

Molecular characterisation of key components of the mucosal immune system in Atlantic salmon (*Salmo salar* L) and transcriptome analysis of responses against the salmon louse (*Lepeophtheirus salmonis*)

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2012

Acknowledgements

The work presented in this thesis is part of a larger project entitled ‘Salmon louse genome sequencing and functional studies on host parasite interactions’, funded through the Functional Genomics (FUGE) platform of the research council of Norway. First of all, I would like to express my heartfelt gratitude to my main supervisor Ivar Hordvik. Words are not enough but to say it briefly: I thank you for your tireless day-to-day guidance on all aspects of my PhD work. My appreciation also goes to co-supervisor and project leader, Frank Nilsen, for his guidance and support. I want to thank all co-authors of the papers included in this thesis for their contributions: I have greatly benefited from discussions and collaboration with you guys, and especial thanks to Aleksei Krasnov for your constructive guidance during the write-up of paper III. The one month time I spent at Nofima was marvelous, packed with intensive laboratory work and lots of fun. Thanks to Linda Andersen: you always had the way out whenever I am lost. I also greatly appreciate people at the Molecular and Computational Biology (MCB) research school, for helping me actively involve with, and for funding several short courses, including a 5 day workshop in Spain. I also thank Paul Thomas for reading through the synopsis.

The office tea/coffee time I had with Fekadu, cooking experiments with Kiniena, and the ski and boat trip adventures and seminars with STIM students, are among the unforgettable experiences.

Last but not least, I would like to thank my family and friends for their prayer and support.



Tariku Markos Tadiso

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Abstract

Mucosal immunity in mammals is mediated mainly by secretory immunoglobulin A (SIgA), which is produced by IgA plasma cells commonly located in the lamina propria, and a transport system involving the polymeric Ig receptor (pIgR). In teleost fish, IgM plays some roles associated with mucosal defence. Very recent findings indicate that IgT, an antibody exclusive to teleosts, might have a special role in mucosal immune responses, and a possible pIgR counterpart has been identified. The salmon louse (*Lepeophtheirus salmonis* Krøyer), an ectoparasitic copepod targeting the skin (and the gill to a lesser extent), has been a major challenge to the aquaculture industry. While the first line of defence against this parasite is crucial, equally important, in the context of vaccine development is the generation of information on the adaptive immune system. Based on this line of reasoning, Atlantic salmon IgT and pIgR were selected as targets for further characterization in the present study. Three distinct IgT heavy chain (τ) sub-variants, with an identity index of 76-80%, were described. The identity index between $\tau 1$ and $\mu 1$ (the first constant domains of the IgT and IgM heavy chains, respectively) in Atlantic salmon is 52%. It is plausible to assume that this relatively high similarity is a result of interactions with common light chains. The relative abundance of τ , μ , and δ transcripts in a series of tissues revealed an overall expression pattern of IgM \gg IgT $>$ IgD. Interestingly, challenge experiments with salmon louse showed 10 fold increase of IgM and IgT mRNA in skin samples, supporting the assumption that these antibodies are involved in mucosal immune responses. The search for pIgR homologues in Atlantic salmon resulted in two pIgR-like candidates: Salsal pIgR and Salsal pIgRL. Meanwhile, a comparative evaluation was made to a series of CD300-like molecules (CMRF-35 like molecules, CLM) reported to the databanks. Salsal pIgR and Salsal pIgRL were identified on the basis of similarity to homologous genes, and like the counterparts in other teleosts they are composed of two Ig superfamily (IgSF) V-like domains, a transmembrane region, a connecting peptide, and a cytoplasmic tail. Two CD300-like molecules in salmon (CLM1 and CLM7) also have the same domain structure, but their cytoplasmic region is predicted to contain putative immunoreceptor tyrosine based inhibition motifs (ITIM), which is a typical feature of CD300A and CD300F in humans. While Salsal pIgR and Salsal pIgRL were expressed in tissues of skin and gill respectively, their expression pattern is not restricted to mucosal tissues, but notably, their expression increased during infection with salmon louse. Further studies are needed to elucidate the transport mechanisms of mucosal antibodies in

salmon. To investigate how the salmon louse evades the immune system of the host, temporal immune gene expression changes in skin, spleen, and head kidney of Atlantic salmon were analysed using microarray and quantitative real-time PCR (qPCR) during the first 15 days post infection (dpi). This window represents the copepodid and chalimus stages of lice development. Transcriptomic responses, recorded already at 1 dpi, were highly complex and large by scale. Many genes showed bi-phasic expression profiles with abrupt changes taking place between 5 and 10 dpi (the copepodid-chalimus transitions). Large group of secretory splenic proteases with unknown roles showed the greatest fluctuations: up-regulated 1-5 dpi and markedly down regulated afterwards. T cell related transcripts showed a short term (1-5 dpi) increase. After 5 dpi, the magnitude of transcriptomic responses decreased markedly in skin. The findings provided an insight into the time windows in the development of the parasite which are critical to the host and where modulation of the host immune system might occur.

List of publications

Paper I

Tadiso T. M., Lie K. K. and Hordvik I. (2011). Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues. *Vet. Immunol. Immunopathol.* **139**, 17-26.

Paper II

Tadiso, T. M., Sharma, A, Hordvik I. (2011). Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon. *Mol. Immunol.* **49**, 462-473.

Paper III

Tadiso T. M., Krasnov A., Skugor S., Afanasyev S., Hordvik I. and Nilsen F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics* **12**, 141.

Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
CDR	Complementarity determining region
CLM	CMRF-35 like molecule
CMS	Cardiomyopathy syndrome
CTL	Cytotoxic T-lymphocyte (s)
D	Diversity (-gene segment)
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
Fc	Fragment crystallizable of Ig
FcR	Fc receptor
Fc μ R	Fc receptor for IgA and IgM
GALT	Gut-associated lymphoid tissue
H	Heavy chain (of Ig)
HSMI	Heart and skeletal muscle inflammation
hu	Human
ICAM	Intracellular adhesion molecule
IEL	Intraepithelial lymphocytes
IFN	Interferon
IMGT	International immunogenetics information system
Ig	Immunoglobulin
IL	Interleukin
ISA	Infectious salmon anemia
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
J	Joining (chain)
kDa	Kilodalton (mol. mass)
L	Light chain (of Ig)

LPS	Lipopolysaccharide
m	Membrane
MAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MDIR	Modular domain immune type receptor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
n	Number in study or group
NA	Not available
NK	Natural killer (cell)
PCR	Polymerase chain reaction
pIgR	Polymeric immunoglobulin receptor
qPCR	Quantitative real-time PCR
RhoG	RAS homologue member G
RAG	Recombination-activating gene
RNA	Ribonucleic acid
RNAi	RNA interference
s	Secreted
S	Secretory
SC	Secretory component
SD	Standard deviation
SEM	Standard error of the mean
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
V	Variable (region, gene segment)
V _H , V _H	Variable region, heavy chain
V _L , V _L	Variable region, light chain

1. Introduction

1.1. Background

The development of comparative genomics, particularly comparative immunology, enormously shaped the investigative approaches that we follow to study the immune system of fish. The field has developed rapidly during the era of gene technology, greatly benefitted from being organised through the international societies of Developmental and Comparative Immunology (ISDCI) and European Organisation of Fish Immunology (EOFFI). The completion of genome sequencing for several organisms has prompted a new era, as elegantly stated by the Nobel laureate Sydney Brenner as ‘The End of the Beginning’ (Brenner, 2000). This ‘beginning’, however, has not yet ended for some important fish species, and there is also a need for systematizing and characterizing immune genes.

Achieving effective protection against pathogens demands an understanding of the immune system of the host and the host pathogen interactions therein. This requires that one must be able to measure or quantify immune related molecules and cells. In the aquaculture industry, there is a need to develop methods for effective monitoring and control of pathogens at low cost to the environment; such as the development of effective vaccines to pathogens that can wholly or partially replace therapeutic treatments. Availability of well-characterised components of the immune system is thus a prerequisite to measure host responses (or to study any other physiological processes per se). Even though a wealth of knowledge is available on well characterised immune genes in fish, there is a need to broaden the spectrum of these elements and therefore one of the objectives of this project was in-parallel characterisation of relevant immune genes, with emphasis on mucosal immunity.

The mucosal immune system protects the body from first-encounter of pathogens, and holds a central place in protective immunity. In mammals, mucosal immune protection is mediated mainly by secretory immunoglobulin A (SIgA)¹ produced by IgA plasma cells commonly located in the lamina propria, and transported into the lumen by the polymeric immunoglobulin receptor (pIgR) (Brandtzaeg et al., 2008; Fagarasan et al., 2010; Kaetzel,

¹ To comply with mucosal immunology nomenclature (Brandtzaeg et al., 2008), a distinction has been made between secretory (denoted by capital letter S) and secreted (s) forms of Igs throughout this thesis.

2005; Woof and Mestecky, 2005). Whereas the intestine occupies a central place in fish mucosal immunology, as a non keratinised mucosal surface, the skin represents a vital defence element of teleost fish, as the aquatic habitat continuously exposes them to a broad spectrum of pathogens, compared to organisms living in the aerial environment (Rakers et al., 2010; Rombout and Joosten, 1998). Despite the fact that there is substantial knowledge in mucosal immunity in fish, much work awaits investigation especially with regard to the key molecules involved and their transport mechanisms. As an economically important species, insight into the mucosal immune system of Atlantic salmon is paramount, as it can aid in understanding and prevention of infection at mucosal surfaces. This study thus examined candidate genes in mucosal immunity and immune response to cutaneous mucosal infection.

