a Stratagene MX3000 thermocycler under the following conditions: Denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, optimal elongation temperature (Table 3-1) for 1 minute, and 72°C for one minute. A dissociation curve program was run on each plate consisting of an initial denaturation step at 95°C for one minute, followed by a ramping up of temperature (at 0.2 °C/sec) from 55°C to 95°C, with continual fluorescence data collection (Stratagene, Agilent Technologies). Data were normalized to a dilution series standard curve run on each plate. Dilution series were made of PCR amplification products of each gene of interest, generated using cDNA copies of mRNA present in a pooled sample extracted from 5 control Chinook salmon kidneys. Data were analyzed using MxPro qPCR software. T-tests were performed on average C_T values from triplicate samples.

3-2. Results and Discussion:

3-2a. PCR of *Loma salmonae* exposed gills at 8 weeks post-exposure (11 weeks post glucan inoculation):

Prevalence of *L. salmonae* infection was determined by PCR analysis of gill samples harvested from 8 glucan-inoculated, parasite-exposed, 8 saline (PBS) inoculated, parasite-exposed, and 8 parasite unexposed control fish harvested at 8 weeks PE. 100% of parasite-exposed fish from both glucan-inoculated and saline-inoculated groups tested positive for *L. salmonae* infection by PCR. All control fish (uninoculated, unexposed) tested negative.

3-2b. Immunohistochemistry of 8 week PE gills (11 weeks PI):

Intensity of *L. salmonae* infection was determined by immunohistochemical analysis of gill samples harvested 8 weeks PE. Total numbers of xenomas from thin sections of the first gill arch of 8 fish from each group were counted and are presented in Table 3-2. Data were analysed using the Mann-Whitney non-parametric test. The intensity of *L. salmonae* infection was significantly reduced in the glucan-inoculated group by ~ 66% ($p = 0.0091$).

IP inoculation of β-1,3/1,6 glucan (100 µg/100 µl) 3 weeks prior to *L. salmonae* exposure resulted in significantly reduced intensity of infection in Chinook salmon, measured by total xenoma counts in gill at 8 weeks PE. Although these results agree with previous studies involving rainbow trout (RBT), (Guselle et al. 2007), prevalence measured among exposed fish in this study does not agree with results from that work, wherein reductions of 60 – 70% from controls were reported. There was no reduction in prevalence among Chinook salmon in this study, with 100% of fish testing positive for *L.*

salmonae infection in both glucan- and PBS-inoculated groups. However, although RBT in that study were also exposed to macerated gill tissue, the numbers of spores were not enumerated, making it possible that the fish in this study were exposed to a higher dose of infective material. In addition, prevalence in this study was measured by PCR of DNA from gill tissue, whereas xenoma counts among the RBT groups were measured by simple observation under a stereomicroscope and/or by haematoxylin and eosin (H & E) staining of histological sections, neither of which are as sensitive as PCR. It is possible that PCR analysis of RBT gills in those studies would reveal higher prevalence among glucan-inoculated fish than was reported.

Conflicting results have been reported regarding the nature and strength of immune responses to glucan exposure (Bridle et al. 2005, Volman et al. 2008). It is possible that the differences reported in those studies were the result of temporal variations in immune response. For example, inclusion of β-glucan to the diets of Pacific white shrimp resulted in a bimodal response, with swift up- or down- regulation of key immune genes ocurring within 24 h, followed by a more delayed response involving other key genes, commencing at 3 to 7 days (Wang et al. 2007). In these shrimp the later-stage changes in gene expression agreed with increases in functional cell-based immune responses such as superoxide dismutase (SOD) activity, phagocytosis, and superoxide anion production (Wang et al. 2007). Temporal variations may result in enhanced immune function for weeks following glucan exposure. RBT inoculated with β-glucan 3 weeks prior to parasite exposure were more resistance to *L. salmonae* infection than RBT inoculated 1 week prior to exposure (Guselle et al. 2007). Similarly, high levels of *in vitro* H_2O_2 production and phagocytic uptake by HK macrophages, as well as serum

lysozyme activity, were measured at 3, and 6 weeks after β-glucan inoculation in Atlantic salmon (Brattgjerd et al. 1994).

Here, intensity of *L. salmonae* was reduced in Chinook salmon inoculated with β-1,3/1,6 glucan 3 weeks prior to parasite exposure. It is not known how long glucan remains present within the peritoneal cavity following IP inoculation, interacting with innate cells that release cytokines or chemokines in order to attract circulating phagocytic cells to that area. In mice, IP inoculated, soluble β-glucan is taken up by macrophages that become localized in the spleen and liver, whereas particulate glucans are taken up by macrophages that remain localized in the peritoneal cavity (Tateishi et al. 1997). Particulate glucan was used in this experiment, but it is unknown whether salmonid immune cells process glucans similarly to those of mice. It is possible that the reduction in *L. salmonae* intensity in glucan-inoculated fish is a result of an accumulation of innate immune cells such as macrophages in the peritoneum, where they may be in a prime position to interact with *L. salmonae* infected cells very early in the infection process. On the other hand, since *L. salmonae* is believed to be transported through the vascular system, perhaps the reduced intensity is due to increased and/or stronger interactions between innate cells and *L. salmonae*-infected cells within highly vascular immune organs such as kidney or spleen.

In addition to chemotaxis of already circulating immune cell populations, it is also possible that the reduction in parasite intensity may be due to increased numbers of innate effectors as a result of glucan-induced hematopoiesis. Tateishi et al found that hematopoiesis, measured by levels of colony stimulating factor (CSF), was induced in glucan exposed mice, although the timeframe of induction varied depending on the nature

of the glucan, with inoculation of soluble glucan resulting in high levels of CSF that persisted for 2 weeks PI, whereas particulate glucan stimulated high levels of CSF that peaked by 2 days and were nearly back to control levels by one week PI (Tateishi et al. 1997).

In fish, IP injection with β-glucan increased the proportion of neutrophils in head kidney at 22 days PI (40% compared with 25% for controls), although the mechanism of this increase was not demonstrated. The same study showed no significant increase in the numbers of macrophages in head kidney (Jorgensen et al. 1993). These researchers did not determine whether the increased proportion of resident HK neutrophils were the result of increased hematopoiesis, decreased numbers of other cell types, or reflected the accumulation of circulating cells. In mice, β-glucan exposure affects the chemotactic capacity of circulating neutrophils, allowing them to pass more readily from the blood into tissues (LeBlanc et al. 2006). These researchers did not measure any increase in the number of circulating neutrophils, although the numbers were measured within hours after glucan exposure. In contrast, Selvaraj et al, working with carp (*Cyprinus carpio),* measured significant, dose-dependent increases in the numbers of circulating total blood leukocytes, along with an increase in the proportion of neutrophils and monocytes 7 days after IP injection with β-glucan (Selvaraj et al. 2005). It would be interesting in the future to measure numbers and proportions of leukocytes within blood and the peritoneal cavity of Chinook salmon at various time points after glucan inoculation.

Here Chinook salmon were exposed to *L. salmonae* after inoculating them with the optimal glucan dose and timing determined for RBT, since the timing of glucan exposure affects the level of protection against *L. salmonae* in that species (Guselle et al.

2007). Glucan inoculation under these conditions was protective in terms of reducing the severity of infection measured by numbers of xenomas in gill tissue. An additional objective of this study was to identify patterns of gene expression in response to glucan inoculation. Head kidney tissue was chosen for gene expression analysis since it plays important roles in immune function and is the primary hematopoietic organ of teleosts.

3-2c. Analysis of gene expression in head kidney at 1, 2, and 3 weeks post-Glucan inoculation (PI):

The numbers of differentially regulated genes were highest at 1 week PI compared with 2 and 3 weeks PI (Table 3-3). Pathways involved with antioxidant defences, innate immune responses, antigen processing and presentation, apoptosis, as well as oxidative metabolism were up-regulated at 1 week PI, whereas complement was the only pathway that appeared to be down-regulated at this time point. Pathways associated with complement as well as antigen processing and presentation via MHC I were both down-regulated at 2 weeks PI. Differential regulation of haematopoietic-associated pathways was not identified in these samples. Complete lists of differentially expressed genes for each week are presented in Appendix 1.

3-2c.1. Defence Responses - Antioxidant defences:

Numerous genes involved in antioxidant defence were up-regulated in the glucan-inoculated samples at 1 week PI (Table 3-4), including genes involved in glutamate and glutathione metabolism. In addition, numerous genes encoding glutathione related enzymes, including peroxiredoxins, glutathione peroxidases (GPX), selenoproteins, glutathione S transferases (GST), retinol dehydrogenases, and multidrug resistance

associated protein (MRP) showed increased expression in glucan-inoculated fish at 1 wk PI (Table 3-4).

Reactive oxygen species (ROS) can be generated by several mechanisms, and the products of these reactions, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), are highly reactive molecules, each capable of damaging cellular macromolecules unless quickly eliminated. ROS can be produced as a continuous byproduct of metabolism in cells, or by enzymes such as cyclooxygenases (COX) or Cytochrome P450 monooxygenases. In addition, ROS are generated as a killing mechanism by specialized enzyme complexes in phagocytic immune cells. The production of ROS begins with the generation of superoxide anion (O_2^-), which is highly reactive and is quickly converted by the enzyme superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Cells employ numerous enzymatic and non-enzymatic anti-oxidant mechanisms as a first line of defence in order to eliminate ROS, but even with rapid detoxification, some cellular damage may occur. A second line of anti-oxidant enzymes act to minimize this damage by preventing free radicals from propagating redox chain reactions. Once they have been detoxified, the metabolites are removed from the cell via energy-dependent efflux pumps (Figure 3-1) (Hayes & McLellan 1999).

Glutathione and glutathione-related enzymes are important components of anti-oxidant defence systems. Reduced glutathione (G-R) is a scavenger of free radicals and plays a part in the reduction of H_2O_2 (Hayes & McLellan 1999). Glutathione-related enzymes are involved in first and second lines of antioxidant defences (Figure 3-1). Glutathione is synthesized in the cell from L-Glutamate and L-Cysteine in a 2 step

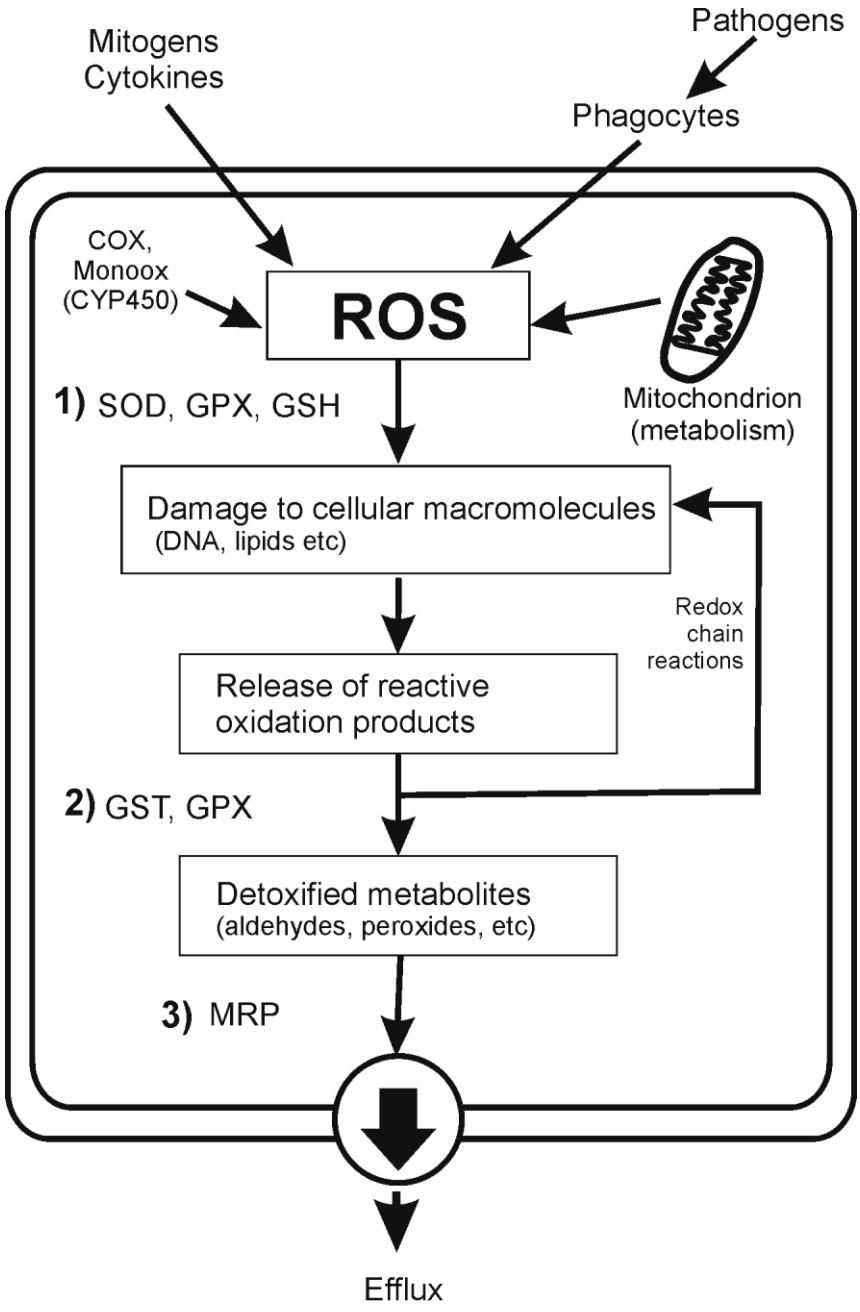

Figure 3-1. Antioxidant mechanisms. Multiple levels of defence against reactive oxygen species (ROS). 1) Enzymes of the first level include superoxide dismutase (SOD), glutathione peroxidases (GPX), reduced glutathione (G-R) and others that act to eliminate the superoxide anion and hydrogen peroxide. Second level enzymes such as glutathione-S-transferases (GST) and GPX detoxify the resulting reactive oxygen products. 3) The resulting metabolites are eliminated from the cell by energy dependent efflux pumps such as the multidrug resistance protein (MRP). Adapted from (Hayes & McLellan 1999).

reaction catalyzed by the actions of γ-glutamyl-L-cysteine and glutathione synthetase. As mentioned, several genes associated with glutamate and glutathione were differentially regulated here. The enzyme alanine aminotransferase (AAT) is involved in glutamate biosynthesis and has been used as a marker for biotoxicity in hepatocytes (Silva et al. 1999a, Silva et al. 1999b). Succinate semialdehyde dehydrogenase is an oxidoreductase that plays a role in glutamate metabolism (Albers & Koval 1961). S-adenosylmethionine synthetase is involved in the biosynthesis of glutathione (Tabor & Tabor 1984). Gamma glutamyltranspeptidase (GGT) is a membrane-bound enzyme involved in glutathione as well as leukotriene metabolism. GGT metabolizes extracellular reduced glutathione, resulting in the generation of ROS and peroxidation of lipids (Maellaro et al. 2000). Ornithine decarboxylase antizyme is responsible for the rapid degradation of ornithine decarboxylase, which is involved in the metabolism of glutathione (Gandre et al. 2002).

Glutathione related enzymes, including peroxiredoxins, glutathione peroxidases (GPX), selenoproteins, and glutathione S transferases (GST) are implicated in protecting cells against peroxidative damage (Hayes & McLellan 1999, Brenneisen et al. 2005). Peroxiredoxins exhibit peroxidase activity towards H_2O_2 (Wood et al. 2003), whereas glutathione peroxidases (GPX) are families of selenium-dependent and selenium-independent enzymes that generate oxidized glutathione while catalysing the reduction of H_2O_2 and organic hydroperoxides to water and alcohols (Hayes & McLellan 1999). For example, GPX1 is a selenium-dependent enzyme found in both the cytosol and mitochondria of cells that is very important in the reduction of H_2O_2 in mice (Haan et al. 1998). Selenoproteins constitute a family of enzymes that have selenium-dependent GPX activity.

Glutathione-S-transferases (GST) are another diverse group of enzymes that participate in cellular detoxification through the conjugation of G-R with reactive epoxides and hydroperoxides formed by the action of ROS on macromolecules (Hayes & McLellan 1999). GSTs also have roles in eicosanoid and prostanoid biosynthesis, and some have selenium-independent GPX activity (Hayes & McLellan 1999). There are two main superfamilies of these enzymes with different functions, the soluble GSTs and the microsomal GSTs. Soluble GSTs act as detoxification enzymes, reacting with the byproducts of ROS-damaged macromolecules, whereas the microsomal GSTs inhibit lipid peroxidation, and some are known to have roles in leukotriene biosynthesis. The microsomal GST superfamily is also called MAPEG, for Membrane-Associated Proteins involved in Eicosanoid and Glutathione metabolism (Hayes & McLellan 1999). Prostaglandin E synthase (PES) is a member of the MAPEG superfamily involved in the biosynthesis of prostaglandin E_2 in humans. Prostaglandins are eicosanoid lipid mediators synthesized from arachidonic acid that play important roles in a variety of organs and tissues, such as vasodilation, smooth muscle relaxation, and inflammation. Prostaglandins act in an autocrine or paracrine fashion through interaction with G-protein-coupled receptors (Funk 2001). In addition, 15-hydroxyprostaglandin dehydrogenase is the key enzyme involved in the breakdown of prostaglandins and may have Prostaglandin E receptor activity (Tai et al. 2002, Otani et al. 2006).

Once antioxidant defence enzymes act to detoxify ROS and their reaction products, these by-products must be eliminated from the cell. Multidrug resistance associated protein (MRP), also called glutathione S-conjugate transporter, is an efflux-

pump responsible for exporting the metabolites generated by anti-oxidant enzymes (Hayes & McLellan 1999).

Retinol dehydrogenases belong to an additional class of glutathione-dependent enzymes represented on this microarray. These enzymes belong to the alcohol dehydrogenase (ADH) family found in bacteria, yeast, invertebrates and vertebrates. Retinol dehydrogenases are involved in the metabolism of retinol, an important molecule for haematopoiesis and the maintenance of immune cells such as neutrophils, macrophages and dendritic cells (Blomhoff 1994, Semba 1998).

Taken together, these results show that antioxidant defences are up-regulated in the kidneys of glucan-inoculated fish at 1 week PI, possibly indicating the development of an oxidative stress response to IP glucan exposure.

3-2c.2. Immune responses – Innate and adaptive:

Several immune-related pathways showed differentially regulated genes at 1 and 2 weeks PI, including numerous innate components, antigen processing and presentation pathway components, and others.

3-2c.3. Immune responses – Complement:

Several components of the complement system exhibited differential expression in kidney from glucan-inoculated fish at 1 and 2 weeks PI. At 1 week PI, C3 showed increased expression, whereas the expression of 2 components of the MAC (C6 and C7), as well as mannose-binding protein, were reduced at the same time point (Table 3-5). By 2 weeks PI, only one complement protein, C3-1, was differentially expressed, exhibiting reduced expression.

The complement system comprises a group of serum proteins that act together to target and kill invading pathogens. There are three methods by which the latter stages of the complement system are initiated; the classical pathway, the alternative pathway, and the lectin pathway. The lectin pathway is initiated by the binding of lectins, such as mannose-binding lectin (MBL), to sugars on carbohydrates or glycoproteins displayed by microorganisms. However they are activated, the three pathways converge at the critical C3 component. Each pathway activates a C3 convertase enzyme, which splits C3 into C3a and C3b. C3a is an anaphylatoxin, which is able to induce inflammation and elicit both innate and adaptive responses. C3b is a potent opsonin able to bind to the surface of pathogens such as parasites, fungi, bacteria and viruses, and also to complement receptors on phagocytic cells, promoting phagocytosis, respiratory burst, and antigen uptake by APCs. C3b is also a component of the next enzyme in the cascade, which cleaves C5 into two products, one of which combines with additional proteins C6, C7, C8, and C9 to form the membrane attack complex (MAC). MAC lyses target cells by inserting directly into the membrane to form pores (Boshra et al. 2006). In previous studies, expression of C3 was also increased in rainbow trout head kidney macrophages after in vivo β-glucan administration (Lovoll et al. 2007).

The expression of 2 components of the MAC (C6 and C7), as well as mannose-binding protein, were reduced at 1 week PI (Table 3-5). It is unknown whether this means that components of the complement pathway were being oppositely regulated. Microarrays measure relative quantities of cDNA generated from the mRNA present in a group of cells at a particular point in time. It is not known whether the RNA present in the cells reflects differential levels of transcription, RNA processing or RNA turnover.

Since sampled tissues contain a heterologous mix of cell types, it is possible that components of the complement system are being regulated differently within particular groups of cells. It is also possible that components of the pathway are being oppositely regulated in order to maintain cellular homeostasis, with reduced expression of some components compensating for the increased expression of others. If so, the up-regulation of C3 and concurrent down-regulation of C6 and C7 components may result in increased levels of the anaphylatoxin C3a (leading to inflammation) and the opsonin C3b, while maintaining steady-state levels of the MAC components.

At 2 weeks PI genes representing lectin, nattectin, as well as alpha 2-macroglobulin (A2M) showed decreased expression at 2 weeks PI (Table 3-5). Nattectin is a C-type lectin able to recruit immune cells such as neutrophils and macrophages during innate intra-peritoneal immune response, where the macrophages then differentiated into functional dendritic cells (DC) in mammals (Saraiva et al. 2009). Alpha 2-macroglobulin may play roles in innate responses, down-regulating immune cell activation as well as promoting the migration of immune cells (Tayade et al. 2005).

3-2c.4. Immune responses – Acute phase proteins:

The expression of ferritin was up-regulated at 1 week PI in glucan-inoculated fish (Table 3-5). Ferritin plays a role in defence responses, sequestering iron, an element that pathogens may require for growth and survival (Ong et al. 2005). Ferritin has been characterized as an acute phase protein that is up-regulated in response to ROS and cytokines produced during conditions of oxidative stress, infection, or inflammation (Koorts & Viljoen 2007, Orino & Watanabe 2008). The up-regulation of acute phase

proteins at 1 week PI may indicate the development of a stress response to glucan inoculation.

3-2c.5. Immune responses - Monooxygenases:

Several monooxygenase enzymes of the cytochrome P450 superfamily were differentially expressed in the glucan-inoculated fish, exhibiting slightly increased expression at 1 week PI (Table 3-5).

Cytochrome P450s (CYP450) belong to a ubiquitous superfamily of enzymes that are responsible for catalyzing a wide range of monooxygenase reactions involving both endogenous and xenobiotic targets (Buhler & Wang-Buhler 1998). Although the majority of research into CYP450s has involved mammalian models, there is evidence for a complex detoxification system in teleosts that includes a wide variety of isoforms with diverse substrates (Miranda et al. 1990, Buhler & Wang-Buhler 1998, Lee et al. 2001). The detoxification reactions of CYP450s can lead to the generation of ROS in cells (Guengerich 2001). In addition, some CYP450s are capable of metabolizing eicosanoids such as prostanoids and leukotrienes (Capdevila & Falck 2002).

Three CYP450 enzymes were differentially expressed, none of which have been extensively studied in fish. CYP450 3A27 isoform of fish shares structural homology with the CYP450 3A subfamily of mammals, which comprise the most abundant CYP 450 form in humans and are involved in the metabolism of a variety of drugs (Lee et al. 2001). CYP450, 46A1 has been described in humans as an enzyme with multiple substrates, including xenobiotic targets, although its functional role in fish remains undescribed (Mast et al. 2003). The substrate specificity of the final CYP450 enzyme up-regulated in the kidney samples from glucan-inoculated fish, CYP450 4V3, remains

largely unknown for either mammals or teleosts. The up-regulation of CYP450s at 1 week post glucan inoculation may be evidence of a role for monooxygenase involvement in glucan processing.

3-2c.6. Immune Responses - Apoptosis:

Several genes related to apoptosis showed increased expression in glucan-inoculated fish at 1 week PI, including p53 apoptosis effector related to PMP-22 (PERP), reticulon 4 and cathepsin B (Table 3-5).

P53 apoptosis effector related to PMP-22, or PERP, is known to be highly expressed in mammalian cells undergoing p53-induced apoptosis, although the requirement for this gene appears to be cell type-specific (Ihrie et al. 2003, Singaravelu et al. 2009). The activity of PERP has not been described in teleost cells. Reticulon 4 is a member of the reticulon family of endoplasmic reticulum (ER)-related proteins with a possible role as an inducer of apoptosis (Sironen et al. 2004). Reticulon 4 is believed to interact with Bcl-1 and/or Bcl-2, reducing their anti-apoptotic effects (Tagami et al. 2000). Cathepsin B has been implicated as a key protease in tumor cell apoptosis under the influence of certain cytokines such as tumor necrosis factor (TNF) (Foghsgaard et al. 2001). Cathepsin B is over-expressed in certain disease contexts that involve tissue remodelling, including some parasitic infections, as well as during inflammatory responses to certain bacterial infections (Del Nery et al. 1997, Kakegawa et al. 2004, Frlan & Gobec 2006). Although several genes associated with apoptosis show increased expression in these samples, it is unknown whether such up-regulation is manifested as cell death in response to glucan-inoculation.

3-2c.7. Immune responses - Antigen processing and presentation:

Components of both the MHC I and MHC II antigen processing and presentation pathways were differentially expressed during this sampling period (Table 3-5). At 1 week PI members of the Cathepsin L family involved in the MHC II pathway showed increased expression. At the same time point several components of the MHCI pathway were up-regulated in glucan inoculated fish, including Beta-2-microglobulin, proteasome associated proteins, and HSP70. In contrast, at 2 weeks PI two genes associated with the MHC I pathway coding for histocompatability antigens as well as the accessory protein tapasin each showed reduced expression (Table 3-5).

Adaptive immunity in vertebrates is characterized by the antigen-specific activation of B- and/or T-lymphocytes displaying antigen-specific receptors on their surface. B-cells bind to their specific antigen directly through their surface Ig, but T-cell receptors recognize specific antigenic peptides only when they are presented in the context of major histocompatibility molecules (MHC). The MHC system comprises two major classes of glycoproteins; MHC I, which is displayed on the surface of self cells and presents antigenic peptides derived from intracellular pathogens, and MHC II, which is confined to specialized antigen presenting cells (APC), and displays antigenic peptides derived from extracellular pathogens. MHC II signalling serves as a link between the adaptive system and the innate systems, influencing the release of particular cytokines that amplify and direct the nature of the response.

3-2c.7.1. Antigen processing and presentation – MHC II:

MHC II is displayed only on specialized APC such as dendritic cells (DC), macrophages, and B-cells. Presentation of antigen by MHC II requires processing

through the exogenous pathway, wherein a pathogen is internalized via phagocytosis and degraded into antigenic peptides within endocytic vesicles (Figure 3-2). MHC II presents antigen to $CD4^+$ T helper (T_H) cells, which release cytokines that amplify and direct the immune response, and thereby act as a bridge linking the innate to the adaptive immune systems. The α- and β chains of MHC II associate with a stabilizing protein called the invariant chain within the rough endoplasmic reticulum, pass through the Golgi body, and are released in vesicles that fuse with the endocytic compartments containing the digested peptides. Within the fused compartments the peptides are inserted into the binding groove formed by the α- and β-chains before being transported to the cell surface for display to T_H cells (Castellino et al. 1997). Cysteine endoproteases of the Cathepsin family (Cathepsin-S, -F & -L) participate in this process, both in invariant chain processing and later in its degradation in order to allow peptide binding (Riese et al. 1998, Shi et al. 2000).

3-2c.7. 2. Antigen processing and presentation – MHC I:

MHC I are displayed on all nucleated self cells and present antigen to $CD8^+$ T_C cells, which, once activated, are able to lyse target cells. "Altered self" cells (tumour cells, virally- or parasite-infected cells) process antigenic peptides for display by MHC I via the endogenous processing pathway. As outlined in Chapter 1, foreign proteins are degraded in the cytosol by a proteasome complex, generating antigenic peptides (Figure 3-2). Class I MHC consists of a heavy chain (HC) containing two domains (α1 and α2) that combine to form an antigenic peptide binding cleft, along with a noncovalently associated β-2 microglobulin (β2M) chain. The HC-β2M complex is assembled within the ER with the aid of chaperone proteins such as calnexin and calreticulin, as well as

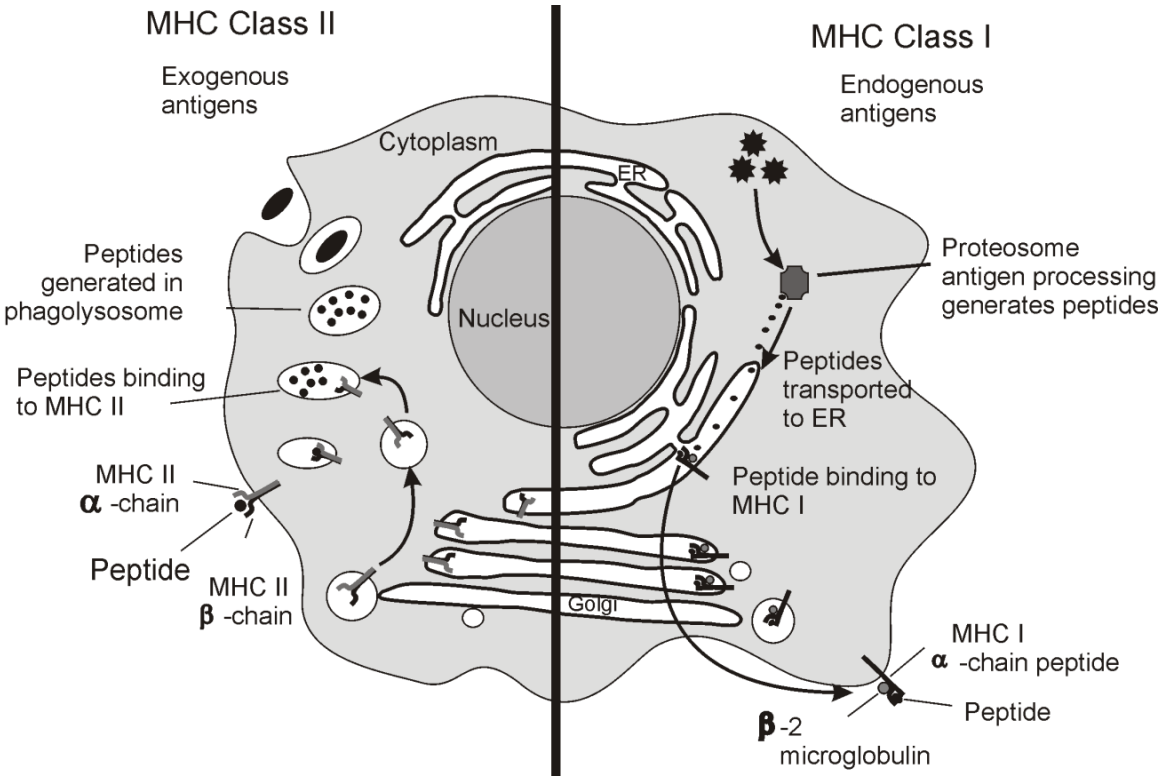

Figure 3-2. Antigen processing and presentation pathways.

MHC Class II - External antigens are processed through the exogenous pathway to be presented in the context of MHC II. Peptides are generated as the pathogen is broken down within increasingly acidic endocytic vacuoles and complexed with MHC II α and β chains. **MHC Class I** - Internal antigens are processed through the endogenous pathway to be presented in the context of MHC I. Antigenic peptides are generated by the proteasome and complexed with MHC I α-chain and β-2 microglobulin. Adapted from: (Parham 2005).

molecular chaperones such as heat shock protein 70 (HSP70). The HC- β2M, associated with calreticulin, forms a complex with the transporter associated with antigen processing (TAP) that transports the processed antigenic peptides from the cytosol into the ER. This process is aided by the TAP-associated glycoprotein, tapasin. Once bound to a peptide, the completed MHC I is transported to the cell surface (Maffei et al. 1997).

3-2c.8. Oxidative metabolism – Glycolysis and the Mitochondrial electron transport chain:

Multiple genes associated with glycolysis and the mitochondrial electron transport chain (METC) were up-regulated at 1 week PI (Table 3-6). Up-regulated genes associated with glycolysis included glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and isocitrate dehydrogenase (ICD). Components of the METC exhibiting increased expression at 1 week PI included components of ComplexI (CI), ComplexIII (CIII), ComplexIV (CIV) and ComplexV (CV), (Table 3-6).

Much of the energy needed by the cell comes from the complete breakdown of carbohydrates such as glucose in order to generate ATP. During glycolysis a 6-carbon glucose is broken down to two 3-carbon molecules of pyruvate which then enter the citric acid cycle (TCA) to be broken down more completely (Figure 3-3). Glyceraldehyde-3-phosphate dehydrogenase, is a key enzyme catalyzing the reversible conversion between glyceraldehyde-3-phosphate and 1,3-bisphosphoglycerate in the first step of the energy-yielding phase of glycolysis (Horton et al. 2006). Pyruvate kinase catalyzes the final step of glycolysis, converting phosphoenolpyruvate to pyruvate, the 3-carbon product of glycolysis. Isocitrate dehydrogenase (ICD) is an enzyme that catalyzes the third step of the TCA cycle: the oxidative decarboxylation of isocitrate to α-ketoglutarate and CO_2 while converting NAD^+ to NADH (Horton et al. 2006).

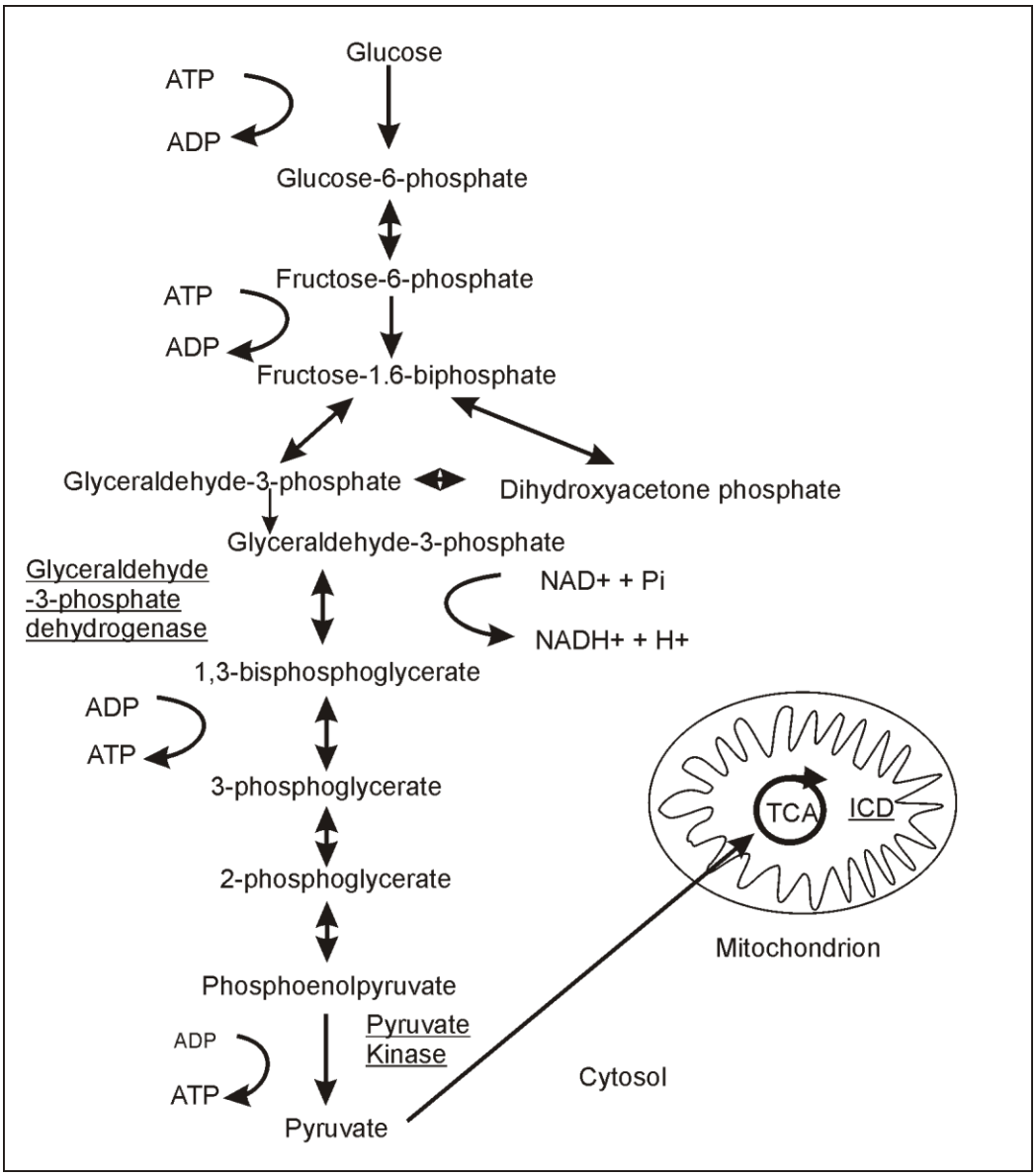

Figure 3-3. Glycolysis and the Citric acid cycle (TCA).

Glucose is broken down in the cytosol during the multistep process of glycolysis, resulting in the release of two 3-carbon molecules of pyruvate. Pyruvate is translocated to the inner mitochondrial matrix for further breakdown in the citric acid cycle (TCA). Differentially regulated enzymes are indicated and underlined, including glyceraldehyde-3 phosphate dehydrogenase, pyruvate kinase, and isocitrate dehydrogenase (ICD).

Although a limited amount of ATP is generated directly within the cytosol, the majority of energy is produced via the transfer of reducing equivalents such as NADH from the cytosol to the mitochondria, where electrons are coupled to ATP production through the mitochondrial electron transport chain (METC) during the process of oxidative phosphorylation. The METC consists of 4 multi-subunit electron transport chain complexes (CI to CIV) along with the ATP synthase (CV) arrayed along the mitochondrial inner membrane (Figure 3-4). Electrons are deposited from NADH or succinate, entering the METC at CI or CII respectively. From there they are transported by ubiquinone and cytochrome c to CIII, and CIV. Molecular oxygen (O_2) acts as the ultimate electron acceptor, and electrons are transferred to O_2 at CIV to form water (H_2O). H^+ ions are transferred across the inner membrane at 3 points (CI, CIII & CIV) during electron transport, creating an electrochemical gradient utilized by CV to generate ATP (Willems et al. 2009).

In addition to its role in energy generation, certain components of the METC are capable of generating ROS, and are responsible for the majority of ROS produced in non-immune cells during normal metabolism. This is in contrast to immune cells, which produce ROS as a killing mechanism by means of specialized membrane associated NADPH oxidase complexes (Bokoch & Knaus 2003). It is possible that the METC components are up-regulated in order to provide more cellular ATP as a consequence of increased energy demands in fish exposed to glucan, resulting in production of ROS, which then triggers compensatory anti-oxidants. In order to maintain cellular redox homeostasis cells must finely regulate the levels of anti-oxidants to ROS.

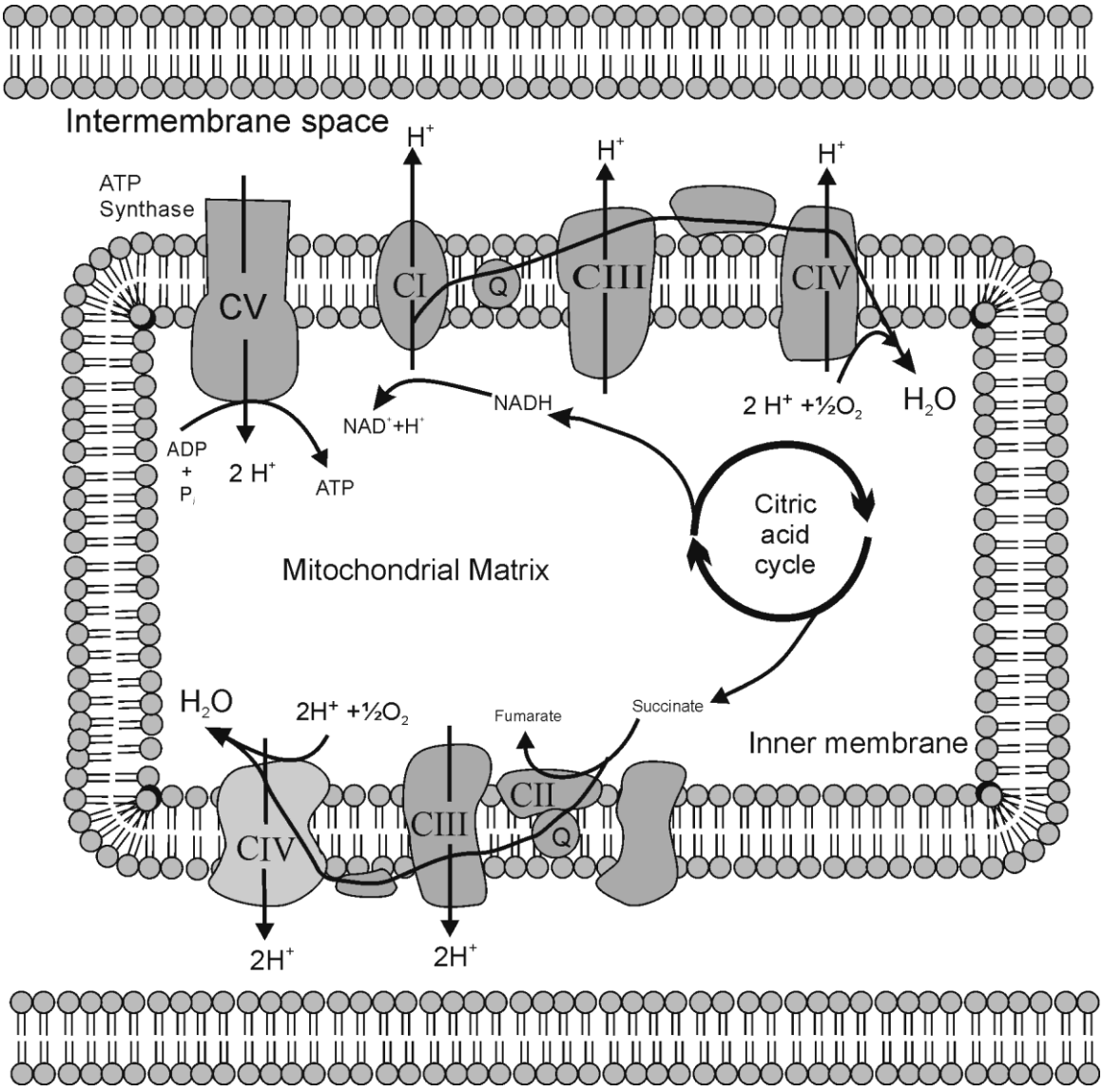

Figure 3-4. The mitochondrial electron transport chain (METC).

