UNIVERSITY OF TROMSØ UIT

FACLTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
NORWEGIAN COLLEGE OF FISHERY SCIENCE

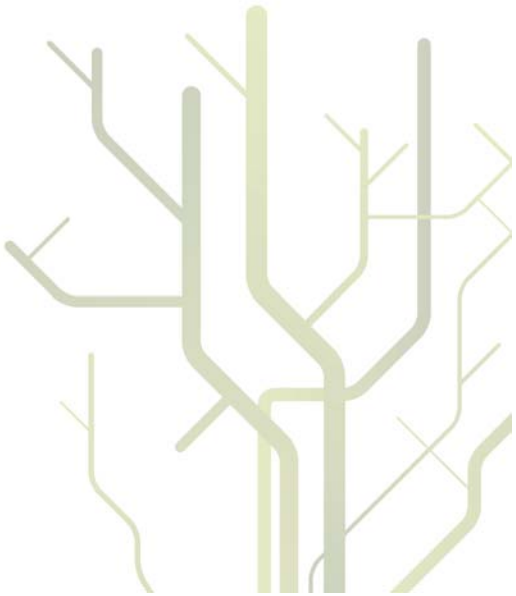
The immune system of Atlantic cod

– with emphasis on intestinal immunology

Makoto Inami

A dissertation for the degree of Philosophiae Doctor

Spring 2011



UNIVERSITY OF TROMSØ

FACLTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
NORWEGIAN COLLEGE OF FISHERY SCIENCE

The immune system of Atlantic cod

– with emphasis on intestinal immunology

Makoto Inami

A dissertation for the degree of Philosophiae Doctor

Spring 2011

Table of contents

Acknowledgements

The work of this thesis was carried out at Bodø University College, University of Tromsø and Wageningen University during 2006-2010. Financial support was provided by the Norwegian Research Council and partly by WIAS grants.

First of all, I would like to express my gratitude to my supervisors; Professor Viswanath Kiron, Associate Professor Merete Bjørgan Schrøder and Professor Jan Rombout. I would like to thank Kiron for providing me the exciting opportunity to study in Norway. Merete, I have appreciated your support, guidance and patience no matter where we are situated. Last but not least, Jan, your endless support, caring, encouragement and positive feedback always gave me the hope and energy to complete my long journey. You are "the" mentor for me.

I would like to thank to my co-authors and colleagues here and there. A huge gratitude goes to Anja, it was absolutely my privilege to learn so many things from you in the lab and outside. Your incredible hospitality and creativity inspire me so much. I always felt at home with you. Our big men, Nico and Henk, thank you for your help with my odd problems and for being there anytime I needed. Mona and Terje, thank you very much for the hard work and efforts on the manuscripts we wrote together! I think we've learned a lot from each other and I really have enjoyed it. Atle, Stein Tore, Rannveig, Vera, Marit and Terese are also acknowledged for their valuable inputs and fruitful discussions. I would like to thank all the current and former members in old IMAB in Tromsø and CBI in Wageningen for including me, the PhD student who randomly comes and goes, and for the great working atmospheres.

Here my gratitude goes to friends all over the world (including friends who are not mentioned below)! Tasha, my Ms. Incredible, I don't even know where to start and don't know how to thank enough. You were first appointed to be a friend of mine by our boss against your will, but we came so far. Your patience, friendship, bottomless positive energy rescued me from a lot of difficult moments during my PhD. I could have already died from PhD disease without you. All the fun and everyday lunch (dinner you say)- and supper (dinner I say)- time we shared kept me as normal human being. Sugar sweet Taianá, thank you for taking care of me so well and for being together "for better or for worse". Thanks to Tim for his big support during the most stressful phase of my PhD. Carla and Wouter, I am so thankful for your friendship and great fun we shared in wild wild Wageningen. I also enjoyed a lot of stimulating midnight-discussions with Carla. Karina, Victoria, Chris, Sigmund and Steingrim thanks for introducing me "Norwegian spirit" a.k.a. drinking, hiking, knitting, drinking, skiing, fishing, drinking and more. I would like to acknowledge Maren, Nina and Liv for their support during the very beginning of my journey. Paula, Konrad, Catarina and Gonçalo, thanks for your amazing hospitality and all the unforgettable moments we experienced are my treasures. Thanks to Reiko, Yuki, Kaori and Izumi for giving me tips of survival skills abroad and for the girls' chats in our mother tongue. And super special thanks goes to Ayaka for 24/7 skype online support and the deep/desperate discussions about science and everything else. Your optimism and geek jokes bring the light to my dark periods.