1.2. Tools and current knowledge in the study of the fish immune system

A key to understanding the immune system of an organism is to be able to assess/measure it. This can be achieved for example by infection or vaccine experiments, utilizing tools that monitor changes in the expression of key molecules. Accordingly, availability of well-characterised genes is usually a prerequisite. Nevertheless, until now, not enough sequence data (genome sequencing and proper annotations) are available for most fish species and thus, current studies in parallel ought to clone and characterise immune relevant genes and provide a basis for future studies. One of the objectives of this PhD project was thus to broaden the spectrum of characterised immune genes in Atlantic salmon. In recent developments, a number of immune effector molecules have been described in teleosts in general and Atlantic salmon in particular. Researchers at our laboratory (including the present work) have characterised a panel of immune related genes, mainly of T cell receptors & co-receptors and immunoglobulins and associated molecules. However, what remains a bottleneck is the lack of established systems and tools corresponding to that of the mammalian systems, for functional studies of the fish immune system. There is lack of specific antibodies against suitable markers, for example, to distinguish sub-populations of lymphocytes. Another major concern is the fact that teleosts are very heterogeneous group, and knowledge obtained on a given species cannot be directly transferred to another: requiring species-specific research.

Study of the temporal and spatial gene expression patterns of biological samples in naïve conditions and in response to stimuli is becoming a routine and compulsory activity for monitoring disease state. To this end, microarrays and qPCR techniques have become appropriate technologies: by making use of multiple gene expression profiling capabilities of microarrays and quantitative performance of qPCR. Transcriptomic data, however, may not always be related to functional information (based on the mammalian model), as one may not know how a given transcript ends up being translated. In this study, an oligonucleotide microarray platform, called STARS (Salmon and Trout Annotated Reference Sequences) was used for gene expression profiling. This platform was specifically designed for the two important salmonid fish Atlantic salmon and rainbow trout, based on the Agilent Technology (Krasnov et al., 2011). While microarray has been used in **paper III** for global gene expression profiling of salmon responses, qPCR has been ubiquitously used in all studies: to determine the relative abundance of the various transcripts in different tissues (**Paper I**, and **Paper II**), to test the abundance of a given transcript in relation to uninfected control (**Paper II** and **Paper III**), and to validate microarray data (**Paper III**).

1.3. Atlantic salmon: a 3D motive to study its immune system

Atlantic salmon is a major contributor to the growing fisheries and aquaculture industry in many countries. In Norway, it takes the lion's share of seafood export. In 2009, it had an export value of 23.6 billion Norwegian kroner (NOK) that exceeded the combined value of all other marine products, which is 21 billion NOK (Norwegian Sea food Export Council, 2010). Moreover, Atlantic salmon has been recognised as an important model system in evolutionary and conservation biology (Garcia de Leaniz et al., 2007). The fact that this precious species has a unique place in the phylogeny makes it a suitable species for comparative studies: their relatively recent genome duplication (and duplicated Ig heavy chain loci) can be mentioned among others (Koop et al., 2008; Yasuike et al., 2010). Atlantic salmon has also been recognised as a potential model for medical research (Majalahti-Palviainen et al., 2000).

1.4. Innate and adaptive immune recognition

The ability of an organism to resist or minimise the impacts of pathogens depends on protective immune responses, which can be both innate and adaptive: the two major systems of immune recognition in mammals and other vertebrates. Immune cells (white blood cells or leukocytes) mediating these reactions are formed from precursor cells (called pluripotent hematopoietic stem cells) in the bone marrow (or most likely the head kidney in teleost fish), which give rise to the myeloid or lymphoid progenitor cells of the innate and adaptive immune system, respectively. Lymphocytes are further divided into B cells (immunoglobulin-positive) and T cells. Innate immunity constitutes the first line of defence against invaders, while adaptive immunity provides a more rigorous immune defence at later stages (and increases with repeated exposure to a particular antigen), as it remembers specific pathogens. A common feature of innate and adaptive immune responses is that they both involve immune receptors, which recognise pathogens. However, the receptor types used to recognise pathogens are distinct (Medzhitov, 2007).

The innate immune system senses pathogens via genetically pre-encoded receptors called pattern recognition receptors (PRR), which are evolved to recognise specific signals common to large groups of microorganisms (Janeway and Medzhitov, 2002). These signals are small molecular motifs conserved within a class of microbes and are referred to as pathogen associated molecular patterns (PAMPs). Toll-like receptors play a major role in pathogen recognition and initiation of inflammatory and immune responses (Janeway and Medzhitov, 2002). PRR activate conserved host defence signalling pathways that control the expression of a variety of immune response genes (Medzhitov and Janeway, 2000). In addition to the usual pathogen associated ligands, several damage-associated molecular patterns (DAMPs) have recently been shown to have immunological importance (Nace et al., 2012). Phagocytes respond to endogenous molecules derived from proteolytic degradation of substances such as collagen, which signal the presence of damage (alarm) via DAMPs. They are also referred to as alarmins (Oppenheim and Yang, 2005). They are part of the extracellular matrix (ECM), which, in addition to provision of structural support and adhesive substrates for the body tissues, has a key role in innate immunity and inflammation (Castillo-Briceno et al., 2009; Pacifici et al., 1991). The resulting proteolytic fragments of collagen after cleavage (for example by MMPs) will activate phagocytes. The role of ECM in serving as hazard signal

has been shown recently in teleost fish, where both collagen and gelatin increased the production of ROS by seabream (Castillo-Briceno et al., 2009). This is particularly important in anti-parasitic defence at cutaneous mucosal surfaces: e.g. cleaved transferrin during sea lice infection is implicated in NO mediated response of salmon macrophages (Easy and Ross, 2009). Inflammatory reactions can be cellular such as phagocytosis and phagocyte activity (including oxidative mechanisms), complement activity: are modulated by many fish parasites, including mainly ciliates, flagellates and myxozoans (Alvarez-Pellitero, 2008). Humoral immune factors (peroxidases, lysozyme, acute-phase proteins) are also implicated in the response to some parasites (Alvarez-Pellitero, 2008; Jones, 2001). B lymphocytes from fish also have potent phagocytic and microbicidal abilities (Li et al., 2006; Zhang et al., 2010).

In adaptive immune recognition, two types of antigen receptors are involved: T cell receptors and B cell receptors. The genes encoding for these receptors are assembled from variable and constant gene fragments in each T or B lymphoid cell through recombination-activating gene (RAG)-protein-mediated somatic recombination (Schatz et al., 1992). Combinatorial and junctional diversification leads to high number of different receptors (Danilova and Amemiya, 2009). Production of a diverse repertoire of receptors, with the potential to recognise almost any antigen, is a result of this process (Schatz et al., 1992).

Key components of the adaptive responses include B lymphocytes, T lymphocytes, and antigen presenting cells. Antibodies, produced by B cells, interact directly with antigens. The T cell receptor, the antigen binding molecule expressed by T cells, on the other hand, recognizes antigens processed and presented by the major histocompatibility complex (MHC) molecules. There are two subpopulations of T cells. T helper cells (which harbor CD4 membrane glycoprotein on their surface) play a crucial role by secreting cytokines and activating B cells; and cytotoxic T cells (displaying CD8) kill target cells (eg. virus infected cells). Clark and Ledbetter (1994) described how B and T cells interact for effective immune protection. Specific recognition of foreign antigen by cell surface Ig induces B cells to proliferate and differentiate into plasma cells (producing soluble Ig to fight infection) or memory B cells which function during repeated challenge with the same antigen (this constitutes the basis for vaccination). This process requires help from T cells: Once activated

by interaction with specific Ag presenting cells, T cells activate B cells by releasing cytokines such as interleukins (Clark and Ledbetter, 1994).

The basic mechanisms and molecular components of immunity in fish and mammals are similar especially in macrophage function, lymphocyte stimulation and characterised humoral factors such as antibodies (Rombout and Joosten, 1998). However, the structure and diversity of immunoglobulin (Ig) genes constitute a major source of variation. While IgG, IgE, and IgA are immunoglobulin classes present in mammals, but not in fish, the newly discovered IgT/Z isotype appear to be specific for teleosts (Danilova et al., 2005; Hansen et al., 2005). IgD and IgM are common in both. The absence of IgG and IgA and their functional equivalents in fish remains an immunological challenge. The biological function of IgD and IgT in teleosts is not clear, except for a recent study in rainbow trout, which claimed IgT to be a mucosal Ig, functioning similarly to the mammalian IgA (Zhang et al., 2010).

Innate and adaptive immune systems are not mutually exclusive, and both work together to enhance effective protection. Proper stimulation of innate effector molecules enhances generation of effective adaptive immunity. Most importantly, the innate immune system has an essential role in the clonal selection of lymphocytes and activation of the adaptive immune responses, whereas the adaptive immune system, in turn, activates innate effector mechanisms in an antigen specific manner (Medzhitov, 2007; Medzhitov and Janeway, 1998). In teleost fish, given the possibility that most fish pathogens can damage their hosts in relatively short period of time (before the building-up of adaptive immunity), the importance of innate defences become of crucial importance (Ellis, 2001; Jones, 2001). It has, however, been emphasised that the innate immune system in vertebrates has been evolved to depend, to some extent, on antigen-specific (adaptive) immunity (e.g. for pathogen clearance) (Medzhitov, 2007; Unanue, 1997).