Multi-subunit enzyme complexes (CI through CV) are arrayed along the inner mitochondrial membrane, wherein electrons are deposited from NADH or succinate. Electron transport along the METC results in the transfer of H^+ ions into the intramembranous space, building an electro-chemical gradient utilized by the CV complex for the generation of ATP. Adapted from:

http://commons.wikimedia.org/wiki/File:Mitochondrial_electron_transport_chain_pl.svg

3-2d. Pathway Analysis:

Overall trends among glucan-inoculated fish are summarized in Table 3-7. It is apparent that a number of important pathways were up-regulated in the kidney at 1 week PI. Numerous genes involved in the antioxidant defence, immune responses, as well as oxidative metabolism pathways were up-regulated, although in each of these cases the majority of genes had increased expression of between 1.5 – 2-fold (Tables 3-4 to 3-6). Researchers have often arbitrarily decided on a 2-fold cut-off as a "meaningful" expression level change in microarray data analysis. However, imposing such arbitrary boundaries may cause researchers to overlook important trends in gene expression. Analysis of data from this study reveals that many genes from the same pathways are exhibiting similar, albeit moderate expression level changes that may add up to a large change overall, and which are interpreted here as meaningful results.

3-2e. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR):

Three genes that were significantly differentially expressed in the microarray study were further tested by qRT-PCR. The results of the qRT-PCR tests agreed with microarray results in terms of directionality of fold change for complement component C7, as well as the Cytochrome P450s 4V3, and 26A1 (Table 3-8). qRT-PCR results for each gene were significant by t-test ($p \leq 0.05$). Genes were chosen for qRT-PCR analysis for the purpose of reinforcing confidence in the presented microarray results and were therefore arbitrarily chosen for their low p-value based on microarray t-tests. The close agreement between microarray fold-changes and qRT-PCR fold changes provide evidence that the microarray results presented here represent the relative levels of mRNA

in kidney cells at the sampling time. Therefore these qRT-PCR results serve to reinforce confidence in the results of this microarray analysis, as intended.

3-3. Conclusions:

IP-inoculation with β-1,3/ 1,6 glucan 3 weeks prior to *L. salmonae* exposure had a protective effect on Chinook salmon, significantly reducing the intensity of infection in inoculated fish, as measured by xenoma counts in the gill, whereas prevalence remained unaffected. These results partially agreed with studies of RBT, in which reductions of both prevalence and intensity were measured. The timing of IP glucan administration played a large role in its efficacy at mitigating *L. salmonae* infection in RBT. Glucan administered to rainbow trout (RBT) 3 weeks prior to *L. salmonae* exposure was more effective at reducing prevalence compared with glucan administered to RBT 1 week prior to parasite exposure. In addition, glucan administered to RBT 1 week after *L. salmonae* exposure resulted in the lowest resistance of all (Guselle et al. 2007). The researchers speculated that handling- and inoculation-induced stress may have resulted in depressed immune responses unrelated to glucan-specific effects. Although glucan was not administered after parasite exposure here, gene expression analysis revealed that in the kidney at 1 week PI many genes involved in antioxidant defence responses showed significant up-regulation, possibly indicating some kind of stress response. However, it is unlikely that any oxidative stress in the glucan-inoculated fish was a consequence of handling, since control fish were handled identically and IP-inoculated with a neutral saline solution. Therefore it can be concluded that the antioxidant response was directly related to glucan exposure and was not a consequence of handling or inoculation stress.

In addition to the the antioxidant pathway, a number of immune and energy related pathways were also up-regulated at 1 week PI. In general, immune pathways showed low-level up-regulation of innate components, a finding which agrees with the accepted model of glucan as an innate immune system stimulant. In addition, the timing of peak response was early, and suggests a lengthy "ramping up" of peak innate immunity, which also agrees with the previous findings in RBT. The fact that fish exposed to glucan are more refractory to *L. salmonae* infection implies increased and/or more effective interactions between immune components and *L. salmonae* early on in the infection. These results did not fully elucidate that process. Increased hematopoiesis was not measured in response to glucan inoculation at these sampling times. Interestingly, there are 2 copies of granulocyte-colony stimulating factor (G-CSF) represented on this array, neither of which were significantly differentially expressed amongst these samples. G-CSF is the major haematopoietic growth factor for neutrophils (Appelberg 2007). These results suggest that hematopoiesis is not being induced within head kidney in response to β-glucan inoculation at the 1, 2, or 3 week sampling times. However, as mentioned, induction of hematopoiesis in glucan exposed mice varied depending on the nature of the glucan, with inoculation of soluble glucan resulting in high levels of CSF for 2 weeks PI, whereas particulate glucan stimulated high levels of CSF that peaked by 2 days and were nearly back to control levels by one week PI (Tateishi et al. 1997). It is possible that haematopoiesis was induced early in these fish in response to particulate glucan inoculation and had returned to control levels by 1 week PI, making the first sampling period too late to identify a strong haematopoietic response. In the future it would be informative to analyze gene expression in response to glucan at time points

very soon after inoculation. It would also be interesting to collect intraperitoneal cells from glucan-exposed fish in order to measure numbers and relative proportions of macrophages, monocytes and neutrophils.

Table 3-1. Primer Sequences for qRT-PCR of selected genes.

Gene	Annealing Temperature (°C)	Primer Sequence (5′ – 3′)
Complement - C7 precursor	58	Forward – CCAGACAGTGCTGTTGCTGT
		Reverse – GAACAGGCCTTGATGCTGAC
CYP450 46A1	55	Forward – GACGCTGCCAAACCCTATTA
		Reverse – GCAGGCTGAAGTCAAACCTC
CYP450 4V3	55	Forward – GTGGTCAGCTTGGGTTCAGT
		Reverse – ACCCTAGCAACGACTTCAGC

Table 3-2. Xenoma counts from gills of *L. salmonae*-infected salmon.

Fish were sampled at 8 weeks post-parasite exposure, 11 weeks post-inoculation. Intensity was measured by total xenoma counts from the first gill arch of Chinook salmon sampled 8 weeks post-exposure to *L. salmonae*. Fish were inoculated with β-glucan or saline 3 weeks prior to *L. salmonae* exposure.

Glucan-exposed fish (# of xenomas/gill arch)	Control fish (# of xenomas/gill arch)
12	30
18	51
21	90
27	102
48	134
54	182
63	206
110	294

Table 3-3. Numbers of differentially expressed genes in glucan inoculated fish.

Differentially expressed genes in glucan-inoculated fish sampled at 1-, 2-, or 3-weeks PI. Normalized levels were expressed as ratios between glucan-inoculated and control (saline-inoculated) fish. Genes significant by t-test ($p \leq 0.05$), with a level of expression of ≥ 1.4-fold up or down were included.

Weeks post-inoculation	# of genes up-regulated (≥ 1.4 fold)	# of genes down-regulated (≥ 1.4 fold)
1	238	58
2	155	36
3	10	5

Table 3-4: Differentially expressed genes involved in antioxidant defence responses. Normalized levels were expressed as ratios between Glucan inoculated and control fish. Genes with a relative expression level of 1.40-fold (up or down) and significance of $p \leq 0.05$ were included, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 3-4. Antioxidant defence responses in Glucan-inoculated fish.

EST #	TC #	Species	# of ESTs	Gene Name	Fold change 1 week		Fold change 2 week	Fold change 3 week
Defence responses - Antioxidant defences								
Glutamate metabolism								
CA064225	TC82084	*Ssal*		Alanine aminotransferase 2	2.49			
Glutathione metabolism								
CK991153	TC82189	*Ssal*		S-adenosylmethionine synthetase isoform type-2	2.28	*		
CA051028	TC87969	*Ssal*		Gamma-glutamyltranspeptidase 1 precursor	1.72			
CB493774	TC143137	*Ssal*		Ornithine decarboxylase antizyme 1	2.21			
CA060381	TC84854	*Ssal*		Ornithine decarboxylase antizyme 1	2.04			
CB512768	TC80214	*Ssal*		Succinate semialdehyde dehydrogenase	1.88			
CB517306	TC64417	*Ssal*		Glucose-6-phosphate 1-dehydrogenase	-1.55	*		
Peroxidase activity								
CB491371	TC147490	*Ssal*		Glutathione peroxidase 1	1.73			
CB510681	TC104291	*Ssal*		Phospholipid hydroperoxide glutathione peroxidise	1.47			
CB509723	TC81840	*Ssal*		Phospholipid hydroperoxide glutathione peroxidise				1.53
CB512125	TC75618	*Ssal*		Peroxiredoxin	1.50			
CA049981	TC81806	*Ssal*	3	Selenoprotein Pa precursor	2.31			
CB502803	TC81389	*Ssal*	2	Selenoprotein Pa precursor	1.96			
CB510462	TC93887	*Ssal*		Selenoprotein Pb precursor	1.58			
CA052444	TC77212	*Ssal*		Putative selenoprotein O			2.00	

Table 3-4, Antioxidant defences continued

EST #	TC #	Species	# of ESTs	Gene Name	Fold change 1 week		2 week	3 week
Glutathione S-transferases								
CB492604	singleton	*O myk*		Glutathione S-transferase A	2.31			
CB497579	TC142140	*O myk*		Glutathione S-transferase P	1.53			
CA050452	TC67699	*Ssal*		Glutathione S-transferase P	1.52			
CB492806	TC146311	*O myk*		Microsomal glutathione S-transferase 3	1.65	**		
Eicosanoid metabolism								
CB496601	TC133159	*O myk*		15-hydroxyprostaglandin dehydrogenase [NAD+]	1.66	*		
CA063917	TC64606	*Ssal*		15-hydroxyprostaglandin dehydrogenase [NAD+]	1.53	*		
CB490484	TC155261	*Ssal*		Prostaglandin E synthase 3	1.51			
Glutathione -dependent enzymes								
CA054079	TC88645	*Ssal*		Retinol dehydrogenase 7	1.74			
CA062348	TC66273	*Ssal*		Retinol dehydrogenase 3	1.67			
NF-κB								
CB492474	singleton	*O myk*		Sequestosome-1 (NF-kappa B regulation)	1.50	**		
Efflux								
CB492627	TC157146	*Ssal*		Multidrug resistance-associated protein 4	1.82			

Table 3-5: Differentially expressed genes involved in Immune defence responses. Normalized levels were expressed as ratios between Glucan inoculated and control fish. Genes with a relative expression level of 1.40-fold (up or down) and significance of $p \leq 0.05$ were included, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 3-5. Immune responses – Differential expression in Glucan-inoculated fish.

EST #	TC #	Species	# of ESTs	Gene Name	Fold change 1 week		Fold change 2 week	Fold change 3 week
Innate Immune responses								
Complement related								
CA039230	TC91703	*Ssal*		Complement C3 precursor	1.74			
CA052383	TC64655	*Ssal*		Complement component C6 precursor	-1.52	*		
CB511778	TC74486	*Ssal*		Complement component C7 precursor	-1.75	*		
Lectins								
CA056667	TC83478	*Ssal*		Mannose-binding protein C precursor	-2.46			
CB510697	TC88932	*Ssal*	2	Lectin precursor	4.46			
CB514743	TC77635	*Ssal*		Lectin precursor			-2.67	
CB507038	TC90114	*Ssal*		Nattectin precursor			-1.51	
CA054857	TC82372	*Ssal*		Alpha-2-macroglobulin precursor			-1.99	
Acute phase proteins - Iron storage								
CA037206	TC81509	*Ssal*		Ferritin, middle subunit	1.63			
CB487639	TC138376	*O myk*		Ferritin, middle subunit	1.63	*		
CB497884	TC139262	*O myk*		Ferritin, middle subunit	1.50			
CK991031	TC110460	*Ssal*		Ferritin, middle subunit	1.46			
Monooxygenases								
CB510407	TC82665	*Ssal*		Cytochrome P450 3A27	1.51	*		
CA063128	TC80794	*Ssal*		Cytochrome P450 4V3	1.50	**		
CA053404	TC63959	*Ssal*		Cytochrome P450 46A1	1.68			

Table 3-5, Immune responses continued.

EST #	TC #	Species	# of ESTs	Gene Name	Fold change 1 week		Fold change 2 week		Fold change 3 week
Apoptosis									
CB502555	TC68344	*Ssal*		p53 apoptosis effector related to PMP-22	2.10				
CB496382	TC135900	*O myk*		Reticulon-4	1.84				
CB497887	TC141789	*O myk*		Reticulon-4	1.49				
CA061548	TC65661	Ssal		Reticulon-4	1.45				
CB496605	TC137976	*Omyk*		Cathepsin B precursor	1.49				
CK990740	singleton	*Ssal*		Cathepsin B precursor	1.49				
CA061726	TC64139	*Ssal*		Programmed cell death protein 6			1.75		
CA040268	TC106665	*Ssal*		Death-associated protein kinase 3			-1.75		
Innate - Adaptive responses - Antigen processing & presentation									
MHC II pathway – Cathepsins									
CB502503	TC85966	*Ssal*	2	Cathepsin L precursor	1.61	*			
CB493844	TC144585	*O myk*		Cathepsin L precursor	1.50				
Adaptive immune responses - Antigen processing & presentation									
MHC I pathway									
CA044407	TC93758	*Ssal*		Oncorhynchus mykiss MHC class Ib antigen			-1.50		
CA044472	TC85510	*Ssal*		BOLA class I histocompatibility Ag, α chain precursor			-1.70	*	
CB489716	TC147651	*O myk*		BOLA class I histocompatibility Ag, α chain precursor					1.54
Beta-2-microglobulin									
CB489043	TC162887	*Ssal*		Beta-2-microglobulin precursor	1.52				
CB516867	TC90785	*Ssal*		Tapasin-related protein precursor			-1.68	*	
Molecular chaperones									
CA051622	TC67000	*Ssal*		Heat shock 70 kDa protein 4L	1.58				
CK991122	TC64609	*Ssal*		Heat shock protein 30			3.03	*	

Table 3-6: Differentially expressed genes involved in energy metabolism and the mitochondrial electron transport chain. Normalized levels were expressed as ratios between Glucan inoculated and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated: * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# indicates a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicate the number of significant spots on the array representing that particular EST that showed the same pattern of regulation.

Table 3-6. Oxidative metabolism and the METC.

EST #	TC #	Species	# of ESTs	Gene Name	Fold-change 1 week	2 week	3 week
Carbohydrate breakdown							
Glycolysis II							
CB497681	TC161099	*O myk*		Glyceraldehyde-3-phosphate dehydrogenase	1.86		
CA039027	TC103056	*Ssal*		Glyceraldehyde-3-phosphate dehydrogenase	1.84		
CK991014	TC71456	*Ssal*		Glyceraldehyde-3-phosphate dehydrogenase	1.80		
CA050886	TC64612	*Ssal*		Glyceraldehyde-3-phosphate dehydrogenase	1.73		
CB491826	TC140481	*Ssal*		Glyceraldehyde-3-phosphate dehydrogenase	1.57		
CA060075	TC97558	*Ssal*		Pyruvate kinase isozymes R/L	1.65		
TCA cycle							
CB498501	TC164636	*Ssal*		Isocitrate dehydrogenase [NADP], mitochondrial precursor	1.58		
Mitochondrial electron transport chain							
Complex I							
CB489039	TC157326	*Omyk*		NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.52		
CA038166	TC87748	*Ssal*		NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.43		
CA057060	TC97352	*Ssal*		NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	1.41		
CA037448	TC63525	*Ssal*		NADH-ubiquinone oxidoreductase chain 2	1.41		
CK990669	TC103937	*Ssal*		NADH-ubiquinone oxidoreductase chain 4	1.44		
Complex III							
CN442526	TC100254	*Ssal*		Cytochrome b	1.56		
CB488683	TC170547	*O myk*		NADH-cytochrome b5 reductase 1		1.52	

Table 3-6, Oxidative metabolism and the METC continued.

EST #	TC #	Species	# of ESTs	Gene Name	Fold-change		
					1 week	2 week	3 week
Transfer from Complex III to Complex IV							
CB496975	TC152680	*Ssal*		Cytochrome c1 heme protein	1.50		
CB497282	TC135291	*Omyk*	2	Cytochrome c	1.56	*	
CA059774	TC85097	*Ssal*		Cytochrome c	1.50		
Complex IV							
CN442493	TC1689958	*O myk*	3	Cytochrome c oxidase subunit 2	1.61	**	
CB514540	TC66612	*Ssal*		Cytochrome c oxidase subunit 3	1.67		
CB499941	TC65457	*Ssal*	2	Cytochrome c oxidase subunit 3	1.50	*	
CK991224	TC170967	*O myk*		Cytochrome c oxidase subunit 3	1.49		
CB492507	TC142741	*O myk*		Cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor	1.43	*	
CB510326	TC67101	*Ssal*	2	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	2.22	*	
CA055851	TC101730	*Ssal*		Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	1.71		
CB497393	TC142918	*O myk*		Cytochrome c oxidase subunit 5B, mitochondrial precursor	1.74		
CA037618	TC101704	*Ssal*		Cytochrome c oxidase subunit 7C, mitochondrial precursor	1.47		
CB493553	TC153883	*Ssal*		Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.53	*	
CK990471	TC88794	*Ssal*		Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.51	*	
CB496944	TC156974	*O myk*		Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.46	*	
CB503935	TC100991	*Ssal*		pfam05392, COX7B, Cytochrome C oxidase chain VIIB	1.57		

Table 3-6, Oxidative metabolism and the METC continued.

EST #	TC #	Species	# of ESTs	Gene Name	Fold-change 1 week	2 week	3 week
Complex V							
CB493612	TC170391	*O myk*		ATP synthase a chain	1.49		
CA061299	TC68270	*Ssal*	2	ATP synthase O subunit, mitochondrial precursor	3.67		
CB492831	TC151218	*O myk*		ATP synthase O subunit, mitochondrial precursor	1.45		
CB497057	TC159447	*O myk*		ATP synthase subunit g	1.43	*	
Complex V							
CB493612	TC170391	*O myk*		ATP synthase a chain	1.49		
CA061299	TC68270	*Ssal*	2	ATP synthase O subunit, mitochondrial precursor	3.67		
CB492831	TC151218	*O myk*		ATP synthase O subunit, mitochondrial precursor	1.45		
CB497057	TC159447	*O myk*		ATP synthase subunit g	1.43	*	
CA062761	TC76289	*Ssal*		ATP synthase lipid-binding protein, mitochondrial precursor	1.59	*	
CB498045	TC138672	*O myk*		ATP synthase lipid-binding protein, mitochondrial precursor	1.51		
CA060182	TC73601	*Ssal*		ATP synthase lipid-binding protein, mitochondrial precursor	1.41		
CK990577	TC78447	*Ssal*		ADP/ATP translocase 2	1.82	*	
CK990363	TC88698	*Ssal*		ADP/ATP translocase 2	1.56		
Additional Redox activity							
CB505109	TC85075	*Ssal*		Electron transfer flavoprotein subunit beta	1.52		
CB497419	TC143892	*O myk*		Putative oxidoreductase yulF	1.73	*	

Table3-7. Differentially regulated pathways from glucan inoculated fish. Fish were sampled at 1, 2 and 3 weeks post-inoculation (PI). Differentially expressed genes were classified into pathways for gene expression analysis. Pathways that included a minimum of 3 differentially regulated genes are summarized below.

Pathway	**1 week PI**	**2 weeks PI**	**3 weeks PI**
Complement	Down	-	-
Lectins	-	Down	-
Antioxidant defences	Up	-	-
Monooxygenases	Up	-	-
Apoptosis	Up	-	-
Acute phase proteins - Iron storage	Up	-	-
Antigen processing & presentation - MHC II	Up	-	-
Antigen processing & presentation - MHC I	Up	Down	-
Oxidative metabolism - Glycolysis	Up	-	-
Oxidative metabolism - METC	Up	-	-

Table 3-8. Comparative differential expression measured by microarray and qRT-PCR. Selected genes were analyzed by qRT-PCR. Expression levels are expressed as fold-change relative to control expression. The expression levels measured by qRT-PCR agree with microarray results in terms of both scale and directionality.

	Expression level	
Gene	Microarray	qRT-PCR
Complement C7 precursor	-1.75	-3.49
Cytochrome P450 46A1	1.68	2.89
Cytochrome P450 4V3	1.5	1.83

Chapter 4

Temporal analysis of gene expression in Chinook salmon infected with *Loma salmonae.*

Introduction

Loma salmonae (Microsporidia) is the causative agent of microsporidial gill disease (MGD) in several members of the Oncorhynchus genus, particularly Chinook salmon (*O. tshawytscha*) and coho salmon (*O. kisutch*) that are farmed in British Columbia, Canada (Speare et al. 1998). Infection results in the formation of spore-filled xenomas in the gill that eventually rupture, provoking a strong inflammatory reaction that may result in severe branchitis and asphyxiation of the host (Kent et al. 1995, Speare et al. 1998). Transmission is direct - spores are released from an infected fish into the surrounding water where they are ingested by a new host. An ingested spore makes contact with the gut epithelium, stimulating the spore to evert its polar tube and inject the infective sporoplasm directly into a host cell. From the epithelium the parasite migrates to the heart and, eventually, the gill, where most xenomas form (Sanchez et al. 2001a). It is believed that the parasite may be transported within host phagocytic cells such as macrophages, although the exact mechanism of transport remains unknown. Vascular transport likely accounts for xenoma formation in other organs such as kidney, liver and spleen. Within the gill infected host cells become severely hypertrophic, and obvious xenoma formation continues for weeks with little or no observable immune response. During this time it is not known how these increasingly deformed cells avoid immune detection and attack.

Eventually the xenomas rupture and an observable immune response develops in the gill, resulting in extensive regions of hyperplasia. Fish that recover from the infection are resistant to reinfection (Kent et al. 1999), indicating the development of immunological memory associated with adaptive immune responses. It has been speculated that phagocytic immune cells may

engulf spores while the infection is being resolved, and transport them to some site of sequestration in an organ such as the kidney, where they are able to continually stimulate immunological memory (Kent et al. 1999).

The objectives of this study were to determine temporal patterns of response to *L. salmonae* infection using genomics tools. Here, the results of gene expression analysis in Chinook salmon infected with *L. salmonae* are presented. The study was designed to utilize a 16,006 feature salmon cDNA microarray developed through the Genome Research on Atlantic Salmon Project (GRASP: http://web.uvic.ca/cbr/grasp; B.F. Koop & W. Davidson; (von Schalburg et al. 2005), containing 13,421 Atlantic salmon features and 2,576 rainbow trout features. cDNA generated from a variety of Chinook salmon tissues had previously been successfully cross-hybridized to this array (Rise et al. 2004, von Schalburg et al. 2005). The microarray study was based on temporal sampling of Chinook salmon gill and anterior kidney tissues after intubation with *L. salmonae*. A subset of differentially-expressed genes identified in the microarray study was further analyzed by quantitative reverse-transcriptase PCR (qRT-PCR).

4-1. Methods

4-1a. Fish husbandry:

Chinook salmon smolts were obtained from Sea Springs Hatchery (Chemainus, BC), and maintained in ambient seawater (10°C – 14°C) in 1000 L flow-through tanks for the duration of the study. Fish were fed a commercial diet daily. Incoming water was treated with a 40 watt UV sterilizer (Pentair Aquatics) at the rate of 20 L/minute.

4-1b. Fish infection and sampling:

Chinook salmon (average wt 23 g.) (n = 75), were exposed to *L. salmonae* by gastric intubation with an inoculum of 1 x 10^6 spores in macerated gill tissue suspended in Hank's

buffered saline solution (HBSS). Spores were activated immediately prior to intubation by dilution with distilled water. In order to minimize handling stress, fish were lightly sedated with tricaine methane sulphonate (TMS) (25 mg/L) prior to intubation. Control fish (n = 75) were similarly handled and intubated with HBSS. Fish from each group were divided between 3 replicate tanks/group. Samples from gill and anterior kidney of 9 exposed and 9 control fish were freshly dissected at 4, 8 and 12 weeks PE for diagnostic PCR, microarrays and qRT-PCR, and tissues from an additional 9 exposed and 9 control fish were sampled for qRT-PCR, following killing by an overdose of tricaine methane sulphonate (TMS). Tissues for microarrays were immediately immersed in 1 ml RNAlater (Invitrogen) and kept at - 20°C until RNA extractions were performed. Tissues from gill, kidney, heart, spleen and liver were sampled for DNA extraction and diagnostic PCR. Freshly dissected tissues were frozen and held at -20°C until DNA extractions were performed. In addition, tissues from gill of the first 9 fish from each group were sampled for immunohistochemistry. Samples were immediately immersed in a 10:1 volume of 10% neutral buffered formalin.

4-1c. DNA extractions and PCR:

DNA was extracted from ~25 mg tissues (gill, kidney, heart, spleen and liver) of 9 fish from each original sampling group, using the Qiagen DNeasy tissue kit according to manufacturer's instructions. 1.5 µl aliquots of extracted DNA (<50 µg/ml) were then used as template in PCR reactions to amplify a 272 base pair fragment of the *L. salmonae* 18S rRNA gene. Each PCR reaction included: 0.625 U Platinum Taq polymerase (Invitrogen), 1x PCR buffer (Invitrogen), 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer (LS1F: 3' - CTG GAT CAG ACC GAT TTA TAT A – 5', LS1R: 5'- ATG ACA TCT CAC ATA ATT GTG A– 3') (Docker et al. 1997), with the remaining reaction volume (25 µl) made up with filter-sterilized,

deionized, double-distilled water (ddH_2O). PCR reactions were performed in a PTC – 200 thermocycler (MJ Research). The PCR consisted of denaturation at 94°C for 1 minute, followed by 45 cycles of 94°C for 35 seconds, 53°C for 20 seconds, and 72°C for one minute, followed by a final extension of 72°C for 7 minutes. PCR products were visualised on a 1.5 % agarose gel containing 0.1 µg/ml SybrSafe (Invitrogen).

In 4 week samples, 3 exposed fish tested positive for *L. salmonae* infection in both heart and gill and were selected for microarray analysis. In 8 and 12 week samples, 3 exposed fish tested strongly PCR positive in every tissue (by gel band intensity), and were selected for microarray analysis.

4-1d. RNA extraction, cDNA generation and labeling:

Gene expression analysis was performed on gill and kidney tissues from 4, 8 and 12 week exposed Chinook salmon. At each time point 3 *L. salmonae*-infected fish that tested strongly positive as described above were selected for microarray analysis. Gill tissue was chosen since it is the ultimate site of xenoma development, allowing the detection of developing immune responses in that tissue over time. Head kidney tissue was also chosen since it plays an important role in fish immunity and consistently yielded good hybridization results in preliminary tests.

For microarray experiments total RNA was extracted from gill and kidney using Trizol, according to a modified manufacturer's protocol (Sigma). Tissues were transferred from RNAlater into one ml Trizol with one 3mm stainless steel ball, incubated at room temperature (RT) for 5 minutes, and then homogenized for 3 minutes at 20 Hz on a Retsch mixer mill (Qiagen). 100 µl of 1-bromo-3-chloropropane were added to the homogenate and the mixture was shaken vigorously for 15 seconds. The mixture was incubated for 3 minutes at RT and

centrifuged at 12,000 x g for 8 minutes. The aqueous phase was transferred to a new tube containing 500 µl of 2-propanol, mixed thoroughly, and incubated for 5 minutes at RT before being centrifuged at 12,000 x g for 5 minutes. The isopropanol was removed and the pellet was washed with 750 ml of ice-cold 75% ethanol, vortexed, and then centrifuged at 7,500 x g for 5 minutes. The ethanol was carefully removed and the pellets were allowed to dry for 10 minutes before they were dissolved in 50 µl of RNase-free water at 55°C for 10 minutes. Extracted RNA was quantified on a Nanodrop spectrophotometer (Thermo Scientific) and stored at – 80°C until used.

Five µg of total RNA were reverse-transcribed into cDNA using the Invitrogen Superscript Indirect labeling kit according to a modified manufacturer's protocol. Briefly, first-strand cDNA was synthesized and precipitated in ethanol overnight. The cDNA was centrifuged at 16,000 x g for 60 minutes at 4°C, washed in 70% EtOH, and air dried before being resuspended in 10 µl of coupling buffer. Cy 3 and Cy 5 monoreactive dye packs (Amersham Biosciences) were each dissolved in 45 µl DMSO and 5 ul of dye were added to the samples and incubated for 2 hours in the dark at RT. 10µl of sodium acetate were added to the labeled samples before they were purified on SNAP columns, pooled, and precipitated in EtOH overnight. Pellets were centrifuged at 16,000 x g for 60 minutes at 4°C, washed once with EtOH, dried, and allowed to resuspend in 27 µl DEPC-treated water for several hours at RT.

4-1e. Microarray hybridization:

Microarray slides were washed twice for 5 minutes in 0.2% SDS, 5 times for 1 minute in ddH_2O, and dried by centrifugation in a 50 ml conical tube for 5 minutes at 514 x g at RT. Dried arrays were immersed in prehybridization buffer (5XSSC, 0.1% SDS, 3%BSA V) for 90 minutes

@ 49°C, washed 3 x 20 sec. in ddH_2O, and then centrifuged dry for 5 minutes at 514 x g at RT. Dry arrays were held at 49°C until hybridized.

Cyanine-labeled samples were mixed with 2X formamide buffer (50% formamide, 8X SSC, 1% SDS, 4X Denhardt's) and 4 µl LNAdT blocker (Genisphere), incubated for 10 minutes @ 80°C, and held at 65°C until hybridized. The microarray slides were placed into Corning hybridization chambers and ddH_2O was added to the reservoirs in order to maintain humidity within the chambers. Pooled cyanine-labeled samples were aliquoted onto the slide and covered with a 22 x 60 mm hybrislip (Invitrogen). Slide chambers were quickly assembled and incubated in a 49°C water bath for 16 hours. The hybridized arrays were washed 1 x 10 minutes (49°C) and 2 x 5 minutes (RT), in 2X SSC/0.1% SDS, followed by 2 x 5 minutes 1X SSC (RT), and finally 4 x 5 minutes in 0.1% SSC (RT). Slides were immediately dried by centrifugation for 5 minutes at 514 x g and held in the dark until scanned.

The hybridized microarrays were scanned using a Perkin Elmer ScanArray Express (Perkin Elmer, Boston, MA), with adjustments to the PMT gain in order to optimize signal/noise ratio in each sample. The images were then quantified using Imagene 5.6.1 (Biodiscovery, El Segundo, CA) and the data were analyzed with GeneSpring 7.3.1 data analysis software (Agilent Technology). Raw data were imported into GeneSpring, dye-swaps were identified, and the pairs were normalized by Lowess normalization with 20% of data used for smoothing. The data set was filtered to eliminate absent or marginal signals and those with values below the average of base/proportional values for control signals. The data from normalized dye swap pairs were exported and re-imported into GeneSpring without further normalizations in order to compensate for GeneSpring's treatment of dye-swap technical replicates as biological replicates.

T-tests were performed on data from each treatment group and genes were retained if they were significantly differently expressed between exposed versus control, (*P*-value of ≤ 0.05), with a fold-change ≥ 1.40 up- or down-regulated. Significantly differentially expressed genes were further classified into pathways using Gene Ontology analysis. T-tests were performed on data from each treatment group and genes were retained if they were significantly differently expressed between exposed versus control, (*P*-value of ≤ 0.05), with a fold-change ≥ 1.40 up- or down-regulated. As mentioned, only fish showing strong infection in every tissue (measured by electrophoresis gel band intensity of PCR product) were chosen for microarray analysis, restricting the sample size to 3. Due to the small number of samples tested, a multiple test correction was not employed in the statistical analysis. Multiple test corrections reduce the likelihood of reporting a false positive (reporting a gene as significantly differentially regulated when it was not), however, employing a multiple test correction then increases the chance of failing to recognize a true positive (eliminating a truly differentially regulated gene from a gene list). Small sample sizes reduce the statistical strength of each result, reducing the resultant p-value, and likely preventing genes from passing stringent multiple test corrections. To compensate for the lack of a multiple test correction, differentially regulated genes were sorted according to gene ontology (GO terms) and grouped into major biological functions or pathways. In addition, similar expression patterns of numerous genes within particular pathways, as well as gene redundancy help to reinforce confidence in the results by acting as internal controls.

4-1f. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

Three genes were selected from amongst all significantly differentially expressed genes identified by microarray analysis (Table 4-1). Genes were selected for qRT-PCR analysis for the purpose of reinforcing confidence in the presented microarray results and were therefore

arbitrarily chosen for their low p-value based on microarray t-tests. Total RNA was extracted from gill or kidney tissues as above using Trizol according to manufacturer's instructions (Invitrogen). Possible contaminating DNA was removed from extracted RNA by an RNeasy on-column DNase digestion according to manufacturer's instructions (Qiagen). cDNA was reverse transcribed using AffinityScript 1st strand cDNA kit according to manufacturer's instructions. Briefly, 1 µg aliquots of RNA were added to a reaction mix consisting of 10 µl 2x First strand master mix, 3 µl Oligo (dT)20 primer (0.1 µg/µl), 1 µl AffinityScript RT/ RNase block enzyme mixture, and RNase/DNase-free water to a total volume of 20 µl. The reaction mixture was incubated at 25°C for 5 minutes to allow primer annealing, followed by synthesis at 42°C for 15 minutes, and the resulting cDNA was immediately placed at 4°C. For qRT-PCR reactions, 1 µl aliquots of cDNA were added to qRT-PCR reaction mixture (12.5 µl 2x Brilliant Sybr green master mix, 0.625 µl each of forward and reverse primers (10 µM), 0.375 µl ROX reference dye, and 9.875 µl ddH_2O) in 96 well plates and amplified in a Stratagene MX3000 thermocycler under the following conditions: Denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, optimal elongation temperature (55°C or 58°C) for 1 minute, and 72°C for one minute. A dissociation curve program was run on each plate consisting of an initial denaturation step at 95°C for one minute, followed by a ramping up of temperature (at 0.2 °C/sec) from 55°C to 95°C, with continual fluorescent data collection (Stratagene, Agilent Technologies). Primer sets are listed in Table 4-1. Data was normalized to a dilution series standard curve run on each plate. Dilution series (10X) were made of PCR amplification products of each gene of interest, generated using cDNA copies of mRNA present in a pooled sample extracted from 5 control Chinook salmon gills or kidneys. Data were analyzed using MxPro qPCR software. T-tests were performed on average C_T values from triplicate samples.

4-1g. Immunohistochemistry:

Thin (5µm) tissue sections were deparaffinized and rinsed for one minute in running tap water. Tissue sections were incubated with 80 µl anti- *L. salmonae* antibody diluted 1:1000 in PBS-T in a humidified chamber for 1 h (Young et al. 2007). Slides were briefly rinsed and then washed in PBS-T (3 x 5 min.) followed by incubation for 45 min with alkaline-phospatase (AP)-conjugated rabbit anti-chicken-IgG (100 µl) diluted 1:50 in PBS-T. Slides were washed as above and then incubated for 30 minutes with Blue-Phos substrate solution (100µl) (KPL). Slides were immersed in water in order to stop the reaction, counterstained with a five-second immersion in eosin, dehydrated in 100% isopropanol (3 x 30 sec.) and xylene (3 x 30 sec), and cover-slipped with Permount.

4-2. Results and discussion

4-2a. Immunohistochemistry:

Gill tissue assayed by immunohistochemistry at 4, 8 and 12 weeks showed progressive xenoma development, with increases in both the numbers and sizes of xenomas. No inflammation was observed in 4 week gill (Figure 4-1a, b), but was evident as lamellar hyperplasia and associated altered gill filament morphology at 8 weeks PE (Figure 4-1c). By 12 weeks PE hyperplasia appeared to have increased (Figure 4-1e). At each of the later time points there were notable differences in the levels of apparent inflammation characterized by lammelar fusion and hyperplasia in different areas of gill from the same fish. Figure 4-1c, and 4-1d represent xenomas in different areas of the first gill arch from a single fish at 8 weeks PE, whereas Figure 4-1e and 4-1f are adjacent areas of gill from a single fish at 12 weeks PE. At 8 and 12 weeks hyperplasia appeared to be localized in patchy areas of the gill and was not predictably associated with xenomas. At 8 and 12 weeks PE some xenomas showed no evidence

of inflammation or immune cell accumulation in immediately adjacent tissue, even though overall the sizes of xenomas appeared to have substantially increased (Figure 4-1d, f). The breakdown of xenomas may represent a prime opportunity for immune recognition. It is likely that the observed inflammation and lamellar hyperplasia develops as xenomas are broken down and individual spores are released.

Although the variability in immune responses between fish was not unexpected, the high levels of variability within the gills of individual fish were somewhat unexpected. It is not known how this may have influenced the microarray results, since the samples would have contained a heterogenous mix of cells from areas of the gill that ranged from relatively normal morphology to areas of severe hyperplasia.

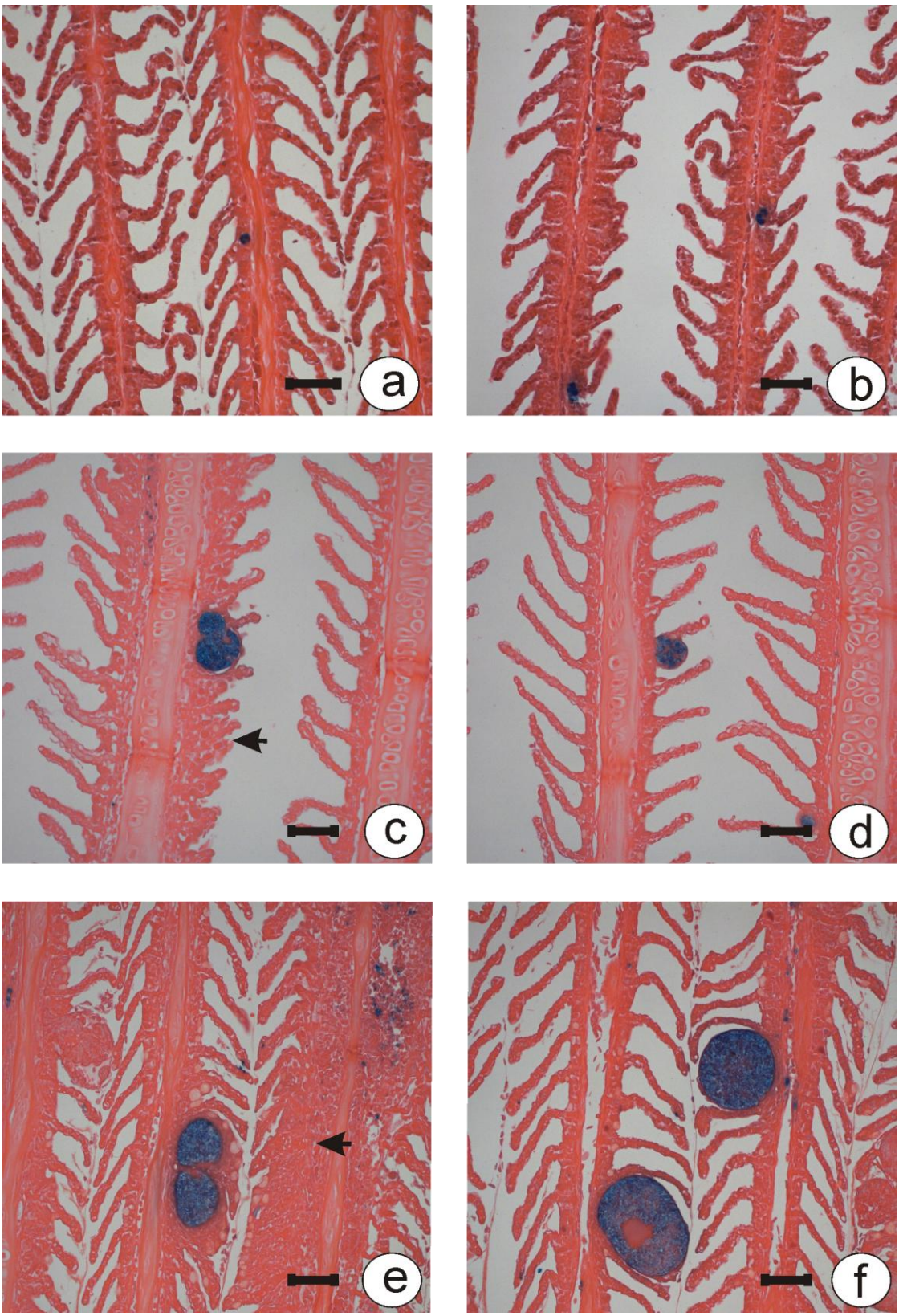

Figure 4-1. *L. salmonae* xenomas in gill tissue at 4, 8 and 12 weeks PE. See legend next page.

Figure 4-1. *Loma salmonae* xenomas in Chinook salmon gill tissue at 4, 8 and 12 weeks PE. *L. salmonae* xenoma progression and associated inflammation in gill tissue at various time points after parasite exposure. At 4 weeks PE the developing xenomas are small, with no observable associated inflammatory response (a, b). By 8 weeks PE *L. salmonae* has developed typical spore-filled xenomas, and possible inflammation is observed in some areas (c) (arrowhead), whereas in other areas of the same gill there is no evidence of inflammation (d). By 12 weeks PE xenomas have grown substantially. Some xenomas appear to have ruptured and many are surrounded by apparent inflammation and lamellar hyperplasia (e) (arrowhead), however, very large xenomas located within the same gill showed little or no inflammatory response in the adjacent tissue (f). Scale bars represent 50 µm.

4-2b. Gene expression analysis:

Temporal analysis of gene expression in *L. salmonae*- infected fish at 4, 8 and 12 week PE revealed that the majority of differential expression in both gill and kidney occurred at 4 weeks PE, with fewer numbers of differentially expressed genes represented at 8 and at 12 weeks (Table 4-2). Also, at 4 weeks PE a larger proportion of genes exhibited reduced expression levels in both gill and kidney, whereas a higher proportion of the differentially expressed genes showed increased expression in both tissues at the later time points (Table 4-2).

Differentially expressed genes were sorted according to gene ontology (GO terms) and grouped into major biological functions or pathways as discussed below. Complete lists of differentially expressed genes are included in Appendix 2.

4-2b.1. Gas transport:

The expression of numerous genes involved with gas transport was increased in gill tissue at 4 weeks PE (Table 4-3). By 8 weeks PE this trend had been reversed, and the expression of genes involved in oxygen transport was reduced in gill and kidney (Table 4-4).