Finally I aim most grateful to my parents for their unlimited generosity and eternal support. To my mom, who sent me hundreds of packages filled with food and love, provided me the courage and energy to overcome the lonesomeness of being so far away from home.

Makoto Inami

稲見麻琴

Tromsø, February 2011

Summary

The objectives of this study were to investigate the defense mechanisms of Atlantic cod (*Gadus morhua* L.) with special emphasis on intestinal mucosal immunology.

Atlantic cod is becoming an important fish species for aquaculture in the North Atlantic region, but it is not exempt from production problems including juvenile losses and diseases. There is still limited information available on the defense mechanisms of cod, especially on mucosal immunity. The mucosal immune system provides the first line of defense against invading pathogens. In the case of fish, environmental antigens or pathogens are encountered directly at skin or gill surfaces or indirectly through normal physiological ingestion. As in higher vertebrates, fish possess gut-associated lymphoid tissues (GALT) though to a lower extent than mammals. The GALT contain many immune competent cells and have a strong uptake capacity in the so-called second gut segment in a variety of fish species. Interestingly, no clear morphological difference in the cod intestine, excluding the rectum and no active antigen uptake was detected. On the other hand, the rectum of cod has high numbers of IgM+ cells and myeloperoxidase containing granulocytes and also showed receptor-mediated uptake of horseradish peroxidise or LTB-GFP. As a consequence, rectum seems to be better equipped against pathogen invasion compared to the intestine.

Vibriosis caused by *Vibrio anguillarum* has been the major bacterial disease in cod, whereas francisellosis caused by facultative intracellular *F. noatunensis* is an ever increasing problem. Investigation of immune responses and the components involved during bacterial infection would help to shed light on how systemic and local immunity are mounted in cod. Real-time quantitative PCR was used to measure mRNA gene expressions of cytokines (*IFN-γ*, *IL-1β*, *IL-8* and *IL-10*), and antimicrobials (*BPI/LBP* and *hepcidin*). During the early phase of *V. anguillarum* infection fast and elevated expression of *IL-1β, IL-8* and *hepcidin* were observed in spleen, whereas rather late up-regulations were observed in foregut and hindgut. Differential immune gene expressions were observed in both foregut and hindgut. After intraperitoneal injection of live *F. noatunensis* bacteria a significant up-regulation of *IFN-γ* and *IL-1β* expression was observed from 15 to 60 days post infection in spleen and head kidney. Up-regulation of *IL-1β* and *IFN-γ* was observed

in spleen whereas intestine and rectum showed a down-regulation in cohabitant fish after 60 days. We have observed that higher antibody responses were mounted in cod compared to injection of inactivated bacteria after *F. noatunensis* challenge. In addition, the immunopathology of francisellosis was investigated by classic histology, immunohistochemistry, enzyme staining and *in situ* hybridization. The granuloma-like inflammatory foci were observed mostly in the spleen, head kidney and heart. The dominant cell types located in the foci were macrophage-like cells, pleomorphic proliferating cells and granulocyte-like cells. The presence of bacteria was seen in macrophage-like cells of the inflammatory foci. With a new antiserum against cod g-type lysozyme, native folded g-type lysozyme could be detected in lymphoid tissues, such as spleen. During the development of inflammatory foci the g-type lysozyme was co-located within the peroxidase positive cells but the identification of these cells needs further attention.

List of papers

Paper I

Makoto Inami, Jan H.W.M. Rombout, Viswanath Kiron and Merete Bjørgan Schrøder. Immune gene expression in the initial phase of *Vibrio auguillarum* infection in cod (*Gadus morhua* L.) Manuscript in preparation

Paper II

Makoto Inami, Anja J. Taverne-Thiele, Merete Bjørgan Schrøder, Viswanath Kiron and Jan H.W.M. Rombout. Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.). Fish & Shellfish Immunology, 2009, 26; 751-759

Paper III

Terje Ellingsen[*], Makoto Inami[*], Mona Cecilie Gjessing, Koen Van Nieuwenhove, Rannveig Larsen, Marit Seppola, Vera Lund and Merete Bjørgan Schrøder. [*]contributed equally

Francisella noatunensis in Atlantic cod (*Gadus morhua* L.); waterborne transmission and immune responses. Accepted manuscript in Fish & Shellfish Immunology

Paper IV

Makoto Inami, Sein Tore Solem, Trond Ø Jørgensen and Atle N Larsen.