1.5. The mucosal immune system

1.5.1. Mucosal surfaces

The body's mucosal surfaces are in a continuous direct contact with the environment. In mammals, the gastrointestinal, respiratory, genital, and urinary surfaces comprise major mucosal organs (Kraehenbuhl and Neutra, 1992). For example, the gut mucosa continuously interacts with food antigens, the gut commensal bacteria, and potential pathogens that enter the host through the intestine (Lambolez and Rocha, 2001). However, there are considerable morphological and functional differences between fish and mammals with regard to mucosal immunity (Bernard et al., 2006; Rombout et al., 2011). Little evidence is available on the presence of inductive sites (antigen capture and presentation) such as the Peyer's patches, sites of antibody production, and organised mucosa associated lymphoid tissues (O-MALT). Despite this, the fish possess a well functioning mucosal immune system (recently reviewed in (Rombout et al., 2011; Salinas et al., 2011)). Whereas the intestine holds a central place in both cases, equally important in bony fish are the gills and the skin as mucosal organs (Press and Evensen, 1999). In contrast to a keratinised skin of mammals, the fish skin is a mucosal organ (Fig. 1a-b), possessing a number of mucus producing goblet cells (Rakers et al., 2010). Compared to organisms living in the aerial environment, the aquatic habitat continuously exposes them (via their skin and gills) to a broad spectrum of pathogens (Rakers et al., 2010; Rombout and Joosten, 1998).

1.5.2. Immune mechanisms at mucosal surfaces of teleost fish

By forming a major barrier, the gut epithelium and its mucous layer trap invading pathogens which are then eliminated when the gut epithelium is shed (Lambolez and Rocha, 2001). Similarly, the skin mucus in fish entraps microorganisms and hinders their entry into the body; the mucus is continuously produced and eliminated carrying away the entrapped pathogens (Ourth, 1980). In the gut, local defence depends partly on T lymphocytes called intraepithelial lymphocytes (IELs), often generated locally, not in the thymus (Saito et al., 1998). The presence of putative T cells or their precursors in the fish gut, together with the RAG-1 expression of intestinal lymphoid cells (Rombout et al., 2011; Rombout et al., 2005), supports this phenomenon. These T cells modulate homeostasis of the gut epithelium through local production of cytokines (Rombout et al., 2011). The development of intestinal

inflammation (enteritis) in the antigen transporting second gut segment and the presence IEL and eosinophils/basophils seem to play a crucial role in fish (Rombout et al., 2011).

The fish skin is a multi-purpose organ. In addition to serving as anatomical and physiological barrier against the external environment, it is also an entry point for many bacteria and viruses, and an important immunological organ. Cutaneous mucus, secreted by goblet cells in the epidermis, contains a number of immune factors. Fish skin serves as a source of pro-inflammatory molecules as well as an active modulator of the local inflammation (Gonzalez et al., 2007a; Gonzalez et al., 2007b). Innate humoral immune components described in fish skin mucus include lysozyme, peroxidases, acute-phase proteins, trypsin like proteases, alkaline phosphatase, and esterases (Alvarez-Pellitero, 2008; Jones, 2001; Palaksha et al., 2008). Many of these immune factors are implicated in the response to some parasites (Alvarez-Pellitero, 2008). Increased expression of interleukin-1b (IL-1b), interferon-g (IFN-g), IL-10 and infiltration of CD3-positive cells in the tail fin epidermis are among characteristic responses of highly susceptible salmon against the ectoparasite *Gyrodactylus salaricus* (Kania et al., 2010). The gill also represents a mucosal organ in fish. It is shown that Atlantic salmon gill tissues contain significant lymphoid accumulations (Haugarvoll et al., 2008; Koppang et al., 2010). Two studies indicated the presence of IgT positive cells in the epithelial lining of the gill lamellae, and IgT and IgM binding to surface structures of the parasite *Ichthyophthirius multifiliis* (Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011).

Fish has a secretory immune system, and can mount cutaneous mucosal immune responses, irrespective of levels in serum (Dickerson and Clark, 1998; Lobb, 1987; Lobb and Clem, 1981a, b; Ourth, 1980; Rombout et al., 1993). Intracutaneous Ab secreting B-lymphocytes represent an integral component of the fish immune system and confer protection against parasitic infections (Dickerson and Clark, 1998; Zhao et al., 2008). In defence against the ectoparasite *I. multifiliis* it has been depicted that tetrameric Abs in skin/mucus bind to parasite surface i-antigens eventually leading to the removal of the parasite (Dickerson and Clark, 1998; Lin et al., 1996). Relatively, more lymphocyte accumulation is expected deeper within the epithelium (Lobb, 1987), which requires some kind of transport. Recent studies show the presence of teleost specific immunoglobulin (IgT) specialised on mucosal defences (Zhang et al., 2010), and expression of a possible transcytosis receptor (pIgR) in mucosal tissues (Hamuro et al., 2007; Rombout et al., 2008). Even though the mechanism how

antibodies from secondary lymphoid tissues are brought into the mucus is not known, it is apparent that antibodies and mainly intraepithelial IgM positive lymphocytes (IEL) are shown to exist. Most importantly, expression of Abs increase considerably with infection or immunization (Lobb, 1987; Maki and Dickerson, 2003; Zhang et al., 2010; Zhao et al., 2008).

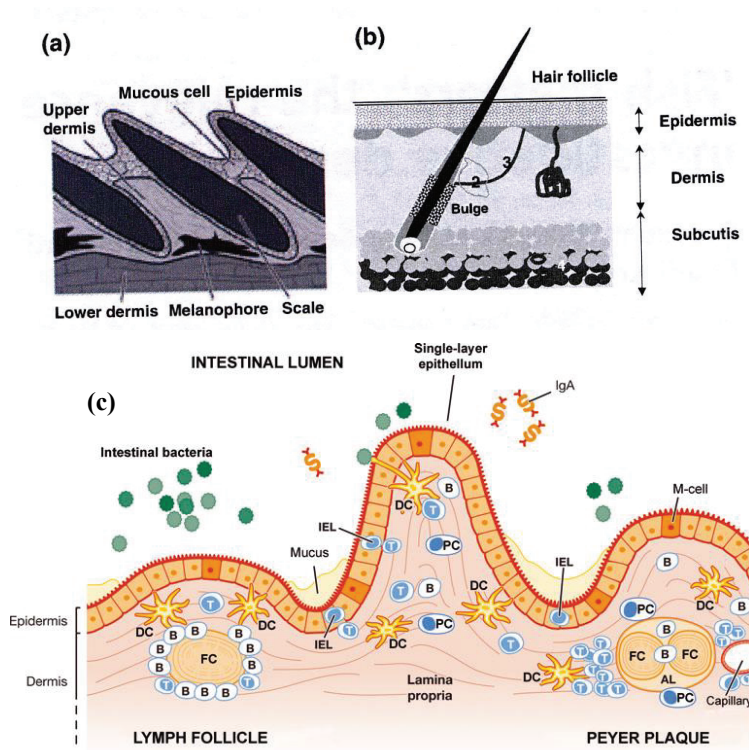


Fig. 1. (a) The structure of teleost skin with dermal scale (Original drawing by Matthias Emde, according to (Schempp et al., 2009)). b) Human skin. 1: sweat gland, 2: sebaceous gland, 3: arrector pili muscle, 4: blood vessel (Rakers et al., 2010). c) The immune system of the gut in human. Proliferative centers (lymph follicles, Peyer plaques) are located directly beneath the monolayer epithelium with high secretory function (production of mucus and IgA antibodies) (Original drawing: Matthias Emde, according to (Wolfe et al., 2009)).

1.5.3. Adaptive immunity at mucosal surfaces

When pathogens first attack mucosal surfaces, antigens (Ag) are sampled and transported across the mucosal epithelium. As lymphoid cells of mucosal tissues are separated from Ag by an epithelial barrier, Ag must be transported across the epithelium by specialised cells. In

mammals, this is accomplished by M-cells, residing at the O-MALT (Kraehenbuhl and Neutra, 1992) (Fig. 1c). B-lymphocytes, following Ag stimulation, leave the O-MALT and migrate to mucosal glandular sites where they differentiate and produce polymeric IgA (pIgA) antibodies. This follows a pIgR mediated transport of pIgA into the lumen. Even though this process has not been clearly described in fish, fish possess a shorter type pIgR and mucosal defence involving polymeric Ig and pIgR is possible. Fish do not have antigen sampling M-cells (Rombout et al., 2011). However, very recently, cells exhibiting the characteristics of M-cells have been reported (Fuglem et al., 2010).

1.5.4. Infections at surfaces of cutaneous mucosa: the case of *Lepeophtheirus salmonis*

The salmon louse (*Lepeophtheirus salmonis* Krøyer) is an economically important ectoparasitic copepod that infects both farmed and wild salmonids in Norway and throughout the northern hemisphere. During the last few years, there has been a dramatic increase in the number of *L. salmonis* in Norwegian fjords. Because of the potential economic and environmental impacts of *L. salmonis*, diverse research has been undertaken in the last three decades. Nevertheless, there are still large gaps in understanding of the host parasite interaction at the molecular level, particularly the mechanisms by which the host avoids the parasite, and how the parasite modulates the immune system of the host. Increased biological knowledge and competence in this field is important with regard to the management of the aquaculture industry and maintenance of wild populations of salmon. The need for new methods of parasite control requires a better understanding of the protective mechanisms. Limitations and decreasing efficiency of chemical treatment stimulate interest to immune responses, which are essential for the development of biological methods of protection. The target site for the salmon louse is the skin (and the gill to a lesser extent), and thus the mucosal immune system is of special interest. While the first line of defence against this parasite is crucial, equally important, in the context of vaccine development is the generation of information on the adaptive immune system, i.e. the part of the immune system that can 'learn', and improve its performance. For ectoparasites such as *I. multifiliis*, it has been shown that antibodies play a key role in immune exclusion of the parasite both from skin and gill tissues. In *L. salmonis*, however, the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known.