4-2b.1.1. Gas transport -Oxygen:

Multiple copies of both alpha and beta haemoglobin subunits showed increased expression in the gill at 4 weeks PE (Table 4-3), whereas haemoglobin expression was reduced in both gill and kidney at 8 weeks PE (Table 4-4). As in mammals, adult haemoglobin of teleosts contains two alpha and 2 beta subunits along with a heme group that binds 4 molecules of oxygen for transport by erythrocytes to the tissues. However, contrary to the erythrocytes of mammals, those of fish retain their nucleus, and both alpha- and beta-globin genes are expressed in circulating and mesonephric erythrocytes of adult teleosts (de Souza & Bonilla-Rodriguez 2007), (Chan et al. 1997), (Maruyama et al. 2004). These results suggest increased gas exchange taking place in the gills of *L. salmonae* infected fish at 4 weeks PE, at a stage when xenomas are just beginning to form. In contrast, reduced haemoglobin expression in gill and kidney at 8 weeks PE may suggest the development of anemia associated with the progressing infection.

4-2b.1.2. Gas transport – CO_2:

The expression of another important gas transport enzyme, carbonic anhydrase (CA), was increased in gill tissue at 4 week PE (Table 4-3). Carbonic anhydrase is an erythrocytic enzyme that is critical in the transport of CO_2 out of cells to be released at the gill. CO_2 from tissues diffuses into RBC where CA converts it into carbonic acid, which then dissociates into H^+ and bicarbonate. The H^+ ions lower the pH of the red blood cell, facilitating the release of oxygen from haemoglobin for diffusion into tissues. Free haemoglobin takes up some of the released hydrogen ions, preventing the intracellular pH from dropping too low. Much of the bicarbonate diffuses out of the cell into the plasma, while some of the HCO_3^- ions react with haemoglobin within the cell to form carbaminohaemoglobin. At the gill these reactions are reversed, allowing CO_2 to be released and eliminated from the blood (Helfman et al. 1997). As with haemoglobin,

the increased expression of carbonic anhydrase in *L. salmonae* infected fish at 4 weeks suggests that increased gas exchange is taking place in those fish.

4-2b.2. Immune responses:

Genes involved with a variety of immune responses were differentially expressed in the gill and kidney as discussed below. In general, the majority of differential expression in the gill occurred at 4 weeks PE, with the down-regulation of immune receptors, interferon-related proteins, apolipoproteins, as well as genes of the MHC I antigen processing and presentation pathways (Table 4-5). In contrast, acute phase protein genes such as ferritin and histone H1 were up-regulated at 4 weeks PE. By 8 weeks PE in the gill there is an up-regulation of cytokines, and genes associated with both the MHC I and MHC II pathways. The MHC I pathway was also up-regulated at 12 weeks PE in the gill (Table 4-5).

In the kidney the majority of differential expression occurred at 4 and 8 weeks PE (Table 4-6). In general, the expression of cytokines, immune receptors, some lectins, interferons and ferritins was increased at 4 and 8 weeks PE in the kidney, whereas apolipoproteins and cytochrome P450s were down-regulated at these time points. Numerous components of both antigen processing and presentation pathways were differentially regulated in the kidney. MHC II was up-regulated at 4 weeks PE, although the associated cathepsin genes were down-regulated at both 4 and 12 weeks PE. Components of the MHC I pathway showed up-regulation at all time points in the kidney. Each of these pathways is discussed in more detail below, with reference to the functions of individual genes.

4-2b.2.1. Immune responses - Cytokines, Chemokines:

A number of cytokines/chemokines were differentially expressed in the gill at various time points, including SCYA113, leukocyte cell-derived chemotaxin 2 (LECT2) and SCYA101

(Table 4-5). C-C chemokines are chemotactic cytokines that are able to attract and activate populations of leukocytes at sites of inflammation (Rossi & Zlotnik 2000). Some C-C chemokines may act as costimulators of T-cells and NK cells (Boring et al. 1997). The C-C chemokine SCYA113 is up-regulated in fish in response to whirling disease (Baerwald et al. 2008). LECT2 has been suggested to act as a chemotactic factor for neutrophils in mammals (Yamagoe et al. 1996, Saito et al. 2004). In addition, studies with LECT2 deficient mice have suggested that this putative cytokine may play a role in the homeostasis of NKT cells (Saito et al. 2004). The increased expression of chemokines in both gill and kidney suggests recruitment and activation of innate responder cells such as neutrophils and NK cells, and may also suggest the development of adaptive immune responses in *L. salmonae* infected fish.

4-2b.2.2. Immune responses – Cell surface receptors:

In the gill, the expression of a number of immune cell-surface receptors was reduced at 4 weeks (Table 4-5), including C-C-chemokine receptor type 6 (CCR6), macrophage receptor MARCO, and Interleukin-13 receptor alpha (IL-13 Rα). In contrast, numerous receptors were up-regulated at each time point in the kidney. At 4 weeks PE receptors associated with T-cell activation, including T-cell receptor alpha chain (TCR-α) and interleukin-2 receptor (IL-2R), showed increased expression in the kidney. A number of immune cell receptors associated with innate response cells also displayed increased expression at 4 weeks PE, including lymphocyte antigen 75 (CD205), as well as novel immune-type receptor 1 (NITR-1). At 8 weeks PE the expression of CD53, CD97, and CD11b was increased in the kidney. At 12 weeks PE in the kidney the expression levels of CD59-like protein 2 and leukocyte immune-type receptor 3

(LITR3) were increased, whereas the expression of interleukin 1 receptor-like 1 (IL1RL1) was reduced (Table 4-6). The function of each of these genes is discussed below.

Effector cells of both the innate and adaptive branches of the immune system rely on cell-surface receptors for adhesion, activation, and cell signalling. CCR6 is a member of the G-protein coupled receptor family (GPCR) that are known to be displayed on the surface of monocytes, lymphocytes, basophils and eosinophils, although its specific function has not been described (Interpro: IPR004067). Macrophage receptor Marco is a scavenger receptor displayed on some populations of macrophages in mammals. Marco is expressed in dendritic cells, where it was induced by pathogen exposure. Marco is known to bind pathogen associated molecular patterns (PAMPs) and plays a role in the uptake and clearance of pathogens by macrophages (Kraal et al. 2000, Grolleau et al. 2003). IL-13 Rα is part of a multi subunit receptor for IL-13, a cytokine with anti-inflammatory properties (Chomarat & Banchereau 1998, Izuhara et al. 2003). In addition, IL-13 may play a role in parasitic infections, potentially antagonizing T_H1 responses in favour of T_H2-mediated responses. Down-regulation of this gene may help promote T_H1 responses, which are thought to be more favourable for the clearance of intracellular pathogens (Sasaki et al. 2000).

Several of the immune cell receptors up-regulated at 4 weeks PE in the kidney may provide evidence of T-cell activation. T-cell receptors are expressed on the surface of naïve T-cells and are composed of paired subunits. The most common TCR variety is made up of alpha and beta subunits that form the antigen specific receptor responsible for specifically binding antigen displayed by MHC (Goldsby et al. 2000). IL-2R is a multi-subunit cell surface receptor that, along with its ligand IL-2, is expressed by activated T-cells. Initial activation is dependent upon interaction of T cell receptor (TCR) with specific antigen/MHC I, aided by ligation of

costimulatory molecules. Once activated, the T-cells express both IL-2R and IL-2, providing positive feedback that results in antigen-specific clonal proliferation of T cells (Minami et al. 1993). Lymphocyte antigen 75 precursor (CD205) is a macrophage mannose receptor family, C-type lectin receptor expressed on dendritic cells. In mammals, CD205 is believed to be involved in the uptake and processing of antigen by these APC (Kato et al. 2006). The up-regulation of immune cell receptors associated with T-cell activation, including TCR-α, IL-2R and CD205 suggests T-cell activition in the kidney at 4 weeks PE.

Surface receptors associated with cells of the innate immune system were also up-regulated at 4 weeks PE in the kidney. NITR-1 is a member of the novel immune-type receptors that have been identified in teleosts. NITR are Ig superfamily members that are putative orthologues of killer immunoglobulin-like receptors (KIR), which act as activation receptors for NK cells in mammals (Litman et al. 2003, Yoder et al. 2004). The increased expression of a number of immune receptors in the kidney at 4 weeks PE provides evidence for the development of an early immune response to *L. salmonae* infection, including both innate and adaptive components, well before any response in the gill can be visually detected.

As mentioned, three immune cell receptors were up-regulated in the kidney at 8 weeks PE. CD53 is a member of the tetraspanin-4 superfamily of cell surface proteins that bind integrins and are involved in signal transduction in T-cells and NK cells (Horejsi & Vlcek 1991). CD97 is a member of the adhesion G-protein-coupled receptors that is expressed on leukocytes and is believed to be involved in the recruitment of inflammatory cells (de Groot et al. 2009). CD11b is an immune cell receptor that complexes with CD18 to make up macrophage 1 antigen, a leukocyte β2 integrin expressed on neutrophils. The ligation of these integrins is important for TNF-mediated calcium oscillations leading to increased respiratory burst in adherent neutrophils

(Richter et al. 1990), as well as facilitating phagocytosis in these cells (Soriano et al. 1998). The increased expression of these immune cell receptors is evidence for the development of immune responses in the kidney at 8 weeks.

In mammals CD59 is a major inhibitor of the membrane attack complex (MAC) of the complement system. CD59 inhibits the MAC by binding to C8 and C9 and preventing them from participating in complex formation. In fish, two isoforms of a CD59-like protein have been discovered (Papanastasiou et al. 2007). Interestingly, trophozoites of the human intestinal parasite *Entamoeba histolytica* express a CD59-like protein on their surface in order to withstand complement attack (Ventura-Juarez et al. 2009). Although there is no evidence that *L. salmonae* employs such a mechanism, it would not be surprising to find similar evasion mechanisms at work for this microsporidian parasite.

LITR are Ig-superfamily members first identified in channel catfish with putative inhibitory and stimulatory functions (Montgomery et al. 2009). This family of innate immune receptors contains Ig domains related to the Fc receptors of mammals, as well as domains with homology to receptors encoded by the leukocyte receptor complex (Stafford et al. 2006). Due to their homology to the innate receptor class PIR in rodents, MHC has been suggested as a possible ligand for LITR (Stafford et al. 2007). IL1RL1 is a member of the Interleukin-1 receptor/toll-like receptor (IL-1/TLR) superfamily of signalling molecules that play roles in defence responses and inflammation (Subramaniam et al. 2002). IL-1 receptor-like proteins have been cloned in *S. salar* (Subramaniam et al. 2002) and there is evidence that IL1RL1 may be involved in T_H responses (Castano et al. 2009).

To summarize, these results indicate a reduction in immune receptor expression in the gill at 4 weeks, a time when *L. salmonae* xenomas are beginning to become established within the

primary and secondary lamellae. In contrast, the expression of immune receptors is up-regulated in the kidney at all time points, suggesting the development of innate and adaptive immune responses.

4-2b.2.3. Immune responses - Lectins:

A number of lectins, including Galectins, C-type, and fish egg lectins were differentially expressed in the gills and kidneys of *L. salmonae* infected fish at various time points. The expression of Galectin 9 was increased in the gill at 4 weeks PE (Table 4-5). In the kidney the expression of fish egg lectins was reduced at 4 weeks PE, whereas CD209 showed increased expression at 8 weeks PE (Table 4-6).

Lectins are proteins that bind specific sugar moieties displayed on the surface of numerous cell types, including pathogens. A variety of lectins have been implicated in innate immune defence, initiating an immune response through the binding of carbohydrates displayed on the cell surfaces of pathogens such as bacteria, viruses, protozoa and fungi. The galectin family of lectins mediate inflammatory reactions and may be involved in recruiting polymorphonuclear leukocytes to sites of inflammation (Matsuura et al. 2009). In addition, galectin-9 may also induce Ca^{2+}-dependent apoptosis in T cells (Vasta et al. 1999, Kashio et al. 2003). Similar to the up-regulation of chemokines at 4 weeks PE in the gill, the up-regulation of galectin-9 suggests the recruitment of innate immune cells to the gill at this early time point.

Fish egg lectins with a variety of carbohydrate specificities were first isolated from the eggs of teleosts, including several *Onchorynchus* species, and some fish egg lectins may be involved in innate immunity (Bildfell et al. 1992, Tateno et al. 2002). For example, of 3 lectins (STL1, STL2, & STL3) first isolated from the eggs of steelhead trout (*O. mykiss*), STL1 was localized in blood leukocytes such as lymphocytes, monocytes, and neutrophils, as well as to the

spleen and tissues involved with mucosal defence in both the gill and intestine (Tateno et al. 2002). Interestingly, the *O. tshawytscha* variant of STL2, a rhamnose binding lectin, was bound in high concentration to the surface of *L. salmonae* spores isolated from the gills of naturally infected wild Chinook salmon (Booy et al. 2005). It is not known what role this lectin plays in fish response to *L. salmonae* infection. However, since rhamnose is rarely found in vertebrates it may suggest that STL2 plays a role in immune response to foreign invaders.

CD209 is a C-type (Ca^{2+}-dependent) lectin that is also called DC-SIGN, for dendritic cell specific ICAM-3 grabbing nonintegrin. The lectin is expressed on dendritic calls, where it binds to ICAM-3 on $CD4^{+}$ T-cells and plays roles in both dendritic cell migration and T-cell activation (Engering et al. 2002). DC-SIGN is also expressed on macrophages, binding to mannose-type pathogen associated molecular patterns (PAMPs) displayed on some pathogens, and activating phagocytosis (Mitchell et al. 2001). The up-regulation of CD209 can be taken as further evidence for the development of an immune response in the kidney at 8 weeks PE.

4-2b.2.4. Immune responses - Interferons:

Several IFN-related genes were differentially expressed in these fish at various time points. In the gill, the expression of interferon-related developmental regulator 1 (IFRD1) and IFN regulatory factor 4 (IRF-4) were decreased at 4 weeks PE, whereas expression of interferon regulatory factor, IRF-1 was increased at 8 weeks PE (Table 4-5). In the kidney IRF-1 was up-regulated at 4 weeks PE along with interferon-induced protein 44 (IFI44), which was up-regulated at 4 and 12 weeks PE. Type I IFN associated proteins interferon-induced protein Gig2 and interferon-induced 17 kDa protein precursor were both down-regulated at 4 weeks PE in the kidney (Table 4-6).

The interferon (IFN) system consists of a large number of secreted cytokines (IFNs) as well as associated proteins that make up an important component of innate defence in vertebrates (Robertson, 2006). Interferon responses can be categorized as belonging to one of 2 patterns, Type I or Type II. Type I IFNs include many structurally similar cytokines that play important roles in immune-surveillance and response to viral infections. Type I IFNs act to promote MHC I expression, inhibit cell proliferation, promote T cell survival, increase NK cell activity, and mediate apoptosis (Perry et al. 2005, Baerwald et al. 2008). Type II IFNs consist of a single cytokine, IFN γ, which has no structural homology to Type I cytokines. Type II IFN promotes increased expression of MHC I and MHC II, enhanced killing activity by macrophages, and induces apoptosis in some cells. In addition to the cytokines themselves, hundreds of additional proteins have been linked to both IFN pathways, acting as regulatory factors, response proteins, etc. (Platanias 2005). Interferon-related developmental regulator 1 (IFRD1) is a histone deacetylase expressed in neutrophils during their differentiation, where it appears to be important for proper functioning of these cells, since effector function of these cells is reduced in IFRD1 deficient mice (Gu et al. 2009).

IFN regulatory factors (IRF) are transcription factors involved in the induction of IFN responsive genes. IRF-4 is expressed in lymphocytes, macrophages and dendritic cells in mammals, and may have roles in both innate and adaptive immune responses (Honma et al. 2005). IRF-4 in macrophages negatively regulates the production of pro-inflammatory cytokines in response to TLR signalling (Honma et al. 2005), and plays a key role in T_H cell differentiation in mammals (Lohoff et al. 2002, Tominaga et al. 2003). In the absence of IRF-4, $CD4^+$ T-cells of mice showed no reduction in IFN-γ production and were capable of generating a T_H1 response. The decreased expression of these IFN-related genes may be associated with a bias

toward a Type II IFN response. IRF-1 is critical for the development of T_H1 responses in mice (Baerwald et al. 2008). Interferon-induced protein 44 (IFI44) expression is induced in human hematopoietic progenitor CD34 cells in response to IFN-γ (Zeng et al., 2009).

Taken together, these results suggest an up-regulation of the Type II IFN pathway and may also indicate the development of a T_H1 cytokine profile in response to *L. salmonae* infection. The T_H1 (Type1) cytokine response is characterized by the production of a cytokine profile that includes IFN-γ, and is important for the development of adaptive cell-mediated responses (Sinigaglia et al. 1999, Salat et al. 2008).

The differential expression of genes related to type I IFNs suggest a down-regulation of that pathway. Type I IFN associated Interferon-induced protein Gig2 is an interferon inducible gene that is thought to play an important role in an antiviral response (Jiang et al. 2009). Interferon-induced 17 kDa protein precursor is processed into ISG15, a protein secreted from monocytes and lymphocytes that is transcriptionally regulated by type I IFNs (D'Cunha et al. 1996).

In summary, the differential expression of interferon-related genes suggest an up-regulation of type II IFNs in *L. salmonae* infected samples, whereas the differential expression of genes related to type I IFNs suggest a down-regulation of that pathway.

4-2b.2.5. Immune responses - Perforin / Granzyme:

In the gill perforin was up-regulated at 4 weeks PE (Table 4-5). In the kidney perforin expression was decreased whereas granzyme expression was increased at 8 weeks PE (Table 4-6). Effector cells of the innate and adaptive immune sytems employ similar killing mechanisms in order to eliminate infected self cells. Natural killer (NK) cells as well as cytotoxic T cells are able to kill their targets via apoptotic mechanisms involving an exocytotic perforin/granzyme

mediated pathway. The effector cells are characterized by the presence of cytoplasmic granules that contain perforin and granzyme proteins. When the effector cell binds to its target, the contents of the granules are discharged by exocytosis. The perforin molecules insert themselves into the target cell membrane, forming a pore that allows the granzyme proteases to enter the cell and induce apoptosis (Muller et al. 2003).

4-2b.2.6. Immune responses - Acute phase proteins – Ferritins:

Ferritins comprise an additional class of proteins that were up-regulated at 4 weeks PE in both gill (Table 4-5) and kidney (Figure 4-6). Ferritins play important roles in iron storage, allowing the element to be bioavailable for use in a variety of cellular functions such as oxygen transport while at the same time sequestering excess iron in a non-toxic form. Unbound iron can be toxic to the cell as it is capable of reacting with hydrogen peroxide through the Fenton reaction to produce hydroxyl radicals ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$) (Orino & Watanabe 2008). Ferritin has been characterized as an acute phase protein that is up-regulated in response to ROS and cytokines produced during conditions of oxidative stress, infection, and / or inflammation (Koorts & Viljoen 2007). The up-regulation of ferritins suggests the development of an early acute-phase response in both gill and kidney in response to *L. salmonae* infection.

4-2b.2.7. Immune responses - Apolipoproteins:

A number of apolipoproteins were differentially regulated in gill and kidney of *L. salmonae* infected fish. In the gill the expression of 14 kDa apolipoprotein was decreased at 4 weeks PE (Table 4-5), whereas Apolipoprotein A-I precursor is up-regulated in the gill at 4 weeks (Table 4-5) and in the kidney at 8 weeks PE (Table 4-6). Lysozyme expression was increased in the kidney at 8 weeks PE (Table 4-6). Apolipoprotein Eb (ApoEb) precursor was down-regulated in the kidney at 4- and 8-weeks PE (Table 4-6).

Apo-14 kDa is a predominant high density lipoprotein (HDL) in fish and is a homologue of mammalian ApoA-II (Choudhury et al. 2009). Mammalian ApoA-I and II comprise HDL and are known to participate in regulation of neutrophils, decreasing cytokine release as well as inhibiting oxidative burst in those cells (Furlaneto et al. 2002). Mammalian HDLs have antimicrobial activity against trypanosomes, acting as platforms for the assembly of immune complexes (Shiflett et al. 2005). Interestingly, the expression of lysozyme is also increased in the kidney. The C-terminal region of carp ApoA-1 exhibits antimicrobial activity, which may be able to synergize with lysozyme activity (Concha et al. 2004). Lysozyme also acts synergistically with antimicrobials such as histone-derived peptides in coho salmon (Patrzykat et al. 2001).

Apo Eb is a member of the Apo E family, which have been implicated in immunoregulation of T lymphocytes, in addition to roles in lipid distribution and cholesterol transport (Mahley 1988). Low density lipoproteins containing Apo E function to activate or inhibit T-cells through their binding to activating or inhibitory receptors (Cuthbert & Lipsky 1984, 1986).

4-2b.2.8. Immune responses - Monooxygenases:

In the gill the expression of CYP450 7A1 was reduced at 4 weeks PE (Table 4-5), whereas CYP450 2K1 showed reduced expression at 4 weeks PE in kidney samples (Table 4-6). Cytochrome P450s (CYP450) belong to a ubiquitous superfamily of enzymes containing over 500 identified members that are responsible for catalyzing a wide range of monooxygenase reactions involving both endogenous and xenobiotic targets (Buhler & Wang-Buhler 1998). Although the majority of research on CYP450s has involved enzymes responsible for detoxification of exogenous compounds such as toxic chemicals, CYP450s acting on endogenous

substrates are known to have important immuno-relevant roles in fatty acid hydroxylation and eicosanoid catabolism. For example, mammalian CYP450s have been identified that are involved in the catabolism of arachidonate derivatives such as prostaglandins and leukotrienes (Powell et al. 1996). CYP450 7A1 is known to play a role in the degradation of cholesterol to bile acids and steroid hormones in the liver of mammals, although its function in fish is unknown (Mast et al. 2005).

The CYP450 2K1 enzymes constitute a fish-specific P450 subfamily with endogenous substrates. CYP450 2K1 was first identified in rainbow trout as the gene coding for the protein CYP450 LMC2, which catalyses the hydroxylation of the medium-chain fatty acid laurate, as well as several longer chain fatty acids (Miranda et al. 1990, Buhler & Wang-Buhler 1998). In mammals, laurate, or lauric acid, is converted to monolaurin, a monoglyceride with several immune-relevant roles, including induction of T-cell proliferation (Hemat 2004). In addition, lauric acid and monolaurin have antiviral, antibacterial and antiprotozoal properties (Hemat 2004, Smith et al. 2008, Yang et al. 2009). For example, certain protozoans such as *Giardia lamblia*, as well as some species of yeast and bacteria may be killed or inactivated by lauric acid and monolaurin (Hemat 2004).

It appears that the expression of CYP450s was decreased in response to *L. salmonae* infection in these fish. In previous research, decreased expression levels were measured for numerous members of the CYP450 family during infection and inflammation, including parasitic infections. In a review by Morgan, a number of examples of parasite-associated CYP450 reductions are detailed. In one such study, rats infected by the hookworm parasite *Ancylostoma ceylanicum* showed decreased levels of CYP450 mRNA in hepatic microsomes (Morgan 1997). In the majority of these studies, however, it is unclear whether the decreased levels of CYP450

mRNAs were the result of decreased transcription levels or a change in RNA processing or turnover. Although CYP450 expression was reduced in the majority of in vitro and in vivo studies, the levels of these enzymes were increased in response to infection and inflammation in a number of other cases, indicating that specific subsets of CYP450s may respond differently during a particular infection process (Morgan 1997). Results from this study support those from the majority of cases, in which CYP450 levels were reduced in response to parasitic infections in vivo.

4-2b.2.9. Immune responses - Histones:

The expression of histone H1 was increased in gill tissue from *L. salmonae*-infected fish at 4 weeks PE (Table 4-5). Histone H1 has been identified as an antimicrobial protein in Atlantic salmon (Richards et al. 2001). Histone H1 is not confined to the nucleus of all cell types and has been identified on the cell surface of human monocytes as well as murine macrophages (Richards et al. 2001). An N-terminal peptide derived from Histone H1 in Atlantic salmon (identified as salmon antimicrobial peptide [SAMP H1]) has been identified as the predominant antimicrobial peptide in skin mucous of that species (Luders et al. 2005).

4-2b.2.10. Immune responses - Antigen processing and presentation:

Adaptive immunity in vertebrates is characterized by specific interactions between T-lymphocytes displaying antigen-specific T-cell receptors on their surface, with antigenic peptides presented in the context of major histocompatibility molecules. The MHC system comprises two major classes of glycoproteins; MHC I, which are displayed on self cells and present antigenic peptides derived from intracellular pathogens, and MHC II, which are displayed on specialized antigen presenting cells (APC) and bind antigenic peptides derived from extracellular pathogens.

4-2b.2.10.a. MHC II:

A number of genes associated with the MHC II pathway, including MHC II, invariant chain, and cathepsins were differentially expressed in these samples. In gill, expression of the pathway is increased at 8 weeks PE (Table 4-5). In kidney, expression of MHC II and invariant chain were increased at 4 weeks, whereas cathepsin expression was decreased at this time point, Cathepsin expression was also decreased at 12 weeks PE in the kidney (Table 4-6).

MHC II presents antigen to $CD4^+$ T helper (T_H) cells, which release cytokines that amplify and direct the immune response. Class II molecules are made up of two non-covalently associated chains, α and β. Presentation by MHC II requires antigen processing through the exogenous pathway, wherein a pathogen is internalized via phagocytosis and degraded into antigenic peptides inside endocytic vesicles (Figure 4-2). The α- and β chains of MHC II associate with a stabilizing protein called the invariant chain within the rough endoplasmic reticulum, pass through the Golgi body and are released in vesicles that fuse with the endocytic compartments containing the digested peptides. Within the fused compartments the peptides are inserted into the binding groove formed by the α- and β-chains before being transported to the cell surface for display to T_H cells (Castellino et al. 1997). Cysteine endoproteases of the Cathepsin family (Cathepsin-S, -F & -L) participate in this process, both in invariant chain processing and later in its degradation in order to allow peptide binding (Riese et al. 1998, Shi et al. 2000).

4-2b.2.10.b. MHC I:

Numerous components of the MHC I antigen processing and presentation pathway were differentially expressed in gill and kidney at each time point. In the gill, genes associated with

MHC I were down-regulated at 4 weeks but up-regulated at 8 and 12 weeks PE (Table 4-5) whereas MHC I pathway expression was up in the kidney at 8 and 12 weeks (Table 4-6).

The MHC I antigen processing and presentation pathway is involved with targeting "altered self" cells for lysis. MHC I is displayed on the surface of all nucleated cells of the body, where they present endogenous antigens to $CD8^+$ cytotoxic (T_C) cells. If the MHC I/antigen complex is recognized as self, T-cells will remain unactivated. However, if the MHC I/antigen binds to nonself-specific T-cell receptor (TCR) on $CD8^+$ T-cells, the T cells will become activated and lyse the altered self cells. Antigenic peptides are processed for display by MHC I via the endogenous processing pathway, where "danger" or foreign proteins are degraded in the cytosol by a proteasome complex, and transported into the ER for assembly with MHC I components (Figure 4-2). Class I MHC consist of a heavy chain (HC) containing two domains (α1 and α2) (which combine to form an antigenic peptide binding cleft), along with a noncovalently associated β-2 microglobulin (β2M) chain. The HC-β2M complex is assembled within the ER with the aid of chaperone proteins such as calnexin and calreticulin. The HC-β2M, associated with calreticulin, forms a complex with the transporter associated with antigen processing (TAP) protein that transports the processed antigenic peptides from the cytosol into the ER. This process is aided by the TAP-associated glycoprotein, tapasin. Once bound to a peptide, the completed MHC I is transported to the cell surface (Maffei et al. 1997).

Expression of MHC I at the cell surface has also been linked to NK cell activation in mammals. Mammalian NK cells become activated via a "missing self" mechanism that relies on a balance of inhibitory and activating receptors displayed on the NK cell surface. Inhibitory receptors recognize determinants on MHC I, independent from presented antigen. If MHC I

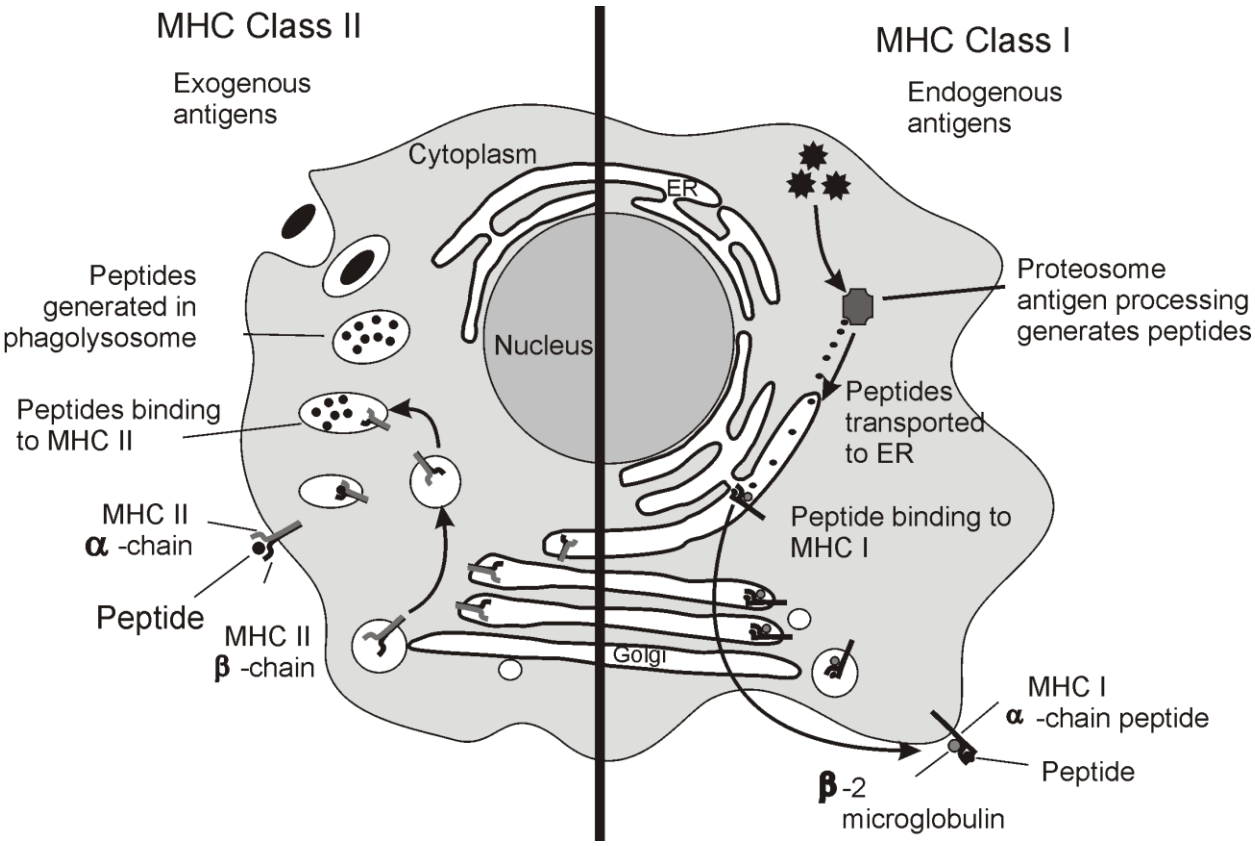

Figure 4-2. Antigen processing and presentation pathways.

MHC Class II - External antigens are processed through the exogenous pathway to be presented in the context of MHC II. Peptides are generated as the pathogen is broken down within increasingly acidic endocytic vacuoles and complexed with MHC II α and β chains. **MHC Class I** - Internal antigens are processed through the endogenous pathway to be presented in the context of MHC I. Antigenic peptides are generated by the proteasome and complexed with MHC I α-chain and β-2 microglobulin. Adapted from: (Parham 2005).

expression is reduced on a potential target cell, then the NK cell may not receive sufficient inhibitory signals and may become activated. Some tumour and virally-infected cells express decreased levels of MHC I, leading to a reduction in inhibitory signals, and allowing activation

of NK cells through activating signals (Yoder et al, 2004; (Shen et al. 2002). Expression of MHCI was reduced in the gills of *L. salmonae* infected fish at 4 weeks PE (Table 4-5), potentially leading to the activation of NK cells in an attempt to lyse parasitized cells.

4-2b.2.10.c. Accessory proteins:

Several genes encoding accessory proteins associated with antigen processing and presentation, including proteasome proteins, heat shock proteins and the accessory proteins TAP and tapasin were differentially expressed at time points in both gill (Table 4-5) and kidney (Table 4-6).

A variety of proteins play accessory roles during the peptide degradation and MHC I-assembly process. Molecular chaperones such as heat shock proteins HSP70, HSP90 and HSP10, as well as the T-complex protein 1-delta are involved in chaperoning proteins and peptides into and out of the ER for degradation by the proteasome and peptide-binding with MHC I (Goldsby et al. 2000).

4-2b.11. Antioxidant defence responses:

Numerous components of antioxidant defence pathways were differentially expressed in both gill and kidney at each time point. In general, a greater number of genes were represented at 4 weeks, with the majority of these showing reduced expression levels in both gill and kidney. In the gill, a number of genes involved in glutathione-related pathways displayed reduced expression at 4 weeks PE, including SOD, as well as a variety of glutathione-related and other anti-oxidant enzymes (Table 4-7).

In the kidney, of a variety of antioxidant-related enzymes and proteins were differentially regulated at each time point, including genes involved in glutathione biosynthesis and

metabolism, as well as redoxins and glutathione-S-transferases (GST). In general, the majority of differentially regulated genes were down-regulated at 4 weeks in the kidney, with mixed responses seen at 8 and 12 weeks PE. At 8 weeks PE in the kidney, the expression of a number of enzymes involved in leukotriene and prostaglandin metabolism was increased, including leukotriene A-4 hydrolase, NADP-dependent leukotriene B4 12-hydroxydehydrogenase and prostaglandin D synthase (Table 4-8).

Antioxidant defences, many involving glutathione, are generated in cells in order to combat reactive oxygen species (ROS) produced during oxidative stress, infections and inflammatory processes. Reactive oxygen species (ROS) can be generated by several mechanisms, and the products of these reactions, the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), are all highly reactive and capable of damaging cellular macromolecules. ROS are generated by specialized enzyme complexes in phagocytic immune cells as a killing mechanism aimed at eliminating pathogens. In addition, ROS can be produced as by-products of metabolism in cells, or by enzymes such as cyclooxygenases (COX) or Cytochrome P450 monooxygenases. The production of ROS begins with the generation of superoxide anion (O_2^-), which is highly reactive and is quickly converted by the enzyme superoxide Dismutase (SOD) to hydrogen peroxide (H_2O_2). Since ROS display indiscriminate toxicity, cells employ numerous enzymatic and non-enzymatic anti-oxidant mechanisms to quickly detoxify and eliminate them, as outlined in Chapter 1. However, even with rapid detoxification some cellular damage may occur, so cells have developed a second line of anti-oxidant enzymes that act to minimize oxidative damage by preventing free radicals from propagating redox chain reactions. Once they have been detoxified, the metabolites are removed from the cell via energy dependent efflux pumps (Figure 4-3).

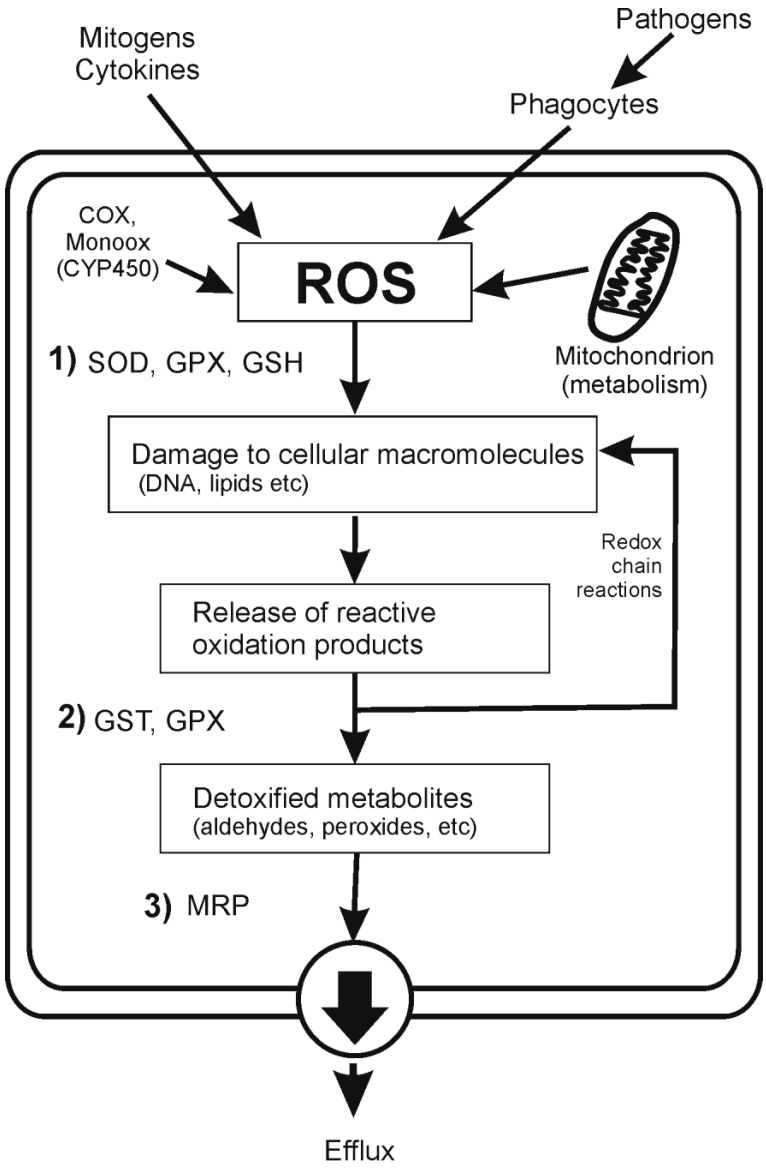

Figure 4-3. Antioxidant mechanisms.

Multiple levels of defence against reactive oxygen species (ROS). 1) Enzymes of the first level include superoxide dismutase (SOD), glutathione peroxidases (GPX), reduced glutathione (G-R) and others that act to eliminate the superoxide anion and hydrogen peroxide. Second level enzymes such as glutathione-S-transferases (GST) and GPX detoxify the resulting reactive oxygen products. 3) The resulting metabolites are eliminated from the cell by energy dependent efflux pumps such as the multidrug resistance protein (MRP). Adapted from (Hayes & McLellan 1999).

Glutathione and glutathione-related enzymes are important components of anti-oxidant defence systems. Peroxiredoxins, thioredoxins, glutathione peroxidases (GPX), selenoproteins, and glutathione S transferases (GST) are all implicated in protecting cells against peroxidative damage (Hayes & McLellan 1999, Brenneisen et al. 2005). Peroxiredoxins are a group of anti-oxidant enzymes that exhibit peroxidase activity towards H_2O_2. Glutathione peroxidases (GPX) are families of selenium-dependent and selenium-independent enzymes that generate oxidized glutathione while catalysing the reduction of H_2O_2 and organic hydroperoxides to water and alcohols (Hayes & McLellan 1999). Selenoproteins form a group of proteins with selenium-dependent GPX activity (Hayes & McLellan 1999).

Glutathione-S-transferases belong to a diverse group of enzymes that participate in cellular detoxification through the conjugation of G-R with reactive epoxides and hydroperoxides produced from the action of ROS on macromolecules during oxidative stress (Hayes & McLellan 1999). GSTs have roles in eicosanoid and prostanoid biosynthesis, and some GSTs have selenium-independent GPX activity (Hayes & McLellan 1999). There are two main superfamilies of these enzymes with different functions, the soluble GSTs and the microsomal GSTs. The soluble class of GST act as detoxification enzymes, reacting with the toxic byproducts of ROS-damaged macromolecules, whereas the microsomal GSTs are known to inhibit lipid peroxidation, and some have roles in leukotriene biosynthesis (Hayes & McLellan 1999). The microsomal GST superfamily is also called MAPEG, for membrane associated proteins involved in eicosanoid and glutathione metabolism (Hayes & McLellan 1999).

The expression of antioxidant-related genes is reduced in a number of disease contexts. For example, GSTs, glutathione peroxidise, and thioredoxin were each down-regulated in Atlantic salmon infected with amoebic gill disease (Wynne et al. 2008). Expression of GSTs is

reduced in Atlantic salmon infected with *Piscirickettsia salmonis* (Rise et al. 2004), as well as saprolegniasis (Roberge et al. 2007). However, glutathione-associated antioxidants may also be upregulated in some infections. For example, glutathione peroxidase was up-regulated in the spleen and liver of Atlantic salmon in response to *Aeromonis salmonicida* (Ewart et al. 2005).

Leukotrienes are important inflammatory mediators, and some, such as leukotriene B4 (LTB_4), are known to have multiple roles depending on the cell type. LTB_4 is produced in cells such as neutrophils and monocytes through the conversion of leukotriene A4 by leukotriene A-4 hydrolase. LTB_4 released from neutrophils and monocytes acts as a chemoattractant for neutrophils (Radmark et al. 1984). NADP-dependent leukotriene B4 12-hydroxydehydrogenase is a member of the alcohol dehydrogenase superfamily responsible for converting leukotriene B4 to 12-oxo-leukotriene, which is much less biologically active (Yokomizo et al. 1996).

Prostaglandins are eicosanoid lipid mediators synthesized from arachidonic acid that play important roles in a variety of organs and tissues, such as vasodilation, smooth muscle relaxation, and inflammation. Prostaglandins act in an autocrine or paracrine fashion through interactions with G-protein-coupled receptors (Funk 2001). Prostaglandin D synthase an enzyme involved in the biosynthesis of Prostaglandin D, a chemical mediator known to be released from activated mast cells in mammals (Matsuoka et al. 2000).

4-2b.12. Oxidative metabolism & Electron transport:

Numerous genes involved with energy metabolism and the transfer of electrons to and through the mitochondrial electron transport chain were differentially regulated in these samples. In the gill and especially the kidney at 4 weeks PE, the expression of a number of genes involved in energy metabolism was decreased (Tables 4-9, 4-10), including genes involved in glycolysis, as well as CI, CIII, CIV and CV of the METC. At 8 weeks PE in the kidney this trend was

reversed, with multiple genes involved in glycolysis and the METC exhibiting increased expression (Table 4-10).

Many of the energy requirements of the cell are met through the breakdown of carbohydrates such as glucose in order to generate ATP. During glycolysis the 6-carbon sugar glucose is broken down to yield two 3-carbon molecules of pyruvate, which then enter the Citric acid (TCA) cycle to be completely broken down to CO_2 (Figure 4-4).

Although a small amount of ATP is produced directly within the cytosol during glycolysis, the majority is generated via the transfer of reducing equivalents such as NADH from the cytosol to the mitochondria, where electrons are coupled to ATP production through the mitochondrial electron transport chain (METC) during the process of oxidative phosphorylation (Lovell 1998).