Characterization of an antiserum against Atlantic cod (*Gadus morhua* L.) g-type lysozyme. Fish & Shellfish Immunology, 2010, 29; 1106-1109

Paper V

Mona Cecilie Gjessing, Makoto Inami[*], Simon Weli[*], Terje Ellingsen, Knut Falk, Erling Olaf Koppang and Agnar Kvellestad. [*]contributed equally

Presence and interaction of inflammatory cells in the spleen of Atlantic cod infected with *Francisella noatunensis.* Accepted manuscript in Journal of Fish Disease

Abbreviations

BPI	bactericidal/permeability increasing protein
C3	complement component 3
CD	clusters of differentiation
CRP	C-reactive protein
c-type lysozyme	chicken-type lysozyme
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FDCs	follicular dendritic cells
GALT	gut-associated lymphoid tissues
GFP	green fluorescent protein
g-type lysozyme	goose-type lysozyme
HRP	horseradish peroxidase
IEL	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILD	Ig-like domain
IPNV	infectious pancreatic necrosis virus
LBP	LPS-binding protein
LPS	lipopolisaccharide
LTB	*Escherichia coli* heat-labile enterotoxin non-toxic B subunit
MALT	mucosa-associated lymphoid tissues
MHC	major histocompatibility complex
MLC	macrophage-like cell
MPO	myeloperoxidase
pIgR	poly Ig receptor
poly I:C	polyinosinic polycytidylic acid
SIgA	secretory IgA
SNV	supranuclear vacuoles
TGF	transforming growth factor
Th	T helper

TLR	toll-like receptor
TNF	tumour necrosis factor
VNN	viral nerve necrosis

1 Introduction

Atlantic cod (*Gadus morhua* L.) belong to family Gadidae, distributed along the continental shelf across most of the North Atlantic Ocean and Norway has long traditions in fishing, eating and marketing of cod. After Atlantic salmon (*Salmo salar* L.) farming, cod farming is considered as a second wave in aquaculture in Norway (Pillay and Kutty, 2005). Farming of Atlantic cod has been commercialized since the early 1980's but has been confronted with several difficulties. These include unsteady market, low prices due to the availability of cheaper wild-caught fish, early sexual maturation and bacterial diseases like vibriosis and more recently francisellosis. For further improvement of the health status of cultured cod a proper knowledge of the cod immune system is required, in particular of the mucosal immune system that provides a first line of defence against invading pathogens and that is also relevant to vaccine development via mucosal route.

1.1 Immune system – Comparison between mammals and fish

All vertebrates have the innate and adaptive immune system to protect themselves against a variety of pathogens (Janeway and Medzhitov, 2002). Innate immunity is a non-specific and fast-reacting defense mechanism which includes physical, cellular and chemical barriers. Microbes are also recognized by the adaptive immune system, a highly specific and diverse system characterized by memory formation. In mammals, the adaptive immune response is highly sophisticated and very well studied.

About 450 million year ago, teleost fish diverged from the tetrapod lineage and it has been hypothesized that evolution of the specific immune response occurred suddenly as a result of gene duplication (Schluter et al., 1999). Although the immune system of teleost is certainly different from that of mammals, there are also common features. Many genes encoding molecules associated with defense mechanisms of fish have counter parts in mammals, suggesting that they represent the evolutionary precursors of key mediators of innate and adaptive immunity (Plouffe et al., 2005).

Antimicrobial peptides play an important role in the first line of defense against invading pathogens (Boman, 2003; Hancock and Diamond, 2000; Zasloff, 2002). They are located in the epithelial cells and mucosal surfaces of the host and contribute

to the inflammatory responses in addition to their direct antibacterial effects. Compared to mammals fish have a relatively smaller diversity of antimicrobial peptides but other innate defense molecules are also found at mucosal surfaces, such as natural antibodies, apolipoproteins, lysozyme, fish specific TLRs (i.e. TLR 22; (Rebl et al., 2010) etc. For instance lysozymes are widely characterized in vertebrates as well as invertebrates (Jolles and Jolles, 1984). In vertebrates including fish, two kinds of lysozyme (chicken-type and goose-type) are reported (Saurabh and Sahoo, 2008).

Cytokines are the key regulators of both innate and adaptive immunity and can be produced by macrophages, T cells and by many other cells (Murphy et al., 2008). Recently, fish genome information is more widely available due to the improvement of sequencing facilities. Whole genome sequencing of fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*), and numerous fish EST databases have led to the detailed studies and identification of fish cytokines. Recently, the cod genome has been fully sequenced and this will in the near future hopefully lead to increased knowledge about the cod immune system (Jakobsen and Lie, 2009).