L. salmonis damage fish by feeding on their mucus, skin, and blood and the wounds increase the risk of secondary infections, and if persistent, the infections can lead to mortality. Overall, *L. salmonis* results in systemic stress and modulation of the immune system and physiological processes (Tully and Nolan, 2002; Wagner et al., 2008). Resistance against *L. salmonis* infections varies among species. Whereas pink (*Oncorhynchus gorbuscha*) and coho salmon (*O. kisutch*) seem to be the most resistant species, higher initial prevalence and intensity of infections are observed in Atlantic salmon and sea trout (*S. trutta*) followed by rainbow trout (*O. mykiss*), chum salmon (*Oncorhynchus keta*), and Chinook salmon (*O. tshawytscha*) (Fast et al., 2002; Jones et al., 2006; Jones et al., 2007; Wagner et al., 2008; Yazawa et al., 2008). It has been hypothesised that this variation is partly attributed to differences in Pacific and Atlantic forms of *L. salmonis* which are believed to be coevolved respectively with Pacific salmon (*Oncorhynchus* spp.) and Atlantic salmonids (*Salmo* spp.) (Yazawa et al., 2008).

2. Main goals of the PhD project

Broadly, this PhD project is aimed at molecular characterisation of key mucosal immune components of Atlantic salmon, and their transcriptional responses following infection by the salmon louse (*L. salmonis*).

Topics covered in the PhD project are:

1. Cloning and characterisation of immunoglobulin tau (IgT), and analysis of the relative expression of Ig isotypes in different tissues.
2. Molecular cloning and expression studies of pIgR and related molecules from Atlantic salmon, and revealing their possible roles in mucosal immune defence.
3. Analysis of immune gene expression (and possible immune modulation) on Atlantic salmon during early infection by the salmon louse.

3. Summary of papers

Paper I

Tadiso T. M., Lie K. K. and Hordvik I. (2011). Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of tau, mu, and delta in different tissues. *Vet. Immunol. Immunopathol.* **139**, 17-26.

The aim of this study was to clone and characterise a key candidate of mucosal immunoglobulins, IgT. In this study, three distinct IgT heavy chain sub-variants, with an identity index of 76-80%, have been described. This is in contrast to IgM and IgD for which two sub variants were reported previously. The similarity between $\tau 1$ and $\mu 1$ in Atlantic salmon is relatively high (identity index of 52%) when compared to the remaining part of the molecules, showing that non-random processes are involved in the evolution of these genes. qPCR assays were designed and evaluated to compare the relative abundance of τ , μ , and δ transcripts in different tissues (head kidney, thymus, spleen, gill, skin, hind gut, brain and muscle) of Atlantic salmon. The analysis revealed relatively high expression of IgM (up to 200 fold more than IgD) followed by IgT (up to 20 fold more than IgD) in most tissues. Head kidney and spleen contained the highest transcript abundance compared to other tissues.

Paper II

Tadiso, T. M., Sharma, A, Hordvik I. (2011). Analysis of polymeric immunoglobulin receptor- and CD300-like molecules from Atlantic salmon. *Mol. Immunol.* **49**, 462-473.

In this study, two pIgR-like molecules of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were cloned and characterised, and a comparative evaluation was made to CD300-like molecules submitted to GenBank (submitted in salmon as CMRF35-like molecules (CLM1, CLM7 and CLM8)). Salsal pIgR and Salsal pIgRL were identified on the basis of similarity to homologous genes in other teleosts. pIgR-like molecules in salmon, like counterparts in other teleosts, are composed of two IgSF V-like domains, a transmembrane region, a connecting peptide, and a cytoplasmic tail. CLM1 and CLM7 in salmon also have the same domain structure, but their cytoplasmic region is predicted to contain putative immunoreceptor tyrosine based inhibition motifs (ITIM), which is a typical feature of CD300A and CD300F proteins in humans. The two V domains of Salsal pIgR and Salsal

pIgRL correspond to the mammalian [D1] and [D5], however, they show the same disulphide bridge topology only with [D1]. While Salsal pIgR and Salsal pIgRL were expressed in tissues of skin and gill respectively, their expression pattern is not restricted to mucosal tissues.

Paper III

Tadiso T. M., Krasnov A., Skugor S., Afanasyev S., Hordvik I. and Nilsen F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics* **12**, 141.

This study addressed the question of what immune transcripts are involved in response to mucosal infections by the salmon louse (*Lepeophtheirus salmonis* Krøyer), an economically important ectoparasitic copepod that infects both farmed and wild salmonids throughout the northern hemisphere. In this study a 21 k oligonucleotide microarray and qPCR were used to examine the temporal immune gene expression changes in skin, spleen, and head kidney of Atlantic salmon during the first 15 days post infection (dpi), representing the copepod and chalimus stages of lice development. Transcriptomic responses, recorded already at 1 dpi, were highly complex and large by scale. Many genes showed bi-phasic expression profiles with abrupt changes taking place between 5 and 10 dpi (the copepod-chalimus transitions). Large group of secretory splenic proteases with unknown roles showed the greatest fluctuations: up regulated 1-5 dpi and markedly down regulated afterwards. T cell related transcripts (T cell receptor alpha, CD4-1, and possible regulators of lymphocyte differentiation) showed a short term (1-5 dpi) up-regulation, suggesting recruitment of T cells of unidentified lineage to the skin probably with innate immune roles. After 5 dpi, the magnitude of transcriptomic responses decreased markedly in skin. Matrix metalloproteinases in all studied organs showed an increase in the second phase. Increase of IgM and IgT transcripts in skin indicated an onset of adaptive humoral immune responses, while MHC I and related transcripts appeared to be down-regulated. The findings gave an insight of the time windows in the development of the parasite which are critical to the host and where modulation of the host immune system might occur.

4. General discussion

The aim of the present work was to identify and characterise key components of mucosal immunity in Atlantic salmon and analyse immune responses of Atlantic salmon to infection by *L. salmonis*. When this study was started, there were not enough data on characterised (mucosal) immune components. Due to the requirement that the PhD should be finished in a three years period, experiments for the papers to be included in this thesis started somewhat in parallel. The papers presented in the thesis are thus organised such that in **paper I** and **II**, the key candidates for mucosal immunity: IgT and pIgR-like molecules were described, including assay design for qPCR studies of all Ig isotypes, pIgR, and CD300-like molecules. **Paper III** explored global gene expression changes due to an ectoparasite invading cutaneous mucosa of salmonids.

When the cloning of a salmon pIgR homolog was initiated, data from other teleosts had just been published, indicating that the mechanisms involved are similar to those in mammals. Thus, it was somewhat unexpected to find an expression pattern of Salsal pIgR and Salsal pIgRL that might point to important differences with regard to possible antibody transport mechanisms in salmon. Regarding the discovery of IgT as a mucosal antibody, which was done in parallel with the present study by another research group, it must be emphasised that the concentration of antibodies in the mucus of fish is still only a fraction of that in mammals. However, the present work has provided important data that further studies can be based on, and identified a possible time window during infection which appear to be essential with regard to immune modulation.

4.1. Sampling and individual variation

One important lesson obtained in this study is that expression levels of Igs and related transcripts is tissue specific. Moreover, there is an obvious individual variation. This variation is again dependent on the tissue type. Whereas the spleen is relatively homogenous and shows little variation, the hind gut, followed by the head kidney (the foremost part of the kidney), show the greatest variation in transcript levels of immunoglobulins and pIgR. This is possibly because of variation in the exact tissue section sampled, as the tissues are heterogeneous with regard to lymphoid accumulations. The head kidney is a lympho-myeloid

compartment serving as both hematopoietic and secondary lymphoid organ (Press and Evensen, 1999).

4.2. Quantitative real-time PCR: The most ubiquitously used method

The most ubiquitously used method in this study was quantitative real-time PCR (qPCR). The important steps to be followed when performing such experiments, among others, are: selection and validation of an internal reference gene, and verifying that the amplification efficiencies of the reference gene and the target genes are similar, followed by statistical analysis of the data. In this study, except for few practical limitations, attempts were made to stick to the MIQE (minimum information for publication of quantitative real-time PCR experiments) guideline (Bustin et al., 2009): Good quality RNA was used, a reference gene was chosen as mentioned in the papers, PCR efficiencies were calculated for selected transcripts, followed by proper statistical analysis. Of particular importance was the design of PCR assays that take into consideration immunoglobulin diversity. The aim of qPCR analysis was to show the relative abundance of the various transcripts in different tissues (**Paper I**, and **Paper II**), and to test the abundance of a given transcript in relation to uninfected control (**Paper II** and **Paper III**).

Statistical analysis of qPCR data is done, in most cases, based on $-\Delta Ct$ or $-\Delta\Delta Ct$ values [$\Delta Ct = Ct$ of target gene (gene of interest) - Ct of reference gene (internal control), while $\Delta\Delta Ct = \Delta Ct$ of sample - ΔCt of calibrator]. However, since relative expression is given as $2^{-\Delta Ct}$, or as $2^{-\Delta\Delta Ct}$, the statistical data should be converted into the linear form, rather than the raw values (Livak and Schmittgen, 2001), i.e., gene expression gives sense if shown as copy number instead of delta Ct values). For example, in the final calculation, the error is estimated by evaluating the $2^{-\Delta\Delta Ct}$ (or $2^{-\Delta Ct}$) term using $\Delta\Delta Ct$ plus or minus the standard deviation (Livak and Schmittgen, 2001). This, however, leads to a range of values that are asymmetrically distributed relative to the average value, a result of converting the results of an exponential process into a linear comparison (Livak and Schmittgen, 2001). To avoid this discrepancy and for simplicity, both figures (obtained using the raw $-\Delta\Delta Ct$ (or $-\Delta Ct$) values as well as $2^{-\Delta Ct}$) were used for comparative purposes in this thesis.