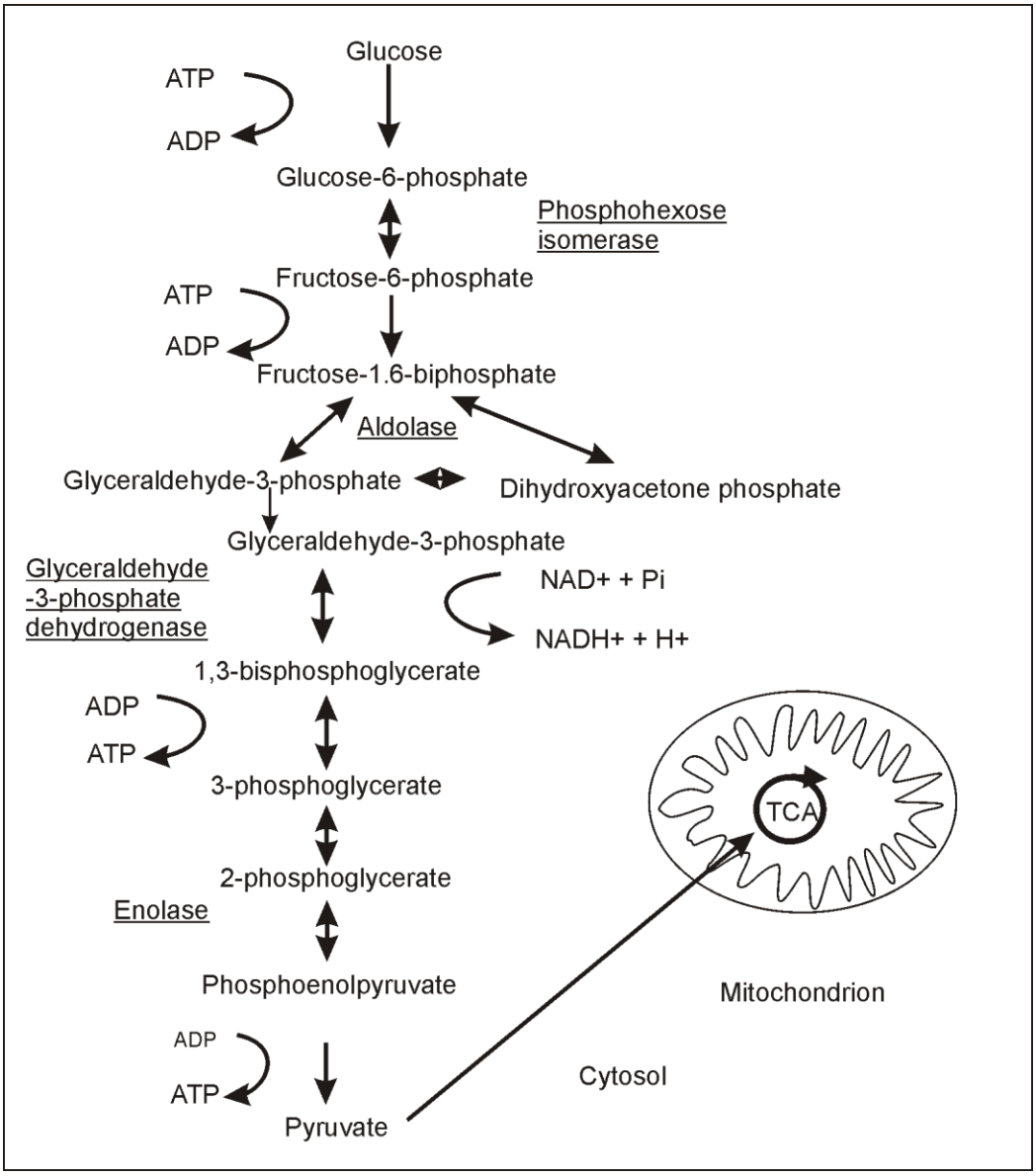

Figure 4-4. Glycolysis and the Citric acid cycle (TCA).

Glucose is broken down in the cytosol during the multistep process of glycolysis, resulting in the release of two 3-carbon molecules of pyruvate. Pyruvate is translocated to the inner mitochondrial matrix for further breakdown in the citric acid cycle (TCA). Differentially regulated enzymes are underlined, including phosphohexose isomerase, aldolase, glyceraldehyde-3 phosphate dehydrogenase, and enolase. Adapted from the Medical Biochemistry Page (http://themedicalbiochemistrypage.org/glycolysis.html)

One of the main mechanisms by which reducing equivalents are transferred from the cytosol to the METC occurs by way of the malate-aspartate shuttle, in which electrons are transferred from the glycolytic pathway to NADH within the inner mitochondria (Figure 4-5). NAD^+ is first reduced to NADH at the 5^{th} step of glycolysis. The NADH is then oxidized to NAD^+ by the enzyme malate dehydrogenase during the reduction of oxaloacetate to malate. The malate enters the mitochondria where the reactions are reversed by mitochondrial malate dehydrogenase, resulting in a supply of NADH within the inner mitochondrial matrix (Lane & Gardner 2005).

L-lactate dehydrogenase is an enzyme that catalyzes the interconversion of lactate with pyruvate. It is known to be abundant in kidney, and may be present in increased concentrations in the blood in response to tissue injury (Cristescu et al. 2008). Pyruvate, the end product of glycolysis, is oxidized to acetyl-CoA, which enters the aerobic citric acid cycle (TCA cycle). This pathway also supplies NADH to the METC to power ATP production in the cell (Lovell 1998). The METC is composed of 4 electron transport chain complexes (CI to CIV) and the ATP Synthase (CV) arrayed along the mitochondrial inner membrane (Figure 4-6). Electrons are deposited from NADH or succinate, entering the METC at CI or CII respectively. From there they are transported by ubiquinone and cytochrome c to CIII and CIV. The ultimate electron acceptor is molecular oxygen (O_2) and electrons are transferred to O_2 at CIV to form water (H_2O). H^+ ions are transferred across the inner membrane at 3 points (CI, CIII & CIV) during electron transport, creating an electrochemical gradient utilized by CV to generate ATP. Each complex consists of multiple subunits, with the largest, CI (also called NADH: ubiquinoneoxidoreductase) consisting of 45 individual proteins (Willems et al. 2009).

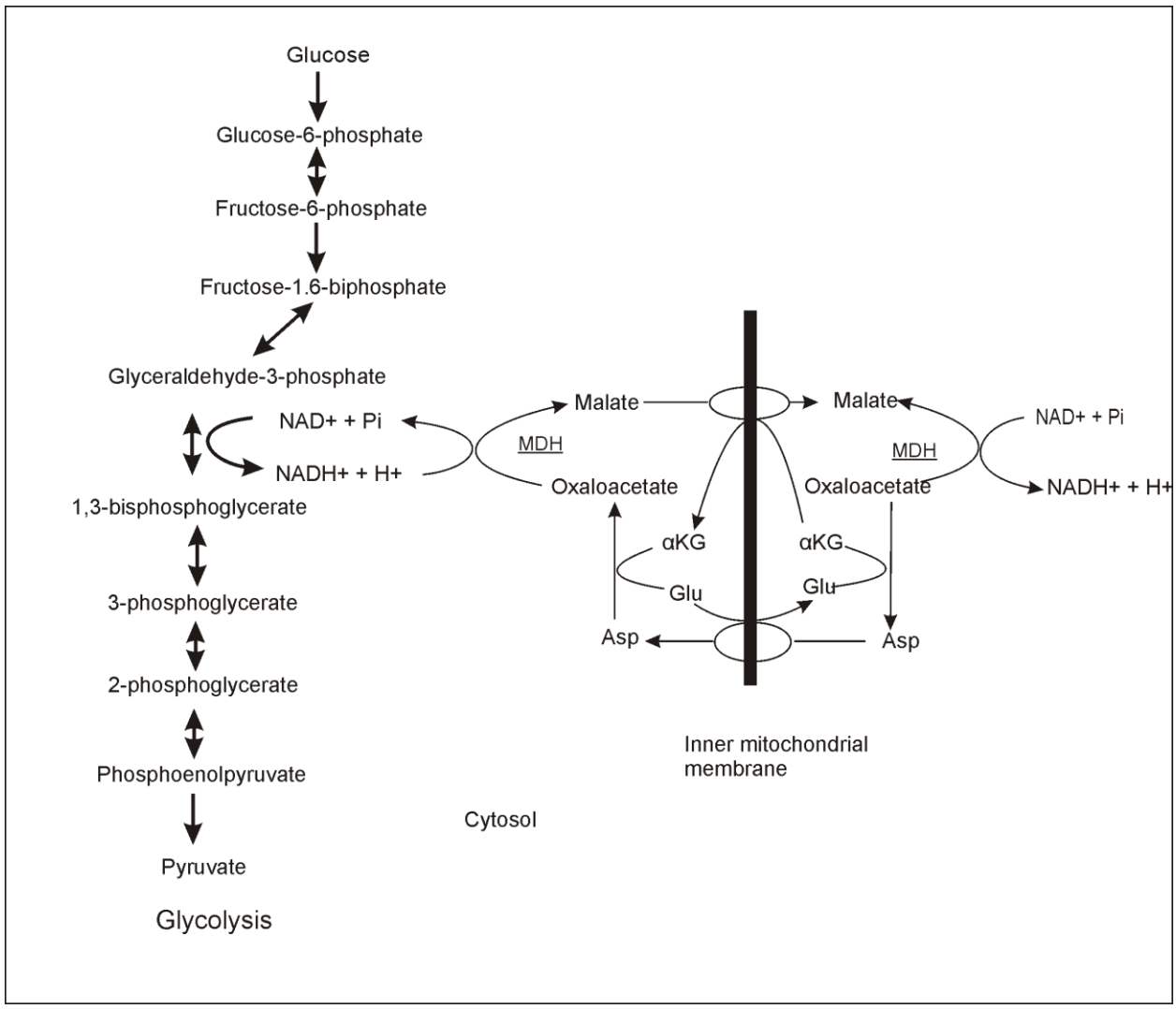

Figure 4-5. Glycolysis and the malate-aspartate shuttle.

Glucose is broken down in the cytosol during the multistep process of glycolysis, resulting in the release of two 3-carbon molecules of pyruvate. Reducing equivalents in the form of NADH are transferred to the inner mitochondrial matrix by way of the malate aspartate shuttle. The shuttle-specific differentially regulated enzyme malate dehydrogenase (MDH) is underlined.

Adapted from the Medical Biochemistry Page (http://themedicalbiochemistrypage.org/glycolysis.html).

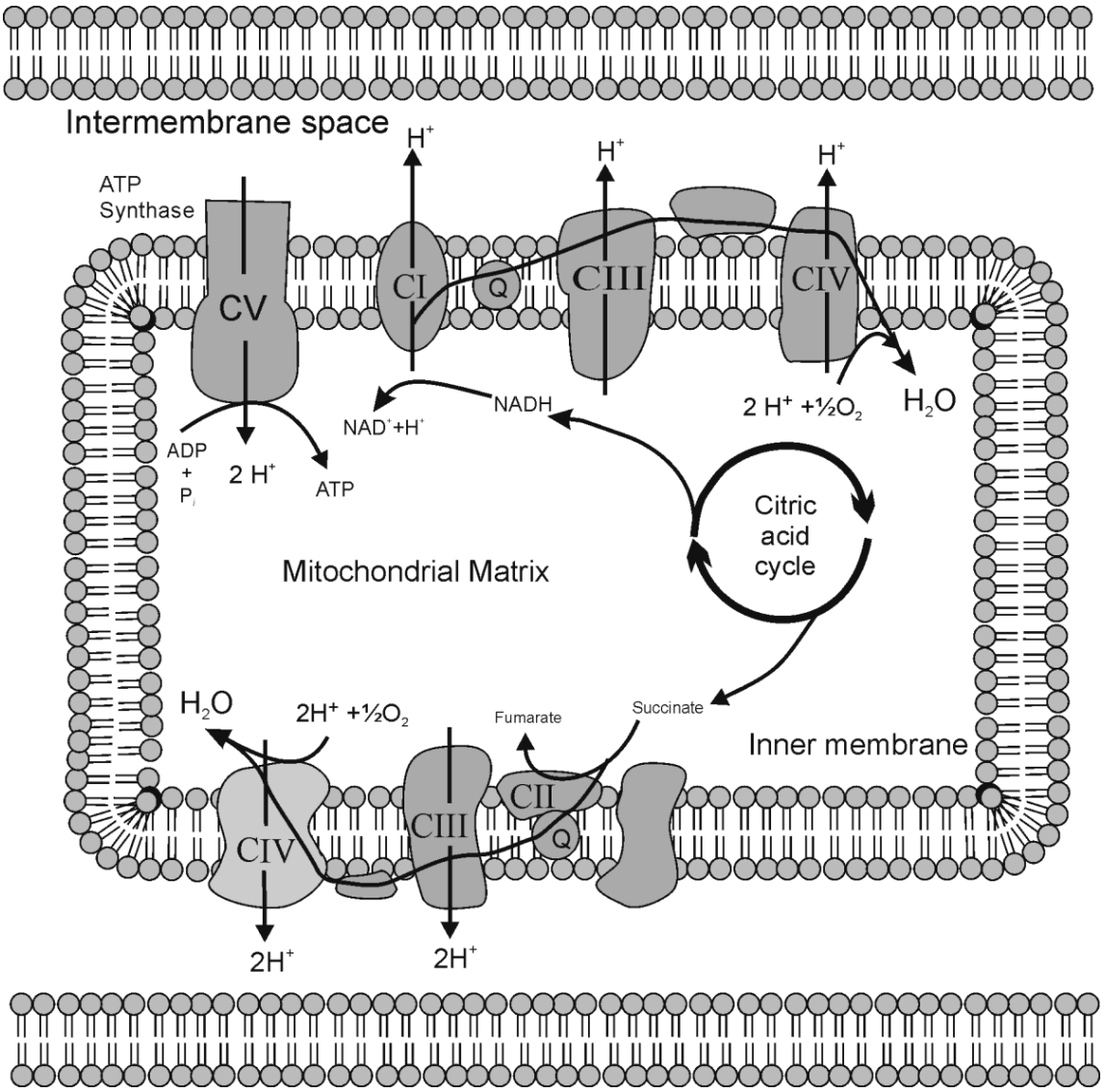

Figure 4-6. The mitochondrial electron transport chain.

Multi-subunit enzyme complexes (CI through CV) are arrayed along the inner mitochondrial membrane, wherein electrons are deposited from NADH or succinate. Electron transport along the METC results in the transfer of H^+ ions into the intramembranous space, resulting in an electro-chemical gradient utilized by the CV complex for the generation of ATP. Adapted from: http://commons.wikimedia.org/wiki/File:Mitochondrial_electron_transport_chain_pl.svg

4-2c. Pathway summaries:

Pathways that were differentially regulated in gill and/or kidney are summarized in Table 4-11. In the gill the majority of differential regulation occurred at 4 weeks PE. Gas transport was up-regulated at 4 weeks, whereas cellular oxidative metabolism and antioxidant defences were down-regulated at that time, suggesting that the increased demand for oxygen at 4 weeks is not related to energy conversion in the gill. A number of immune related pathways were also down-regulated at 4 weeks post exposure in the gill, including immune receptors, Type II interferons, as well as the MHC I antigen processing and presentation pathways (Table 4-11). As mentioned, the display of fewer MHC I on the cell surface may trigger innate effector cells, although at 4 weeks an immune response was not observed by immunohistochemistry. At 8 weeks PE in the gill MHC II expression was up-regulated, whereas the MHC I pathway was up-regulated at both 8 and 12 weeks PE.

In the kidney the majority of differential regulation occurred at 4 and 8 weeks PE. At 4 weeks the expression of cellular oxidative metabolism and antioxidant pathways were down-regulated, whereas both of these pathways were up-regulated by 8 weeks PE. A number of immune related pathways were up-regulated in the kidney, including immune receptors, which were up at 4 and 8 weeks PE (Table 4-6). MHC II was up-regulated at 4 weeks PE, whereas the MHC I pathway showed up-regulation at 4, 8 and 12 weeks PE in the kidney. The up-regulation of MHC I as well as numerous immune cell receptors associated with both innate and adaptive immune components, supports the development of cell-based innate and adaptive immune responses.

4-2d. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

Three genes that were significantly differentially expressed in the microarray study were further tested by qRT-PCR. The results of the qPCR tests agreed with microarray results in terms of both scale and direction of fold change for both Cytochrome C oxidase polypeptide VIa from kidney, as well as β-2 microglobulin from kidney, and were significant by t-test ($p \leq 0.05$). However, although the directionality of fold-change was also consistent between the qRT-PCR and microarray results for CYP 450 2K1 from kidney, these results were not significant (Table 4-12). As discussed above, numerous studies have linked decreased levels of Cytochrome P450s to infection and /or inflammation (Morgan 1997). Although averaged C_T values for all 18 samples showed decreased expression levels, a high degree of variability was recorded between fish for CYP 450 2K1 expression, ranging from 2.5 to 10.4-fold down-regulated in *L. salmonae*-infected vs control fish. Given these results it would be interesting to determine whether there is any correlation between infection levels and CYP 450 expression in individual fish.

4-3. Conclusions:

The results of gene expression analysis of gill and kidney from *L. salmonae*-infected fish suggest that immune responses to the parasite involve both innate and adaptive cell-based factors, as expected. Results also suggest that early in the infection there is a repression of host responses that may be parasite-driven. A number of immune and defence-related genes were down-regulated in the gill at 4 weeks PE, suggesting the possibility of parasite-mediated suppression of host responses. In contrast, immune receptors and cytokines/chemokines were slightly up-regulated in the kidney at 4 weeks PE, possibly indicating some amount of immune recognition occurring there early in the infection. The fact that the majority of differential expression occurred early (at 4 weeks PE) in the kidney and gill was unexpected. Sample times

were originally chosen that were anticipated to precede and then coincide with the timing of the strongest immune response. However, relatively few immune-and defence-related genes were differentially expressed in 12 week samples, when the majority of immune response was anticipated to occur.

It is not known whether differential expression or lack of it, especially at later time points, could be the result of tank effects. It is possible, especially after an incubation period of 12 weeks, that the fish in each tank were responding to tank conditions that may have altered or masked *L. salmonae*-specific effects. Tank effects have been demonstrated to influence the results from disease challenge tests in Atlantic salmon exposed to infectious pancreatic necrosis virus (Kjoglum et al. 2005).

The magnitude of responses at each time point was also smaller than expected. If all genes with less than two-fold differential expression were eliminated from the gene lists, very few genes would remain. Although the two-fold measure is arbitrary, stronger levels of differential regulation in general were anticipated. On the other hand, the relatively low levels of differential expression are less unexpected when discussed in the context of the immunohistochemical results that show an apparent range of responses within individual fish.

Small sample sizes limited the statistical strength of this study. Since microarray analysis was limited to heavily infected fish, the analysis was confined to 3 samples per group, a relatively small number by increasingly stringent microarray study standards. The small number of samples prevented the application of a multiple test correction to the data. For this reason the decision was made to report only overall trends, pathways in which multiple genes were differentially expressed. Given these factors, the data contained herein must be regarded as preliminary, highlighting overall patterns or trends in gene expression. However, the fact that

numerous genes exhibit similar expression within particular pathways, coupled with the redundancy of numerous genes, act as internal controls and lead to the conclusion that these pathways are indeed being differentially regulated. In the future it will be important to perform gene expression analysis on larger numbers of samples, allowing greater numbers of genes to pass stringent analysis. In addition, it would be interesting to analyze gene expression in *L. salmonae* infected fish demonstrating a range of infection levels, particularly comparing differential expression between fish displaying heavy vs. light infection levels.

Table 4-1. Primer sequences for qRT-PCR analysis of selected genes.

Gene	Annealing Temperature (°C)	Sequence (5′ – 3′)
Cytochrome c oxidase polypeptide VIa	58	Forward – ATTAGCTGCTGCATCACACG
		Reverse – TGCTGCATCTTCATGTAGGC
Cytochrome P450 2K1	58	Forward – TCGGTGCATAGGCTACTTCC
		Reverse – GCTGCAGACAGATGCTGAAA
B-2 Microglobulin	55	Forward – TCATTTACAGCGCGGTGGAGT
		Reverse – GTCTGTCTGCTTGGCGTCTGG

Table 4-2. Numbers of differentially expressed genes in *L. salmonae* infected Chinook.
Numbers of differentially expressed genes in tissues from fish sampled at 4, 8 and 12 weeks post-exposure to *L. salmonae*. Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes significant by t-test ($p \leq 0.05$), with an expression level of ≥ 1.4-fold up or down were included. Tissues sampled at 4 weeks showed both larger numbers of differentially regulated genes as well as a higher proportion of genes with decreased expression levels.

Time	Tissue	# of genes Up-regulated (≥ 1.4 fold)	# of genes Down-regulated (≥1.4 fold)
4 weeks	Gill	151	261
	Kidney	141	192
8 weeks	Gill	112	28
	Kidney	187	82
12 weeks	Gill	50	26
	Kidney	88	67

Table 4-3. Gas transport in Gill - Differential expression in *L. salmonae* infected fish. Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-3. Gas transport in gill of L. salmonae infected fish.

EST #	TC #	# of ESTs	Species	Gene Name	Fold change 4 weeks		Fold change 8 weeks		Fold change 12 weeks	
Gas transport – Oxygen										
Hemoglobins										
CB493961	TC157030		*Omyk*	Hemoglobin subunit alpha	2.37					
CA060701	TC96087	3	*Ssal*	Hemoglobin subunit alpha	1.98					
CB500796	TC98087		*Ssal*	Hemoglobin subunit alpha	1.91					
CB492226	TC158289		*Omyk*	Hemoglobin subunit alpha	1.79					
CA049294	TC98290	2	*Ssal*	Hemoglobin subunit alpha	1.51					
CB498419	TC168031		*Omyk*	Hemoglobin subunit alpha-1	2.06	*				
CB516893	TC75282		*Ssal*	Hemoglobin subunit alpha-4	2.10					
CA058361	TC111235		*Ssal*	Hemoglobin subunit alpha-4			-3.01	*		
CB509758	TC111330	2	*Ssal*	Hemoglobin subunit beta	2.11					
CB505738	TC86835	3	*Ssal*	Hemoglobin subunit beta-1	1.79					
CB497335	TC166638		*Omyk*	Hemoglobin subunit beta-1	1.74					
CK990883	Singleton		*Ssal*	Hemoglobin subunit beta-2	1.61					
CB497309	TC166982		*Omyk*	Hemoglobin subunit beta-4	1.83	**				
Gas transport - Carbon dioxide										
Carbonic anhydrase										
CB494301	TC132430		*Omyk*	Carbonic anhydrase	1.96					
CA039360	TC90409		*Ssal*	Omyk mRNA for carbonic anhydrase 2	1.91					
CB501928	TC106131		*Ssal*	Omyk mRNA for carbonic anhydrase 2	1.69	*				
CB494032	TC144422		*Omyk*	Carbonic anhydrase					1.55	*

Table 4-4. Gas transport in kidney - Differential expression in *Loma salmonae* infected fish.

Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-4. Gas transport in kidney of *L. salmonae* infected fish.

EST #	TC #	# of ESTs	Species	Gene Name	Fold change		
					4 weeks	8 weeks	12 weeks
Gas Transport – Oxygen							
Hemoglobins							
CB501216	TC98290		*Ssal*	Hemoglobin subunit alpha		-1.76 *	
CA058361	TC111235	2	*Ssal*	Hemoglobin subunit alpha-4		-1.84	
CK990457	TC86835	2	*Ssal*	Hemoglobin subunit beta-1		-1.94	

Table 4-5. Immune responses in Gill - Differential expression in *L. salmonae* infected fish.

Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-5. Immune responses in gill of *L. salmonae* infected fish.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		Fold change 8 week	Fold change 12 week
Immune responses								
Cytokine & chemokine related								
CB503743	TC83144		*Ssal*	CC chemokine SCYA113	1.52			
CA767983	TC767983		*Ssal*	Small inducible cytokine SCYA105	-1.60	*		
CB510320	TC76958		*Ssal*	Small inducible cytokine SCYA101			2.31	
CX984314	TC67619		*Ssal*	Leukocyte cell-derived chemotaxin 2 precursor			2.17	
Immune receptors								
CB498131	TC146207		*Omyk*	C-C chemokine receptor type 6	-1.68			
CA062570	TC77714		*Ssal*	Interleukin-13 receptor alpha-2 chain precursor	-1.53			
CB512396	TC86306		*Ssal*	Macrophage receptor MARCO	-1.60	*		
Lectins								
CB514743	TC77635		*Ssal*	Lectin precursor	1.98	*		
CA052520	TC67158		*Ssal*	Galectin-9	1.90			
CA042766	TC68648		*Ssal*	C-type lectin				-1.94
Interferon related								
CA052204	TC84989		*Ssal*	Interferon-related developmental regulator 1	-1.50			
CB511902	TC70566		*Ssal*	Interferon regulatory factor 4	-1.86			
CA063565	TC63801		*Ssal*	Interferon regulatory factor 1			2.20	
Perforin / Granzyme								
CB505667	TC92159		*Ssal*	Perforin	1.78			

Table 4-5, Immune responses in gill continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		Fold change 8 week		Fold change 12 week
Acute phase proteins - Iron storage									
CB498077	TC169278	2	*Omyk*	Ferritin, heavy subunit	1.61	*			
CK990586	TC79453	2	*Ssal*	Ferritin, heavy subunit	1.58				
CB493178	TC133183		*Omyk*	Ferritin, heavy subunit	1.50				
CB489257	TC165223		*Omyk*	Ferritin, heavy subunit	1.40				
CB506201	TC81509		*Ssal*	Ferritin, middle subunit					1.48
Monooxygenases									
CA043801	TC70592		*Ssal*	Cytochrome P450 7A1	-1.77				
Apolipoproteins									
CK990692	TC79505		*Ssal*	Apolipoprotein A-I precursor	1.53				
CB510157	TC104333		*Ssal*	14 kDa apolipoprotein	-1.73				
CA044883	TC104333		*Ssal*	14 kDa apolipoprotein	-1.98	*			
Histone H1									
CK991241	TC140848	2	*Ssal*	Histone H1	2.13	**			
Innate - adaptive: Antigen processing & presentation									
MHC II									
CA060476	TC74995		*Ssal*	H-2 class II histocompatibility antigen, A-B alpha chain precursor			1.51		
CB502659	TC74875	4	*Ssal*	H-2 class II histocompatibility antigen gamma chain			1.92		
CK991089	TC95444		*Ssal*	H-2 class II histocompatibility antigen, I-E beta chain precursor					1.72
CB492871	TC146925		*Omyk*	Oncorhynchus mykiss mRNA for MHC class II alpha			1.50		
CB511842	TC64122		*Ssal*	Invariant chain-like protein 14-1 [Oncorhynchus mykiss]			1.75	**	

Table 4-5, Immune responses in gill continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		8 week		12 week	
Cathepsins										
CK990496	TC85966	2	*Ssal*	Cathepsin L precursor	-1.63	*				
CB511609	TC66765		*Ssal*	Cathepsin L precursor			1.94			
CK990241	TC82713	2	*Ssal*	Cathepsin S precursor			2.38			
CB493711	TC157214		*Omyk*	Cathepsin S precursor			2.17			
Adaptive immune responses - Antigen processing & presentation										
MHC I										
CA044026	TC96744		*Ssal*	BOLA class I histocompatibility antigen, alpha chain	-1.44					
CA057048	TC63530	2	*Ssal*	BOLA class I histocompatibility antigen, alpha chain	-1.60					
CK990795	singleton		*Ssal*	Class I histocompatibility antigen, F10 alpha chain	-1.81					
CA039982	TC71964		*Ssal*	Salmo trutta MHC class I heavy chain			1.99			
CA050346	TC91027		*Ssal*	Oncorhynchus mykiss genes, MHC class I a region					1.41	*
Beta-2-microglobulin										
CB498391	TC162887	2	*Omyk*	Beta-2-microglobulin precursor			1.58	*		
CB501401	TC63514		*Ssal*	Beta-2-microglobulin precursor			1.54			
CN442516	TC63514		*Ssal*	Beta-2-microglobulin precursor					1.49	**
Proteasome										
CA060658	TC65407		*Ssal*	Proteasome subunit alpha type 1	1.89	*				
CA050422	TC105755		*Ssal*	Proteasome activator complex subunit 2			2.12			

Table 4-5, Immune responses in gill continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change		
					4 week	8 week	12 week
Molecular chaperones							
CB497602	TC497602		*Omyk*	Heat shock cognate 71 kDa protein	1.40		
CB493960	TC132347		*Omyk*	Heat shock protein HSP 90-beta			1.51
CA057590	TC70302		*Ssal*	T-complex protein 1 subunit delta	-1.53		
Accessory proteins							
CA062112	TC111774		*Ssal*	O mykiss TAP1			1.54
CA039925	Singleton		*Ssal*	O mykiss TAP1			1.47
CA052144	TC110472		*Ssal*	O mykiss TAP1			1.43
CA050694	TC110415		*Ssal*	Salmo salar tapasin (TAPBP) mRNA	-1.62		

Table 4-6. Immune responses of kidney - Differential expression in *L. salmonae* infected fish.

Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-6. Immune responses in kidney of *L. salmonae* infected fish.

- EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		8 week		12 week	
Innate immune responses										
Cytokine & chemokine related										
CB503743	TC83144		*Ssal*	CC chemokine SCYA113	1.68		3.00			
Immune receptors										
CB516976	TC64231		*Ssal*	T cell receptor alpha chain [*S. salar*]	1.54					
CA050786	TC64800		*Ssal*	Interleukin-2 receptor subunit beta precursor	1.49	*				
CA057993	TC69460		*Ssal*	CD-205 - Lymphocyte antigen 75 precursor	1.61	*				
CA058310	TC81110		*Ssal*	Novel immune-type receptor 1 [*O. mykiss*]	1.53					
CB497885	TC151498		*Omyk*	CD53 - Leukocyte surface antigen			1.58			
CB516935	TC74531		*Ssal*	CD97 antigen precursor			2.08			
CB493651	TC139742		*Omyk*	CD11B precursor (TNF receptor superfamily)			1.58			
CK990487	TC68337		*Ssal*	CD59-like protein 2					3.67	
CA051480	TC84196		*Ssal*	Leukocyte immune-type receptor 3					1.42	**
CB516701	TC64573		*Ssal*	Interleukin-1 receptor-like 1 precursor					-1.84	*
Lectins										
CB505565	TC71866		*Ssal*	Serum lectin isoform 3 precursor	2.09					
CB507188	TC79641		*Ssal*	Lectin precursor	1.51					
CB503169	TC64451		*Ssal*	Fish-egg lectin	-2.39	**				
CA061489	TC68524	2	*Ssal*	Fish-egg lectin	-2.46					
CA056108	TC63493		*Ssal*	CD209 antigen-like protein E			2.13	*		

Table 4-6, Immune responses in kidney continued.

- EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		8 week		12 week	
Interferon-related										
CB500977	singleton		*Ssal*	Interferon regulatory factor 7					3.22	
CB511472	TC64779		*Ssal*	Omyk interferon regulatory factor 1 gene	1.78					
CA064287	TC77528		*Ssal*	Interferon-induced protein 44	1.72					
CA064171	TC64374		*Ssal*	Interferon-induced protein 44					3.51	*
CA058271	TC73780		*Ssal*	Interferon-inducible protein Gig2	-2.21					
CB494193	TC139559		*Omyk*	Interferon-induced 17 kDa protein precursor	-2.10					
Perforin / Granzyme										
CB502941	TC85651		*Ssal*	Perforin			-1.53	*		
CB514320	TC75241		*Ssal*	Granzyme K precursor			1.52			
Lysozyme										
CB511680	TC81054	2	*Ssal*	Lysozyme C II precursor			2.37			
Acute phase proteins - Iron storage										
CB508626	TC67649		*Ssal*	Ferritin, lower subunit	2.77					
CB494485	TC158455		*Omyk*	Ferritin, heavy subunit	1.44					
Apolipoproteins										
CA057824	TC100220		*Ssal*	Apolipoprotein Eb precursor	-1.50	*				
CA058895	TC91898		*Ssal*	Apolipoprotein Eb precursor	-2.08					
CB497987	TC161762		*Omyk*	Apolipoprotein Eb precursor	-1.55					
CB498630	TC155447		*Omyk*	Apolipoprotein Eb precursor			-1.80			
CB510571	TC78259		*Ssal*	Apolipoprotein A-I-1 precursor			1.68			

Table 4-6, Immune responses in kidney continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		8 week		12 week	
Monooxygenases										
CA061778	TC75328		*Ssal*	Cytochrome P450 2K1	-10.40	*				
CB496520	TC150232		*Omyk*	Cytochrome P450 2K1	-7.84	*				
CA062367	TC80421		*Ssal*	Cytochrome P450 2K1	-2.49					
CB501070	TC75404		*Ssal*	Cytochrome P450 1A3			1.54			
Innate - adaptive: Antigen processing & presentation										
MHC II										
CB502631	TC74875	2	*Ssal*	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.90	*				
CB497250	TC142161		*Omyk*	H-2 class II histocompatibility antigen gamma chain	1.58					
CA043705	TC95444	6	*Ssal*	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.90	*				
CN442529	TC84000		*Ssal*	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.91					
CK990275	TC64856		*Ssal*	HLA class II histocompatibility antigen gamma chain	1.70					
CB492871	TC146925		*Omyk*	Oncorhynchus mykiss mRNA for MHC class II alpha	1.43	*				
CB511603	TC64122		*Ssal*	Invariant chain-like protein 14-1 [Oncorhynchus mykiss]	1.71					
Cathepsins										
CB511870	TC73883		*Ssal*	Cathepsin F precursor	-1.52					
CB502503	TC85966		*Ssal*	Cathepsin L precursor	-1.94					
CB497670	TC143390		*Omyk*	Cathepsin L precursor	-1.78					
CB502503	TC85966		*Ssal*	Cathepsin L precursor					-1.78	**
CB511609	TC66765		*Ssal*	Cathepsin L precursor					-1.64	*
CK990727	TC85966		*Ssal*	Cathepsin L precursor					-1.43	

Table 4-6, Immune responses in kidney continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week	8 week		12 week
Adaptive immune responses - Antigen processing & presentation								
MHC I								
CA040172	TC77434		*Ssal*	Class I histocompatibility antigen, F10 alpha chain precursor		1.47	**	
CA044472	TC85510		*Ssal*	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor		1.83		
CB502985	TC93533		*Ssal*	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor		1.71		
CA044026	TC96744		*Ssal*	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor		1.78		
CB502804	TC92265	2	*Ssal*	MHC class I [Salmo salar]		1.62		
CA060492	TC89267		*Ssal*	Oncorhynchus mykiss genes, MHC class I a region		1.42		
CA044407	TC93758		*Ssal*	Oncorhynchus mykiss MHC class Ib antigen (UDA)		1.89		
CA039982	TC71964		*Ssal*	Salmo trutta MHC class I heavy chain (Satr-UBA)		1.71		
Beta-2-microglobulin								
CA043324	TC63545		*Ssal*	Beta-2-microglobulin precursor	1.51			
CB505594	TC63514		*Ssal*	Beta-2-microglobulin precursor	1.61			
CB501401	TC63514	6	*Ssal*	Beta-2-microglobulin precursor		1.52	*	
CK990545	TC111297		*Ssal*	Beta-2-microglobulin precursor		1.47	*	
CK990806	TC108939		*Ssal*	Beta-2-microglobulin precursor		1.52	*	
CB489043	TC162887	3	*Omyk*	Beta-2-microglobulin precursor		1.56	*	
CB505897	TC63545		*Ssal*	Beta-2-microglobulin precursor		1.50		
CB500763	TC64964		*Ssal*	Beta-2-microglobulin precursor		1.48		
CK990799	singleton		*Ssal*	Salmo salar clone BE7 beta-2 microglobulin		1.46		

Table 4-6, Immune responses in kidney continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week	8 week	12 week	
Proteasome								
CA052445	TC65742		*Ssal*	Proteasome subunit beta type 6 precursor	1.58			
CB511841	TC63754		*Ssal*	Proteasome subunit beta type 7 precursor	1.48			
CA038117	TC64616		*Ssal*	Proteasome subunit alpha type 6			1.75	**
CA055186	TC63754		*Ssal*	Proteasome subunit beta type 7 precursor			1.41	
CB516784	TC74134		*Ssal*	26S protease regulatory subunit 6B			1.76	
CK990887	TC102502		*Ssal*	26S protease regulatory subunit 6B			1.77	
CA063091	TC74911		*Ssal*	26S proteasome non-ATPase regulatory subunit 12			1.55	
CB517754	TC64365		*Ssal*	Proteasome activator complex subunit 3			1.61	*
Accessory proteins								
CA770328	TC66045		*Ssal*	Tapasin precursor	1.52			
CA054410	TC81494		*Ssal*	Derlin-1			1.48	*

Table 4-7. Antioxidant defencess in gill - Differential expression in *L. salmonae* infected fish.

Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-7. Antioxidant defence responses in gill of *L. salmonae* infected fish.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		8 week	12 week
Antioxidant defences								
CA054957	TC63709		*Ssal*	Superoxide dismutase [Cu-Zn]	-1.75			
CA060381	TC84854		*Ssal*	Ornithine decarboxylase antizyme 1	-1.53	*		
CB493774	TC143137		*Omyk*	Ornithine decarboxylase antizyme 1	-1.68	*		
Redoxins								
CA054618	TC63616		*Ssal*	Peroxiredoxin-1				-2.05
CB493639	TC132824		*Omyk*	Phospholipid hydroperoxide glutathione peroxidase	-1.43			
CA041451	TC65381		*Ssal*	Thioredoxin			2.20	
CB488515	TC160100		*Omyk*	Similar to thioredoxin domain containing 14			-1.50	
Glutathione-S-transferases								
CA057678	TC65309		*Ssal*	Glutathione S-transferase Mu 3	1.61			
Leukotrienes & Prostaglandins								
CB494012	TC142778		*Omyk*	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	-1.43			

Table 4-8. Antioxidant defences in kidney - Differential expression in *L. salmonae* infected fish. Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-8. Antioxidant defence responses in kidney of *L. salmonae*-infected fish

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		Fold change 8 week		Fold change 12 week	
Antioxidant defences										
CK991153	TC82189		*Ssal*	S-adenosylmethionine synthetase isoform type-2	-1.67	*				
CK990365	TC74925		*Ssal*	Glutathione reductase, mitochondrial precursor					2.01	*
Redoxins										
CB505875	TC75618		*Ssal*	Peroxiredoxin	1.77					
CB509722	TC66216		*Ssal*	Glutathione peroxidase 3 precursor					-1.43	
CA042641	TC78532		*Ssal*	Selenoprotein H	1.42	**				
CB510644	TC81806	2	*Ssal*	Selenoprotein Pa precursor	-2.32	*				
CA061844	TC65283		*Ssal*	15 kDa selenoprotein precursor					1.61	*
CA050544	TC98076		*Ssal*	Selenium-binding protein 2					-1.94	
CB498613	TC146297		*Omyk*	Thioredoxin-like selenoprotein M precursor	-1.89					
CA057296	TC103339		*Ssal*	Thioredoxin			1.54			
CB511879	TC105788		*Ssal*	Thioredoxin-like protein 2			-1.46	*		
Glutathione-S-transferases										
CB492604	singleton		*Omyk*	Glutathione S-transferase A	-2.26	**				
CA052963	TC65218		*Ssal*	Glutathione S-transferase A	-2.23	*				
CB496788	TC144409		*Omyk*	Glutathione S-transferase A	-2.42					
CB496493	TC142140		*Omyk*	Glutathione S-transferase P	-1.50					
CB510883	TC73524		*Ssal*	Glutathione S-transferase theta-1	-1.56					
CB492806	TC146311		*Omyk*	Microsomal glutathione S-transferase 3	-1.43					
CB492382	TC142151		*Omyk*	Maleylacetoacetate isomerase/glutathione transferase zeta	-1.84	*				

Table 4-8, Antioxidant defence responses in kidney continued.

EST #	TC #	# of ESTs	Species	Gene name	Fold change 4 week		Fold change 8 week		Fold change 12 week	
Leukotrienes & Prostaglandins										
CA058848	TC88866	2	*Ssal*	Leukotriene A-4 hydrolase			1.52	*		
CB494012	TC142778		*Omyk*	NADP-dependent leukotriene B4 12-hydroxydehydrogenase			1.48	*		
CB509870	TC108525		*Ssal*	Prostaglandin D synthase [Omyk]			1.58			
CB493062	TC138217		*Omyk*	Alcohol dehydrogenase class 3					1.40	*
CB517743	TC63686		*Ssal*	Salmo salar aryl hydrocarbon receptor 2 gamma					1.75	*
NADPH oxidase-related										
CB506394	TC65699		*Ssal*	Cytochrome b5	1.73					
CK990859	TC67084		*Ssal*	Isocitrate dehydrogenase [NADP], mitochondrial precursor	-1.44	**				
CB498501	TC164636		*Omyk*	Isocitrate dehydrogenase [NADP], mitochondrial precursor	-1.50	*				

Table 4-9. Energy metabolism and Mitochondrial Electron Transport in Gill - Differential expression in *L. salmonae* infected fish. Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-9. Energy metabolism and METC in gill of *L. salmonae* infected fish

EST #	TC #	# of ESTs	Species	Gene Name	Fold change 4 weeks		8 weeks	12 weeks	
Carbohydrate breakdown									
Glycolysis II									
CB501837	TC98271	2	*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase	1.99	*			
Mitochondrial electron transport chain									
CIV									
CA041318	TC76869		*Ssal*	Vacuolar ATP synthase subunit G 1	-1.66				
CA053755	TC78327		*Ssal*	Vacuolar ATP synthase subunit H	-1.53	*			
CB497797	TC140154		*Omyk*	Vacuolar ATP synthase 16 kDa proteolipid subunit				1.43	*
Additional redox proteins									
CA053721	TC70898		*Ssal*	Ubiquinone biosynthesis monooxygenase COQ6	-1.55				

Table 4-10. Energy metabolism and Mitochondrial Electron Transport in kidney - Differential expression in *L. salmonae* infected fish. Normalized levels were expressed as ratios between *L. salmonae* infected and control fish. Genes were included with a relative expression level of 1.40-fold (up or down). Significance - $p \leq 0.05$, unless otherwise indicated by * ($p \leq 0.01$) or ** ($p \leq 0.001$). Genes are grouped according to major pathways. EST# indicates the expressed sequence tag sequence identifier. TC# is a sequence identifier from TIGR (http://compbio.dfci.harvard.edu/tgi/). # of ESTs indicates the number of significant spots on the array representing that particular EST that show the same pattern of regulation.