In general, fish have a similar repertoire of cytokines as mammals, but the function of each fish cytokine is not so well established as for mammalian cytokines (Secombes et al., 1996). Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are the best characterized cytokines in fish (Scapigliati et al., 2000; Secombes et al., 1996) but also other interleukins, chemokines and interferons are reported in fish (Robertsen, 2006; Seppola, 2008). Several immune genes have been characterized and analysed from cod in the recent years, including cytokines, chemokines and interferon stimulated genes (Borza et al., 2010; Feng et al., 2009a; Furnes et al., 2009; Seppola et al., 2008; Seppola et al., 2007).

Phagocytes such as macrophages, neutrophils and monocytes play an essential role in the activation of both the innate and adaptive immune system. In mammals, macrophages arise from a common precursor in the bone marrow, circulating in the blood stream as monocytes and migrating into the organs transformed into various types of tissue macrophages. Already decades ago macrophages in fish have been described and reviewed (Ellis, 1977; Secombes and Fletcher, 1992) . They are shown to have a long life compared to neutrophils and play a crucial role in innate immune responses. In many cases macrophages appear to be the most common cell type with

inflammations frequently described as granulomas in fish such as Atlantic cod and Atlantic halibut (*Hippoglossus hippoglossus*) (Gudmundsdóttir et al., 2006; Roberts, 2001). In addition, these phagocytic cells secrete a diverse array of cytokines to initiate innate and adaptive responses (Djaldetti et al., 2002; Kapetanovic and Cavaillon, 2007). Recently, B cells are suggested to have phagocytic activity in rainbow trout (*Oncorhynchus mykiss* Walbaum), Atlantic salmon and Atlantic cod (Li et al., 2006; Øverland et al., 2010).

Two essential features of the adaptive immune system are immunoglobulins and the major histocompatibility complex (MHC) (Schluter et al., 1999). In humans, MHC class I and II genes are linked and located on the same chromosome, but in teleost they are found on separate chromosomes (Flajnik et al., 1999). Interestingly, the MHC class II molecule is speculated to be absent in Atlantic cod (Pilström et al., 2005) whereas the MHC class I genes were expressed in unusually high amounts in cod (Persson et al., 1999). Also, the recent sequencing of the cod genome has revealed that cod seem to have a very unique immune system that is clearly distinct from other sequenced species (Jakobsen and Lie, 2009). Mammals have five types of immunoglobulin (IgA, IgD, IgE, IgG and IgM), whereas in fish only IgM and IgD have been described (Flajnik, 2002). Teleost IgM is found mainly in tetramic form, but some fish species have it as a monomer, dimmer or even half-meric form (Bang et al., 1996; Lobb and Clem, 1981; Szalai et al., 1994), and in sheephead (*Semicossyphus pulcher*) a dimeric mucosal IgM with secretory component has been reported (Lobb and Clem, 1981), whereas in mammals IgM forms pentamers joined by a J chain. In teleosts J chains are not described yet, but recently a new isotype of immunoglobulin was reported in cyprinids, rainbow trout and fugu, and named IgZ in zebrafish (Danilova et al., 2005; Flajnik, 2005) and carp (*Cyprinus carpio*) (Savan et al., 2005a), IgT in trout (Hansen et al., 2005) and IgH in fugu (Savan et al., 2005b). Trout IgT claims to be specialized in mucosal immunity and IgT responses to a gut parasite are restricted to the intestine (Zhang et al., 2010). On the other hand, not every teleost species, like channel catfish (*Ictalurus punctatus*) (Bengtén et al., 2006), seem to have the sequence of IgZ or IgT.

One of the largest differences between the mammalian and piscine adaptive immune system is memory formation. Compared to mammals the memory response is modest in fish. The presence of enhanced secondary responses in mammals is closely related to the logarithmic increase in IgG during the class switch from IgM to IgG. On

the contrary, IgG is not found in fish and no class switch seems to be possible (Magor et al., 1999). This does not mean that piscine immune system is per definition less effective than that of mammals, but it might be that fish do not require a better or more sophisticated immune system (Kaattari, 1994). The ambient temperature is a critical factor affecting the immune system in cold-blooded animals like fish. Low temperature for the individual species are immunosuppressive (Bly and Clem, 1992) and may even induce tolerance in some fish species (Wishkovsky and Avtalion, 1987). Fish in aquaculture systems may be even more affected by temperature because they are not able to migrate from suboptimal temperatures (Watts et al., 2001).