The $-\Delta\Delta Ct$ or $2^{-\Delta\Delta Ct}$ method is often used where there are two sets of samples to be compared (e.g. a test and a control sample), or by the use of a calibrator (that show the highest or the lowest expression from the groups). When the objective was direct comparison of gene expression in different tissues, as in **Paper I**, and partly **Paper II**, this method has not been utilised because it is not always easy choosing which tissue to use as a calibrator (in this case, simply because the different Ig, pIgR-like, and CLM genes show tissue specific variation). Moreover, the use of a calibrator sample does not always give a biologically meaningful result (Livak and Schmittgen, 2001). Thus, all analyses were based on the $2^{-\Delta Ct}$ method, without a calibrator.

In this study, pair-wise comparison of transcript abundance (based on percentage copy # relative to EF1A or $2^{-\Delta Ct}$) of τ , μ , and δ within the various tissues was done by calculating the probability associated with the student's t-test (2 tailed) using the Microsoft Excel spreadsheet program (**Paper I**). Based on this test, the most abundant Ig transcript in all tissues examined was IgM followed by IgT. In all tissues tested, IgD (= mIgD) transcripts were minimal. Similarly, a t-test for the overall Ig transcript abundance in various tissues revealed that relative Ig transcript levels were significantly higher in head kidney and spleen when compared to all other tissues ($P < 0.05$). Transcript levels of each gene was compared between tissues by one way ANOVA, and individual 95% CIs for mean based on pooled StDev of $-\Delta Ct$ values (**Paper I**, Appendix 1) and using $2^{-\Delta Ct}$ values (**Paper II**) was calculated using Minitab 14 software. From this it was possible to see the individual variation of transcripts studied (mainly IgM) especially in the hind gut and head kidney. In **paper III**, qPCR was used for validation of microarray results and analyses of additional transcripts and tissues not included in the microarray. In **paper II** (partly) and **Paper III**, to test for difference between control and test samples at each sampling point, the data were presented as mean $-\Delta\Delta Ct \pm SE$. $-\Delta\Delta Ct$ was calculated as: $-\Delta\Delta Ct = -(\Delta Ct_{Test} - \Delta Ct_{Control})$, where $\Delta Ct = (Ct_{Target} - Ct_{EF1A})$. The probability related to student's t-test (2 sample) was calculated for each transcript.

4.3. Antibody isotypes

Still its basic components being conserved in many species, the adaptive immune system involving immunoglobulins is considerably dynamic in terms of Ig loci organisation, Ig structure, and means to generate diversity (Danilova and Amemiya, 2009).

Generally, antibodies comprise Ig heavy (IgH) and light (IgL) chains. Whereas the variable region of the IgH chain (VH) determines the antigenic specificity, differences in the IgH constant regions (IgC) determine the antibody isotypes. Most mammals harbor gene segments encoding for five antibody isotypes: IgM, IgD, IgG, IgA, and IgE. In teleosts, however, only IgM, IgD, and IgT gene segments have been found. The latter is a teleost specific antibody described very recently in many fish species, including Atlantic salmon (**Paper I**). In addition, variable numbers of antibody subclasses exists in different species. Amphibians possess IgX (which seems to be an IgA equivalent), and IgY (analogous to mammalian IgG) (Hadge and Ambrosius, 1984; Mussmann et al., 1996). Cartilaginous fish (sharks, skates and rays), in addition to two forms of IgM, have two isotypes of IgNAR, a dimer which does not associate with IgL chain; and IgW, an isotype phylogenetically related to IgD (Berstein et al., 1996; Dooley and Flajnik, 2006; Hordvik et al., 1999). IgH loci are organised in two major ways, named cluster (in cartilaginous fish) and translocon. The latter is a typical feature of IgH loci in tetrapods (amphibians, reptiles, birds, and mammals) where an array of VH genomic segments is followed by diversity (DH) and joining (JH) segments. Located downstream of these regions are segments encoding the CH. Antibodies are then generated by VH-DH-JH-CH rearrangements. In bony fish, the heavy chains are in translocon configuration (Warr, 1995), whereas the pattern varies among species. Except for minor differences in C ζ / τ gene locations and channel catfish which is devoid of the IgT/Z genes, generally, the VH segments lie together in tandem upstream of the constant region in the following manner: [VH-DH-JH-C ζ / τ -(VH)-DH-JH-C μ -C δ] (recently reviewed in (Hikima et al., 2011)). While IgT in rainbow trout is located within the VH gene region (Hansen et al., 2005); in zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), and three-spined stickleback (*Gasterosteus aculeatus*), the IgT/Z genes are sandwiched between the VH and DH-JH-C μ -C δ regions: IgT/Z, IgM, and IgD all share the same VH regions, while IgT/Z has its own DH and JH segments (Danilova et al., 2005; Deza et al., 2009; Savan et al., 2005) (Fig. 2).

In Atlantic salmon, there are two highly similar IgM and IgD transcripts, corresponding to the two duplicated IgH loci (A and B) (Hordvik et al., 2002), whereas three IgT sub-variants with an identity index of 76-80% have been found (**Paper I**). A more detailed study indicated the presence of multiple C_τ genes upstream of the C_μ region, with three of them (two in locus A, and one in locus B) being functional (Yasuike et al., 2010) (Fig. 2). The study also indicated that the IgT sub-variants exhibit some differences in their expression patterns. In Atlantic salmon and brown trout (*Salmo trutta*), the two IgM subpopulations were shown to vary in their reaction to a monoclonal antibody (mAb): the two sub-variants differ with respect to an additional cysteine at the C-terminal of the fourth constant domain ($\mu 4$), whereas the mAb reacts with the third constant domain (Hordvik, 1998; Hordvik et al., 2002; Kamil et al., 2011). But so far differential expression of these genes has not been reported.

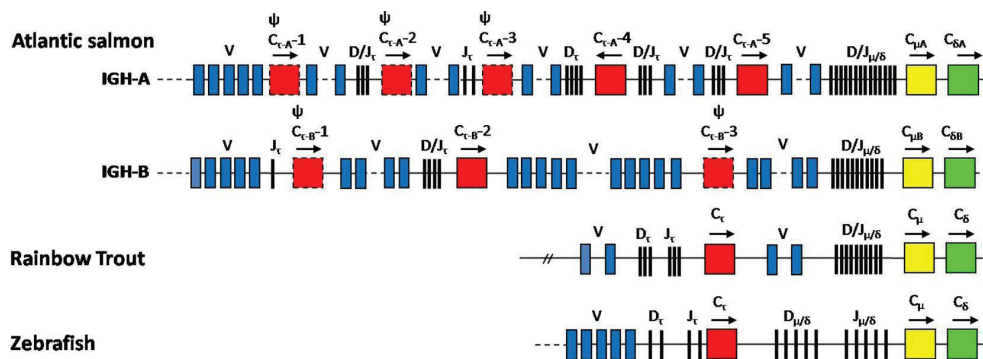


Fig. 2 IgH loci organisation of Atlantic salmon, rainbow trout and zebrafish. The Atlantic salmon duplicated IgH loci, IGH-A (670 kb) and IGH-B (710 kb), were completely sequenced by (Yasuike et al., 2010). Dashed-line boxes indicate the CH pseudogenes (ψ). Transcription directions are shown by arrowhead. Adapted from (Yasuike et al., 2010).

Ig isotypes in mammals occur solely in a monomeric (IgG, IgD, and IgE) or polymeric form (IgM), while IgA is found in both forms (Woof and Mestecky, 2005). In teleosts, IgM predominantly occurs as a tetramer, and its monomeric units are composed of two identical light chains (~25KDa) and two identical heavy chains (~75KDa) (Pilstrom and Bengten, 1996).

Immunoglobulins have two basic forms which determine their function. Membrane bound (m) Igs serve as antigen receptors on the surface of B cells, while secreted (s) Igs (antibodies secreted into blood and other body fluids) function as immune effector molecules (Pilstrom and Bengten, 1996). Teleost B lymphocytes express mIgM as a monomer and secrete soluble IgM as a tetramer (Miller et al., 1998). Lymphocytes in fish also express transcripts encoding IgT, which may represent a novel BCR. IgT is shown to be a monomer (in serum), and a polymer (in gut) (Zhang et al., 2010). Both membrane and secreted transcripts are generated from the IgT and IgM genes, while most teleosts appear to express primarily (or only) membrane bound IgD (discussed in **Paper I**; Fig. 3). Channel catfish, however, do not have the IgT genes, while both secreted and membrane forms of IgD have been reported (Bengten et al., 2002). The heavy chains of both secreted and membrane anchored forms of salmon IgT include four constant Ig domains, $\tau 1$ - $\tau 4$, like the μ chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, $\mu 1$ - $\mu 3$. A typical feature of IgD of most teleosts is a chimeric $C_{\mu 1}$ - C_{δ} structure, where $\mu 1$ is incorporated between the variable Ig domain and the δ chain. Internal duplications of the C_{δ} exons generates a longer IgD molecule. In mammals, secreted IgD is involved in mucosal immunity (Chen and Cerutti, 2010, 2011), and in channel catfish $IgM^{-}IgD^{+}$ cells were described (Chen et al., 2009; Edholm et al., 2010). These cells show variations among individuals. Very recent studies show special exon usage of IgD in zebrafish (Zimmerman et al., 2011), and splicing patterns resulting in a secreted form of IgD (sIgD) in rainbow trout (Ramirez-Gomez et al., 2011). Conventionally, secreted Igs are formed by splicing to the transmembrane exon, whereas trout sIgD is produced via transcription through the splice site at the end of the $\delta 7$ domain and continuing into the intron until a stop codon is reached, resulting in an 11 amino acid secretory tail (Ramirez-Gomez et al., 2011). This sIgD arrangement differs from the one observed in channel catfish, which is generated from a separate sIgD terminal exon (Bengten et al., 2002; Wilson et al., 1997).