Table 4-10. Oxidative metabolism & METC in kidney of *L. salmonae* infected fish

EST #	TC #	# of ESTs	Species	Gene Name	4 weeks		8 weeks		12 weeks
Carbohydrate breakdown									
Glycolysis I									
CA060235	TC63768		*Ssal*	Glucose-6-phosphate isomerase			1.59	*	
CB493962	TC138846		*Omyk*	Fructose-bisphosphate aldolase B	-2.81	*			
CA056945	TC63694	2	*Ssal*	Fructose-bisphosphate aldolase B	-3.69	*			
CA043730	TC101020		*Ssal*	Fructose-bisphosphate aldolase B	-1.55				
CB514705	TC64599		*Ssal*	Fructose-bisphosphate aldolase A			1.62	*	
CB517144	TC73208		*Ssal*	Fructose-bisphosphate aldolase A			1.50		
Glycolysis II									
CA050886	TC64612	2	*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase	-3.77	*			
CA768062	TC103056	2	*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase	-3.15				
CB498361	TC142854		*Omyk*	Glyceraldehyde-3-phosphate dehydrogenase	-3.05				
CK991014	TC71456	2	*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase	-2.86	*			
CB491826	TC140481		*Omyk*	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	-3.27				
CA051897	TC99126		*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase			1.87		
CB492813	TC134469	2	*Omyk*	Glyceraldehyde-3-phosphate dehydrogenase			1.81		
CA047126	TC99126		*Ssal*	Glyceraldehyde-3-phosphate dehydrogenase			1.68		
CA061915	TC85529		*Ssal*	Enolase			1.41	*	

Table 4-10, Oxidative metabolism & METC in kidney continued.

EST #	TC #	# of ESTs	Species	Gene Name	4 weeks		8 weeks	12 weeks
Malate - aspartate shuttle								
CB497834	TC167187		*Omyk*	Malate dehydrogenase, cytoplasmic	-1.47	*		
CB493498	TC167187		*Omyk*	Malate dehydrogenase, cytoplasmic			1.63	
Pyruvate conversion								
CA057499	TC64172		*Ssal*	L-lactate dehydrogenase B chain			1.58	
CA057499	TC90894		*Ssal*	L-lactate dehydrogenase B chain			1.63	
Mitochondrial electron transport chain								
Complex I								
CB494115	TC137118		*Omyk*	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11	-1.43	*		
CB493855	TC150932		*Omyk*	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mito precursor	-1.83			
CA046322	TC85599		*Ssal*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.53			
CB497043	TC169334		*Omyk*	NADH dehydrogenase [ubiquinone] flavoprotein 3, mito precursor			1.90	
CA041405	TC64051		*Ssal*	Ubiquinone biosynthesis protein COQ9, mitochondrial precursor				1.41
CB490489	TC132624		*Omyk*	NADH-ubiquinone oxidoreductase chain 1				2.23
CK990926	TC87748		*Ssal*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4				-1.50
CB516972	TC70184		*Ssal*	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mito precurs				-1.58
Complex III								
CB492393	TC135291		*Omyk*	Cytochrome c			1.69	
Complex IV								
CN442538	TC109170		*Ssal*	Cytochrome c oxidase subunit 1	-1.42			
CB496924	TC145971		*Omyk*	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	-1.58			

Table 4-10, Oxidative metabolism & METC in kidney continued.

EST #	TC #	# of ESTs	Species	Gene Name	4 weeks		8 weeks	12 weeks	
Complex IV									
CA042792	TC86974		*Ssal*	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	-11.02	*			
CB496806	TC156955		*Omyk*	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	-5.23	*			
CA037498	TC102407		*Ssal*	Cytochrome c oxidase polypeptide VIc precursor	-1.43	*			
CB497287	TC142242		*Omyk*	Cytochrome c oxidase subunit VIIa-related protein, mito precursor			1.45		
CA052300	TC67464		*Ssal*	Cytochrome c1 heme protein, mitochondrial precursor			1.48		
Complex V									
CB498523	TC165851		*Omyk*	ATP synthase gamma chain, mitochondrial precursor	-1.79				
CA042951	TC84544		*Ssal*	ATP synthase gamma chain, mitochondrial precursor			1.57		
CB514424	TC65005		*Ssal*	Vacuolar ATP synthase 16 kDa proteolipid subunit			1.67		
CA058181	TC65849		*Ssal*	ATP synthase mitochondrial F1 complex assembly factor 2, mito precursor				2.18	
CA042578	TC70614		*Ssal*	ATP synthase delta chain, mitochondrial precursor				-1.43	
CA053755	TC78327		*Ssal*	Vacuolar ATP synthase subunit H			1.40		
Additional redox proteins									
Oxidoreductase									
CA052648	TC71148		*Ssal*	Putative oxidoreductase yulF	-8.61	*			
CB499596	TC65814		*Ssal*	Hypothetical oxidoreductase yoxD			-1.54		
CB488311	TC149807		*Omyk*	Glutaryl-CoA dehydrogenase, mitochondrial precursor	-1.67				
Accessory proteins									
Mitochondrial molecular chaperones									
CA036918	TC69298		*Ssal*	10 kDa heat shock protein, mitochondrial				2.02	**
CA042054	TC76806		*Ssal*	C-factor	-1.73	*			

Table 4-11. Pathways differentially regulated during *Loma salmonae* infection.

Differentially expressed genes were classified into pathways for gene expression analysis. Pathways that included a minimum of 3 differentially regulated genes are summarized below.

Gill	**Time post-exposure**		
Pathways	4 weeks	8 weeks	12 weeks
Gas transport	Up	-	-
Immune receptors	Down	-	-
Acute phase proteins – Iron storage	Up		
MHC II	-	Up	-
MHCI	Down	Up	Up
Antioxidant defences	Down	-	-
Oxidative metabolism & METC	Down	-	-

Kidney	**Time post-exposure**		
Pathways	4 weeks	8 weeks	12 weeks
Gas transport	-	Down	-
Immune receptors	Up	Up	-
Type II Interferons	Up	-	-
Apolipoproteins	Down		
Monooxygenases	Down		
MHC II	Up	-	Down
MHCI	Up	Up	Up
Antioxidant defences	Down	Up	-
Oxidative metabolism & METC	Down	Up	-

Table 4-12. Comparative differential expression measured by microarray and qRT-PCR.

Selected genes were analyzed by qRT-PCR. Expression levels are expressed as fold-change relative to control expression. The expression levels measured by qRT-PCR agree with microarray results in terms of both scale and directionality.

	Expression level		**Significant?**
Gene	Microarray	qPCR	
Cytochrome C oxidase	-5.23	-6.19	Yes
β-2 microglobulin	1.51	1.39	Yes
Cytochrome P450 2K1	(-2.5) - (-10.4)	-2.98	No

Chapter 5

Summary and Conclusions

The research presented here represents the results from a variety of efforts aimed at increasing understanding of the host-parasite relationship between Chinook salmon (*Oncorhynchus tshawytscha*) and the microsporidian parasite *Loma salmonae*. The objectives of the study included improving early detection and diagnostic tools, increasing understanding of potential immuno-prophylactic treatments, as well as measuring immune and other defence responses of Chinook salmon during *L. salmonae* infection. This chapter is separated into sections in which the results supporting each objective are briefly summarized and discussed in the context of current research.

5-1. Development of *Loma salmonae*-specific IgY from chickens:

The first objective of this study was the development of tools to allow early detection and diagnosis of *L. salmonae* within tissues. Chicken-derived IgY polyclonal antibodies specific for *L. salmonae* proved an abundant and highly specific detection tool that allows the localization of pre-xenoma stages of *L. salmonae* within gill tissue of Chinook salmon as early as 4 weeks PE, when such stages would be impossible to detect by traditional staining methods. IgY staining provides an alternative to traditional detection methods, which include polymerase chain reaction (PCR) of tissue or staining of histological sections with non-specific stains such as haemotoxylin and eosin (H & E) or Giemsa. Immunohistochemistry based on the use of the IgY reagent developed here has important advantages over these traditional methods. Although PCR allows very sensitive detection, this method only detects parasite DNA and allows no localization of *L. salmonae* within tissues. The traditional stains H & E and giemsa allow visualization of tissues,

but since the stains are not specific for *L. salmonae* they do not allow localization of parasite stages such as small xenomas, individual spores or presporogonic stages during early development. In contrast, the chicken-derived IgY described here binds specifically to *L. salmonae* and allows the parasite to be localized and visualized within tissues beginning at a very early pre-xenoma stage of development.

Since the development of this anti-*L. salmonae* polyclonal IgY, applications for chicken-derived IgY have continued to increase, and recent descriptions can be found for the use of IgY in both research and medical applications. For example, the research and diagnostic potential has been described for IgY specific for *Mycobacterium avium*, subspecies *paratubercolosis*, the cause of paratuberculosis in ruminants and also potentially implicated as a causative agent of Crohn's disease in humans (Shin et al. 2009). Another recent application for IgY involves their use as highly specific targeting mechanisms toward breast cancer cells when conjugated with single-walled carbon nanotubes (Xiao et al. 2009). This report represents the first application of IgY in the diagnosis of a fish pathogen and demonstrates that the value of this approach as an alternative to traditional polyclonal antibodies and histochemical stains.

5-2. The immuno-prophylactic effects of β-1,3/1,6 glucan on Chinook salmon exposed to *L. salmonae*

β-glucans have immune-modulating effects in fish and continue to be studied in a variety of species for their potential to prevent or reduce disease. The second objective of this research was to increase the current level of understanding of β-glucans as potential immune-modulators in teleosts, specifically the effect of β-1,3/1,6 glucan in reducing the prevalence or severity of *L. salmonae* infection in Chinook salmon. Rainbow trout (RBT) intra-peritoneally (IP) inoculated with β-1,3/1,6 glucan and later *per os* exposed to *L. salmonae* showed reductions in both

prevalence and intensity of infection. In this work Chinook salmon were similarly inoculated with β-glucan, followed by *L. salmonae* exposure at 3 weeks post-inoculation (PI). Prevalence and intensity of infection were measured by PCR and immunohistochemistry following a further incubation period of 8 weeks. Inoculation of 100 µg of β-1,3/1,6 glucan resulted in reduced intensity of *L. salmonae* infection (~66%) in Chinook salmon as measured by xenoma counts in gill tissue at 8 weeks post-parasite exposure, although prevalence was not reduced in the glucan-inoculated fish. It is likely that the greater sensitivity of diagnostic methods (PCR) employed here may partly explain the apparent disparity between the present results and those described earlier for RBT. Regardless of the unchanged prevalence levels, the reduced intensity of infection for Chinook salmon supports the need for additional studies designed to further elucidate the protective effects of β-glucan.

In addition to demonstrating protection against *L. salmonae* elicited in Chinook salmon by β-glucan, this study also sought to elucidate possible mechanisms of protection by analyzing gene expression in the salmon in response to intra-peritoneal (IP) inoculation with β-1,3/1,6-glucan. cDNA microarray analysis of kidney tissue from glucan-inoculated fish at 1, 2, and 3 weeks PI revealed the up-regulation of a number of pathways at 1 week PI, including antioxidant defences and both MHC I and MHC II antigen processing and presentation pathways. Although no evidence was found for increased haematopoiesis at these sample times, it is possible that any such increases occurred too early to allow detection at the first sampling period.

Results presented here represent the first microarray analysis of Chinook salmon tissues in response to glucan exposure. Previously, most gene expression analyses in response to β-glucan in fish have employed qRT-PCR to measure differential regulation of a relatively small number of genes. In recent studies where microarrays were applied, results may have been confounded

by additional experimental conditions such as concurrent exposure to an inflammation-inducing agent (Djordjevic et al. 2009).

Gene expression in response to putative immune-modulators such as β-glucan has been studied in a number of fish and mammalian models (Rodriguez et al. 2009, Torosantucci et al. 2009). Several receptors for β-glucan have been described, including complement receptor 3 and Dectin-1 (Sun & Zhao 2007). Receptors for β-glucan are expressed on the surface of Atlantic salmon macrophages permitting the binding and interalization of glucans (Engstad & Robertsen 1994). The nature of internalized glucans may affect subsequent phagocytic cell responses. In one study mouse macrophages that internalized particulate glucans remained localized within the peritoneal cavity, whereas macrophages that had taken up soluble glucans localized to the spleen and liver (Tateishi et al. 1997). If Chinook salmon macrophages respond in a similar way, the particulate β-glucan in this study may have been phagocytosed by macrophages that remained localized within the peritoneal cavity, potentially releasing cytokines able to recruit and activate additional macrophages and innate effectors to the site. If so, these cells may have retained a heightened responsiveness that allowed swift and strong recognition of and reaction to nascent *L. salmonae* infection. It is unknown at what stage or in what form such recognition could take place, since *L. salmonae* is thought to be transported within a host cell from the site of initial infection (the gut) to the ultimate site of xenoma formation. Perhaps resident macrophages of the gut area are the first host cells colonized by *L. salmonae*. If so, it is possible that prior activation by soluble factors released from phagocytes after their interaction with glucan allowed the host macrophages to kill *L. salmonae* more effectively. On the other hand, perhaps the original host cells are themselves engulfed by activated macrophages further on from the original site of

infection. In this scenario migrating host cells would come into contact with glucan-activated macrophages, which would presumably kill the infected host cell along with the parasite.

There is evidence that β-glucan inoculation promotes the production of cytokines involved in the development of cell-based immune response. Zebrafish IP-inoculated with β-glucan three times (6, 4, and 2 days) prior to bacterial challenge demonstrated enhanced resistance to *Aeromonas hydrophila* infection (Rodriguez et al. 2009). The expression of IFN-γ was increased in the glucan-inoculated fish, suggesting that β-glucan may modulate the expression of IFN-γ, a key cytokine involved in macrophage activation and induction of a T_H1 response to intracellular pathogens (Rodriguez et al. 2009). An effective immune response to *L. salmonae* is believed to involve the development of T_H1-based cellular immunity. It is possible that the β-glucan-induced resistance to *L. salmonae* infection observed here in Chinook is related to the development of a T_H1-directed response that results in a population of immune cells able to kill *L. salmonae* early in the infection, whether directly or within a host cell. Although the cDNA microarray utilized here did not contain any IFN-γ specific features, it will be important in the future to include IFN-γ as a target gene in qRT-PCR studies designed to measure the expression of immune-related genes in β-glucan-inoculated Chinook salmon.

Glutathione-related antioxidant defences comprised a key pathway that was up-regulated 1 week following glucan injection in Chinook salmon. Expression of the antioxidant enzyme super-oxide dismutase (SOD) has previously been demonstrated in spleen for 15 days following β-glucan administration in grass carp (*Ctenopharyngodon idella*) (Kim et al. 2009). Similarly, here, numerous components of antioxidant related pathways were up-regulated at 7 days (1 wk) PI in Chinook salmon kidney, although SOD was not differentially expressed, and differential regulation of antioxidant genes was no longer detected at 2 weeks PI. β-glucans in the peritoneal

cavity are presumably taken up by phagocytic cells, which may be stimulated to produce reactive oxygen species through the respiratory burst. Increased antioxidant expression may be necessary to combat ROS and other reactive by-products of these oxidative killing mechanisms.

It is clear that IP-inoculation with β-1,3/1,6 glucan has an immune-modulating effect on Chinook salmon, allowing a more effective response to *L. salmonae* and thereby reducing the parasite load in the fish. The possibility that particulate β-glucan in the peritoneal cavity is engulfed by phagocytic cells, initiating a respiratory burst that triggers the up-regulation of antioxidant-related genes in the kidney requires further research.

5-3. Gene expression changes in Chinook salmon responding to *Loma salmonae* infection

Members of the *Oncorhynchus* genus, including Chinook salmon and rainbow trout (RBT), infected with *L. salmonae* are capable of mounting an effective immune response that allows them to recover from the infection. In addition, recovered individuals display resistance to re-infection. However, despite ongoing research efforts the nature of immune responses and resistance in both species remains unclear. The third objective of this research was to measure immune and other defence responses of Chinook salmon in response to the microsporidian parasite *L. salmonae*. The analysis of gene expression of Chinook salmon heavily infected with *L. salmonae* represents a third significant research project described in this dissertation. Gill and kidney tissues were analyzed using an Atlantic salmon based cDNA microarray platform at 4, 8 and 12 weeks post exposure (PE). The results from this study represent an initial view into the genome-wide expression patterns of immune and other defence responses to an intra-cellular parasite.

In the kidney, numerous immune- and defence-related genes were differentially regulated at each of the time points. Throughout the time course of 4, 8 and 12 weeks after parasite exposure several immune-related pathways showed differential regulation in the kidney, supporting the development of both innate and adaptive, cell-based immune responses, as indicated by the up-regulation of immune receptors, type II interferons, as well as MHC I and MHC II antigen processing and presentation pathways. In contrast, antioxidants, monooxygenases, as well as apolipoproteins were down-regulated at 4 weeks PE in the kidney tissue.

In the gill a number of immune- and defence-related pathways were down-regulated at 4 weeks PE, including immune receptors, MHCI, as well as antioxidant defences. In contrast, gas transport pathways were up-regulated in the gill at 4 weeks PE. At 8 and 12 weeks PE in the gill few immune-related genes showed differential expression, although *L. salmonae* xenomas become progressively larger and begin to break down during this time period and the secondary lamellae display increasing regions of hyperplasia. Only antigen processing and presentation pathways were differentially expressed at 8 and 12 weeks PE, with MHC I showing up-regulation at both 8 and 12 weeks PE, whereas MHC II was up-regulated only at 8 weeks PE. Although specific patterns of immune response differed between gill and kidney early in the infection process, the differential regulation of key immune components indicate the development of an immune response in Chinook salmon in response to *L. salmonae*.

5-4. General discussion

Although *L. salmonae* has been studied in RBT and Chinook for more than two decades, relatively little is known about the nature of host cells and other components that contribute to an

effective immune response. Studies suggest the development of cell-based innate and adaptive immune responses to intracellular parasites. Although small sample sizes mean that the results reported here must be regarded as preliminary, these findings suggest that genes associated with both innate and adaptive cell-based immunity were differentially regulated in response to *L. salmonae* infection, supporting the development of innate and adaptive cell-based responses in Chinook salmon.

Interestingly, patterns of immune response differed between gill and kidney early in the infection process. Some initial depression of immune gene expression was measured in the gill at 4 weeks PE, whereas a number of receptors and other immune-related genes were up-regulated in the kidney at the same time point. It is possible that these expression profiles reflect differences in immune recognition occurring within each tissue as the parasite migrates through the vascular system to the gill. Some percentage of host cells migrating or circulating within the vascular system may become trapped by reticulo-endothelial cells in the kidney prior to xenoma formation in the gill. If so, this may present an initial window of immune recognition. In contrast, xenomas develop and grow in the gills of susceptible species such as Chinook salmon and RBT for several weeks before they begin to break down. During this time the parasite appears to maintain a strictly intra-cellular existence, likely preventing immune recognition and activation. The down-regulation of immune receptors and other components in the gill may reflect a localized parasite-mediated host suppression that allows *L. salmonae* to remain undetectable by immune surveillance components. In contrast, when mature spores are ready to be disseminated it is in the parasite's best interest for xenomas to be disrupted. It is not known whether the breakdown of the xenoma structure is parasite or host driven, but however it is

initiated, the dissolution of xenomas likely represents an optimal opportunity for immune recognition.

Once recognition has occurred, immune cells accumulate in the gill, and this stage is characterized by phagocytic cells engulfing freed spores. Recovered fish develop strong resistance to reinfection that may continue for upwards of a year. Atlantic salmon are refractory to *L. salmonae*, and do not develop xenomas in the gill, although the parasite is able to enter the fish and infection proceeds to the development of merogonic stages in the heart. Macrophages of Atlantic salmon have significantly better ability to phagocytose mature *L. salmonae* spores in vitro than do macrophages of Chinook salmon, suggesting that macrophages mediate a possible mechanism of resistance to the parasite in Atlantic salmon (Shaw et al. 2001). Phagocytic uptake of microsporidian spores as well as yeast particles by Atlantic salmon macrophages may be influenced by opsonising factors such as complement and/or antibody (Shaw et al. 2001). Antibodies have also been implicated as a potential opsonin in relation to phagocytic uptake of *L. salmonae* in RBT. Passive transfer of sera from *L. salmonae*-exposed RBT to naive RBT, followed by parasite exposure, was shown to delay but not prevent xenoma formation, suggesting increased opsonisation by *L. salmonae* –specific antibody, resulting in improved phagocytic uptake by macrophages (Sanchez et al. 2001b).

Loma salmonae spores have been documented within head kidney of Chinook salmon at 22 weeks PE, after xenomas were fully cleared from the gill (Kent et al. 1999). The authors speculated that these spores had been transported to the reticulo-endothelial system of the kidney by phagocytic cells clearing spores from ruptured xenomas in the gill. Within the kidney spores may provide a continual immune stimulus, promoting and prolonging a memory response. Both neutrophils and macrophages have been observed in gills of naturally infected Chinook salmon

with xenomas at varying stages of resolution. Although *L. salmonae* spores were observed within both types of phagocytic cells, there was no evidence of spore degradation within neutrophils. In contrast, macrophages appeared to be actively degrading engulfed spores. If phagocytic cells display similar patterns of response within the peritoneal cavity, it is possible that both neutrophils and macrophages are capable of responding to and engulfing *L. salmonae,* either directly or within infected cells, but that effective killing of the parasite may be limited to macrophages. If so, macrophages previously activated by glucan may be able to degrade *L. salmonae* more effectively, resulting in the reductions in parasite intensity observed for β-glucan-inoculated fish. The finding that glucan inoculation results in reduced numbers of xenomas in fish exposed to *L. salmonae* 3 weeks post-inoculation, along with the lack of haematopoiesis observed in the kidney, suggests the importance of macrophages, which are long-lived cells compared to neutrophis (Appelberg 2007). This does not necessarily imply that neutrophils do not play an important role in the immune response to *L. salmonae*. Although most recognized for their phagocytic role, there is evidence that mammalian neutrophils may act similarly to dendritic cells, shuttling pathogens to lymphoid organs. In addition, mammalian neutrophils secrete cytokines such as IFN-γ and IL-12 that may promote and direct a T_H1 response and the recruitment of T-cells into an infectious site.

The results from these studies contribute to a growing body of knowledge involving patterns of gene expression of fish in response to parasitic infections. Gene expression measured by qRT-PCR and cDNA microarrays has contributed to the accumulation of data for numerous host-parasite models, including *Neoparamoeba* sp., *Gyrodactylus salaris*, and *Lepeophtheirus salmonis*, in Atlantic salmon (*Salmo salar*), *Myxobolus cerebralis* in rainbow trout (*O. mykiss*), as well as *Ptychobothrium* sp. in carp (*Cyprinus Carpio* L.).

Gene expression has been studied in Atlantic salmon in response to amoebic gill disease (AGD) caused by protozoan *Neoparamoeba* sp. (Wynne et al. 2008, Young et al. 2008) as well as in RBT in response to whirling disease (*M. Cerebralis*) , via cDNA microarray technology. Data from these host/parasite models implicate the involvement of numerous innate immune components, as well as antioxidant-related genes (Skugor et al. 2008, Wynne et al. 2008). Interestingly, a number of antioxidant-related genes were down-regulated in AGD affected Atlantic salmon gills, including glutathione-S-transferases, glutathione peroxidases, and thioredoxins (Wynne et al. 2008). The general down-regulation of transcripts in AGD affected gills was hypothesized to reflect parasite mediation of gene expression (Wynne et al. 2008). Similarly, here, antioxidant defences were down-regulated in gill and kidney of *L. salmonae*-infected Chinook at 4 weeks PE, followed by an up-regulation in the kidney at 8 weeks PE. Pathway analysis of tissues at 4 weeks PE revealed the down-regulation of numerous defence-related pathways in the gill, perhaps also suggesting a parasite-mediated suppression of defence responses during the initial period of parasite colonization and migration to the gill. Dampening of factors associated with immune and other defence responses is a strategy employed by parasites and other pathogens in order to increase their chances of survival (Fallon & Mangan 2007, Wynne et al. 2008, Young et al. 2008). Although such a strategy has not previously been described for *L. salmonae*, further study of the apparent down-regulation of key immune genes early in the infection process may confirm parasite mediation of host responses.

Although numerous genes related to glutathione-based antioxidant defences were down-regulated here, the majority showed relatively small fold changes, with expression levels ranging from (– 1.43) to (– 2.42) fold in gill and kidney at 4 weeks PE. Interestingly, these levels were similar to those reported for antioxidant related genes in AGD-infected Atlantic salmon, which

ranged from (– 1.50) to (-2.30) fold down-regulated (Wynne et al. 2008). This pattern was observed for most of the differentially regulated immune- and defence-related genes in Chinook salmon, the majority of which exhibited small scale changes in expression rather than large (>2-fold) changes. Although a two-fold expression level cut-off is often arbitrary applied in genome-wide expression studies, it is likely that relatively small-scale changes in expression levels of numerous genes, as reported here, add up to significant differences in pathway expression.

In contrast to the down-regulation of antioxidants in Chinook and Atlantic salmon discussed above, antioxidants may be up-regulated in response to pathogen challenge. For example, up-regulation of antioxidants has been described in carp infected with an intestinal parasite *Ptychobothrium* sp. (Dautremepuits et al. 2003). Antioxidants may be up-regulated in order to combat the potentially harmful effects of reactive oxygen species (ROS) produced by phagocytic cells as a mechanism for pathogen killing. Similarly antioxidant-related genes in glucan-inoculated fish may be up-regulated in order to control ROS produced by macrophages and neutrophils after glucan uptake. If so, it is possible that the down-regulation of antioxidant-related genes observed early on in the course of *L. salmonae* infection in gill and kidney reflects either a lack of ROS producing cells or the lack of ROS production in them.

An important aspect of this study is that it represents one of the first analyses of gene expression in salmon in response to an intracellular parasite of fish. Of the host/parasite pairs mentioned, both *Gyrodactylus salaris* and *Lepeoptheirus salmonis* are ectoparasites that attach to the epidermis of their salmonid host species. *G. salaris* is a monogenean ectoparasite of salmonids that parasitizes the fins, skin, and gills of Atlantic salmon (Kania et al. 2007, Kania et al. 2009). *L. salmonis* is a marine copepod ectoparasite of salmonids (Wagner et al. 2008). Immune responses of fish in response to ectoparasites are likely to involve different mechanisms

than responses to intracellular parasites. Intracellular residence may reduce exposure of the pathogen to host defence mechanisms that rely on recognition of "non-self", or "danger" signals for their activation. Mammalian responses to intracellular parasitic infection are primarily cell-mediated and include both innate and adaptive effector cells. Evidence from the study presented here supports the development of cell-based immunity, with the up-regulation of genes associated with innate phagocytic cells such as monocyte/macrophages, granulocytic neutrophils and cytotoxic natural killer cells, as well as genes associated with T-cells that may indicate the development of an adaptive response.

The results reported here were grouped into functional pathways that provide a broad view of patterns of expression occurring within a tissue at several time points. The differential regulation of multiple genes from a particular pathway permits a more confident assessment that the pathway is truly being up-, or down-regulated. For example, in this study the large number of differentially regulated genes from glutathione-related antioxidant pathways was not anticipated, and would not have been discovered during the course of a qRT-PCR study targeting only immune-related genes. Although the small number of samples included in these analyses necessitates that these results be considered preliminary, the patterns shown here may present a starting point for future studies.

As these results and those from transcriptome analyses of AGD-infected Atlantic salmon show, genome-wide expression profiling utilizing cDNA microarray technology allows the detection of broad patterns of genetic response, whereas qRT-PCR allows a more quantitative measure of differential expression of a relatively small number of targeted genes. Although qRT-PCR studies have provided clues as to the nature of immune responses in salmonids and other teleosts, the gene-specific nature of qRT-PCR may limit researchers' ability to uncover

broader patterns of gene expression. In order to perform a qRT-PCR study, researchers must choose a relatively small number of genes to analyse. Choices of appropriate genes are often based on known pathways and may be derived from preconceived ideas or expectations as to the nature of the genetic responses. Although data from qRT-PCR studies are valuable, such narrow focus may prevent the discovery of the roles of genes and pathways not traditionally associated with the disease state or immune response.

Another important aspect of the study reported here involves the time-course design. Experiments designed to capture data at a single time point do not address trends in gene expression over the course of a disease or infection. Expression profiling by microarray analysis represents a snapshot of mRNA within cells at one point in time. A time course design allows several such snapshots to be analysed and compared, potentially highlighting trends in the expression data. For example, analysis of gene expression at 4 weeks PE revealed differential expression of numerous genes in both the gill and kidney, whereas relatively few genes were differentially regulated by 12 weeks PE. These results were unexpected, as it was anticipated that the majority of differential expression in the gill would be observed later in the infection, when an observable tissue response is developing.

Although microarray analysis allows researchers to capture a snapshot of mRNA within cells at the time of sampling, there are multiple levels of translational control at work within the cell, including mRNA turnover. All mRNA is not necessarily translated into functional protein, adding additional uncertainty to data interpretation. Proteomics, or the study of the expression, functions, and interactions of proteins within cells, may be an eventual alternative to the current gene expression analysis technologies for the study of disease responses in fish. Current genomic information for fish is largely based on sequence homology to mammals such as mice

and humans, and fish-specific proteome information lags even further behind. However, protein information will no doubt continue to expand, eventually allowing expanded proteomics studies of fish.

5-4. Conclusions and Future Directions

The data presented here support the conclusion that IP-inoculation of β-1,3/1,6 in Chinook salmon has an immune-modulating effect, making fish more resistant to *L. salmonae* infection, and that this resistance may be based on the activation of innate phagocytic cells capable of generating ROS. In the future, it will be important to continue this work. Studies designed to identify elements of the Chinook salmon response to glucan may include identifying and localizing the cells within the peritoneum that interact with glucan. Assuming they are phagocytic cells, are they primarily macrophages, neutrophils, or both? Are any other cell types involved? Once glucan particles are internalized, where do the cells go? Do they localize to other organs or remain within the peritoneal cavity? And most importantly, do these cells then interact with *L. salmonae* directly, or do they secrete activation factors that heighten the response of other immune cells to the parasite early on in an infection?

Future research may involve additional microarray analyses of gene expression in glucan-inoculated fish. If such work is undertaken, it would be important to include larger numbers of samples in order to improve statistical power required for data analysis. In addition, a modification to the design employed here would involve comparing both glucan-inoculated and control (PBS-inoculated) fish to un-inoculated controls in order to control for the potential immuno-stimulatory effects of inoculation.

It is difficult to come to strong conclusions regarding gene expression patterns in Chinook salmon in response to *L. salmonae* infection. Several factors may have hampered the

interpretation of results in *L. salmonae*-infected fish. First, small sample sizes limited the statistical power for gene expression analysis. As such, potential patterns of gene expression may have been masked. In addition, high inter- (as evidenced by highly variable expression values by qRT-PCR) as well as intra-fish variability (as suggested by histology) may have contributed to the masking of strong responses within individual fish. Lastly, because only heavily infected fish were examined, this may have precluded the possibility of differential responses in fish capable of more rapidly eliminating the infection.

However limited the analysis may have been, the trends observed represent successful contributions towards increased understanding of immune and defence responses to parasitic infections. Most interestingly, the apparent down-regulation of a number of important immune and antioxidant pathways in the gill and possibly also the kidney at 4 weeks PE suggests the intriguing possibility of parasite-mediated host immune suppression. Future research should be conducted to analyze gene expression in the gills of *L. salmonae*-infected Chinook. These studies should include the testing of a larger number of samples taken over a number of earlier time points after parasite exposure. In addition, PCR and histology should be performed prior to microarray analysis in order to separate heavily-infected from moderate or lightly-infected fish. This would allow the data from heavily infected fish to be compared with that from fish possibly mounting a more effective immune response. The expanded gene expression analysis combined with concurrent functional-based assays would allow researchers to bridge the gap between genetic and functional responses, and perhaps shed a brighter light on Chinook salmon responses to *Loma salmonae*.

Parasitic infections have proven to be some of the most challenging for researchers, and intracellular parasites remain among the most successful and fascinating of organisms. As

research efforts into human parasitic diseases such as malaria and leishmaniasis have shown, intracellular parasites can be extraordinarily complex. Like other intracellular parasites, *Loma salmonae* is highly effective at what it does: entering a host, growing, reproducing and eventually being released in order to repeat the process, all while provoking limited immune responses. Given the complexities of this and other host/parasite interactions, it will likely take much more work on the part of many dedicated researchers before a thorough understanding of Chinook salmon responses to *Loma salmonae* is achieved.

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Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

Complete list of differentially expressed genes from Glucan-inoculated fish sampled at 1 week post-inoculation (PI). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB510697	Lectin precursor	4.46	0.0255
CA061299	ATP synthase O subunit, mitochondrial precursor	3.67	0.0266
CB509813	Lectin precursor	3.32	0.0422
CB514012	Sodium- and chloride-dependent GABA transporter ine	2.81	0.0428
CA058748	UNKNOWN	2.79	0.0379
CB493657	UNKNOWN	2.78	0.0380
CA058048	PDZ domain-containing protein 1	2.61	0.0496
CA064225	Alanine aminotransferase 2	2.49	0.0464
CA062954	UNKNOWN	2.33	0.0419
CA055857	Epidermal growth factor receptor pathway substrate 8-like protein 3	2.33	0.0326
CA058593	PDZ domain-containing protein 1	2.33	0.0292
CA042069	Sorbitol dehydrogenase	2.33	0.0447
CA060022	ADM2 precursor	2.32	0.0436
CB492604	Glutathione S-transferase A	2.31	0.0446
CA049981	Selenoprotein Pa precursor	2.31	0.0175
CK991153	S-adenosylmethionine synthetase isoform type-2	2.28	0.0071
CB510326	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	2.22	0.0048
CB493774	Ornithine decarboxylase antizyme 1	2.21	0.0497
CA057835	Sodium-dependent neutral amino acid transporter B(0)	2.21	0.0491
CB498862	Selenoprotein Pa precursor	2.16	0.0234
CA061732	UNKNOWN	2.15	0.0001
CB502555	p53 apoptosis effector related to PMP-22	2.10	0.0456
CB511159	UNKNOWN	2.07	0.0085
CB488506	cyclin-dependent kinase inhibitor 1C (p57, Kip2) [Danio rerio]	2.06	0.0285
CA060381	Ornithine decarboxylase antizyme 1	2.04	0.0326
CA044941	UNKNOWN	2.04	0.0422
CA055137	Cadherin-17 precursor	2.02	0.0377
CK990329	UNKNOWN	1.99	0.0172
CB491193	glycogen synthase kinase binding protein [Danio rerio]	1.98	0.0123
CB502803	Selenoprotein Pa precursor	1.96	0.0386
CA051222	Organic cation transporter 3	1.96	0.0450
CA042256	UNKNOWN	1.94	0.0081
CA044003	UNKNOWN	1.91	0.0022

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA042402	Apolipoprotein B-100 precursor	1.89	0.0091
CB512768	Succinate semialdehyde dehydrogenase, mitochondrial precursor	1.88	0.0246
CK990881	Salmo salar somatolactin 180 splice variant mRNA, complete cds, alternatively spliced	1.88	0.0286
CA048145	Selenoprotein Pa precursor	1.86	0.0497
CB497681	Glyceraldehyde-3-phosphate dehydrogenase	1.86	0.0438
CB496382	Reticulon-4	1.84	0.0364
CA039027	Glyceraldehyde-3-phosphate dehydrogenase	1.84	0.0440
CK990577	ADP/ATP translocase 2	1.82	0.0077
CB492627	Multidrug resistance-associated protein 4	1.82	0.0303
CA057703	Tetraspanin-1	1.81	0.0255
CB509993	Brain protein 44-like protein	1.80	0.0211
CA043312	pfam06077, LR8, LR8 protein.	1.80	0.0495
CK991014	Glyceraldehyde-3-phosphate dehydrogenase	1.80	0.0357
CA056048	UNKNOWN	1.75	0.0030
CA055327	UNKNOWN	1.75	0.0200
CA044104	Selenoprotein Pa precursor	1.75	0.0306
CA039230	Complement C3 precursor	1.74	0.0141
CB505771	pfam06077, LR8, LR8 protein.	1.74	0.0461
CA054079	Retinol dehydrogenase 7	1.74	0.0166
CB497393	Cytochrome c oxidase subunit 5B, mitochondrial precursor	1.74	0.0349
CB491371	Glutathione peroxidase 1	1.73	0.0265
CK990916	UNKNOWN	1.73	0.0151
CB497419	Putative oxidoreductase yulF	1.73	0.0054
CA050886	Glyceraldehyde-3-phosphate dehydrogenase	1.73	0.0261
CA055409	UNKNOWN	1.72	0.0277
CA051028	Gamma-glutamyltranspeptidase 1 precursor	1.72	0.0340
CB506413	Phosphotriesterase-related protein	1.72	0.0067
CA044982	ES1 protein homolog, mitochondrial precursor	1.71	0.0320
CA055851	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	1.71	0.0448
CB498020	Glutaryl-CoA dehydrogenase, mitochondrial precursor	1.69	0.0290
CB485926	C-Myc-binding protein	1.68	0.0001
CB494290	Adenylosuccinate synthetase isozyme 1	1.68	0.0467
CB503035	Tetraspanin-7	1.68	0.0109
CB510905	Brain protein 44-like protein	1.67	0.0426
CA062348	Retinol dehydrogenase 3	1.67	0.0390
CB514540	Cytochrome c oxidase subunit 3	1.67	0.0233
CK990983	Basigin precursor	1.66	0.0192

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB496601	15-hydroxyprostaglandin dehydrogenase [NAD+]	1.66	0.0043
CA051541	UNKNOWN	1.66	0.0248
CB492806	Microsomal glutathione S-transferase 3	1.65	0.0001
CB510764	UNKNOWN	1.65	0.0201
CA060075	Pyruvate kinase isozymes R/L	1.65	0.0239
CB510300	Haptoglobin precursor	1.64	0.0115
CA059976	Brain protein 44-like protein	1.64	0.0483
CA037206	Ferritin, middle subunit	1.63	0.0237
CB487639	Ferritin, middle subunit	1.63	0.0066
CB497940	UDP-glucuronosyltransferase	1.63	0.0228
CA040160	UNKNOWN	1.63	0.0043
CN442493	Cytochrome c oxidase subunit 2	1.61	0.0011
CA061697	SJCHGC06004 protein [Schistosoma japonicum]	1.61	0.0094
CA037505	UNKNOWN	1.61	0.0113
CB502503	Cathepsin L precursor	1.61	0.0168
CB492498	Gamma crystallin M2	1.61	0.0024
CB517456	UNKNOWN	1.61	0.0223
CB511033	Diamine acetyltransferase 1	1.60	0.0130
CK990727	Cathepsin L precursor	1.60	0.0109
CB506140	Proteasome activator complex subunit 1	1.60	0.0493
CA040124	UNKNOWN	1.59	0.0489
CB515217	Endonuclease domain-containing 1 protein precursor	1.59	0.0354
CB517934	ARF GTPase-activating protein GIT2	1.59	0.0438
CA055404	Dehydrodolichyl diphosphate synthase	1.59	0.0044
CA050455	Alpha-N-acetylgalactosaminidase	1.59	0.0106
CA062761	ATP synthase lipid-binding protein, mitochondrial precursor	1.59	0.0027
CB488847	Brix domain-containing protein 1	1.59	0.0052
CA058954	DNA mismatch repair protein Mlh3	1.59	0.0315
CB488311	Glutaryl-CoA dehydrogenase, mitochondrial precursor	1.58	0.0196
CN442496	Cytochrome c oxidase subunit 2	1.58	0.0156
CB498501	Isocitrate dehydrogenase [NADP], mitochondrial precursor	1.58	0.0496
CB510462	Selenoprotein Pb precursor	1.58	0.0379
CA051622	Heat shock 70 kDa protein 4L	1.58	0.0121
CB491826	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	1.57	0.0357
CB503935	pfam05392, COX7B, Cytochrome C oxidase chain VIIB	1.57	0.0184
CB507933	DPH3 homolog	1.57	0.0194
CB497282	Cytochrome c	1.56	0.0067
CA043661	Delta(5)/Delta(6) fatty acid desaturase	1.56	0.0337

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CN442526	Cytochrome b	1.56	0.0269
CK990363	ADP/ATP translocase 2	1.56	0.0169
CA051867	Oncorhynchus mykiss StAR mRNA for steroidogenic acute regulatory protein, complete cds	1.56	0.0245
CA043750	Homeodomain-only protein	1.55	0.0387
DN047382	Sodium-dependent phosphate transporter 1-A	1.54	0.0325
CB516203	UNKNOWN	1.53	0.0127
CN442492	Cytochrome c oxidase subunit 2	1.53	0.0092
CB493553	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.53	0.0115
CA062147	UNKNOWN	1.53	0.0136
CB502028	Guanine nucleotide-binding protein G(T) gamma-T1 subunit precursor	1.53	0.0357
CA058370	Coiled-coil-helix-coiled-coil-helix domain-containing protein 7	1.53	0.0251
CB498320	cd00213, S-100/ICaBP-like, S-100/ICaBP-like domain; S-100/intestinal calcium binding domain (ICaBP);	1.53	0.0248
CB497579	Glutathione S-transferase P	1.53	0.0165
CA063917	15-hydroxyprostaglandin dehydrogenase [NAD+]	1.53	0.0058
CB505109	Electron transfer flavoprotein subunit beta	1.52	0.0192
CB512398	Abhydrolase domain-containing protein 12	1.52	0.0015
CB489043	Beta-2-microglobulin precursor	1.52	0.0434
CB504722	High mobility group protein B3	1.52	0.0004
CA052464	UNKNOWN	1.52	0.0327
CA054880	Dipeptidyl-peptidase 2 precursor	1.52	0.0217
CB489039	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.52	0.0265
CA050452	Glutathione S-transferase P	1.52	0.0217
CB498045	ATP synthase lipid-binding protein, mitochondrial precursor	1.51	0.0148
CA042009	Membrane-associated progesterone receptor component 1	1.51	0.0418
CB490484	Prostaglandin E synthase 3	1.51	0.0219
CK990471	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.51	0.0036
CB510407	Cytochrome P450 3A27	1.51	0.0108
CB496975	Cytochrome c1 heme protein, mitochondrial precursor	1.50	0.0221
CB510297	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	1.50	0.0483
CB493844	Cathepsin L precursor	1.50	0.0301
CB494139	Coatomer subunit delta	1.50	0.0059
CA053705	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	1.50	0.0246
CB499941	Cytochrome c oxidase subunit 3	1.50	0.0044
CA044555	UNKNOWN	1.50	0.0468
CB497884	Ferritin, middle subunit	1.50	0.0362