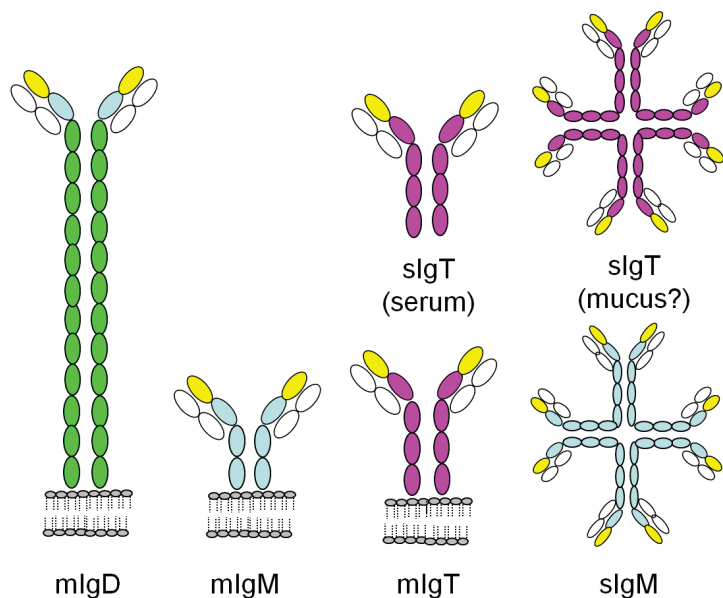


Fig 3. Secreted and membrane forms of immunoglobulins in teleost fish. Both membrane and secreted transcripts are generated from the IgT and IgM genes, while most teleosts appear to express only membrane bound IgD. The heavy chains of both secreted and membrane anchored forms of salmon IgT include four constant Ig domains, $\tau 1$ - $\tau 4$, like the μ chains of other vertebrates. However, the membrane form of IgM includes only three constant Ig domains, $\mu 1$ - $\mu 3$. Internal duplications of the C δ exons generates a longer IgD molecule. $\mu 1$ is incorporated into the IgD heavy chain, between the variable Ig domain and the δ chain. IgT is monomeric in serum but in gut mucus, it has been indicated to be polymeric, similar to IgM. The heavy chain variable regions are shown by yellow ovals, and constant regions (CH domains) are represented by colored ovals: baby blue, bright pink and bright green, respectively for μ , τ , and δ .

4.4. Expression of Ig isotypes in different tissues

The relative abundance of τ , μ , and δ transcripts in a series of tissues revealed an overall expression pattern of IgM \gg IgT $>$ IgD (**Paper I**). This trend has also been maintained in many fish species e.g. (Zimmerman et al., 2011). This asserts the fact that IgM is the predominant antibody class in most teleosts. Even though the abundance of IgT transcripts in samples of mucosal tissues (skin, gill, and hind gut) is relatively weak, IgT enormously responds against infection, supporting the assumption that these antibodies are involved in mucosal immune responses. The expression level of IgD is somewhat similar (or lower) to membrane IgM (mIgM), while secreted IgM (sIgM) transcripts are abundant in most tissues.

The spleen and the head kidney, serving as major hematopoietic tissues (Pilstrom and Bengten, 1996; Press and Evensen, 1999), contain the highest transcript levels of immunoglobulins. However in the hindgut and head kidney, there is a large individual variation in contrast to the spleen which shows the least variability in Ig transcript levels among individuals (Appendix 1). Even though the transcriptional control of Ig isotypes leading to tissue specific expression is not clearly described in fish (Hikima et al., 2011; Pilstrom and Bengten, 1996), in mammals, tissue specificity of Ig gene expression is regulated at least by three DNA sequence elements: the enhancer, the promoter, and undefined intragenic sequences (Grosschedl and Baltimore, 1985).

4.5. pIgR-like and similar molecules in Atlantic salmon

The polymeric immunoglobulin receptor (pIgR) is a transmembrane glycoprotein mediating transport of polymeric immunoglobulins (IgA, and to a lesser extent IgM) across mucosal epithelium (Kaetzel, 2005). In **paper II**, two pIgR-like molecules of Atlantic salmon (Salsal pIgR and Salsal pIgRL) were cloned and characterised (Appendix 2 A and B), and a comparative evaluation was made to CD300-like molecules submitted to GenBank (submitted in salmon as CMRF35-like molecules (CLM1, CLM7 and CLM8)). Whereas the extracellular region of higher vertebrate's pIgR is composed of five or four immunoglobulin superfamily (IgSF) variable-like (V) domains (Braathen et al., 2007; Krajci et al., 1992; Kulseth et al., 1995; Piskurich et al., 1995; Wieland et al., 2004), teleost pIgR (including Atlantic salmon) is a shorter type with two domains (**Paper II**). In both cases, the V domains are followed by a transmembrane region, a connecting peptide, and a cytoplasmic tail. The two domains in teleost pIgR correspond to [D1] and [D5] of the mammalian counterparts. Teleost pIgR polypeptides were identified on the basis of homology to the mammalian counterparts, and also share some functional properties, even though their expression is not restricted to mucosal tissues (discussed in **paper II**). However, there is difficulty in establishing homology because of differences in the domain structures. Teleost pIgR, in addition to being shorter, share very low amino acid sequence identity to pIgR of other vertebrates. Moreover, **Paper II** identified structurally and phylogenetically similar protein products in Atlantic salmon. Teleost pIgR also share substantial percent identities to the V domains of CD300A molecules, despite sharing a very low overall amino acid sequence identity. Searching by the IMGT/DomainGapAlign (

DB/cgi/DomainGapAlign.cgi) tool (Ehrenmann et al., 2010) using [D1] and [D2] of Salsal pIgR and Salsal pIgRL also results in CD300A hits on top. There is also some similarity in the connecting region (eg. *O*-glycosylation). However, BLAST search clearly indicated that the best hits are pIgRs. Two things must be clear here. Firstly, as CD300 molecules contain only one domain, their overall identity is low and they don't appear in BLAST searches. Secondly, pIgR do not possess activating or inhibitory motifs which is a typical feature of CD300 molecules (Clark et al., 2009). However, teleost pIgR V domains have characteristic disulphide bridge topology (Cx₇C motif) that makes them resemble the mammalian pIgR [D1], and V domains of salmon CLM (partly), human CD300A, FcαμR, and NKp44 (Fig. 4).

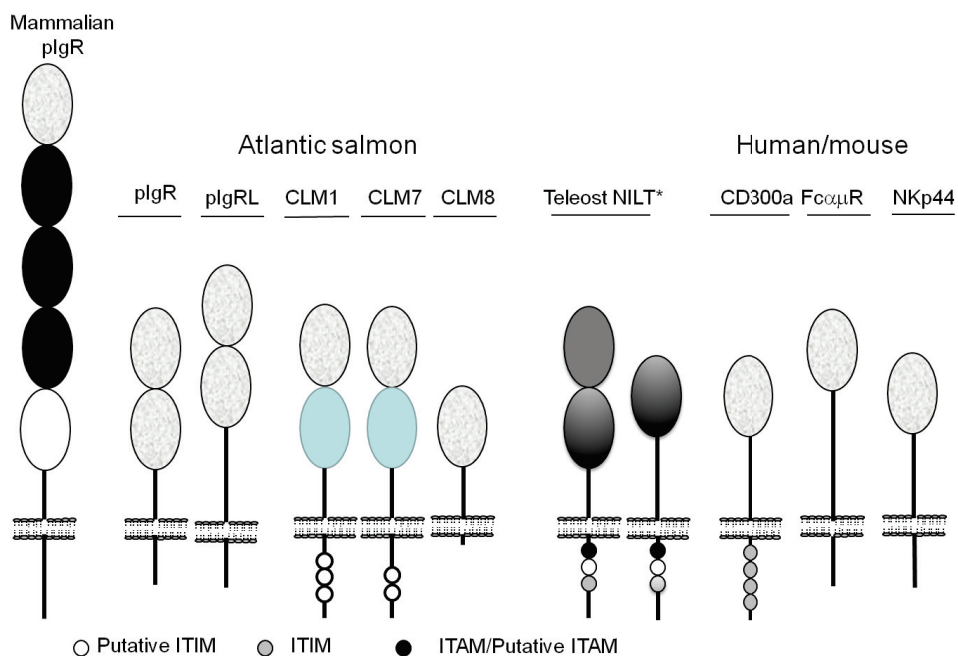


Fig. 4. Schematic representation comparing the main features of pIgR, pIgRL, and CLM V domains with other molecules. Mammalian pIgR contains 5 extracellular domains, with [D1] and [D5] being the corresponding domains of teleost counterparts. Domains with Cx₇C motifs are shown in light gray texture. Salmon pIgR, pIgRL, CLM (partly), human CD300, FcαμR, and NKp44 V domains have Cx₇C motifs which make them resemble the mammalian [D1]. Cytoplasmic signaling regions are indicated. *There are diverse NILT genes in teleosts with varying number and composition of V-like domains and signaling motifs.