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB496704	Alpha-N-acetylgalactosaminidase	1.50	0.0032
CA052218	Protein MAL2	1.50	0.0226
CA044244	PREDICTED: similar to C29E4.12 isoform 11 [Canis familiaris]	1.49	0.0097
CA059774	Cytochrome c	1.49	0.0351
CB492474	Sequestosome-1	1.49	0.0009
CB496605	Cathepsin B precursor	1.49	0.0269
CB493612	ATP synthase a chain	1.49	0.0302
CK990740	Cathepsin B precursor	1.49	0.0409
CK990262	Cold-inducible RNA-binding protein	1.49	0.0091
CK991224	Cytochrome c oxidase subunit 3	1.49	0.0075
CA768741	UNKNOWN	1.49	0.0019
CB497887	Reticulon-4	1.49	0.0225
CA058351	UTP--glucose-1-phosphate uridylyltransferase	1.48	0.0428
CK991017	Cytochrome c oxidase subunit 3	1.48	0.0113
CB503670	Transmembrane protein 33	1.48	0.0270
CB511278	unknown protein [Siniperca chuatsi]	1.48	0.0441
CB516797	Vacuolar ATP synthase subunit G 1	1.48	0.0116
CB494476	Adenosylhomocysteinase B	1.48	0.0459
CB512542	Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial precursor	1.48	0.0242
CK990708	ATP synthase O subunit, mitochondrial precursor	1.48	0.0138
CB496978	Myosin heavy chain, fast skeletal muscle	1.47	0.0030
CA054964	Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor	1.47	0.0019
CA038358	Proteasome subunit alpha type 2	1.47	0.0064
CB492654	L-xylulose reductase	1.47	0.0360
CB510681	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor	1.47	0.0165
BU965787	60S ribosomal protein L29	1.47	0.0432
CB515170	FAM128B protein [Homo sapiens]	1.47	0.0305
CB501235	UNKNOWN	1.47	0.0267
CA050658	Aspartate aminotransferase, mitochondrial precursor	1.47	0.0336
CB507244	UNKNOWN	1.47	0.0123
CA037618	Cytochrome c oxidase subunit 7C, mitochondrial precursor	1.47	0.0148
CA043106	Transmembrane protein 9B precursor	1.46	0.0181
CA040156	Integral membrane protein GPR137B	1.46	0.0305
CB510698	UNKNOWN	1.46	0.0405
CB515027	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	1.46	0.0488
CA052884	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor	1.46	0.0150

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CK990732	Trypsin precursor	1.46	0.0221
CK991031	Ferritin, middle subunit	1.46	0.0284
CA058527	PREDICTED: similar to AD030 [Gallus gallus]	1.46	0.0069
CB496944	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	1.46	0.0059
CB503486	Alpha-enolase	1.46	0.0054
CA058014	Negative elongation factor A	1.45	0.0139
CA059155	UNKNOWN	1.45	0.0060
CB493494	BolA-like protein 2	1.45	0.0271
CA061548	Reticulon-4	1.45	0.0385
CA051741	Nucleolar GTP-binding protein 1	1.45	0.0488
CB492831	ATP synthase O subunit, mitochondrial precursor	1.45	0.0133
CA037733	Angiogenin-2 precursor	1.45	0.0045
CA044034	Adenylosuccinate synthetase isozyme 1	1.45	0.0293
CB494642	Glutathione S-transferase kappa 1	1.45	0.0445
CB512686	Hydroxyacylglutathione hydrolase	1.45	0.0084
CB517460	UNKNOWN	1.45	0.0239
CA058589	Transcription factor HES-5	1.45	0.0216
CK990669	NADH-ubiquinone oxidoreductase chain 4	1.44	0.0241
CA055866	Phosphotriesterase-related protein	1.44	0.0452
CA058552	UNKNOWN	1.44	0.0061
CB501848	UNKNOWN	1.44	0.0351
CB497411	M-phase phosphoprotein 8	1.44	0.0081
CA059811	UNKNOWN	1.44	0.0396
CA044851	LYR motif-containing protein 5	1.44	0.0151
CB515564	Myosin-Ic	1.43	0.0053
CB492393	Cytochrome c	1.43	0.0204
CB492507	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor	1.43	0.0088
CB506406	UNKNOWN	1.43	0.0080
CB515793	AN1-type zinc finger protein 6	1.43	0.0249
CA038166	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.43	0.0223
CA053442	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	1.43	0.0068
CB497057	ATP synthase subunit g, mitochondrial	1.43	0.0045
CB515926	UNKNOWN	1.43	0.0025
CA055243	Transmembrane protein 50A	1.43	0.0035
CB494682	Protein kinase C and casein kinase substrate in neurons protein 3	1.42	0.0450
CB516804	Transmembrane 4 L6 family member 1	1.42	0.0109

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA051534	UNKNOWN	1.42	0.0354
CB509786	Protein Z-dependent protease inhibitor precursor	1.42	0.0415
CA044952	Acyl-CoA-binding protein	1.42	0.0188
CK991135	3-ketoacyl-CoA thiolase, mitochondrial	1.42	0.0105
CA037448	NADH-ubiquinone oxidoreductase chain 2	1.41	0.0204
CA060182	ATP synthase lipid-binding protein, mitochondrial precursor	1.41	0.0238
CB503739	Uncharacterized protein C15A10.05c	1.41	0.0064
CB504181	UNKNOWN	1.41	0.0437
CA042290	Oncorhynchus mykiss liver-expressed antimicrobial peptide 2A (LEAP-2A) mRNA, complete cds	1.41	0.0277
CA051121	Homo sapiens calcium homeostasis endoplasmic reticulum protein (CHERP), mRNA	1.41	0.0045
CA060874	Protein MCM10 homolog	1.41	0.0196
CB515458	Slc9a3r2 protein [Danio rerio]	1.41	0.0034
CK991050	S.salar mRNA for prolactin	1.41	0.0488
CA057060	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	1.41	0.0128
CK990870	Hemoglobin subunit beta-1	1.41	0.0350
CB515538	Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	1.40	0.0484
CA051402	Pyruvate kinase muscle isozyme	1.40	0.0143
CN442537	Cytochrome c oxidase subunit 1	1.40	0.0280
CK991230	Oncorhynchus mykiss low molecular mass polypeptide complex subunit 2 (LMP2) mRNA, complete cds	1.40	0.0087
CA038333	60S ribosomal protein L39	1.40	0.0319
CB514320	Granzyme K precursor	1.40	0.0419
CA063128	Cytochrome P450 4V3	1.40	0.0012
CA044390	UNKNOWN	1.40	0.0048
CB503360	60S ribosomal protein L9	1.40	0.0114
CB517011	DNA replication complex GINS protein PSF1	1.40	0.0278
CB490998	N(2),N(2)-dimethylguanosine tRNA methyltransferase	1.40	0.0118
CA037026	Transcription factor 15	1.40	0.0177
CB498104	Si:dkey-78d16.1 protein [Danio rerio]	1.40	0.0367
CB493990	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	0.70	0.0494
CB512496	UNKNOWN	0.70	0.0191
CA051026	UNKNOWN	0.70	0.0434
CB488440	Syntaxin-binding protein 2	0.70	0.0345
CB512170	Pre-mRNA-processing-splicing factor 8	0.70	0.0489
CB496703	Structural maintenance of chromosomes protein 4	0.70	0.0007
CB517969	Schistosoma japonicum SJCHGC04882 protein mRNA, complete cds	0.70	0.0120

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA050583	PREDICTED: similar to KIAA1682 protein [Danio rerio]	0.70	0.0136
CB511814	UNKNOWN	0.69	0.0371
CB499014	UNKNOWN	0.69	0.0480
CB509853	UNKNOWN	0.69	0.0373
CB500057	UNKNOWN	0.69	0.0062
CA048359	Ubiquitin carboxyl-terminal hydrolase 37	0.69	0.0259
CA057439	immunoglobulin light chain, isotype 2 [Gadus morhua]	0.69	0.0384
CA051966	Nuclear pore complex protein Nup88	0.69	0.0297
CA063266	Peroxisomal proliferator-activated receptor A-interacting complex 285 kDa protein	0.69	0.0221
CB498325	UNKNOWN	0.68	0.0420
CA052383	Complement component C6 precursor	0.68	0.0026
CB512373	Barrier-to-autointegration factor	0.68	0.0038
CB512653	UNKNOWN	0.68	0.0426
CB516815	Autophagy-related protein 9A	0.68	0.0469
CB495313	60S ribosomal protein L6	0.68	0.0416
CA037272	UNKNOWN	0.67	0.0044
CA058040	UNKNOWN	0.67	0.0345
CA056078	Putative endonuclease C1F12.06c	0.67	0.0129
CK991349	UNKNOWN	0.67	0.0360
CA045433	Plastin-3	0.67	0.0115
CB515204	39S ribosomal protein L19, mitochondrial precursor	0.67	0.0194
CA037834	Protein transport protein SEC61 subunit gamma	0.67	0.0500
CA769653	UNKNOWN	0.66	0.0438
CA058127	Fibronectin	0.66	0.0194
CA059679	Matrix metalloproteinase-9 precursor	0.66	0.0500
CB517238	UNKNOWN	0.66	0.0143
CK991339	Papilin precursor	0.66	0.0078
CB516981	Coatomer subunit gamma-2	0.66	0.0221
CB514307	UNKNOWN	0.66	0.0482
CA054582	Placental protein 11 precursor	0.66	0.0454
CK990570	UNKNOWN	0.66	0.0413
CA060266	Transmembrane emp24 domain-containing protein 10 precursor	0.66	0.0016
CA054237	Coproporphyrinogen III oxidase, mitochondrial precursor	0.66	0.0351
CA048851	Ig mu chain C region membrane-bound form	0.65	0.0373
CA055055	UNKNOWN	0.65	0.0389
CB517306	Glucose-6-phosphate 1-dehydrogenase	0.64	0.0102
CA049298	UNKNOWN	0.64	0.0081
CA058493	UNKNOWN	0.64	0.0436

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		1 week	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA053215	Gamma-tubulin complex component 2	0.63	0.0085
CA058371	Enhancer of filamentation 1	0.63	0.0383
CA053911	Cingulin	0.62	0.0015
CA059371	UNKNOWN	0.62	0.0036
CA062923	Homo sapiens mRNA; cDNA DKFZp686O2148 (from clone DKFZp686O2148)	0.62	0.0328
CA056354	UNKNOWN	0.62	0.0331
CA050145	Enhancer of mRNA-decapping protein 4	0.62	0.0023
CB502963	UNKNOWN	0.61	0.0220
CA060030	UNKNOWN	0.61	0.0283
CK990556	60S ribosomal protein L13	0.61	0.0117
CB494032	Carbonic anhydrase	0.59	0.0205
CA057830	PREDICTED: similar to prostate stem cell antigen precursor-like [Danio rerio]	0.58	0.0493
CB511778	Complement component C7 precursor	0.57	0.0087
CA061486	Peroxisomal trans-2-enoyl-CoA reductase	0.55	0.0027
CB510980	Barrier-to-autointegration factor	0.54	0.0175
CB498642	VIG-2 [Oncorhynchus mykiss] >gi|10304385|gb|AAG16231.1| VHSV-induced protein 2 [Oncorhynchus mykiss]	0.53	0.0321
CA060011	Barrier-to-autointegration factor	0.53	0.0159
CA056667	Mannose-binding protein C precursor	0.41	0.0415

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

Complete list of differentially expressed genes from Glucan-inoculated fish sampled at 2 weeks post-inoculation (PI). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB511618	UNKNOWN	3.26	0.0363
CA058832	UNKNOWN	3.06	0.0163
CK991122	Heat shock protein 30	3.03	0.0037
CA046376	Salvelinus fontinalis differentially regulated trout protein 1 mRNA, complete cds	2.91	0.0343
CB486238	Microtubule-associated proteins 1A/1B light chain 3C precursor	2.83	0.0446
CB509536	Lipocalin precursor	2.68	0.0414
CB515902	pfam00201, UDPGT, UDP-glucoronosyl and UDP-glucosyl transferase	2.64	0.0328
CB507065	UNKNOWN	2.60	0.0195
BU965639	UNKNOWN	2.42	0.0084
CA046838	UNKNOWN	2.38	0.0280
CK991073	Hemoglobin subunit alpha	2.34	0.0157
CK990230	Protein phosphatase 1 regulatory subunit 14A	2.32	0.0238
CA046004	UNKNOWN	2.28	0.0127
CA060049	Uncharacterized protein C11orf49 homolog	2.26	0.0135
CA043350	UNKNOWN	2.24	0.0182
CA047117	UNKNOWN	2.23	0.0060
CA062133	Forkhead box protein D3-A	2.21	0.0169
CA038288	Serum albumin 1 precursor	2.20	0.0337
CB511872	UNKNOWN	2.07	0.0151
CK990782	UNKNOWN	2.05	0.0385
CB498535	cd01481, vWA_collagen_alpha3-VI-like, VWA_collagen alpha 3(VI) like:	2.05	0.0173
CA768473	Oncorhynchus mykiss mRNA for putative ribosomal protein L39 protein	2.01	0.0010
CA052444	PREDICTED: similar to putative selenoprotein O [Gallus gallus]	2.00	0.0122
CB492258	UNKNOWN	2.00	0.0060
CK990461	Salmo salar isolate Ss2_GH1 growth hormone I gene, complete cds	1.99	0.0355
CK990428	UNKNOWN	1.98	0.0413
CB488440	Syntaxin-binding protein 2	1.97	0.0318
CB512075	UNKNOWN	1.96	0.0433
CB507068	UNKNOWN	1.96	0.0112
CA064491	UNKNOWN	1.96	0.0232

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA050381	PREDICTED: Canis familiaris similar to germinal histone H4 gene (LOC611231), mRNA	1.95	0.0323
CB508826	oocyte protease inhibitor-2 [Oncorhynchus mykiss]	1.95	0.0017
CA062678	Guanine nucleotide-binding protein G(o) subunit alpha 1	1.95	0.0090
CB509497	Protein JTB precursor	1.92	0.0096
CB511777	UNKNOWN	1.91	0.0097
CB508571	UNKNOWN	1.90	0.0129
CA051380	Preimplantation protein 3	1.88	0.0068
CB508763	Microtubule-associated proteins 1A/1B light chain 3C precursor	1.87	0.0094
CA054835	UNKNOWN	1.87	0.0068
CA047345	Salmon (S.salar) growth hormone gene, complete cds	1.85	0.0168
CB512370	UNKNOWN	1.84	0.0118
CA036575	Oncorhynchus mykiss oocyte protease inhibitor-2 (OPI-2) mRNA, complete cds	1.83	0.0208
CA037221	Transcription initiation factor IIB	1.82	0.0083
CA047492	PREDICTED: similar to splicing coactivator subunit SRm300 [Homo sapiens]	1.82	0.0179
CA063854	UNKNOWN	1.79	0.0138
CA050344	Ependymin-1 precursor	1.78	0.0101
CA047398	UNKNOWN	1.78	0.0272
CA057697	UNKNOWN	1.77	0.0117
CB496355	Parvalbumin beta 2	1.77	0.0424
CK991183	Red-sensitive opsin	1.77	0.0316
CB493542	UNKNOWN	1.76	0.0150
CA057345	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene,	1.75	0.0364
CA061726	Programmed cell death protein 6	1.75	0.0297
CA037858	UNKNOWN	1.75	0.0136
CA054568	UNKNOWN	1.75	0.0396
CA042068	Deleted in malignant brain tumors 1 protein precursor	1.73	0.0397
CA046437	UNKNOWN	1.73	0.0435
CB500083	UNKNOWN	1.73	0.0110
CA054937	UNKNOWN	1.72	0.0010
CB501971	UNKNOWN	1.72	0.0017
CA038137	UNKNOWN	1.69	0.0460
CK991248	S.salar mRNA for parvalbumin beta (clone 14.1)	1.68	0.0000
CB498511	Trifunctional enzyme subunit alpha, mitochondrial precursor	1.68	0.0186
CK990890	UNKNOWN	1.68	0.0233
CA037368	Salvelinus alpinus mRNA for cystein inhibitor protein (salarin gene)	1.68	0.0057

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA052494	Secretory carrier-associated membrane protein 4	1.68	0.0027
CB508490	UNKNOWN	1.67	0.0240
CA058654	Retinol-binding protein I, cellular	1.66	0.0334
CB511073	Retinal pigment epithelium-specific 65 kDa protein	1.66	0.0367
CA059761	Microspherule protein 1 [Danio rerio]	1.65	0.0039
CK991180	Mucin-5B precursor	1.65	0.0473
CA044766	UNKNOWN	1.65	0.0190
CA038928	hCG22882, isoform CRA_a [Homo sapiens]	1.65	0.0177
CA050350	Taeniopygia guttata clone 0058P0050A03 meningioma (disrupted in balanced transloon) 1-like mRNA, complete sequence	1.64	0.0244
CB505730	guanylin precursor [Anguilla anguilla]	1.64	0.0267
CB501332	GDP-L-fucose synthetase	1.64	0.0150
CB510887	Creatine kinase M-type	1.64	0.0026
CB507157	UNKNOWN	1.63	0.0354
CA042082	Thiamin pyrophosphokinase 1	1.63	0.0003
CA036891	TP53RK-binding protein	1.63	0.0314
CB500069	PQ-loop repeat-containing protein 2	1.63	0.0175
CA045552	UNKNOWN	1.63	0.0363
CA044616	UNKNOWN	1.62	0.0396
CB508555	UNKNOWN	1.61	0.0179
CA768774	UNKNOWN	1.61	0.0260
CA047095	UNKNOWN	1.60	0.0450
CA050879	Transmembrane protein 106B	1.60	0.0368
CA051457	Ubiquitin thioesterase OTUB2	1.60	0.0410
CK991057	Trafficking protein particle complex subunit 4	1.60	0.0392
CB501257	UNKNOWN	1.59	0.0245
CA052003	UNKNOWN	1.59	0.0310
CA036655	Si:dkey-78d16.1 protein [Danio rerio]	1.59	0.0109
CA061453	UNKNOWN	1.58	0.0256
CK990548	Fatty acid-binding protein, liver	1.58	0.0007
CB492971	Troponin T, fast skeletal muscle isoforms	1.57	0.0252
CK990936	UNKNOWN	1.57	0.0113
CA046875	Salmon glycoprotein hormone alpha-subunit mRNA, complete cds	1.57	0.0375
CA038189	Insulin-like growth factor-binding protein complex acid labile chain precursor	1.56	0.0224
CA044970	UNKNOWN	1.56	0.0374
CB497759	Putative uncharacterized protein C10orf130	1.55	0.0330
CA058493	UNKNOWN	1.55	0.0138

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA059314	Probable D-tyrosyl-tRNA(Tyr) deacylase 1	1.55	0.0201
CA036636	Cell cycle checkpoint protein RAD1	1.55	0.0010
CB501075	Guanine nucleotide-binding protein G(T) gamma-T1 subunit precursor	1.55	0.0399
CA059602	Exosome complex exonuclease RRP4	1.55	0.0146
CA051331	Oryzias latipes hox gene cluster, complete cds, contains hoxCa	1.54	0.0190
CA062414	Uncharacterized protein KIAA1849	1.54	0.0091
CB511182	UNKNOWN	1.54	0.0103
CK990374	UNKNOWN	1.53	0.0281
CB496693	Transcription initiation factor IIA gamma chain	1.53	0.0212
CA047656	UNKNOWN	1.53	0.0096
CA047510	UNKNOWN	1.53	0.0308
CA052532	UNKNOWN	1.53	0.0472
CA059343	Poly [ADP-ribose] polymerase 12	1.52	0.0260
CA061875	snRNA-activating protein complex subunit 3	1.52	0.0183
CB488683	NADH-cytochrome b5 reductase 1	1.52	0.0449
CK991080	E3 ubiquitin-protein ligase LNX	1.52	0.0160
CA054585	UNKNOWN	1.52	0.0479
CB509843	Deoxyribonuclease gamma precursor	1.52	0.0152
CA057076	Cyclic nucleotide-gated channel rod photoreceptor subunit alpha	1.52	0.0399
CA057340	PREDICTED: similar to solute carrier family 39 (zinc transporter), member 3 isoform a [Macaca mulatta]	1.51	0.0106
CA064443	UNKNOWN	1.51	0.0093
CB510729	Nattectin precursor	1.51	0.0256
CA046308	UNKNOWN	1.51	0.0486
CB512109	Glucocorticoid receptor DNA-binding factor 1	1.50	0.0105
CB494706	Triosephosphate isomerase	1.50	0.0148
CB498337	UNKNOWN	1.49	0.0129
BU965791	UNKNOWN	1.49	0.0320
CK991084	UNKNOWN	1.49	0.0204
CB492127	UNKNOWN	1.49	0.0008
CA039629	UNKNOWN	1.48	0.0277
CB508771	Zinc finger protein 331	1.48	0.0344
DN047829	precerebellin-like protein [Oncorhynchus mykiss]	1.48	0.0136
CB510869	UNKNOWN	1.48	0.0295
CB509932	UNKNOWN	1.48	0.0055
CA047235	UNKNOWN	1.47	0.0499
CA051663	UNKNOWN	1.47	0.0478

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA055935	Probable protein BRICK1	1.46	0.0252
CA048073	UNKNOWN	1.46	0.0372
CA036813	UNKNOWN	1.46	0.0456
CA037053	UNKNOWN	1.45	0.0198
CA039482	Endothelial differentiation-related factor 1 homolog	1.45	0.0373
CK990261	UNKNOWN	1.45	0.0014
CB492348	Type-4 ice-structuring protein LS-12 precursor	1.45	0.0042
CA037037	Fatty acid-binding protein, liver	1.45	0.0283
CA053605	Bifunctional polynucleotide phosphatase/kinase	1.44	0.0397
CA767795	Mannosyl-oligosaccharide glucosidase	1.43	0.0482
CB501879	UNKNOWN	1.43	0.0020
CA044638	UNKNOWN	1.43	0.0171
CB507227	TATA-box-binding protein	1.43	0.0314
CB501435	UNKNOWN	1.43	0.0257
CA050726	UNKNOWN	1.42	0.0276
CA042344	UNKNOWN	1.41	0.0234
CA050743	4F2 cell-surface antigen heavy chain	1.41	0.0286
CA047329	Importin-7	1.40	0.0469
CB493644	GINS complex subunit 3 [Danio rerio]	1.40	0.0443
CA038734	Protein involved in cell morphogenesis and proliferation, associated with protein kinase Cbk1p	0.70	0.0255
CA060811	Oocyte zinc finger protein XLCOF7.1	0.70	0.0115
CA055080	Core histone macro-H2A.2	0.69	0.0398
CB511945	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor	0.69	0.0300
CB502503	Cathepsin L precursor	0.69	0.0455
CA041210	MARCKS-related protein	0.68	0.0054
CA057201	UNKNOWN	0.68	0.0167
CA055391	N-myc-interactor	0.68	0.0449
CK990740	Cathepsin B precursor	0.68	0.0096
CB516060	UNKNOWN	0.67	0.0412
CB517058	Oncorhynchus kisutch transgene insertion site	0.67	0.0489
CA044407	Oncorhynchus mykiss MHC class Ib antigen (UDA) mRNA, UDA*0301 allele, complete cds	0.67	0.0203
CB507038	Nattectin precursor	0.66	0.0379
CB499297	UNKNOWN	0.66	0.0136
CA063453	DNA polymerase iota	0.65	0.0166
CA056639	U4/U6 small nuclear ribonucleoprotein Prp4	0.64	0.0107
CA064453	Intron-binding protein aquarius	0.64	0.0052
CA056303	CREB/ATF bZIP transcription factor	0.63	0.0112

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		2 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB488781	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.63	0.0363
CA060097	3-phosphoinositide-dependent protein kinase 1	0.63	0.0050
CB515457	Lysyl-tRNA synthetase	0.63	0.0013
CA061687	GTP-binding nuclear protein Ran	0.62	0.0076
CB493991	Complement C3-1	0.62	0.0202
CB512367	Oncorhynchus tshawytscha follicle stimulating hormone beta subunit (FSHbeta) gene, promoter and complete cds	0.61	0.0086
CA058538	FYVE finger-containing phosphoinositide kinase	0.61	0.0020
CA060741	Uncharacterized protein C1orf77	0.60	0.0261
CK991047	FKBP12-rapamycin complex-associated protein	0.60	0.0029
CB517543	PRA1 family protein 3	0.60	0.0365
CB516716	immunoglobulin tau heavy chain secretory form [Oncorhynchus mykiss]	0.60	0.0165
CB516867	Tapasin-related protein precursor	0.60	0.0017
CA044472	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	0.59	0.0045
CB486275	Mitochondrial import inner membrane translocase subunit Tim13	0.58	0.0285
CA040268	Death-associated protein kinase 3	0.57	0.0236
CK990466	EGF-like module-containing mucin-like hormone receptor-like 1 precursor	0.57	0.0197
CA054739	UNKNOWN	0.54	0.0427
CB515523	UNKNOWN	0.50	0.0106
CA054857	Alpha-2-macroglobulin precursor	0.50	0.0172
CB514743	Lectin precursor	0.38	0.0446

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

Complete list of differentially expressed genes from Glucan-inoculated fish sampled at 3 weeks post-inoculation (PI). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		3 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB498627	Sodium channel subunit beta-3 precursor	1.97	0.0305
CA058771	Nucleolysin TIAR	1.91	0.0352
CK990772	UNKNOWN	1.83	0.0282
CB489380	Signal peptide peptidase-like 2A	1.83	0.0439
CB516214	Danio rerio SRY (sex determining region Y)-box 4b (sox4b), mRNA >gi|40352711|gb|BC064664.1|	1.82	0.0200
CA055511	Loss of heterozygosity 11 chromosomal region 2 gene A protein	1.60	0.0093
CA054949	Skeletal muscle and kidney-enriched inositol phosphatase	1.56	0.0286
CB489716	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.54	0.0356
CK991281	UNKNOWN	1.54	0.0297
CB509723	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor	1.53	0.0316
CA049903	Nuclear receptor coactivator 4	1.52	0.0401
CB514706	Tudor and KH domain-containing protein	1.50	0.0432
CA048944	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform	1.50	0.0173
CB506526	Dolichol-phosphate mannosyltransferase	1.50	0.0328
CA057516	Cytochrome P450 4F3	1.49	0.0319
CA041695	UNKNOWN	1.49	0.0163
CA054886	Fizzy-related protein homolog	1.48	0.0154
CB516212	UNKNOWN	1.48	0.0179
CB492603	Alpha-actinin-2	1.48	0.0142
CB510601	Protein RCC2 homolog	1.47	0.0463
CA057720	UNKNOWN	1.46	0.0465
CA058095	Gamma-tubulin complex component 4	1.46	0.0217
CB510809	UNKNOWN	1.44	0.0283
CA055841	Interleukin-1 receptor-associated kinase 3	1.43	0.0217
CB500831	PREDICTED: similar to CC chemokine SCYA113 [Danio rerio]	1.42	0.0129
CB511293	UNKNOWN	1.42	0.0302
CB512117	Methylosome protein 50	1.42	0.0472
CB510106	Histone acetyltransferase type B catalytic subunit	1.42	0.0093
CB499371	Coiled-coil domain-containing protein 76	1.41	0.0256
CA050953	UNKNOWN	1.40	0.0389
CK990540	UNKNOWN	0.70	0.0125
CB508440	Zinc finger protein 333	0.70	0.0496

Appendix 1 - Differentially expressed genes from glucan-inoculated Chinook salmon

		3 weeks PI	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB515872	UNKNOWN	0.70	0.0269
CB492938	UNKNOWN	0.70	0.0029
CB514281	Neuronal membrane glycoprotein M6-b	0.70	0.0174
CB509799	Proteoglycan-4 precursor	0.70	0.0062
CA053216	UNKNOWN	0.70	0.0391
CK990396	UNKNOWN	0.70	0.0442
CB510291	Niemann-Pick C1-like protein 1 precursor	0.69	0.0000
CB507068	UNKNOWN	0.69	0.0058
CB498416	Hemoglobin subunit beta-1	0.69	0.0238
CB509909	UNKNOWN	0.69	0.0398
CA050893	P2X purinoceptor 7	0.69	0.0401
CK990980	UNKNOWN	0.69	0.0436
CA059264	Heparan sulfate glucosamine 3-O-sulfotransferase 2	0.68	0.0219
CA062678	Guanine nucleotide-binding protein G(o) subunit alpha 1	0.68	0.0227
CA044054	Prostaglandin G/H synthase 2 precursor	0.68	0.0279
CB511453	Cytochrome c oxidase subunit VIIa-related protein, mitochondrial precursor	0.67	0.0211
CA770029	Xylulose kinase	0.67	0.0060
CB508450	UNKNOWN	0.66	0.0431
CA054120	Probable global transcription activator SNF2L2	0.65	0.0456
CA063474	UNKNOWN	0.65	0.0096
CB511649	novel immune-type receptor 4 [Oncorhynchus mykiss]	0.64	0.0220
CA041914	UNKNOWN	0.64	0.0401
CA050489	Xenopus tropicalis cDNA clone IMAGE:7749006	0.62	0.0063
CB497636	Pepsin A-4 precursor	0.61	0.0067

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

Complete list of differentially expressed genes from gills of *Loma salmonae* infected fish sampled at 4 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Gill - 4 weeks	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA054223	UNKNOWN	12.16	0.024
CA055007	Heterogeneous nuclear ribonucleoprotein H	2.40	0.024
CB493961	Hemoglobin subunit alpha	2.37	0.040
CA041067	5-aminolevulinate synthase, erythroid-specific, mitochondrial precursor	2.31	0.012
CB510950	UNKNOWN	2.26	0.024
CA062377	Coiled-coil domain-containing protein 82	2.23	0.014
CK991241	Histone H1	2.13	0.001
CB509758	Hemoglobin subunit beta	2.11	0.047
CB516893	Hemoglobin subunit alpha-4	2.10	0.012
CB493678	Acylphosphatase-2	2.07	0.044
CB498419	Hemoglobin subunit alpha-1	2.06	0.003
CA051865	Coiled-coil domain-containing protein 124	2.06	0.005
CB496964	Histone H1	2.06	0.017
CA042530	6-phosphofructokinase, liver type	2.03	0.004
CB505759	Midasin	2.01	0.022
CA060239	Caspase-8 precursor	2.01	0.022
CB501837	Glyceraldehyde-3-phosphate dehydrogenase	1.99	0.002
CB514743	Lectin precursor	1.98	0.003
CA060701	Hemoglobin subunit alpha	1.98	0.014
CB494301	Carbonic anhydrase	1.96	0.027
CA053773	UNKNOWN	1.93	0.046
CA042535	40 kDa peptidyl-prolyl cis-trans isomerase	1.93	0.037
CA054908	ETS-related transcription factor Elf-2	1.93	0.006
CB500796	Hemoglobin subunit alpha	1.91	0.038
CA039360	Oncorhynchus mykiss mRNA for carbonic anhydrase 2, complete cds	1.91	0.032
CB500653	UNKNOWN	1.90	0.029
CB498181	Kelch repeat and BTB domain-containing protein 10	1.90	0.028
CA052520	Galectin-9	1.90	0.016
CA060658	Proteasome subunit alpha type 1	1.89	0.010
CA052843	H.sapiens gene PACAP for pituitary adenylate cyclase activating polypeptide	1.88	0.007
CB496985	Troponin C, skeletal muscle	1.88	0.021
CA047136	UNKNOWN	1.88	0.043
CA060279	U6 snRNA-associated Sm-like protein LSm4	1.87	0.014

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB493388	Myosin light chain 1, cardiac muscle	1.87	0.048
CB496983	Homeobox protein PRH	1.87	0.000
CA051864	Protein OS-9 precursor	1.87	0.047
CK991021	Tripeptidyl-peptidase 1 precursor	1.86	0.010
CK990254	Human G protein-coupled receptor (GPR2) gene, partial cds	1.84	0.005
CA052835	Rab GTPase-binding effector protein 2	1.84	0.041
CB496981	DNA-directed RNA polymerases I, II, and III subunit RPABC2	1.84	0.011
CB502332	UNKNOWN	1.83	0.040
CB497309	Hemoglobin subunit beta-4	1.83	0.000
CA053830	Kynurenine--oxoglutarate transaminase 3	1.82	0.003
CB507520	Alpha-adducin	1.79	0.016
CB492226	Hemoglobin subunit alpha	1.79	0.029
CA050568	UNKNOWN	1.79	0.022
CB511907	UNKNOWN	1.79	0.036
CB505738	Hemoglobin subunit beta-1	1.79	0.042
CB508129	UNKNOWN	1.78	0.004
CB505667	perforin [Ctenopharyngodon idella]	1.78	0.022
CA049300	Eukaryotic translation initiation factor 3 subunit 7	1.77	0.040
CB517228	Crystallin J1B	1.76	0.048
CA049963	Unc-13 homolog D	1.75	0.043
CB512119	ATPase family AAA domain-containing protein 2	1.75	0.040
CA057131	UNKNOWN	1.74	0.022
CB513665	Immunoglobulin lambda-like polypeptide 1 precursor	1.74	0.008
CB487151	Mitochondrial uncoupling protein 2	1.74	0.013
CB497335	Hemoglobin subunit beta-1	1.74	0.047
CB512370	UNKNOWN	1.72	0.004
CA058619	UNKNOWN	1.71	0.017
CK990563	Hemoglobin subunit beta-1	1.71	0.036
CA056915	Microtubule-actin cross-linking factor 1, isoform 4	1.71	0.002
CA064277	Hemoglobin subunit alpha	1.71	0.032
CB514000	Protein-lysine 6-oxidase precursor	1.70	0.037
CA061786	UNKNOWN	1.70	0.028
CB497307	Biotinidase precursor	1.70	0.039
CB509524	Complement factor B precursor	1.69	0.000
CB501928	Oncorhynchus mykiss mRNA for carbonic anhydrase 2, complete cds	1.69	0.009
CK990609	BCAS2 protein homolog	1.67	0.027
CA058163	Hemoglobin subunit beta-1	1.66	0.017
CA050824	Cytosolic purine 5'-nucleotidase	1.66	0.006

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA768258	preproguanylin [Anguilla japonica]	1.65	0.036
CK991341	UNKNOWN	1.65	0.005
CB487032	Tubulin beta-2C chain	1.65	0.048
CA062768	PRA1 family protein 2	1.65	0.022
CA056091	Platelet glycoprotein Ib beta chain precursor	1.64	0.041
CA059811	UNKNOWN	1.64	0.005
CA043128	Meprin A subunit beta precursor	1.64	0.009
CA056798	Atrial natriuretic peptide receptor B precursor	1.63	0.034
CA058605	Cystathionine beta-synthase	1.62	0.006
CB497762	Myosin light chain 1, skeletal muscle isoform	1.62	0.039
CA043079	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	1.62	0.024
CA039745	Hemoglobin subunit alpha	1.61	0.018
CK990883	Hemoglobin subunit beta-2	1.61	0.037
CB498077	Ferritin, heavy subunit	1.61	0.010
CA057678	Glutathione S-transferase Mu 3	1.61	0.028
CA058632	Recoverin	1.60	0.032
CA058820	UNKNOWN	1.60	0.006
CB512475	UNKNOWN	1.59	0.043
CB505201	similar to C. Elegans protein F17C8.5 [Homo sapiens] >gi|119606160|gb|EAW85754.1| hypothetical LOC339123, isoform CRA_a [Homo sapiens]	1.58	0.019
CK990586	Ferritin, heavy subunit	1.58	0.030
CB514188	Collagen alpha-2(VI) chain precursor	1.58	0.012
CA064525	Platelet endothelial cell adhesion molecule precursor	1.57	0.016
CB493667	Phosphoglycerate mutase 1	1.56	0.017
CK990656	UNKNOWN	1.56	0.024
CB494364	Collagen alpha-1(I) chain precursor	1.55	0.018
CB501353	Transketolase	1.55	0.012
CB501478	Danio rerio ATPase, Na+/K+ transporting, beta 1a polypeptide, mRNA (cDNA clone MGC:55540 IMAGE:2642288), complete cds	1.55	0.040
CA058102	CCAAT/enhancer-binding protein epsilon	1.55	0.038
CA058869	Oncorhynchus mykiss Na/K ATPase alpha subunit isoform 2 mRNA, complete cds	1.54	0.011
CA062865	NACHT, LRR and PYD domains-containing protein 5	1.54	0.014
CB492469	Nicotinamide riboside kinase 2	1.54	0.009
CB496691	ATP synthase subunit alpha, mitochondrial precursor	1.53	0.013
CB498122	UNKNOWN	1.53	0.018
CK990692	Apolipoprotein A-I precursor	1.53	0.021
CB502660	7-dehydrocholesterol reductase	1.53	0.019

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB503743	PREDICTED: similar to CC chemokine SCYA113 [Danio rerio]	1.52	0.035
CA051001	Glyceraldehyde-3-phosphate dehydrogenase	1.52	0.046
CA058522	UNKNOWN	1.52	0.047
CB497206	Mbp protein [Danio rerio]	1.52	0.029
CA049294	Hemoglobin subunit alpha	1.51	0.036
CB493478	Calponin-3	1.51	0.009
CA063916	Nicotinamide riboside kinase 2	1.51	0.031
CA060347	Ribokinase	1.50	0.020
CB493178	Ferritin, heavy subunit	1.50	0.043
CA046509	UNKNOWN	1.50	0.030
CA064425	TPA: TPA_exp: Oncorhynchus mykiss RTN11 (RTN11) mRNA, complete cds; and 3' UTR	1.49	0.039
CA037818	UNKNOWN	1.49	0.039
CB507253	pfam00909, Ammonium_transp, Ammonium Transporter Family	1.48	0.027
CB511513	Paxillin	1.47	0.048
CK990242	High mobility group protein B2	1.47	0.007
CB492420	Myosin light chain 3, skeletal muscle isoform	1.46	0.021
CB494322	Eukaryotic translation initiation factor 5	1.46	0.040
CB509406	Phosphoribosyl pyrophosphate synthetase-associated protein 1	1.44	0.050
CA051332	Ferritin, heavy subunit	1.44	0.012
CB493071	UNKNOWN	1.44	0.034
CB494545	Ig kappa-b4 chain C region	1.44	0.045
CB492722	Uncharacterized protein C16orf14 homolog	1.44	0.031
CA047018	PREDICTED: similar to disulfide isomerase [Macaca mulatta]	1.43	0.042
CB497598	UNKNOWN	1.43	0.031
CK991259	Gastrotropin	1.43	0.047
CB511959	Solute carrier organic anion transporter family member 2B1	1.43	0.026
CA050427	UNKNOWN	1.43	0.002
CA042382	UNKNOWN	1.43	0.012
CB517765	Toll-like receptor 7 precursor	1.42	0.039
CA061243	UNKNOWN	1.42	0.030
CA050293	Hemoglobin subunit beta	1.42	0.032
CB501216	Hemoglobin subunit alpha	1.42	0.008
CK991235	Ferritin, heavy subunit	1.42	0.010
CB499831	fragile site-associated protein [Homo sapiens]	1.41	0.017
CK991234	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	1.41	0.024
CA044003	UNKNOWN	1.41	0.016
CK990254	Human G protein-coupled receptor (GPR2) gene, partial cds	1.41	0.020

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB490699	Salmo salar hyperosmotic glycine rich protein mRNA, complete cds	1.41	0.009
CA050894	DPH4 homolog	1.41	0.048
CB489257	Ferritin, heavy subunit	1.40	0.027
CB497602	Heat shock cognate 71 kDa protein	1.40	0.034
CB511552	UNKNOWN	1.40	0.033
CA058206	Eukaryotic translation initiation factor 3 subunit 7	1.40	0.045
CA039922	Galactocerebrosidase precursor	1.40	0.035
CB516835	Centromere protein Q	1.40	0.025
CA036980	UNKNOWN	0.71	0.028
CA062812	Ribosomal protein S6 kinase alpha-5	0.71	0.003
CB487382	Peroxisome assembly factor 1	0.71	0.042
CA040733	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	0.71	0.005
CB516524	Calcium/calmodulin-dependent protein kinase type II gamma chain	0.71	0.031
CA055117	UNKNOWN	0.71	0.035
CB514944	UNKNOWN	0.71	0.016
CB492187	Probable tumor suppressor protein MN1	0.71	0.039
CA057359	UNKNOWN	0.71	0.012
CB503191	Anterior gradient protein 2 homolog precursor	0.71	0.027
CA059121	Component of gems 4	0.71	0.035
CA064154	UNKNOWN	0.71	0.007
CA058536	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	0.71	0.038
CA054084	Interferon-induced protein with tetratricopeptide repeats 5	0.71	0.024
CA040282	Dipeptidyl peptidase 9	0.70	0.007
CA063628	PREDICTED: similar to Pold2 protein [Danio rerio]	0.70	0.009
CA058973	Ethanolamine kinase 1	0.70	0.031
CA061065	UNKNOWN	0.70	0.018
CA044989	Plastin-1	0.70	0.027
CB496873	UNKNOWN	0.70	0.007
CA050655	26S protease regulatory subunit 8	0.70	0.037
CB517779	Proto-oncogene tyrosine-protein kinase FER	0.70	0.017
CB497970	Complement C3-1	0.70	0.020
CA052005	UNKNOWN	0.70	0.038
CB490426	Ubiquitin-conjugating enzyme E2 T	0.70	0.002
CK990863	UNKNOWN	0.70	0.040
CB511650	Keratin, type I cytoskeletal 13	0.70	0.018
CB516892	RAC-beta serine/threonine-protein kinase-A	0.70	0.008
CA041425	Cyclin-dependent kinase 4 inhibitor B	0.70	0.037
CA055144	Protein kinase C eta type	0.70	0.020

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB494012	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	0.70	0.029
CB493639	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor	0.70	0.013
CB498603	UNKNOWN	0.70	0.046
CA039925	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.70	0.007
CA058036	bloodthirsty [Chaenocephalus aceratus]	0.70	0.013
CA044026	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	0.70	0.019
CA063517	Ras-related C3 botulinum toxin substrate 3 precursor	0.70	0.001
CB498431	Ena/VASP-like protein	0.70	0.021
CB493904	Endothelin-converting enzyme 1	0.69	0.043
CA063454	Attractin precursor	0.69	0.030
CA055151	UNKNOWN	0.69	0.001
CB500553	UNKNOWN	0.69	0.016
CA064519	Type 2A phosphatase activator TIP41	0.69	0.047
CB499750	Zinc finger protein 180	0.69	0.020
CA061371	UNKNOWN	0.69	0.015
CA054065	UNKNOWN	0.69	0.034
CA048905	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5 precursor	0.69	0.039
CA053678	Protein Tob1	0.69	0.002
CA046116	pfam02758, PAAD_DAPIN, PAAD/DAPIN/Pyrin domain.	0.69	0.012
CA047557	Homeobox protein PKNOX1	0.69	0.028
CA037220	UNKNOWN	0.68	0.023
CA061021	UNKNOWN	0.68	0.026
CA060972	EGF-like module-containing mucin-like hormone receptor-like 1 precursor	0.68	0.015
CB517888	UNKNOWN	0.68	0.025
CA040847	UNKNOWN	0.68	0.009
CA039532	Beta crystallin A3-1	0.68	0.028
CB498855	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.68	0.039
CK991319	UNKNOWN	0.68	0.014
CB499669	Serine/threonine-protein kinase Nek9	0.68	0.022
CB500860	Nuclear body protein SP140	0.68	0.019
CA060172	PREDICTED: similar to limb-bud and heart [Homo sapiens]	0.68	0.008
CA053608	Glutaminyl-peptide cyclotransferase precursor	0.68	0.022
CA055897	UNKNOWN	0.68	0.018

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA039107	Ras-related protein Rab-1A	0.68	0.028
CB496743	Nucleoporin NDC1	0.68	0.038
CB509890	Natterin-like protein	0.68	0.017
CB492663	UNKNOWN	0.68	0.036
CA060909	Protein C14orf166	0.68	0.005
CB512132	Probable G-protein coupled receptor 3	0.68	0.027
CA039055	Complement factor B precursor	0.67	0.039
CB514758	UNKNOWN	0.67	0.027
CA059819	RNA-binding protein 12	0.67	0.016
CA042130	Fatty-acid amide hydrolase 1	0.67	0.021
CB502944	Neuroblastoma suppressor of tumorigenicity 1 precursor	0.67	0.006
CA040378	Tubulin polymerization-promoting protein family member 3	0.67	0.037
CA061271	UNKNOWN	0.67	0.037
CA061044	Serine protease 23 precursor	0.67	0.019
CB506798	UNKNOWN	0.67	0.001
CA052325	Prefoldin subunit 2	0.67	0.034
CA058225	Adaptin ear-binding coat-associated protein 2	0.67	0.039
CK990796	osteopontin-like protein [Oncorhynchus mykiss]	0.67	0.018
CB510567	UNKNOWN	0.67	0.050
CK990962	UNKNOWN	0.67	0.002
CB496394	novel protein [Xenopus tropicalis]	0.67	0.041
CA044626	UNKNOWN	0.67	0.044
CA056636	Ribose-phosphate pyrophosphokinase II	0.67	0.005
CA052204	Interferon-related developmental regulator 1	0.67	0.035
CA064075	UNKNOWN	0.66	0.003
CA042156	UNKNOWN	0.66	0.012
CB492138	UNKNOWN	0.66	0.002
CA060163	Ribosomal protein S6 kinase beta-2	0.66	0.001
CK990808	type I keratin S8 [Oncorhynchus mykiss]	0.66	0.010
CA042703	UNKNOWN	0.66	0.027
CB505840	Oncorhynchus mykiss mRNA for carbonic anhydrase 1, complete cds	0.66	0.047
CB511977	Centaurin-beta 1	0.66	0.049
CA060629	UNKNOWN	0.66	0.031
CB512326	UNKNOWN	0.66	0.023
CA055399	UNKNOWN	0.66	0.027
CA055885	Receptor-transporting protein 3	0.66	0.006
CA057888	UNKNOWN	0.66	0.010
CA054967	Receptor-transporting protein 3	0.66	0.008

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB503968	Protein FAM3C precursor	0.66	0.016
CA058272	PREDICTED: similar to predicted protein [Danio rerio]	0.66	0.021
CA055267	Lithognathus mormyrus clone lmos8p04c12 mRNA sequence	0.66	0.009
CK990874	UNKNOWN	0.66	0.005
CB510091	MHC class I [Salmo salar]	0.66	0.027
CA062381	UNKNOWN	0.66	0.008
CB494687	UNKNOWN	0.66	0.041
CA052552	Mus musculus B-cell translocation gene 1, anti-proliferative (Btg1), mRNA Mus musculus B-cell translocation gene 1, anti-proliferative, mRNA (	0.66	0.000
CA041246	PREDICTED: similar to small inducible cytokine SCYA105 [Danio rerio]	0.66	0.011
CA046761	Hemoglobin subunit alpha-4	0.66	0.002
CK990329	UNKNOWN	0.65	0.005
CB496828	envelope protein [Atlantic salmon swim bladder sarcoma virus] >gi|76786464|gb|ABA54983.1| envelope protein [Atlantic salmon swim bladder sarcoma virus]	0.65	0.001
CK990238	Lens fiber membrane intrinsic protein	0.65	0.008
CA060381	Ornithine decarboxylase antizyme 1	0.65	0.001
CA062570	Interleukin-13 receptor alpha-2 chain precursor	0.65	0.033
CA057590	T-complex protein 1 subunit delta	0.65	0.016
CA053755	Vacuolar ATP synthase subunit H	0.65	0.007
CB501822	UNKNOWN	0.65	0.048
CA044002	UNKNOWN	0.65	0.042
CA041059	UNKNOWN	0.65	0.026
CA049907	Selenide, water dikinase 2	0.65	0.032
CA062832	UNKNOWN	0.65	0.021
CA061160	COP9 signalosome complex subunit 8	0.65	0.006
CB494271	Protein PHS1	0.65	0.049
CA040852	Leucine-rich repeat-containing protein 8D	0.65	0.008
CB507975	UNKNOWN	0.65	0.047
CB512198	Bile acid CoA:amino acid N-acyltransferase	0.65	0.002
CA060630	UNKNOWN	0.65	0.003
CA043931	cAMP-dependent protein kinase type II-alpha regulatory subunit	0.65	0.006
CA053721	Ubiquinone biosynthesis monooxygenase COQ6	0.65	0.020
CA058204	UNKNOWN	0.65	0.038
CB496550	COP9 signalosome complex subunit 5	0.65	0.001
CA064082	BDNF/NT-3 growth factors receptor precursor	0.65	0.009
CB493095	Arachidonate 5-lipoxygenase-activating protein	0.64	0.002
CB493963	LIM domain-binding protein 3	0.64	0.022

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA060222	6-phosphofructokinase type C	0.64	0.042
CB506393	Cytosolic sulfotransferase 2	0.64	0.015
CA059156	SON protein	0.64	0.008
CA053276	UNKNOWN	0.64	0.008
CA052321	Histone H1.0-B	0.64	0.022
CA062136	FK506-binding protein 1B	0.64	0.006
CA040534	UNKNOWN	0.64	0.014
CA062979	PREDICTED: similar to ZC477.3a [Canis familiaris]	0.64	0.006
CA053097	UPF0414 transmembrane protein C20orf30 homolog	0.64	0.016
CB510482	Collagen alpha-1(VI) chain precursor	0.64	0.011
CB496965	UNKNOWN	0.64	0.003
CA036636	Cell cycle checkpoint protein RAD1	0.64	0.011
CA044550	UNKNOWN	0.63	0.027
CA057335	UNKNOWN	0.63	0.005
CA040396	Forkhead box protein K2	0.63	0.025
CA059962	Kelch-like protein 6	0.63	0.021
CB512548	UNKNOWN	0.63	0.012
CA056207	UNKNOWN	0.63	0.047
CB517751	Takifugu rubripes HoxCa gene cluster, complete sequence	0.63	0.011
CA062163	OX-2 membrane glycoprotein precursor	0.63	0.019
CA058580	UNKNOWN	0.63	0.013
CB518105	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	0.63	0.014
CA060321	Copper-transporting ATPase 2	0.63	0.033
CA058813	UNKNOWN	0.63	0.033
CK991138	UNKNOWN	0.63	0.014
CA061018	UNKNOWN	0.63	0.006
CA063017	Nuclear factor 7, ovary	0.63	0.012
CK990882	UNKNOWN	0.63	0.043
CA054059	UNKNOWN	0.63	0.046
CB512396	Macrophage receptor MARCO	0.63	0.010
CA767983	PREDICTED: similar to small inducible cytokine SCYA105 [Danio rerio]	0.63	0.009
CB493128	UNKNOWN	0.63	0.032
CB498955	Ras-related protein Rab-14	0.63	0.007
CA063143	UNKNOWN	0.63	0.010
CA057218	UNKNOWN	0.63	0.008
CA057975	Microtubule-associated protein RP/EB family member 1	0.63	0.008
CA046957	UNKNOWN	0.62	0.002
CA057048	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	0.62	0.023

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA056190	UNKNOWN	0.62	0.014
CA058161	Oncorhynchus mykiss IgH.A locus, partial sequence	0.62	0.001
CA058306	UNKNOWN	0.62	0.002
CA044612	Sorting nexin-16	0.62	0.024
CB516605	Myosin-binding protein C, cardiac-type	0.62	0.002
CA061958	UNKNOWN	0.62	0.035
CB511319	Homo sapiens F-box protein SEL10 (SEL10) mRNA, complete cds	0.62	0.006
CA057911	UNKNOWN	0.62	0.009
CA042961	Eukaryotic peptide chain release factor subunit 1	0.62	0.020
CB515794	Pituitary tumor-transforming gene 1 protein-interacting protein precursor	0.62	0.017
CA044775	UNKNOWN	0.62	0.023
CA063337	UNKNOWN	0.62	0.016
CA058022	Arfaptin-1	0.62	0.002
CA039963	UNKNOWN	0.62	0.017
CA050376	UNKNOWN	0.62	0.001
CB511693	Thrombospondin-3b precursor	0.62	0.046
CB515222	UNKNOWN	0.62	0.029
CB517447	UNKNOWN	0.62	0.006
CA062930	UNKNOWN	0.62	0.001
CA050694	Salmo salar tapasin (TAPBP) mRNA, complete cds	0.62	0.019
CA058947	Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase	0.61	0.001
CA047944	Microtubule-associated proteins 1A/1B light chain 3A precursor	0.61	0.004
CB511711	UNKNOWN	0.61	0.019
CK990496	Cathepsin L precursor	0.61	0.011
CB502503	Cathepsin L precursor	0.61	0.036
CA052316	UNKNOWN	0.61	0.006
CA042655	Microtubule-associated protein RP/EB family member 1	0.61	0.003
CA063116	UNKNOWN	0.61	0.003
CB518113	UNKNOWN	0.61	0.001
CA058806	Actin-related protein 2/3 complex subunit 5-like protein	0.61	0.001
CA036813	UNKNOWN	0.61	0.000
CB516627	Serine hydroxymethyltransferase, cytosolic	0.61	0.024
CA061818	UNKNOWN	0.61	0.022
CA041318	Vacuolar ATP synthase subunit G 1	0.60	0.025
CB517046	GTP:AMP phosphotransferase mitochondrial	0.60	0.041
CB496908	UNKNOWN	0.60	0.010

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB512520	Nck-associated protein 1	0.60	0.017
CB517726	Polypyrimidine tract-binding protein 1	0.60	0.020
CA040745	S.salar CHA(6.3) gene	0.60	0.005
CA057705	Regulator of G-protein signaling 18	0.60	0.004
CA060108	UNKNOWN	0.60	0.019
CB492392	Tripeptidyl-peptidase 2	0.60	0.024
CA051653	UNKNOWN	0.60	0.040
CA051610	UNKNOWN	0.60	0.005
CA051158	Histone deacetylase 10	0.60	0.032
CB498131	C-C chemokine receptor type 6	0.60	0.016
CB493774	Ornithine decarboxylase antizyme 1	0.60	0.006
CA041312	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 2	0.59	0.012
CB517268	UNKNOWN	0.59	0.008
CA056994	Transmembrane protein 169	0.59	0.004
CA059058	UNKNOWN	0.59	0.030
CB517728	UNKNOWN	0.59	0.019
CA054194	UNKNOWN	0.59	0.034
CA046167	UNKNOWN	0.59	0.032
CB497785	Serine/threonine-protein kinase LMTK1	0.59	0.037
CB492980	Collagen alpha-1(VI) chain precursor	0.59	0.004
CA055778	UNKNOWN	0.59	0.037
CA057061	UNKNOWN	0.59	0.003
CA054090	Salvelinus fontinalis decoy TNF receptor mRNA, complete cds	0.59	0.013
CK990484	UNKNOWN	0.59	0.003
CB512095	PDZ and LIM domain protein 3	0.59	0.023
CA058532	UNKNOWN	0.58	0.011
CB496792	Collagen alpha-1(VI) chain precursor	0.58	0.013
CB502615	Phospholipase AdRab-B precursor	0.58	0.014
CA064284	UNKNOWN	0.58	0.042
CA040167	UNKNOWN	0.58	0.006
CA064439	UNKNOWN	0.58	0.014
CA043338	UNKNOWN	0.58	0.046
CA060028	Mitogen-activated protein kinase organizer 1	0.58	0.016
CB510303	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor	0.58	0.006
CA060226	UNKNOWN	0.58	0.031
CB510157	putative 14 kDa apolipoprotein [Platichthys flesus]	0.58	0.035
CB515453	UNKNOWN	0.58	0.001
CA056284	ARMET protein precursor	0.58	0.012

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA045948	UNKNOWN	0.57	0.035
CA051691	UNKNOWN	0.57	0.014
CA053162	UNKNOWN	0.57	0.003
CA047542	UNKNOWN	0.57	0.033
CA057681	UNKNOWN	0.57	0.009
CA054957	Superoxide dismutase [Cu-Zn]	0.57	0.024
CB497376	Small nuclear ribonucleoprotein F	0.57	0.028
CA051654	UNKNOWN	0.57	0.002
CA059425	UNKNOWN	0.57	0.014
CA043801	Cytochrome P450 7A1	0.57	0.022
CA047650	UNKNOWN	0.56	0.043
CB515126	PREDICTED: similar to MYCBP associated protein [Gallus gallus]	0.56	0.002
CA062844	Septin-5	0.56	0.005
CB511922	Vasoactive intestinal polypeptide receptor 2 precursor	0.56	0.022
CA063885	UNKNOWN	0.56	0.005
CB510525	Guanine nucleotide-binding protein G(t) subunit alpha	0.55	0.023
CK990795	Class I histocompatibility antigen, F10 alpha chain precursor	0.55	0.024
CB502824	Phospholipase AdRab-B precursor	0.55	0.002
CA039985	UNKNOWN	0.55	0.012
CB514295	hCG15426, isoform CRA_a [Homo sapiens]	0.55	0.010
CB516202	Sterile alpha motif domain-containing protein 9-like	0.55	0.024
CA060310	UNKNOWN	0.55	0.032
CA061284	Bifunctional purine biosynthesis protein PURH	0.55	0.016
CA056678	Chronic lymphocytic leukemia deletion region gene 6 protein homolog	0.54	0.008
CB506177	UNKNOWN	0.54	0.009
CB515647	PREDICTED: similar to DOCK180-related Cdc42 guanine nucleotide exchange factor [Danio rerio]	0.54	0.015
CA063769	Alpha-mannosidase 2C1	0.54	0.023
CB496839	BET1 homolog	0.54	0.029
CB511902	PREDICTED: similar to interferon regulatory factor 4 [Danio rerio]	0.54	0.042
CA041550	UNKNOWN	0.54	0.011
CB511857	BNIP2 motif-containing molecule at the C-terminal region 1	0.53	0.044
CA056706	Tektin-4	0.53	0.001
CB501836	UNKNOWN	0.53	0.037
CB498117	AP-2 complex subunit mu-1	0.52	0.011
CB511678	UNKNOWN	0.52	0.023
CA039983	UNKNOWN	0.52	0.041
CA042256	UNKNOWN	0.51	0.018

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA044883	putative 14 kDa apolipoprotein [Platichthys flesus]	0.51	0.004
CA057765	Glutamate-rich WD repeat-containing protein 1	0.50	0.002
CB511952	RING finger protein 170	0.49	0.010
CN442546	Translationally-controlled tumor protein	0.44	0.037

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

Complete list of differentially expressed genes from kidney of *Loma salmonae* infected fish sampled at 4 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA047642	Retinoschisin precursor	2.80	0.025
CB508626	Ferritin, lower subunit	2.77	0.040
CA061876	UNKNOWN	2.67	0.013
CA042092	Trypsin-1 precursor	2.35	0.004
CK991035	Zinc finger and BTB domain-containing protein 11	2.26	0.044
CB512203	Uromodulin precursor	2.22	0.014
CA057457	Lysosome-associated membrane glycoprotein 3 precursor	2.20	0.000
CB515390	Vitellogenin precursor	2.13	0.015
CB505565	serum lectin isoform 3 precursor [Salmo salar]	2.09	0.028
CA052274	UNKNOWN	1.96	0.019
CB505647	Apolipoprotein Eb precursor	1.96	0.007
CB508083	cyclin-dependent kinase inhibitor 1C (P57)	1.94	0.010
CN442529	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.91	0.030
CK990907	S.salar mRNA for MHC-Sasa class II B (clone c157)	1.91	0.025
CA046169	UNKNOWN	1.91	0.045
CB502763	UNKNOWN	1.91	0.033
CA043705	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.90	0.040
CB502631	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.90	0.045
CK991089	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.89	0.030
CB515720	Eomesodermin	1.85	0.006
CB514060	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.83	0.017
CB515267	Integral membrane protein 2C	1.81	0.048
CB511472	Oncorhynchus mykiss interferon regulatory factor 1 (IRF-1) gene, promoter region and partial sequence	1.78	0.034
CB505875	Peroxiredoxin	1.77	0.016
CB510574	PREDICTED: Danio rerio similar to sreb2, transcript variant 2 (LOC794099), mRNA	1.76	0.003
CA053172	Calsequestrin-1 precursor	1.74	0.003
CB506394	Cytochrome b5	1.73	0.041
CB506027	H-2 class II histocompatibility antigen gamma chain	1.73	0.010
CA057214	Fatty acyl-CoA hydrolase precursor, medium chain	1.72	0.002
CA045898	UNKNOWN	1.72	0.043
CA064287	Interferon-induced protein 44	1.72	0.026
CB511603	invariant chain-like protein 14-1 [Oncorhynchus mykiss]	1.71	0.021
CB512309	UNKNOWN	1.70	0.021

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA052525	Protein FAM38A	1.70	0.046
CK990275	HLA class II histocompatibility antigen gamma chain	1.70	0.048
CA040529	Peptidyl-prolyl cis-trans isomerase-like 2	1.68	0.018
CA058437	UNKNOWN	1.68	0.021
CB503743	PREDICTED: similar to CC chemokine SCYA113 [Danio rerio]	1.68	0.043
CB512316	UNKNOWN	1.67	0.040
CB491461	Transcription factor E2F4	1.65	0.003
CB489974	ZPC2 [Oryzias latipes]	1.65	0.003
CB517869	UNKNOWN	1.65	0.032
CA052549	UNKNOWN	1.64	0.000
CA058095	Gamma-tubulin complex component 4	1.64	0.012
CA769845	MHC class II [Salmo salar]	1.63	0.027
CB511037	Intestinal mucin-like protein	1.63	0.045
CB516721	PREDICTED: similar to elastin microfibril interfacer 3 [Rattus norvegicus]	1.63	0.049
CA057993	Lymphocyte antigen 75 precursor	1.61	0.007
CB505594	Beta-2-microglobulin precursor	1.61	0.037
CA044333	Elongation of very long chain fatty acids protein 2	1.61	0.015
CB488427	Small nuclear ribonucleoprotein Sm D2	1.61	0.012
CA050443	Salvelinus fontinalis C1q-like adipose specific protein mRNA, complete cds	1.60	0.010
CA061904	Short-chain dehydrogenase/reductase 3	1.59	0.019
CA052445	Proteasome subunit beta type 6 precursor	1.58	0.043
CB497250	H-2 class II histocompatibility antigen gamma chain	1.58	0.028
CA046723	UNKNOWN	1.58	0.035
CB485951	Heat shock 70 kDa protein cognate 4	1.57	0.034
CB494563	UNKNOWN	1.57	0.014
CB515870	B-cell lymphoma 6 protein	1.57	0.020
CK990877	UNKNOWN	1.56	0.029
CB510631	H-2 class II histocompatibility antigen gamma chain	1.56	0.008
CA064476	Delta-aminolevulinic acid dehydratase	1.56	0.006
CB494403	Creatine kinase M-type	1.55	0.042
CB496891	40S ribosomal protein S5	1.55	0.023
CA059974	Cyclic AMP-dependent transcription factor ATF-6 alpha	1.55	0.041
CA057058	Histone-arginine methyltransferase CARM1	1.55	0.020
CA038063	unknown protein [Siniperca chuatsi]	1.55	0.021
CB516976	T cell receptor alpha chain [Salmo salar]	1.54	0.017
CA051473	Homo sapiens IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast), mRNA (cDNA clone MGC:910 IMAGE:3546883), complete cds	1.54	0.001

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA058516	UNKNOWN	1.53	0.009
CA036615	High mobility group protein B2	1.53	0.008
CB506008	Protein kinase C alpha type	1.53	0.029
CA058393	UNKNOWN	1.53	0.044
CA046322	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	1.53	0.048
CA058310	novel immune-type receptor 1 [Oncorhynchus mykiss]	1.53	0.032
CB510046	UNKNOWN	1.52	0.043
CA055541	UNKNOWN	1.52	0.016
CA055272	UNKNOWN	1.52	0.031
CB488401	Glycine cleavage system H protein, mitochondrial precursor	1.52	0.036
CB488244	Zinc finger protein 271	1.52	0.048
CA770328	Tapasin precursor	1.52	0.016
CB513891	UNKNOWN	1.51	0.008
CB507188	Lectin precursor	1.51	0.039
CA043324	Beta-2-microglobulin precursor	1.51	0.015
CB511972	Anosmin-1 precursor	1.51	0.008
CA047947	Takifugu rubripes protocadherin gene locus 2, complete cds, alternatively spliced	1.50	0.048
CA046676	UNKNOWN	1.50	0.003
CA064401	UNKNOWN	1.49	0.004
CA050786	Interleukin-2 receptor subunit beta precursor	1.49	0.010
CB492384	Creatine kinase B-type	1.48	0.038
CB509631	UNKNOWN	1.48	0.027
CB511841	Proteasome subunit beta type 7 precursor	1.48	0.037
CB516159	Tumor suppressor p53-binding protein 1	1.47	0.038
CA036488	Fatty acid-binding protein 1, liver	1.47	0.015
CA054654	40 kDa peptidyl-prolyl cis-trans isomerase	1.47	0.046
CA060830	UNKNOWN	1.46	0.003
CA051861	UNKNOWN	1.46	0.039
CA053500	Receptor-type tyrosine-protein phosphatase epsilon precursor	1.46	0.014
CA770331	UNKNOWN	1.46	0.036
CA064242	RING finger protein 213	1.46	0.043
CA060097	3-phosphoinositide-dependent protein kinase 1	1.45	0.033
CA043885	Profilin-2	1.45	0.033
CB507747	Homeobox protein OTX1	1.45	0.009
CA063057	Y-box-binding protein 2-A	1.45	0.041
CB517236	Rab-interacting lysosomal protein	1.45	0.041
CA041367	UNKNOWN	1.45	0.012
CA058374	tRNA	1.44	0.044

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB494485	Ferritin, heavy subunit	1.44	0.025
CA053070	Keratinocytes-associated transmembrane protein 2 precursor	1.44	0.049
CA053415	Signal transducer and activator of transcription 4	1.44	0.024
CB513907	Transcriptional regulator Myc-b	1.44	0.036
CA047656	UNKNOWN	1.44	0.013
CA051676	Eomesodermin homolog	1.44	0.049
CA059238	Polyadenylate-binding protein 4	1.43	0.033
CB502196	WD repeat protein 23	1.43	0.028
CB492871	Oncorhynchus mykiss mRNA for MHC class II alpha (onmy-DAA*02 gene)	1.43	0.010
CA050726	UNKNOWN	1.43	0.039
CA054877	UNKNOWN	1.43	0.001
CA051247	Splicing factor 3A subunit 2	1.43	0.023
CA050593	T-cell immunoglobulin and mucin domain-containing protein 4 precursor	1.43	0.027
CA036801	Heterogeneous nuclear ribonucleoprotein A/B	1.43	0.017
CA059909	UNKNOWN	1.42	0.018
CA054237	Coproporphyrinogen III oxidase, mitochondrial precursor	1.42	0.023
CB513912	UNKNOWN	1.42	0.020
CB514206	UNKNOWN	1.42	0.012
CA040510	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	1.42	0.046
CK991039	UNKNOWN	1.42	0.038
CA051334	UNKNOWN	1.42	0.045
CA042641	Selenoprotein H	1.42	0.000
CA062966	UNKNOWN	1.42	0.005
CA044332	Protein disulfide-isomerase A2 precursor	1.42	0.001
CB509970	Oncorhynchus mykiss IgH.A locus, partial sequence	1.42	0.042
CA062951	Ig kappa chain V-IV region JI precursor	1.42	0.050
CA047235	UNKNOWN	1.41	0.016
CB511125	Integrin beta-2 precursor	1.41	0.016
CA051170	Probable ATP-dependent RNA helicase DDX27	1.41	0.010
CK990263	Collagen alpha-2(I) chain precursor	1.41	0.009
CA042123	Protein NipSnap3A	1.40	0.036
CA060011	Barrier-to-autointegration factor	1.40	0.005
CN442538	Cytochrome c oxidase subunit 1	0.70	0.002
CA037498	Cytochrome c oxidase polypeptide VIc precursor	0.70	0.005

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA052094	Oncorhynchus mykiss fructose-1,6-bisphosphatase-like mRNA, partial sequence	0.70	0.030
CB492806	Microsomal glutathione S-transferase 3	0.70	0.028
CB494115	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial precursor	0.70	0.005
CB496707	Brain protein 44-like protein	0.70	0.002
CA052206	PQ-loop repeat-containing protein 2	0.70	0.018
CA064541	Collagen alpha-1(I) chain precursor	0.70	0.033
CK990859	Isocitrate dehydrogenase [NADP], mitochondrial precursor	0.70	0.000
CA042803	pfam07051, OCIA, Ovarian carcinoma immunoreactive antigen (OCIA).	0.69	0.005
CN442488	Oncorhynchus mykiss clone OSU IL-8 receptor (IL-8R) mRNA, complete cds	0.69	0.042
CB515644	Interferon regulatory factor 3	0.68	0.027
CB504181	UNKNOWN	0.68	0.010
CA051158	Histone deacetylase 10	0.68	0.013
CB497834	Malate dehydrogenase, cytoplasmic	0.68	0.011
CK991229	UNKNOWN	0.68	0.014
CA062755	Oncorhynchus nerka connective tissue growth factor (CTGF) gene, partial sequence	0.68	0.043
CB517028	Lysosome membrane protein 2	0.68	0.048
CB509536	Lipocalin precursor	0.67	0.037
CB496493	Glutathione S-transferase P	0.67	0.025
CA061491	PREDICTED: rTS beta protein isoform 3 [Pan troglodytes]	0.67	0.020
CA058608	UNKNOWN	0.67	0.019
CB498691	Sulfotransferase family cytosolic 2B member 1	0.67	0.012
CB516919	Extracellular matrix protein 1 precursor	0.67	0.014
CA056491	3-hydroxyanthranilate 3,4-dioxygenase	0.67	0.009
CA050630	PREDICTED: similar to Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A [Danio rerio]	0.67	0.037
CA057824	Apolipoprotein Eb precursor	0.67	0.004
CB498501	Isocitrate dehydrogenase [NADP], mitochondrial precursor	0.66	0.008
CB500108	UNKNOWN	0.66	0.043
CA056962	Ubl carboxyl-terminal hydrolase 18	0.66	0.048
CA060184	Arginase-1	0.66	0.020
CA053442	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	0.66	0.009
CB511870	Cathepsin F precursor	0.66	0.044
CA058472	UNKNOWN	0.66	0.036
CB512101	UNKNOWN	0.65	0.049
CA064171	Interferon-induced protein 44	0.65	0.037

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB492938	UNKNOWN	0.65	0.042
CA058425	Carboxypeptidase A1 precursor	0.65	0.028
CA062987	Isocitrate dehydrogenase [NADP], mitochondrial precursor	0.65	0.034
CB515883	Sodium/potassium-transporting ATPase subunit beta-233	0.65	0.038
CA036595	UNKNOWN	0.65	0.030
CB510653	Salvelinus alpinus metallothionein B gene, introns 1 and 2 and partial cds	0.65	0.027
CB497767	Acyl-coenzyme A thioesterase 4	0.65	0.007
CA057951	UNKNOWN	0.65	0.043
CA054858	Splicing factor, arginine/serine-rich 4	0.65	0.048
CB496732	Signal recognition particle 9 kDa protein	0.64	0.042
CB497987	Apolipoprotein Eb precursor	0.64	0.024
CA043730	Fructose-bisphosphate aldolase B	0.64	0.045
CB510883	Glutathione S-transferase theta-1	0.64	0.039
CA036673	Plasma retinol-binding protein I	0.64	0.003
CA041505	UNKNOWN	0.64	0.019
CB510517	Sodium/potassium-transporting ATPase subunit alpha-1 precursor	0.64	0.033
CA037667	60S ribosomal protein L13a	0.64	0.045
CA043777	Equilibrative nucleoside transporter 3	0.64	0.015
CA056620	Salmo salar hyperosmotic protein 21 (Shop21) gene, Shop21-2 allele, complete cds	0.63	0.022
CB496924	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial precursor	0.63	0.030
CB510670	UNKNOWN	0.63	0.029
CA044003	UNKNOWN	0.63	0.039
CB512768	Succinate semialdehyde dehydrogenase, mitochondrial precursor	0.63	0.015
CA049938	Prefoldin subunit 5	0.63	0.017
CA053164	Sacsin	0.63	0.022
CA049405	Ras association domain-containing protein 1	0.62	0.035
CB508001	Sorbitol dehydrogenase	0.62	0.032
CB503644	L-xylulose reductase	0.61	0.047
CA062436	Sodium/potassium-transporting ATPase subunit beta-233	0.61	0.024
CA063291	UNKNOWN	0.60	0.002
CA055682	Uncharacterized protein C16orf14 homolog	0.60	0.046
CB514228	Oncorhynchus mykiss hepatic glucose transporter GLUT2 mRNA, complete cds	0.60	0.039
CB518106	Sodium/nucleoside cotransporter 1	0.60	0.032
CB488311	Glutaryl-CoA dehydrogenase, mitochondrial precursor	0.60	0.039
CB496570	B-cadherin precursor	0.60	0.010

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA048877	Plakophilin-3	0.60	0.041
CA045238	Harmonin	0.60	0.013
CK991153	S-adenosylmethionine synthetase isoform type-2	0.60	0.011
CA037589	N-acetylglucosamine-1-phosphotransferase subunit gamma precursor	0.60	0.039
CA044978	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	0.60	0.045
CA041385	Proactivator polypeptide precursor	0.59	0.031
CB514900	Eukaryotic translation initiation factor 4B	0.59	0.028
CA041439	UNKNOWN	0.59	0.046
CA051034	Mitotic spindle assembly checkpoint protein MAD2B	0.59	0.038
CA052870	AP-1 complex subunit mu-2	0.58	0.045
CA042054	C-factor	0.58	0.004
CA055600	Membrane-associated progesterone receptor component 1	0.58	0.038
CA054630	Sodium/potassium-transporting ATPase subunit alpha-1 precursor	0.58	0.042
CA042916	Alkaline phosphatase, tissue-nonspecific isozyme precursor	0.57	0.047
CA055131	Solute carrier family 25 member 39	0.57	0.028
CA058254	PREDICTED: similar to prostate stem cell antigen precursor-like [Danio rerio]	0.57	0.007
CA058323	Claudin-3	0.57	0.040
CB494013	Transforming growth factor-beta-induced protein ig-h3 precursor	0.56	0.012
CB497670	Cathepsin L precursor	0.56	0.029
CB498673	Gastricsin precursor	0.56	0.030
CB498523	ATP synthase gamma chain, mitochondrial precursor	0.56	0.018
CA043350	UNKNOWN	0.55	0.026
CA056535	UNKNOWN	0.55	0.022
CB493855	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial precursor	0.55	0.042
CA056419	UNKNOWN	0.55	0.013
CB510911	Complement C1q-like protein 2 precursor	0.55	0.024
CA052560	Probable E3 ubiquitin-protein ligase HERC6	0.54	0.018
CB492382	Maleylacetoacetate isomerase	0.54	0.002
CB505680	UNKNOWN	0.54	0.030
CB514361	Danio rerio insulin-like growth factor binding protein 5, mRNA (cDNA clone MGC:76889 IMAGE:6520159), complete cds	0.53	0.015
CA042788	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.53	0.028
CB508872	GDP-L-fucose synthetase	0.53	0.039

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB492865	Anterior gradient protein 2 homolog precursor	0.53	0.038
CB498613	Thioredoxin-like selenoprotein M precursor	0.53	0.041
CA050478	UNKNOWN	0.52	0.019
CB502503	Cathepsin L precursor	0.52	0.047
CB495027	40S ribosomal protein S3	0.52	0.034
CA057830	PREDICTED: similar to prostate stem cell antigen precursor-like [Danio rerio]	0.51	0.002
CB497426	60S ribosomal protein L38	0.51	0.050
CA057188	Ectonucleotide pyrophosphatase/phosphodiesterase 6 precursor	0.51	0.050
CB492197	Metallothionein A	0.51	0.007
CA054101	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.51	0.046
CK990445	PREDICTED: similar to KIAA1593 protein [Danio rerio]	0.50	0.024
CA039027	Glyceraldehyde-3-phosphate dehydrogenase	0.50	0.035
CK990918	Anterior gradient protein 2 homolog precursor	0.49	0.023
CB492722	Uncharacterized protein C16orf14 homolog	0.49	0.020
CB516765	Fish-egg lectin	0.48	0.040
CA058895	Apolipoprotein Eb precursor	0.48	0.040
CB494193	Interferon-induced 17 kDa protein precursor	0.48	0.025
CB493770	Arginase-1	0.47	0.000
CA043085	Aminoacylase-1	0.46	0.020
CA058271	PREDICTED: similar to interferon-inducible protein Gig2 [Danio rerio]	0.45	0.037
CB511159	UNKNOWN	0.45	0.006
CA052963	Glutathione S-transferase A	0.45	0.002
CB492604	Glutathione S-transferase A	0.44	0.001
CB516720	Serine--pyruvate aminotransferase, mitochondrial precursor	0.44	0.005
CA052666	Epidermal growth factor receptor kinase substrate 8-like protein 3	0.44	0.004
CB510644	Selenoprotein Pa precursor	0.43	0.004
CK991354	Keratin, type I cytoskeletal 13	0.43	0.016
CB488151	Elongation factor 1-alpha	0.42	0.034
CA059885	Solute carrier family 22 member 20	0.42	0.029
CB503169	Fish-egg lectin	0.42	0.001
CA049981	Selenoprotein Pa precursor	0.42	0.025
CB496788	Glutathione S-transferase A	0.41	0.038
CB498320	cd00213, S-100/ICaBP-like, S-100/ICaBP-like domain; S-100/intestinal calcium binding domain (ICaBP);	0.41	0.018

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA061489	Fish-egg lectin	0.41	0.043
CB510408	Glyceraldehyde-3-phosphate dehydrogenase	0.41	0.005
CA057703	Tetraspanin-1	0.40	0.012
CA062367	Cytochrome P450 2K1	0.40	0.018
CB506050	Aspartoacylase-2	0.40	0.010
CA064176	Ubiquitin-like protein 1	0.39	0.045
CB491527	Histone H2AV	0.38	0.046
CB499972	Peroxisomal membrane protein 2	0.38	0.000
CB514460	Glyceraldehyde-3-phosphate dehydrogenase	0.38	0.038
CK990462	Anterior gradient protein 2 homolog precursor	0.38	0.011
CA055137	Cadherin-17 precursor	0.37	0.001
CK990492	Sodium/potassium-transporting ATPase subunit alpha-1 precursor	0.37	0.021
CB518118	Serine/threonine-protein kinase Sgk2	0.36	0.045
CB493962	Fructose-bisphosphate aldolase B	0.36	0.006
CA063811	Collectrin precursor	0.35	0.043
CA053670	Glucosidase 2 subunit beta precursor	0.35	0.004
CA062587	Solute carrier family 12 member 3	0.35	0.049
CK991014	Glyceraldehyde-3-phosphate dehydrogenase	0.35	0.037
CB503528	UDP-glucuronosyltransferase	0.34	0.011
CA060621	UNKNOWN	0.33	0.007
CA063623	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial precursor	0.33	0.030
CB498361	Glyceraldehyde-3-phosphate dehydrogenase	0.33	0.030
CA768062	Glyceraldehyde-3-phosphate dehydrogenase	0.32	0.013
CA042581	Alpha-tectorin precursor	0.32	0.001
CK991283	UDP-glucuronosyltransferase 2A1 precursor	0.31	0.009
CA042561	PDZ domain-containing protein 1	0.31	0.002
CA058620	Methyltransferase type 11 [Sphingomonas wittichii RW1]	0.31	0.009
CB491826	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	0.31	0.023
CB493105	UNKNOWN	0.31	0.008
CA062426	Fructose-bisphosphate aldolase B	0.30	0.024
CA056406	Transmembrane protease, serine 2	0.30	0.048
CB509760	Acidic mammalian chitinase precursor	0.29	0.019
CK990930	UNKNOWN	0.29	0.036
CA055857	Epidermal growth factor receptor pathway substrate 8-like protein 3	0.29	0.023
CB503840	UNKNOWN	0.28	0.007
CA056945	Fructose-bisphosphate aldolase B	0.27	0.007
CA058609	Methyltransferase type 11 [Sphingomonas wittichii RW1]	0.27	0.006

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 4 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA050886	Glyceraldehyde-3-phosphate dehydrogenase	0.27	0.006
CB512664	Solute carrier family 22 member 8	0.25	0.041
CA061403	Alanine aminotransferase 2	0.25	0.004
CB494088	Guanidinoacetate N-methyltransferase	0.23	0.023
CB493657	UNKNOWN	0.23	0.015
CA058048	PDZ domain-containing protein 1	0.22	0.037
CB510892	Guanidinoacetate N-methyltransferase	0.21	0.004
CB514012	Sodium- and chloride-dependent GABA transporter ine	0.21	0.048
CA058986	Formimidoyltransferase-cyclodeaminase	0.20	0.012
CA064225	Alanine aminotransferase 2	0.19	0.009
CB496806	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	0.19	0.003
DR695512	UNKNOWN	0.18	0.003
CA056849	UNKNOWN	0.16	0.019
CB514478	Serine--pyruvate aminotransferase, mitochondrial precursor	0.15	0.007
CB496520	Cytochrome P450 2K1	0.13	0.004
CA063374	Solute carrier family 22 member 20	0.12	0.001
CB514448	Major facilitator superfamily domain-containing protein 4	0.12	0.005
CA052648	Putative oxidoreductase yulF	0.12	0.004
CA061778	Cytochrome P450 2K1	0.10	0.003
CA042792	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor	0.09	0.008
CB514166	Major facilitator superfamily domain-containing protein 4	0.07	0.000
CA054117	RING finger protein 113A	0.05	0.006

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected fish

Complete list of differentially expressed genes from gills of *Loma salmonae* infected fish sampled at 8 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Gill - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA061572	Lysyl-tRNA synthetase	3.98	0.025
CB511988	Signal peptidase complex subunit 3	3.76	0.006
CA058254	PREDICTED: similar to prostate stem cell antigen precursor-like [Danio rerio]	2.91	0.036
CB498458	Collagen alpha-1(I) chain precursor	2.66	0.033
CK990864	Cystatin-B	2.57	0.029
CA056691	Methylglutaconyl-CoA hydratase, mitochondrial precursor	2.54	0.037
CA055521	CCAAT/enhancer-binding protein beta	2.52	0.003
CB510151	UNKNOWN	2.48	0.020
CA041110	UNKNOWN	2.40	0.022
CK990241	Cathepsin S precursor	2.38	0.015
CB502538	Protein FAM49B	2.36	0.035
CB501389	UNKNOWN	2.36	0.046
CB510320	small inducible cytokine SCYA101 [Paralabidochromis chilotes]	2.31	0.016
CB515162	Pleiotrophic factor-beta-1 precursor	2.22	0.024
CA063565	Interferon regulatory factor 1	2.20	0.046
CB492758	novel protein [Xenopus tropicalis]	2.20	0.011
CA041451	Thioredoxin	2.20	0.020
CA045508	Protein disulfide-isomerase precursor	2.18	0.027
CB494323	Eukaryotic initiation factor 4A-I	2.18	0.004
CX984314	Leukocyte cell-derived chemotaxin 2 precursor	2.17	0.043
CB493711	Cathepsin S precursor	2.17	0.018
CB502879	differentially regulated trout protein 1 [Oncorhynchus mykiss]	2.14	0.015
CB502697	Tetraspanin-8	2.12	0.005
CA050422	Proteasome activator complex subunit 2	2.12	0.036
CA061434	26S proteasome non-ATPase regulatory subunit 7	2.05	0.049
CA053623	Cathepsin S precursor	2.03	0.022
CB506394	Cytochrome b5	2.02	0.013
CA051673	Si:dkey-78d16.1 protein [Danio rerio]	2.02	0.021
CA039982	Salmo trutta MHC class I heavy chain (Satr-UBA) mRNA, Satr-UBA*0501 allele, partial cds	1.99	0.039
CA062588	UNKNOWN	1.98	0.014
CA050114	Myosin-9	1.98	0.033
CB514949	Tubulin beta-1 chain	1.98	0.020

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected fish

		Gill - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA057499	L-lactate dehydrogenase B chain	1.97	0.028
CA063187	UNKNOWN	1.94	0.043
CB511609	Cathepsin L precursor	1.94	0.026
CB502659	H-2 class II histocompatibility antigen gamma chain	1.92	0.041
CB499527	Myosin-9	1.90	0.018
CB498580	Myosin light polypeptide 6	1.90	0.046
CB502869	Protein deltex-2	1.87	0.046
CA059260	UNKNOWN	1.83	0.029
CB494398	Protein disulfide-isomerase A3 precursor	1.81	0.014
CA052753	UNKNOWN	1.80	0.011
CB498286	PREDICTED: similar to expressed sequence AV312086 [Canis familiaris]	1.78	0.002
CB497444	Actin, alpha cardiac	1.78	0.047
CA050489	Xenopus tropicalis cDNA clone IMAGE:7749006	1.77	0.047
CB498249	Protein BTG1	1.77	0.013
CB497324	Translocon-associated protein subunit beta precursor	1.75	0.031
CB511842	invariant chain-like protein 14-1 [Oncorhynchus mykiss]	1.75	0.001
CN442551	NADH-ubiquinone oxidoreductase chain 1	1.73	0.036
CB488101	Cold-inducible RNA-binding protein	1.73	0.033
CA040068	pfam06077, LR8, LR8 protein.	1.73	0.013
CB499697	Matrix metalloproteinase-9 precursor	1.72	0.012
CB501298	cd01327, KAZAL_PSTI, Kazal-type pancreatic secretory trypsin inhibitors (PSTI)	1.71	0.023
CA043183	UNKNOWN	1.71	0.042
CA060555	H-2 class II histocompatibility antigen gamma chain	1.71	0.039
CB499769	Cold-inducible RNA-binding protein	1.70	0.021
CB512514	Ubiquitin-conjugating enzyme E2 L3	1.69	0.024
CA051835	Nucleosome assembly protein 1-like 1	1.68	0.007
CA769983	H-2 class II histocompatibility antigen gamma chain	1.67	0.042
CA050718	Tubulin alpha chain	1.67	0.013
CA054828	Ig mu chain C region membrane-bound form	1.65	0.002
CB510848	Histone H1.0	1.65	0.036
CB517961	Histone H3.3	1.65	0.030
CB493056	GTP-binding nuclear protein Ran	1.65	0.025
CA041701	Protein SET	1.64	0.048
CB502720	Cofilin-2	1.64	0.018
CB511634	Cell division control protein 42 homolog precursor	1.64	0.026
CK990431	UNKNOWN	1.63	0.035
CA040196	Integral membrane protein 2B	1.60	0.000