Spleen tissue was used to isolate Salsal pIgR and Salsal pIgRL cDNA. To check if cDNA of pIgR varies among different tissues (spleen, skin, gut or liver), EST sequences of Atlantic salmon pIgR as well as trout pIgR were compared for the presence of single nucleotide polymorphisms (SNPs) or deletions and insertions. No differences in pIgR sequences from muscle, spleen, thymus, and ‘mixed tissue’ were found. However, a tandem repeat in the connecting region differs in pIgR cDNA from Norway and Canada, as well as between salmon and trout pIgR.

CMRF35-like molecules (CLM), which are designated as CD300, are a family of proteins having 7 members in humans and 9 members in mouse (Clark et al., 2009). In Atlantic salmon, they have been reported to GenBank as CLM1, CLM7, and CLM8 (Leong et al. 2010), and their *in silico* analysis has been presented in **Paper II** (and Appendix 3 A-C). CLM1 and CLM7 have a similar domain structure to that of teleost pIgR, but owing to the presence of putative ITIM in their cytoplasmic region, it is likely that they belong to the inhibitory CD300 members (CD300A and CD300F) in humans (Cantoni et al., 1999; Clark et al., 2009; Shi et al., 2006). BLAST search showed that CLM8 in salmon is most similar to CLM8 in rats but doesn’t have the inhibitory motifs of rat CLM8: this indicates the possibility of splice variants or domain deletions. Moreover, Clark et al. (2009) emphasised the fact that these molecules (CD300 versus CLM) are not necessarily functional orthologues; for example, the CD300c gene orthologue is CLM6 but CD300c is more functionally similar to CLM4 (Reviewed in (Clark et al., 2009)). It should be noted that these molecules are complex and their naming is not yet clearly standardised.

The two pIgR-like protein sequences contain a connecting region rich in polar amino acids serine and threonine (comprising ~28% of the connecting region) which are very prone to posttranslational modifications such as *O*-linked glycosylation and phosphorylation. The two sequences, however, show some differences; this region is restricted to the connecting region and considerably longer in Salsal pIgRL (**Paper II**). The connecting region in salmon CLM7 (21% of the region) and CLM8 (27%) also contain these residues. Interestingly, the presence of these amino acids is correlated with a longer connecting region (Appendix 3 A-C), supporting the idea that these *O*-glycosylation residues help maintain a longer stalk structure. In contrast, *in silico* analysis revealed that mammalian pIgR barely contains these residues. This also poses a question because *O*-glycosylation causes resistance against proteolytic

cleavage (Kim et al., 1994), which is a crucial step for delivering secretory Igs to mucosal surfaces (Asano et al., 2004; Kaetzel, 2005). The above observations, added with differences in the expression patterns of mammalian and teleost (putative) pIgRs, implies important differences with regard to possible antibody transport mechanisms between mammals and teleosts.

4.6. Mucosal antibodies and pIgR

In mammals, mucosal immunity is mediated mainly by secretory IgA, and it is accomplished by the mucosal B cell system and secretory component (SC) of polymeric Ig receptor (pIgR) (Brandtzaeg, 1995). In teleost fish, IgM is the predominant antibody present in mucosal immune tissues. Very recent findings indicate that IgT, an antibody exclusive to teleosts, might have a special role in mucosal immune responses (Zhang et al., 2010; Zhang et al., 2011). In common carp, IgZ2 (a chimera molecule composed of μ 1 and ζ 4) shows preferential expression at mucosal sites (Ryo et al., 2010). In cutaneous mucus of teleosts, B cells and antibody secreting cells have been identified (Dickerson and Clark, 1998; Hatten et al., 2001; Zhao et al., 2008). In gut mucus, however, little data is available on the presence of Igs (Hatten et al., 2001; Rombout et al., 2011). The strong proteolytic environment in the gut mucus may account for this (Hatten et al., 2001). In this regard, it is worth noting that pIgR at mucosal surfaces of mammals has a great role of protecting Igs from proteolytic degradation (Kaetzel, 2005). In mammals, secreted IgD is also involved in mucosal immunity, and in channel catfish IgM⁻IgD⁺ cells were described at mucosal surfaces suggesting that catfish secreted IgD might have mucosal immune functions (Edholm et al., 2010).

In mammals, following the binding of IgA to pIgR, most of the IgA involved in immunity, produced locally at submucosal sites, is transported into external secretions as a pIgR-IgA complex (Kaetzel, 2005). Then, the secretory component is cleaved. Free SC and IgA play here a protective role (Fig 5). Likewise, the presence of IgM and IgT on mucosal surfaces of teleosts depicts local production of Igs or the presence of associated transcytosis receptors, mainly of pIgR. A possible pIgR counterpart has been identified in different fish species, including Atlantic salmon (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008; Zhang et al., 2010). Even though the functional aspects of teleost pIgR particularly the

association of the secretory component (SC) with poly Igs has not been clearly described, pIgR and IgT/IgM mediated mucosal defence is possible.

Studies show that mucosal antibodies are locally produced, which is evidenced partly because production of mucosal antibodies is independent of that of levels in serum (Lobb and Clem, 1981a; Rombout et al., 1993). Structural and functional differences (e.g. differences in immunoreactivity to a MAb) between serum and mucus Igs in carp depicted that they are independent (Rombout et al., 1993). Migration of antibodies is one possibility, but this has not been confirmed by *in vivo* functional studies, which is partly hampered by the availability of antibodies that can identify relevant Abs. Due to possible physiological barriers, polymeric high molecular weight Abs such as IgM may not easily migrate into the skin mucus, which make immunisation difficult (Dickerson and Clark, 1998; Lin et al., 1996).

Even though the levels of antibodies at mucosal tissues of teleost fish are low compared to the mammalian counterparts, this magnitude is dynamic and increases enormously with infection or immunization (Zhang et al., 2010; Zhao et al., 2008). For some ectoparasites such as *I. multifiliis*, it has been shown that antibodies play a crucial role in immune exclusion of the parasite both from skin and gill tissues. Presence of IgT positive cells in the epithelial lining of the gill lamellae of rainbow trout was documented in one study (Olsen et al., 2011). Another study documented IgT and IgM binding to surface structures of the parasite *I. multifiliis* early during infection in the gills of immune rainbow trout (von Gersdorff Jorgensen et al., 2011). The target antigens of this parasite are surface proteins called immobilizing antigens or i-antigens to which mucosal antibodies will bind eventually leading to removal of the parasite (Dickerson and Clark, 1998; Lin et al., 1996). In *L. salmonis*, however, the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known. Transcriptomic data in **paper III** suggested involvement of IgT and IgM antibodies. Zhang et al. (2010) also showed the role of IgT in anti-parasitic defence against gut mucosal parasites. It is thus plausible to assume that antibodies are good candidates of immune effector molecules against parasites. However, involvement of other immune factors is possible (see Section 4.7), and this is something to be dealt with in detail in future studies.

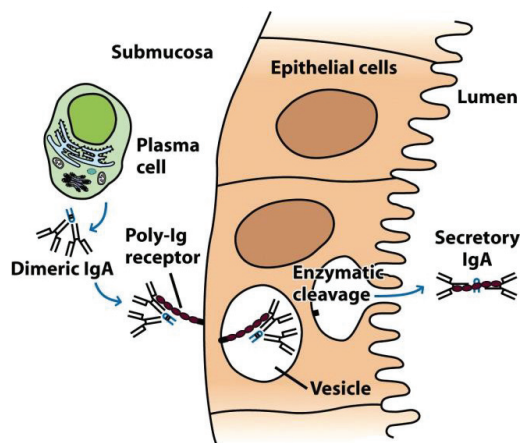


Fig. 5. Formation of secretory IgA in mammals. Following the binding of IgA to pIgR, most of the IgA involved in immunity, produced locally at submucosal sites, is transported into external secretions as a pIgR-IgA complex (Kaetzel, 2005). Then, the secretory component is cleaved at the lumen. Here, Free SC and IgA play innate and adaptive immune functions. Source: Kindt et al. (2007)

4.7. Host parasite interactions at mucosal surfaces: the case of *L. salmonis*

L. salmonis are host specific ectoparasitic copepods known to affect salmonid fish, and cause significant losses. However, there is considerable inter-species variation in the immunological responses and ability of salmonids to reject the parasite. **Paper III** addressed transcriptomic (microarray and qPCR) immune responses of Atlantic salmon during the time window encompassing the copepod and chalimus stages of lice development.

The fact that Atlantic salmon is more susceptible to infections by *L. salmonis*, compared to closely related species, such as sockeye and coho salmon, is well established. A possible evidence for this is the failure of Atlantic salmon to clear infections, while resistant salmonids get rid of lice few days after challenge. Weak inflammation at the site of attachment was regarded as a plausible explanation for this (reviewed in (Wagner et al., 2008)). Susceptibility of Atlantic salmon to *L. salmonis* infections may also be due to the composition of skin mucosal agents that block or stimulate the release of proteases and other agents to the skin (Fast et al., 2003). Lice produce prostaglandin E synthase (PGE2), trypsin-like proteases, and other products that suppress the immune system of Atlantic salmon (Firth

et al., 2000). Significant reduction of oxidative and phagocytic activities of macrophages (Mustafa et al., 2000), and reduced transcription of IL-1 β and COX-2 in lice infected salmon has been reported (Fast et al., 2007; Fast et al., 2006). Experiments conducted on LPS stimulated Atlantic salmon macrophage-like SHK-1 cells have shown that PGE(2) inhibits the expression of MHC I and II and pro-inflammatory cytokine interleukin-1 beta (Fast et al., 2005). PGE2 is a potent vasodilator found on most parasite secretions and has a significant effect on immunity; for example, prostaglandin dependent suppression of lymphocyte proliferation is documented (Papadogiannakis et al., 1985). The aforementioned factors indicate that early responses seem to be critical in this host parasite system. In addition, as the louse depends on the host after its attachment, clearing infections by immobilising the parasites at early stages would be a great advantage to the host, which barely happens in Atlantic salmon. **Paper III** thus addressed early responses 1-15 days post challenge.