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected fish

		Gill - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB489293	UNKNOWN	1.60	0.020
CA050722	Histone H3.3	1.59	0.031
CB498391	Beta-2-microglobulin precursor	1.58	0.024
CA049279	E3 ubiquitin-protein ligase TRIM63	1.57	0.028
CB499376	Peptidyl-prolyl cis-trans isomerase	1.57	0.016
CA054958	UNKNOWN	1.56	0.002
CK991141	Histone H3.3	1.55	0.017
CB501401	Beta-2-microglobulin precursor	1.54	0.025
CA056106	Serine/threonine-protein kinase OSR1	1.53	0.027
CA037500	UNKNOWN	1.53	0.009
CA056224	UNKNOWN	1.53	0.026
CB494704	Thymosin beta-11	1.53	0.009
CB496576	Beta-2-microglobulin precursor	1.53	0.010
CB509675	Actin, alpha sarcomeric/cardiac	1.52	0.036
CB511399	Transgelin-3	1.52	0.047
CB502196	WD repeat protein 23	1.52	0.041
CA041695	UNKNOWN	1.51	0.013
CB502487	H-2 class II histocompatibility antigen gamma chain	1.51	0.041
CB492112	UNKNOWN	1.51	0.014
CA060476	H-2 class II histocompatibility antigen, A-B alpha chain precursor	1.51	0.044
CB492871	Oncorhynchus mykiss mRNA for MHC class II alpha (onmy-DAA*02 gene)	1.50	0.042
CB514457	Myosin light polypeptide 6	1.50	0.046
CA055371	GTP-binding protein SAR1a	1.49	0.043
CA062450	UNKNOWN	1.48	0.044
CB510775	UNKNOWN	1.48	0.042
CA056866	Tubulin beta-2B chain	1.48	0.013
CA042924	Glyceraldehyde-3-phosphate dehydrogenase	1.47	0.004
CB518072	Ubiquitin	1.47	0.039
CK991246	Elongation factor 1-alpha, oocyte form	1.47	0.023
CB496514	Protein disulfide-isomerase A3 precursor	1.46	0.009
CA064406	Induced by contact to basement membrane 1 protein homolog	1.45	0.016
CB499941	Cytochrome c oxidase subunit 3	1.45	0.049
CK990286	Ubiquitin	1.44	0.047
CN442545	Cytochrome c oxidase subunit 3	1.44	0.019
CA063314	Eukaryotic translation initiation factor 4H	1.43	0.025
CB497481	Actin-related protein 2/3 complex subunit 4	1.43	0.006
CB510672	FK506-binding protein 1A	1.42	0.010
CB516924	Myosin light polypeptide 6	1.42	0.004

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected fish

		Gill - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CK991284	Elongation factor 1-alpha, oocyte form	1.41	0.048
CA061961	UNKNOWN	1.41	0.039
CB496999	UNKNOWN	1.41	0.006
CB497425	osteopontin-like protein [Oncorhynchus mykiss]	1.41	0.002
CB497383	Proteasome subunit alpha type 2	1.41	0.006
CA044728	UNKNOWN	0.70	0.006
CB504138	UNKNOWN	0.69	0.017
CA046194	Salmo salar RAF1a gene for serine/threonine protein kinase RAF1a, partial cds	0.69	0.047
CB500603	Heterogeneous nuclear ribonucleoprotein L	0.67	0.036
CB488515	PREDICTED: similar to thioredoxin domain containing 14, [Monodelphis domestica]	0.67	0.039
CB499359	Trafficking protein particle complex subunit 2	0.65	0.007
CA062246	Methionine aminopeptidase 2	0.65	0.004
CA054109	Oncorhynchus mykiss IgH.A locus, partial sequence	0.64	0.023
CB492655	DNA-binding protein inhibitor ID-1	0.63	0.037
CA058605	Cystathionine beta-synthase	0.61	0.048
CB493209	Isocitrate dehydrogenase [NADP], mitochondrial precursor	0.60	0.004
CA051144	UNKNOWN	0.60	0.020
CA042459	Salmo salar Na,K-ATPase alpha subunit isoform 1b/ii (ATP1A1B/ii) gene, partial cds	0.60	0.010
CA062601	Serine/threonine-protein kinase RIO3	0.57	0.047
CB511019	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	0.53	0.015
CB498855	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.52	0.039
CA062226	RP11-297I6.1 [Homo sapiens]	0.45	0.016
CB496792	Collagen alpha-1(VI) chain precursor	0.44	0.021
CA062018	UNKNOWN	0.41	0.043
CA050376	UNKNOWN	0.41	0.049
CB496828	envelope protein [Atlantic salmon swim bladder sarcoma virus] >gi|76786464|gb|ABA54983.1| envelope protein	0.39	0.037
CA039175	UNKNOWN	0.38	0.037
CA047857	UNKNOWN	0.35	0.041
CA058361	Hemoglobin subunit alpha-4	0.33	0.003
CA057131	UNKNOWN	0.30	0.029
CA047918	Vesicular integral-membrane protein VIP36 precursor	0.26	0.005
CA057663	Zinc-binding protein A33	0.24	0.042
CA059593	UNKNOWN	0.20	0.047

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

Complete list of differentially expressed genes from kidney of *Loma salmonae* infected fish sampled at 8 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA052439	Ras GTPase-activating protein-binding protein 1	2.46	0.037
CA054405	Ras GTPase-activating protein-binding protein 1	1.68	0.008
CB515604	RNA-binding protein EWS	1.60	0.018
CA062324	Heterogeneous nuclear ribonucleoprotein A3 homolog 1	1.55	0.009
CB515851	Seryl-tRNA synthetase, cytoplasmic	1.89	0.008
CA063945	Asparaginyl-tRNA synthetase, cytoplasmic	1.66	0.013
CB517585	Exosome complex exonuclease MTR3	1.49	0.020
CB496548	Inorganic pyrophosphatase	1.67	0.036
CB518099	Pyruvate kinase muscle isozyme	1.67	0.046
CA054312	Pyruvate kinase muscle isozyme	1.53	0.041
CA051402	Pyruvate kinase muscle isozyme	1.45	0.012
CA050175	Transcription factor Sp3	1.81	0.040
CB496654	Barrier-to-autointegration factor	0.47	0.020
CB499798	Myb-binding protein 1A-like protein	1.40	0.021
CB516494	Transforming growth factor-beta-inducible early growth response protein 3	0.70	0.029
CA055985	Max-binding protein MNT	0.59	0.002
CA055521	CCAAT/enhancer-binding protein beta	1.64	0.046
CA055219	CCAAT/enhancer-binding protein beta	1.42	0.024
CA054749	Mitochondrial 28S ribosomal protein S30	1.63	0.014
CB505864	40S ribosomal protein S27	0.65	0.017
CA058206	Eukaryotic translation initiation factor 3 subunit 7	0.64	0.016
CB514561	Protein disulfide-isomerase precursor	2.03	0.044
CA037557	125 kDa kinesin-related protein	0.46	0.020
CA054211	Plastin-2	1.62	0.040
CB514443	Lymphocyte-specific protein 1	1.60	0.004
CA039933	Plastin-2	1.56	0.006
CA051041	Coactosin-like protein	1.49	0.002
CB492837	Coactosin-like protein	1.48	0.045
CA057584	Complement factor D precursor	1.66	0.003
CA063027	Transaldolase	1.59	0.039
CA057987	Triosephosphate isomerase	1.50	0.002
CA060308	Transaldolase	1.48	0.047
CB497546	Transaldolase	1.42	0.018
CA063511	Transaldolase	1.41	0.049

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA060167	3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	0.47	0.046
CA058389	Creatine kinase B-type	1.49	0.014
CA055298	Proteasome subunit beta type 4 precursor	1.46	0.037
CA055186	Proteasome subunit beta type 7 precursor	1.41	0.035
CA038117	Proteasome subunit alpha type 6	1.75	0.000
CK991247	Protein disulfide-isomerase A3 precursor	1.64	0.041
CA045102	Protein disulfide-isomerase A3 precursor	1.43	0.012
CB486366	Proliferation-associated protein 2G4	1.44	0.039
CB497383	Proteasome subunit alpha type 2	1.46	0.040
CA060697	Proteasome subunit alpha type 3	1.45	0.042
CA060658	Proteasome subunit alpha type 1	0.65	0.025
CA058848	Leukotriene A-4 hydrolase	1.52	0.002
CA048688	Leukotriene A-4 hydrolase	1.43	0.037
CB514705	Fructose-bisphosphate aldolase A	1.62	0.003
CB517144	Fructose-bisphosphate aldolase A	1.50	0.027
CA060235	Glucose-6-phosphate isomerase	1.59	0.004
CA051897	Glyceraldehyde-3-phosphate dehydrogenase	1.87	0.012
CB492813	Glyceraldehyde-3-phosphate dehydrogenase	1.81	0.029
CB498678	Glyceraldehyde-3-phosphate dehydrogenase	1.71	0.034
CA047126	Glyceraldehyde-3-phosphate dehydrogenase	1.68	0.033
CB498382	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	1.73	0.021
CA060790	Phosphoglucomutase-1	1.72	0.029
CA061915	Enolase	1.41	0.007
CB517819	Glycogen phosphorylase, muscle form	1.51	0.024
CB491412	T-lymphokine-activated killer cell-originated protein kinase homolog	0.59	0.032
CA060609	Ubiquitin-conjugating enzyme E2 G1	0.58	0.042
CA048909	Cyclin-dependent kinase inhibitor 1B	0.49	0.047
CB517178	Docking protein 2	0.63	0.040
CA058242	Moesin	1.78	0.037
CB493651	Tumor necrosis factor receptor superfamily member 11B precursor	1.58	0.014
CA038429	Lamin-B2	1.71	0.016
CA062008	Nucleoporin p54	1.42	0.024
CB488319	Uncharacterized protein C8orf76	2.36	0.013
CB492854	Fatty acid-binding protein, brain	1.45	0.029
CA064032	Catenin delta-2	1.44	0.012
CA053755	Vacuolar ATP synthase subunit H	1.40	0.039
CB492393	Cytochrome c	1.69	0.011

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA052300	Cytochrome c1 heme protein, mitochondrial precursor	1.48	0.017
CA049318	Hemoglobin subunit alpha-4	0.66	0.015
CB501216	Hemoglobin subunit alpha	0.57	0.047
CA058361	Hemoglobin subunit alpha-4	0.54	0.010
CA058163	Hemoglobin subunit beta-1	0.53	0.024
CK990457	Hemoglobin subunit beta-1	0.52	0.038
CB498012	SPARC precursor	0.47	0.046
CB517934	ARF GTPase-activating protein GIT2	2.68	0.003
CA055322	Lymphocyte cytosolic protein 2	1.97	0.019
CA064428	6-phosphogluconate dehydrogenase, decarboxylating	1.84	0.039
CA058076	Ribosomal L1 domain-containing protein 1	1.71	0.031
CA042951	ATP synthase gamma chain, mitochondrial precursor	1.57	0.025
CA063091	26S proteasome non-ATPase regulatory subunit 12	1.55	0.028
CA060411	Transitional endoplasmic reticulum ATPase	1.52	0.002
CB490812	F-box only protein 3	1.52	0.033
CA050102	Protein kinase C-binding protein 1	1.48	0.041
CB517811	Ubiquitin carboxyl-terminal hydrolase isozyme L5	1.44	0.020
CB493362	Nucleoside diphosphate kinase B	1.43	0.033
CK990727	Cathepsin L precursor	0.70	0.029
CA044216	Cyclin-dependent kinases regulatory subunit 1	0.70	0.035
CA063144	52 kDa repressor of the inhibitor of the protein kinase	0.68	0.046
CB509669	Mitogen-activated protein kinase kinase 1-interacting protein 1	0.65	0.040
CA060279	U6 snRNA-associated Sm-like protein LSm4	0.63	0.041
CA062714	Eukaryotic translation initiation factor 4 gamma 1	1.93	0.009
CB493017	Serine/threonine-protein kinase 19	1.58	0.037
CA059948	Rho GDP-dissociation inhibitor 1	1.46	0.030
CA056326	Importin subunit alpha-2	1.82	0.010
CB499584	Importin subunit alpha-2	1.41	0.001
CA060052	Conserved oligomeric Golgi complex component 1	0.66	0.033
CA036918	10 kDa heat shock protein, mitochondrial	2.02	0.001
CB493461	Endoplasmin precursor	1.79	0.038
CB494192	Phosphoglycerate kinase	1.45	0.015
CK991335	T-complex protein 1 subunit theta	1.99	0.044
CB498771	T-complex protein 1 subunit epsilon	1.44	0.003
CA055371	GTP-binding protein SAR1a	1.63	0.014
CA061469	GTP-binding protein SAR1a	1.61	0.004
CB509813	Lectin precursor	0.25	0.019
CB510697	Lectin precursor	0.19	0.042
CA056108	CD209 antigen-like protein E	2.13	0.006

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA058595	Lymphatic vessel endothelial hyaluronic acid receptor 1 precursor	0.66	0.046
CB498203	Pleiotrophic factor-alpha-2 precursor	0.70	0.045
CA047948	Pleiotrophic factor-alpha-2 precursor	0.59	0.036
CA062222	Ankyrin-1	0.61	0.008
CB507390	Eukaryotic translation initiation factor 1A, X-chromosomal	1.71	0.002
CA058810	Eukaryotic translation initiation factor 1A, X-chromosomal	1.70	0.026
CB497043	NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial precursor	1.90	0.024
CB514320	Granzyme K precursor	1.52	0.047
CB494012	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	1.48	0.010
CB512614	Protein arginine N-methyltransferase 8	1.41	0.003
CB517754	Proteasome activator complex subunit 3	1.61	0.002
CB492396	Cytosolic 5'-nucleotidase III	0.66	0.010
CB494688	Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family	0.62	0.007
CB511879	Thioredoxin-like protein 2	0.68	0.009
CB503548	Protein transport protein Sec61 subunit alpha	1.46	0.025
CB499596	Hypothetical oxidoreductase yoxD	0.65	0.042
CB493498	Malate dehydrogenase, cytoplasmic	1.63	0.017
CA057499	L-lactate dehydrogenase B chain	1.63	0.027
CA055946	L-lactate dehydrogenase B chain	1.58	0.016
CB516784	26S protease regulatory subunit 6B	1.76	0.016
CK990887	26S protease regulatory subunit 6B	1.77	0.042
CB512514	Ubiquitin-conjugating enzyme E2 L3	1.49	0.011
CB496563	14-3-3 protein beta/alpha-2	1.46	0.024
CA051043	DnaJ homolog subfamily C member 5	0.69	0.004
CB510537	Nucleoside diphosphate kinase A	1.56	0.008
CK990592	Metallothionein B	0.65	0.039
CB507722	Metallothionein B	0.51	0.008
CB514424	Vacuolar ATP synthase 16 kDa proteolipid subunit	1.67	0.024
CB501070	Cytochrome P450 1A3	1.54	0.035
CB508872	GDP-L-fucose synthetase	0.69	0.032
CB503743	PREDICTED: similar to CC chemokine SCYA113 [Danio rerio]	3.00	0.036
CB510481	60S ribosomal protein L4-B	2.52	0.029
CB509250	Salmo salar zonadhesin-like gene, complete cds and 3' UTR	2.39	0.003
CB511680	Lysozyme C II precursor	2.37	0.040
CA054167	Lysozyme C II precursor	2.35	0.033
CA052549	UNKNOWN	2.09	0.010
CB516935	CD97 antigen precursor	2.08	0.047

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB500378	UNKNOWN	2.04	0.048
CA058804	ARMET protein precursor	1.91	0.015
CA051927	UNKNOWN	1.89	0.016
CA044407	Oncorhynchus mykiss MHC class Ib antigen (UDA) mRNA, UDA*0301 allele, complete cds	1.89	0.024
CA044472	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.83	0.012
CA060888	UNKNOWN	1.80	0.004
CB511108	Neighbor of COX4	1.80	0.019
CA044026	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.78	0.017
CA044159	ARMET protein precursor	1.76	0.011
CK990704	ES1 protein homolog, mitochondrial precursor	1.74	0.045
CB505921	UNKNOWN	1.72	0.034
CB502985	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.71	0.017
CA039982	Salmo trutta MHC class I heavy chain (Satr-UBA) mRNA, Satr-UBA*0501 allele, partial cds	1.71	0.046
CA052146	Integral membrane protein 2C	1.71	0.011
CA052045	Complement component C7 precursor	1.71	0.047
CB510571	Apolipoprotein A-I-1 precursor	1.68	0.036
CA044472	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.66	0.041
CB502804	MHC class I [Salmo salar]	1.62	0.016
CK990235	Immunoglobulin lambda-like polypeptide 1 precursor	1.59	0.044
CB514421	Protein ADRM1	1.58	0.004
CB509870	prostaglandine D synthase [Oncorhynchus mykiss]	1.58	0.045
CB497885	PREDICTED: similar to Leukocyte surface antigen CD53 (Cell surface glycoprotein CD53) (Tetraspanin-25) (Tspan-25)	1.58	0.028
CB497119	UNKNOWN	1.56	0.011
CB489043	Beta-2-microglobulin precursor	1.56	0.026
CA051689	Growth hormone-inducible transmembrane protein	1.55	0.018
CK990973	oocyte protease inhibitor-2 [Oncorhynchus mykiss]	1.54	0.031
CA057296	Thioredoxin	1.54	0.034
CA041110	UNKNOWN	1.54	0.019
CK990752	UNKNOWN	1.53	0.025
CB501401	Beta-2-microglobulin precursor	1.52	0.002
CB517149	Major vault protein	1.52	0.042
CB512542	Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial precursor	1.52	0.033
CK990806	Beta-2-microglobulin precursor	1.52	0.008
CK990626	Beta-2-microglobulin precursor	1.52	0.028

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB493295	Myosin heavy chain, fast skeletal muscle	1.52	0.021
CB492176	Receptor expression-enhancing protein 5	1.51	0.021
CA055201	PREDICTED: similar to CaM-KII inhibitory protein [Macaca mulatta]	1.51	0.017
CA053924	Beta-2-microglobulin precursor	1.50	0.034
CK991314	Beta-2-microglobulin precursor	1.50	0.002
CB505897	Beta-2-microglobulin precursor	1.50	0.031
CA057967	UNKNOWN	1.49	0.035
CA043807	Transmembrane protein 150 precursor	1.49	0.009
CB502959	Danio rerio SET translocation (myeloid leukemia-associated) B (setb), mRNA >gi|37681942|gb|AY398416.1| Danio rerio clone RK071A2C04 myeloid leukemia-associated SET translocation protein (SET) mRNA, complete cds	1.49	0.014
CB502763	UNKNOWN	1.49	0.039
CB497392	Oncorhynchus mykiss clone Glan 1 transposon Tc1-like transposase pseudogene mRNA, complete sequence	1.48	0.037
CB500763	Beta-2-microglobulin precursor	1.48	0.031
CA060212	Immunoglobulin lambda-like polypeptide 1 precursor	1.48	0.036
CN442516	Beta-2-microglobulin precursor	1.48	0.050
CA062814	UNKNOWN	1.47	0.035
CB501051	Receptor expression-enhancing protein 5	1.47	0.017
CB505679	Tetraspanin-8	1.47	0.037
CK990545	Beta-2-microglobulin precursor	1.47	0.003
CA040172	Class I histocompatibility antigen, F10 alpha chain precursor	1.47	0.001
CB500982	Beta-2-microglobulin precursor	1.47	0.047
CA062818	UNKNOWN	1.46	0.008
CK990799	Salmo salar clone BE7 beta-2 microglobulin (B2m) mRNA, complete cds	1.46	0.044
CB496576	Beta-2-microglobulin precursor	1.46	0.028
CB497287	Cytochrome c oxidase subunit VIIa-related protein, mitochondrial precursor	1.45	0.036
CA055854	UNKNOWN	1.45	0.021
CA042157	Beta-2-microglobulin precursor	1.44	0.007
CA064059	Thymosin beta-11	1.44	0.024
CK991138	UNKNOWN	1.44	0.035
CA056866	Tubulin beta-2B chain	1.44	0.011
CB517208	Coronin-1A	1.43	0.010
CB510736	DNA-binding protein inhibitor ID-2	1.43	0.004
CA059339	Tubulin beta-1 chain	1.43	0.032
CB515910	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.43	0.025

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CK990907	S.salar mRNA for MHC-Sasa class II B (clone c157)	1.43	0.019
CA061886	Natterin-like protein	1.42	0.025
CB501187	ORF077R [Rock bream iridovirus] >gi|62421268|gb|AAX82388.1| ORF79R [Orange-spotted grouper iridovirus]	1.42	0.002
CA060492	Oncorhynchus mykiss genes, MHC class I a region, complete and partial cds	1.42	0.021
CA039238	Oncorhynchus nerka connective tissue growth factor (CTGF) gene, partial sequence	1.42	0.046
CA055608	Splicing factor, arginine/serine-rich 16	1.42	0.026
CA059629	Transmembrane emp24 domain-containing protein 2 precursor	1.42	0.022
CA769697	glucose-regulated protein 94 [Paralichthys olivaceus]	1.41	0.030
CK990451	UNKNOWN	1.41	0.038
CA061639	UNKNOWN	1.41	0.040
CA052529	PREDICTED: similar to Hook-related protein 1 [Danio rerio]	1.40	0.032
CK991229	UNKNOWN	0.70	0.026
CA063400	Trafficking protein particle complex subunit 5	0.70	0.010
CA044489	Salvelinus alpinus GnRH1 gene, partial cds	0.70	0.000
CB498161	Anterior gradient protein 2 homolog precursor	0.70	0.014
CB510653	Salvelinus alpinus metallothionein B gene, introns 1 and 2 and partial cds	0.70	0.039
CA051373	Oncorhynchus mykiss clone GC41 MHC class I antigen (Onmy-U41p) pseudogene, partial sequence; and proteosome subunit LMP7/PSMB8 (LMP7/PSMB8) gene	0.69	0.028
CB505771	pfam06077, LR8, LR8 protein.	0.69	0.011
CK990802	UNKNOWN	0.68	0.045
CB509079	Oncorhynchus mykiss 12S ribosomal RNA, complete sequence; tRNA-Val, complete sequence and 16S ribosomal RNA, complete sequence, mitochondrial genes for mitochondrial products	0.67	0.024
CB510986	Platelet-derived growth factor receptor-like protein precursor	0.67	0.008
CA061025	UNKNOWN	0.67	0.017
CB517743	Salmo salar aryl hydrocarbon receptor 2 gamma (AhR2g) mRNA, complete cds	0.67	0.013
CB516815	Autophagy-related protein 9A	0.67	0.039
CA054775	UNKNOWN	0.67	0.033
CA056525	clone A24K23, A41I8 of Tetraodon nigroviridis	0.66	0.004
CB516179	Oncorhynchus mykiss Na/K ATPase alpha subunit isoform 3 mRNA, complete cds	0.66	0.005
CB509610	60S ribosomal protein L35	0.66	0.019
CB500057	UNKNOWN	0.66	0.020
CA055542	UNKNOWN	0.66	0.035
CB496792	Collagen alpha-1(VI) chain precursor	0.66	0.035

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 8 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB515627	UNKNOWN	0.66	0.016
CB514577	Reticulon-3	0.65	0.025
CB502941	perforin [Ctenopharyngodon idella]	0.65	0.005
CA057905	Arginine/serine-rich coiled coil protein 1	0.65	0.020
CB498112	Heat shock factor-binding protein 1	0.65	0.025
CA046805	UNKNOWN	0.63	0.048
CK990717	UNKNOWN	0.63	0.026
CN442486	UNKNOWN	0.63	0.014
CA058605	Cystathionine beta-synthase	0.63	0.010
CB514543	UNKNOWN	0.62	0.026
CB515824	Sushi domain-containing protein 1 precursor	0.62	0.027
CA043106	Transmembrane protein 9B precursor	0.61	0.002
CB498787	Oncorhynchus mykiss mRNA for putative ribosomal protein L39 protein	0.60	0.014
CA045710	UNKNOWN	0.60	0.005
CA042057	UNKNOWN	0.60	0.013
CB505818	Oncorhynchus mykiss carbonyl reductase/20beta-hydroxysteroid dehydrogenase B gene, complete cds	0.58	0.012
CB498630	Apolipoprotein Eb precursor	0.56	0.033
CA054530	UNKNOWN	0.55	0.043
CK990320	UNKNOWN	0.54	0.002
CB510414	PREDICTED: similar to ankyrin 2,3/unc44 [Strongylocentrotus purpuratus]	0.47	0.024
CB500684	Collagen alpha-1(I) chain precursor	0.44	0.009
CA053722	UNKNOWN	0.42	0.040
CA062494	Extracellular matrix protein 1 precursor	0.37	0.049
CA046687	Oncorhynchus kisutch glycoprotein hormone alpha 1 subunit mRNA, partial cds	0.35	0.007
CB488520	Anaphase-promoting complex subunit CDC26	0.35	0.012

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

Complete list of differentially expressed genes from gills of *Loma salmonae* infected fish sampled at 12 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Gill - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA062845	UNKNOWN	2.19	0.049
CA056199	Diamine acetyltransferase 1	1.96	0.039
CA062902	Reticulon-3	1.93	0.001
CB502577	Glutamine synthetase	1.92	0.007
CB512278	Transposable element Tcb1 transposase	1.83	0.001
CA058090	Tropomyosin beta chain	1.74	0.049
CB494195	Reticulon-3	1.73	0.020
CK991089	H-2 class II histocompatibility antigen, I-E beta chain precursor	1.72	0.049
CA055946	L-lactate dehydrogenase B chain	1.72	0.012
CB498950	Basigin precursor	1.66	0.019
CA040969	UNKNOWN	1.64	0.021
CA040013	UNKNOWN	1.64	0.028
CA042684	Eukaryotic translation initiation factor 5	1.60	0.044
CB490715	ATP-dependent DNA helicase 2 subunit 1	1.59	0.032
CB489874	TIM21-like protein, mitochondrial precursor	1.57	0.010
CA056760	Salmo trutta single nucleotide polymorphism (SNP)	1.57	0.032
CB494032	Carbonic anhydrase	1.55	0.005
CA062112	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	1.54	0.044
CA057695	UNKNOWN	1.54	0.044
CA059348	similar to Genbank # Accession Number AB073376 non-LTR retrotransposable element partially supported by	1.53	0.026
CA060152	Elongation factor Tu, mitochondrial precursor	1.53	0.033
CA042983	Diamine acetyltransferase 1	1.52	0.026
CA050741	UNKNOWN	1.52	0.047
CB493960	Heat shock protein HSP 90-beta	1.51	0.029
CA059723	ORF2-encoded protein [Danio rerio]	1.50	0.000
CA042135	Membrane-spanning 4-domains subfamily A member 12	1.50	0.009
CN442516	Beta-2-microglobulin precursor	1.49	0.001
CB513914	UNKNOWN	1.48	0.035
CB506201	Ferritin, middle subunit	1.48	0.020
CA060239	Caspase-8 precursor	1.48	0.037
CB498222	Actin, alpha cardiac	1.47	0.018

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA039925	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	1.47	0.047
CA060085	UNKNOWN	1.47	0.017
CB505581	Transcriptional activator protein Pur-alpha	1.46	0.008
CA054305	UNKNOWN	1.45	0.000
CB517566	UNKNOWN	1.45	0.006
CA044026	BOLA class I histocompatibility antigen, alpha chain BL3-7 precursor	1.45	0.004
CB510468	Ubiquitin	1.45	0.015
CB517910	Serine/threonine-protein kinase Nek6	1.44	0.034
CB498325	UNKNOWN	1.44	0.008
CB499836	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase PTEN	1.44	0.024
CA064431	UNKNOWN	1.43	0.006
CA052144	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	1.43	0.003
CB497797	Vacuolar ATP synthase 16 kDa proteolipid subunit	1.43	0.009
CA045434	UNKNOWN	1.43	0.013
CA058956	UNKNOWN	1.42	0.002
CB490012	UNKNOWN	1.42	0.002
CA769653	UNKNOWN	1.41	0.013
CA050346	Oncorhynchus mykiss genes, MHC class I a region, complete and partial cds	1.41	0.004
CA056070	UNKNOWN	1.40	0.015
CB492180	UNKNOWN	0.70	0.049
CA057000	UNKNOWN	0.69	0.037
CA052881	TGF-beta-inducible nuclear protein 1	0.69	0.043
CB500232	UNKNOWN	0.68	0.038
CB516924	Myosin light polypeptide 6	0.68	0.001
CA037944	Plasma retinol-binding protein I	0.68	0.020
CA041280	UNKNOWN	0.68	0.021
CB510540	Claudin-4	0.67	0.043
CB512340	Elongation factor 1-delta	0.66	0.031
CB499401	UNKNOWN	0.66	0.015
CB510525	Guanine nucleotide-binding protein G(t) subunit alpha	0.66	0.012
CB493147	Sesbania drummondii clone SSH-1_01_F12_T3 mRNA sequence	0.66	0.023

Appendix 2 - Differentially expressed genes from gills of *Loma salmonae*-infected Chinook salmon

		Gill - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA063705	UNKNOWN	0.66	0.005
CA054747	WD repeat domain 52, isoform CRA_a [Homo sapiens]	0.66	0.006
CB493233	39S ribosomal protein L28, mitochondrial precursor	0.63	0.034
CA057617	Surfeit locus protein 4	0.63	0.030
CB489859	PREDICTED: similar to rhotekin-2 [Bos taurus]	0.62	0.021
CA050681	UNKNOWN	0.62	0.003
CB504002	40S ribosomal protein S18	0.61	0.019
CA038049	UNKNOWN	0.60	0.001
CA769898	40S ribosomal protein S26	0.60	0.025
CA058854	Mon2 protein [Mus musculus]	0.60	0.047
CA060189	UNKNOWN	0.59	0.044
CA042766	smart00034, CLECT, C-type lectin (CTL) or carbohydrate-recognition domain (CRD);	0.52	0.014
CB512193	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.51	0.032
CA054618	Peroxiredoxin-1	0.49	0.049
CB497076	CCAAT/enhancer-binding protein delta	0.49	0.018

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

Complete list of differentially expressed genes from kidney of *Loma salmonae* infected fish sampled at 12 weeks post-exposure (PE). Normalized levels were expressed as ratios between glucan-inoculated and control fish. Genes with a relative expression level of 1.4-fold (up or down) and significance of $p \leq 0.05$ were included (t-test p-value).

		Kidney - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA055344	UNKNOWN	7.13	0.030
CA053221	Adipophilin	5.35	0.004
CB509876	Gamma crystallin M2	5.15	0.038
CA052297	Coagulation factor X precursor	4.38	0.042
CB497763	Complement C3-1	4.33	0.027
CK990487	CD59-like protein 2 [Oncorhynchus mykiss]	3.67	0.044
CA064171	Interferon-induced protein 44	3.51	0.002
CB500977	Interferon regulatory factor 7	3.22	0.031
CB502142	WD repeat protein 37	2.92	0.006
CB515650	UNKNOWN	2.90	0.006
CB510421	Deoxyribonuclease gamma precursor	2.89	0.005
CB491596	Retrotransposable element Tf2 155 kDa protein type 1	2.72	0.040
CA050861	Focal adhesion kinase 1	2.70	0.021
CB510272	PREDICTED: Danio rerio titin-like (ttnl), mRNA	2.61	0.046
CA044122	2-acylglycerol O-acyltransferase 2-A	2.31	0.043
CB494267	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	2.28	0.042
CB515751	UNKNOWN	2.25	0.012
CB490489	NADH-ubiquinone oxidoreductase chain 1	2.23	0.024
CB501073	Dynein light chain 2, cytoplasmic	2.21	0.030
CA058181	ATP synthase mitochondrial F1 complex assembly factor 2, mitochondrial precursor	2.18	0.037
CB510230	UNKNOWN	2.12	0.001
CB491548	egg envelope component ZPAX [Xenopus laevis]	2.12	0.028
CA047260	Secretogranin-3 precursor	2.08	0.034
CB492603	Alpha-actinin-2	2.02	0.032
CK990365	Glutathione reductase, mitochondrial precursor	2.01	0.002
CB514960	UNKNOWN	1.98	0.036
CB507899	Endothelial lipase precursor	1.95	0.009
CB514188	Collagen alpha-2(VI) chain precursor	1.90	0.023
CB488430	Eukaryotic translation initiation factor 4E type 2	1.89	0.005
CK990848	Insulin-like growth factor-binding protein 6 precursor	1.87	0.020

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CB502991	BTB/POZ domain-containing protein 6	1.86	0.005
CA052198	Pre-mRNA-processing factor 39	1.86	0.028
CK990747	UNKNOWN	1.85	0.001
CA058263	viperin [Oncorhynchus mykiss]	1.83	0.007
CA057541	RNA polymerase II subunit A C-terminal domain phosphatase SSU72	1.83	0.037
CA039573	Polypyrimidine tract-binding protein 2	1.83	0.049
CB500823	Salmo salar dsRNA activated Z-DNA binding protein kinase (PKZ) mRNA, complete cds	1.80	0.008
CA056901	UNKNOWN	1.79	0.017
CK991203	Adseverin	1.79	0.026
CB516447	G/T mismatch-specific thymine DNA glycosylase	1.76	0.014
CB517743	Salmo salar aryl hydrocarbon receptor 2 gamma (AhR2g) mRNA, complete cds	1.75	0.005
CA047310	UNKNOWN	1.74	0.022
CA037319	UNKNOWN	1.74	0.012
CB496571	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	1.73	0.033
CB507142	UNKNOWN	1.73	0.022
CA059009	UNKNOWN	1.73	0.015
CA037782	UNKNOWN	1.72	0.018
CB511934	Sorting and assembly machinery component 50 homolog	1.70	0.041
CB508304	rRNA-processing protein FCF1 homolog	1.69	0.010
CB515885	Methionine aminopeptidase 1	1.66	0.001
CA769238	UNKNOWN	1.65	0.023
CB514361	Danio rerio insulin-like growth factor binding protein 5, mRNA (cDNA clone MGC:76889 IMAGE:6520159), complete cds	1.65	0.041
CA770384	PAB-dependent poly(A)-specific ribonuclease subunit 2	1.64	0.032
CA062062	Hyccin	1.62	0.003
CB510906	UNKNOWN	1.61	0.030
CB511896	Mitochondrial import inner membrane translocase subunit Tim17-A	1.61	0.037
CA061844	15 kDa selenoprotein precursor	1.61	0.008
CA046002	UNKNOWN	1.61	0.035
CB497626	SRA stem-loop-interacting RNA-binding protein, mitochondrial precursor	1.61	0.035
CB514560	Factor VIII intron 22 protein	1.60	0.020
CB509935	type II keratin E2 [Oncorhynchus mykiss]	1.58	0.047
CA063099	UNKNOWN	1.58	0.049
CB515631	Protein-tyrosine sulfotransferase 2	1.57	0.043
CB493199	alpha1-microglobulin/bikunin precursor (AMBP) [Xenopus laevis]	1.57	0.016
CB494269	Acidic mammalian chitinase precursor	1.57	0.020

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA062784	Poly [ADP-ribose] polymerase 14	1.55	0.035
CA041811	Transducin beta-like 2 protein	1.55	0.007
CA056026	Transmembrane protein 87A precursor	1.54	0.014
CB496633	39S ribosomal protein L9, mitochondrial precursor	1.54	0.017
CA052177	UNKNOWN	1.54	0.037
CB514413	UNKNOWN	1.53	0.006
CB500788	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	1.51	0.013
CB500418	Far upstream element-binding protein 3	1.51	0.016
CA061524	Echinoderm microtubule-associated protein-like 3	1.51	0.008
CK990542	UNKNOWN	1.48	0.027
CA062203	UNKNOWN	1.48	0.010
CA054410	Derlin-1	1.48	0.007
CA043395	DCN1-like protein 5	1.47	0.044
CB515424	UNKNOWN	1.46	0.028
CB507385	Metalloproteinase inhibitor 2 precursor	1.45	0.017
CA053479	Mitochondrial ribosomal protein S23	1.44	0.018
CA060347	Ribokinase	1.44	0.042
CA063133	GA-binding protein alpha chain	1.43	0.016
CB510330	Exosome complex exonuclease RRP46	1.42	0.011
CA051480	leukocyte immune-type receptor 3 [Ictalurus punctatus]	1.42	0.001
CB506255	Organic cation/carnitine transporter 2	1.41	0.036
CA041405	Ubiquinone biosynthesis protein COQ9, mitochondrial precursor	1.41	0.015
CB493062	Alcohol dehydrogenase class 3	1.40	0.008
CA042578	ATP synthase delta chain, mitochondrial precursor	0.70	0.041
CB509722	Glutathione peroxidase 3 precursor	0.70	0.026
CA057016	NF-kappa-B-repressing factor	0.70	0.038
CA050278	Acyl-protein thioesterase 2	0.69	0.021
CA056159	Arrestin domain-containing protein 2	0.69	0.013
CA063757	WD repeat protein 82	0.69	0.005
CA058076	Ribosomal L1 domain-containing protein 1	0.69	0.030
CA044186	UNKNOWN	0.69	0.030
CA056779	Ig kappa chain V-I region Walker precursor	0.69	0.008
CA040681	Oncorhynchus mykiss SYPG1 (SYPG1), PHF1 (PHF1), and RGL2 (RGL2) genes, complete cds; DNaseII pseudogene, complete sequence; LGN-like, PBX2 (PBX2), NOTCH-like, TAP1 (TAP1), and BRD2 (BRD2) genes, complete cds; and MHCII-alpha and Raftlin-like pseudogenes, complete sequence	0.68	0.032
CB510049	Heat shock cognate 70 kDa protein	0.68	0.042
CB516593	UNKNOWN	0.68	0.016

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA057721	DNA fragmentation factor subunit beta	0.68	0.042
CA052649	UNKNOWN	0.67	0.024
CB510503	Oncorhynchus mykiss mRNA for type II keratin E1 (E1 gene)	0.67	0.024
CA053957	UNKNOWN	0.67	0.037
CB515401	PREDICTED: Pan troglodytes synaptotagmin I, transcript variant 11 (SYT1), mRNA	0.67	0.016
CA056691	Methylglutaconyl-CoA hydratase, mitochondrial precursor	0.67	0.029
CK990926	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	0.67	0.027
CA053813	Ig kappa chain V-III region MOPC 63 precursor	0.67	0.032
CA058612	Uncharacterized protein YOR154W precursor	0.67	0.016
CA043986	UNKNOWN	0.66	0.021
CA056972	Importin subunit alpha-2	0.66	0.023
CB498109	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related	0.66	0.018
CA058844	Stabilin-2 precursor	0.66	0.027
CA050328	UNKNOWN	0.66	0.017
CB516919	Extracellular matrix protein 1 precursor	0.66	0.002
CB498385	PREDICTED: similar to expressed sequence AV312086 [Canis familiaris]	0.65	0.001
CA051091	Regulator of G-protein signaling 1	0.65	0.011
CA046109	60S ribosomal protein L4-B	0.64	0.037
CA063277	Glutamine synthetase	0.64	0.044
CA061001	Astatotilapia burtoni early growth response 1 mRNA, complete cds	0.63	0.036
CA052211	Decorin precursor	0.63	0.000
CB516972	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial precursor	0.63	0.038
CA770277	Proteasome subunit alpha type 7	0.63	0.022
CA055216	Zinc finger and SCAN domain-containing protein 2	0.63	0.035
CA064165	Protein disulfide-isomerase A4 precursor	0.63	0.010
CB514296	UNKNOWN	0.62	0.010
CB509655	Myosin heavy chain, fast skeletal muscle	0.62	0.010
CA052744	Large neutral amino acids transporter small subunit 2	0.62	0.046
CB512110	Ig kappa chain V-IV region JI precursor	0.62	0.023
CB517724	Sodium/potassium-transporting ATPase subunit alpha-1	0.61	0.008
CA053129	High mobility group-T protein	0.61	0.000
CB511609	Cathepsin L precursor	0.61	0.003
CK990239	UNKNOWN	0.61	0.020
CB515142	putative nucleic acid binding protein [Paralichthys olivaceus]	0.60	0.004
CB514524	DNA replication licensing factor mcm5	0.60	0.032

Appendix 2 - Differentially expressed genes from kidney of *Loma salmonae*-infected Chinook salmon

		Kidney - 12 weeks PE	
Genbank #	**Description**	**Normalized**	**t-test P-value**
CA051200	UNKNOWN	0.60	0.030
CA048126	NEDD4 family-interacting protein 2	0.59	0.003
CA054110	Tether containing UBX domain for GLUT4	0.59	0.025
CA045270	U3 small nucleolar ribonucleoprotein protein IMP4	0.58	0.031
CA043100	Vacuolar protein sorting-associated protein 33B	0.58	0.026
CA063639	Seryl-tRNA synthetase, mitochondrial precursor	0.56	0.011
CB502503	Cathepsin L precursor	0.56	0.000
CA058311	Heterogeneous nuclear ribonucleoprotein A/B	0.56	0.030
CA060888	UNKNOWN	0.55	0.049
CA053610	Stathmin-3	0.55	0.045
CB516701	Interleukin-1 receptor-like 1 precursor	0.54	0.010
CA057815	Tissue factor pathway inhibitor 2 precursor	0.53	0.029
CK991353	Dipeptidyl-peptidase 1 precursor	0.52	0.029
CA050544	Selenium-binding protein 2	0.51	0.032
CA055438	BarH-like 2 homeobox protein	0.50	0.027
CA063487	Splicing factor, arginine/serine-rich 9	0.49	0.025
CA768033	Coatomer subunit alpha	0.49	0.046
CB507685	Mitofusin-1	0.44	0.001
CB502684	Ependymin precursor	0.39	0.021
CA060828	UNKNOWN	0.39	0.032
CA060167	3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	0.34	0.031