Despite the high lice load found by the end of the experiment (15 dpi), transcriptomic analyses showed dramatic gene expression changes immediately after infection in the target sites and in the spleen, indicative of rapid local and systemic sensing. This suggested that low resistance of Atlantic salmon to lice appears to be accounted for by the character of immune response rather than the scale of the response. Interestingly, most of the differentially expressed genes are not those that are commonly included in studies of salmon immunity. Thus, it was difficult to detect these changes based on the candidate genes approach. An unexpected finding was involvement of splenic proteases. Dramatic expression changes of a group of genes encoding functionally related proteins implied their important role, which remains completely unknown.

4.7.1. The copepodid-chalimus transition is a critical stage in host immune gene regulation

Salmon louse develops through ten stages. Even though the pre-adult and adult louse can move unrestricted on the host surface, and subsequently result in an increased virulence, the copepodid is the infectious stage of *L. salmonis* and is able to settle and recognise a relevant host. During the copepodid-chalimus transition, the louse penetrates the salmon epidermis, and the response at this stage appears to be very critical. Interestingly, the transcriptome changes observed at this transition supported this phenomenon (**Paper III**). The findings clearly depicted early sensing at day one post infection and subsequent regulation of a diverse

array of genes. A remarkable finding was the presence of groups of genes with similar profiles: early up (or down)-regulation followed by strong down (or up)-regulation between days 5 and 10 both in skin and spleen. The large switch during the copepodid-chalimus transition (5 and 10 dpi) suggested that this is a possible time-window where modulation of the host immune response occurs.

4.7.2. Early expression of putative T cell (-like) transcripts and their regulators

Transcriptomic responses to lice were also characterised by an early increase of T cell specific transcripts in the skin, which totally ceases after day 5. Their functional roles remain to be identified, while a number of questions were raised including from where they originate (head kidney or the thymus) or if they are locally produced. The former possibility is plausible because a number of T cell related transcripts have shown depletion in parallel. On the other hand, activation of RAG and other regulators of transcription of T cells suggest that they can be locally produced. The next question is what happened to those transcripts after 5 dpi? One possibility is that they are blocked by the parasite. A good example here is the parasite *Trypanosoma evansi* induces tyrosine phosphatase mediated killing of lymphocytes (Antoine-Moussiaux et al., 2009). Due to the enormous abilities of parasites to trigger highly polarized CD4+ T cell subset responses, which depends on the host genetic background, T lymphocyte-parasite interactions have been regarded as crucial subjects of research (Jankovic et al., 2001; Sher and Coffman, 1992).

4.7.3. Splenic proteases: important anti-lice strategies or vice-versa?

Somewhat unexpectedly, in the spleen, a group of related transcripts encoding for secretory proteases showed an early (1-5 dpi) up-regulation followed by strong down-regulation in the second phase (10-15 dpi) (**Paper III**, Appendix 4). In parallel with the up-regulation of proteases day 1-5 dpi in the spleen, some of their precursor molecules also increased in the skin, which go off completely after day 10. Furthermore, a large number of transcripts for secretory proteins were down-regulated in parallel with proteases at 10 dpi. It is tempting to speculate that the first phase can be stimulation where proteolytic enzymes are produced, which migrate to the target site in the second phase. Nevertheless, there are important questions which remain unanswered, including where these enzymes reside and what their roles are, and where they migrate to? It can be deduced, however, that production of

extracellular proteases in spleen is an important anti-louse strategy. In invertebrates, a serine protease cascade (analogous to the complement system in mammals) is responsible for initiating an innate immune response following parasitic infection (Kanost et al., 2004; Volz et al., 2005). Conversely, the observation in *L. salmonis* can also be an anti-host strategy by the parasite. Apart from producing these proteases themselves, it is suspected that salmon louse have the ability to stimulate the release of such enzymes from the host (in this case the spleen) which possibly are transported to the skin/mucus surface. Studies show that in response to infection with salmon louse, low molecular weight (18-25 kDa) trypsin-like proteases were detected in the skin mucus of infected fish, but not in healthy fish (Ross et al., 2000). These are suggested to be produced by louse to aid feeding and suppress the immune system of Atlantic salmon (Firth et al., 2000). However, it is not possible to rule out the possibilities that these proteases can be of host origin, migrated from the spleen (or other organs) to the skin/mucus. Nevertheless, this deserves a thorough biochemical investigation at protein level including immunohistochemistry and enzyme assays.

It is worth mentioning here that the target antigens for many parasites are (cysteine) proteases: e.g. in *T. cruzi* causing Chagas disease (Cazorla et al., 2008; Doyle et al., 2007; Stempin et al., 2008), and in *Schistosomiasis mansoni* which causes human schistosomiasis (Abdulla et al., 2007). These immunomodulatory peptides inhibit a number of immune mechanisms including macrophage functions. Cysteine proteases also represent potential antigens in anti-parasitic DNA vaccines (Jorgensen and Buchmann, 2011). Proteases thus seem to occupy a central role in future host-parasite interaction studies and thus deserve a closer investigation.

The overall pattern of transcriptomic responses (**paper III**) indicated regulation of group of genes, such as transcripts encoding for splenic proteases, secretory proteins, ECM proteins, pro-inflammatory molecules, etc., all indicative of a holistic response rather than a few special genes. Besides, an opposite regulation in virus responsive genes suggested a connection between louse and viral infection, which requires further studies including a co-challenge experiment.

Finally, the question of how louse modulates the mucosal immune components of Atlantic salmon has been postulated. 1) *By hampering local production/recruitment of T lymphocytes.*

Transient (1-5 dpi) increase of T cell receptor alpha, CD4-1, and possible regulators of lymphocyte differentiation didn't last after 5 dpi indicating possible blockage of lymphocytes by the parasite. 2) *Decreased abundance of transcripts involved in antigen presentation.* This is perhaps an important indication of immune modulation observed especially in head kidney and skin, as witnessed by decreased transcript levels of MHCI and B2M consistently throughout the experiment. In addition, a number of heat shock proteins (HSPs) are down regulated early in the skin and the spleen. In humans, it has been shown that HSPs play a role in delivering antigens to antigen presenting cells (Nishikawa et al., 2008). The down regulation of these transcripts may thus imply that the fish might not be able to mount the necessary responses. 3) *Stimulation of production and or depletion of splenic proteases* as discussed above. In parallel examination of host immune responses both in resistant and susceptible species is thus crucial to analyse host immune modulation in terms of the above parameters.

5. Conclusions and future perspectives

Enhancing effective protection against pathogens is a crucial but challenging task, and requires a deeper understanding of the immune system of the host, and the host-pathogen interactions therein. In this study, information on components of the mucosal immune system in Atlantic salmon has been gathered including design of qPCR assays that consider immunoglobulin diversity in salmon; this can greatly assist monitoring of Ig gene expression and immune responses. Secondly, pIgR like molecules and their possible role in mucosal immunity have been revealed. Thirdly, transcriptional responses of Atlantic salmon to an economically important ectoparasite *L. salmonis* were determined.

In *L. salmonis*, even though the target antigens as well as the protective mechanisms which lead to the exclusion of the parasite are not known, from other ectoparasites such as *I. multifiliis*, it can be deduced that antibodies can have potential roles. Transcriptomic data also suggested involvement of IgT and IgM antibodies. The role of IgT in anti-parasitic defence against gut mucosal parasites has also been documented. Nevertheless, it must be emphasised that the concentration of mucosal antibodies in fish is still only a fraction of that in mammals. This magnitude, however, is dynamic and increases enormously with infection or immunization. On the other hand, due to a potential anti-host effects of proteolytic enzymes, coupled with the unexpected transcriptomic fluctuations of splenic proteases observed in response to *L. salmonis* infection, it is not possible to exclude involvement of other immune factors as well, and this is something to be dealt with in detail in future studies. An important aspect being investigated in parallel is regulation of genes in *L. salmonis* during the same time-window of infection. This will enable the identification of possible antigens of the louse that can be targeted for vaccination. A recent study in related species *Caligus rogercresseyi* documented that a novel gene named my32 (having a high identity to *L. salmonis* akirin-2) has been found to be a promising target for vaccination against sea lice (Carpio et al., 2011). Study of the immune response of Atlantic salmon in the absence of immunomodulatory substances can also be done by blocking the responsible gene/s (gene silencing) by using molecular technologies such as RNA interference (RNAi). This method has been found working in *L. salmonis* where knock down of putative PGE synthase has been tested

(Campbell et al., 2009). A compilation of genomic resources is now available to study the biology, immunology, and other aspects of sea lice (Yasuike et al., 2011).

The completion of genome sequencing for many species now allows the use of modern techniques within proteomics, and functional studies which previously were restricted to human immunology: for systematizing and characterizing immune genes, and developing tools to study the immune system in different fish species such as in (<http://www.umass.edu/vetimm/catfish/index.html>).

The present work has provided important data that further studies can be based on, and identified a possible time window during infection which appear to be essential with regard to immune modulation. Altogether, the information gathered in this thesis is believed to add to the existing knowledge in the immunology of infection of Atlantic salmon that will assist further research to the effective control of pathogens in general, and *L. salmonis* in particular.

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