1.2 Mucosal immune system

The mucosal immune system is a part of the immune system that responds to and protects against microbes that enter the body through mucosal surfaces. The mucosal immune system is composed of collections of lymphoid cells and antigen-presenting cells in the epithelia and lamina propria of mucosal surface. It provides three main functions: protecting the mucus membrane against infection, preventing the uptake of antigens, microorganisms, and other foreign materials, and moderating the organism's immune response to that material (Holmgren and Czerkinsky, 2005). In the case of fish, environmental antigens or pathogens are encountered directly at skin or gill surfaces or indirectly through normal physiological ingestion. As in higher vertebrates, fish possess mucosa-associated lymphoid tissues (MALT), but are less organised than described for mammals. The gut-associated lymphoid tissues (GALT) contain many immune-competent cells and have been well studied in carp, salmonids (Atlantic salmon and trout), European sea bass (*Dicentrarchus labrax*) and gilt-head sea bream (*Sparus aurata* L.) (Rombout et al., 2010). However, not much is known yet about functional aspects of the mucosal immune system of fish.

1.2.1 Mucosal immunity in mammals

The mucosal tissues of the body, such as the intestine, are exposed continuously to enormous amounts of different antigens, such as food, commensal organisms and pathogens. Potential immune responses to the antigen load are controlled by distinct components of the mucosal immune system, which is the largest

immune system in the body and processes many unique features (Brandtzaeg and Pabst, 2004). This includes M cells that are found in the follicle-associated epithelium of the organised lymphoid nodules, known as Peyer's patches (Fig. 1). M cells have the ability to take up antigen from the lumen of the small intestine via endocytosis or phagocytosis, and then deliver it via transcytosis to the underlying antigen presenting-(dendritic), B and T cells. Lymphocytes primed in the MALT acquire specific homing receptors, allowing them to redistribute preferentially back to mucosal surfaces as effector cells. The MALT also generate mucosa-specific effector responses. The adaptive immune responses in mucosal tissues is characterized by the secretory dimeric IgA, and by the presence of distinct populations of effector T cells whose functional and phenotypic properties are highly influenced by their anatomical location.

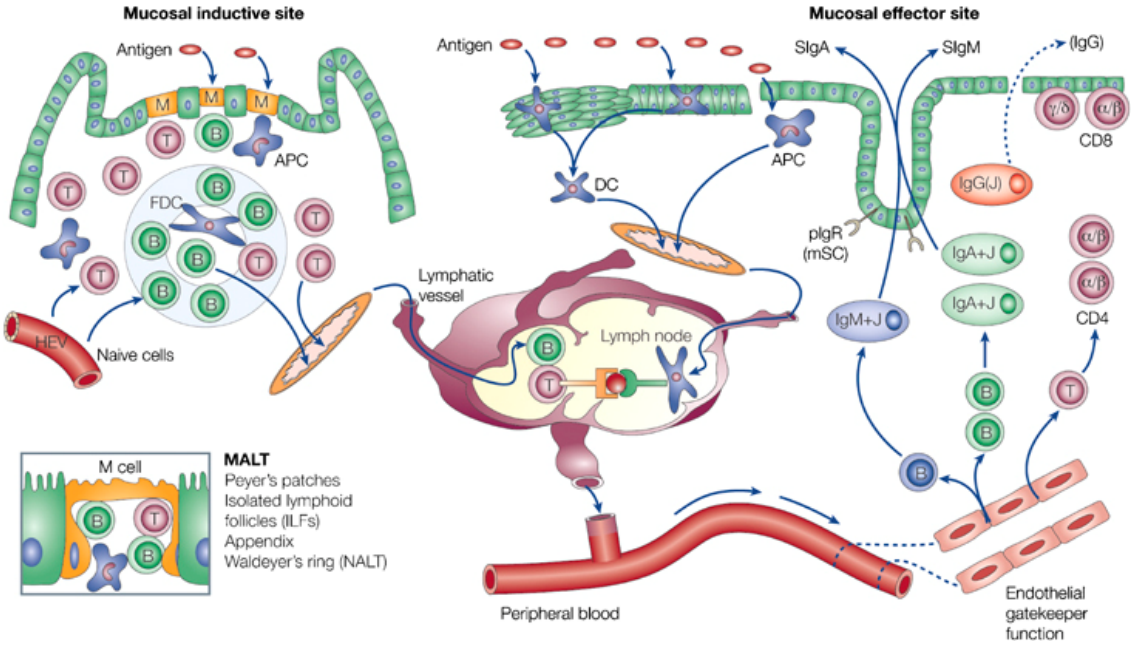


Figure 1 Depiction of the human mucosal immune system. Inductive sites for mucosal immunity are constituted by regional MALT with their B-cell follicles and M-cell including dendritic cells, macrophages, B cells, and follicular dendritic cells. The gut lamina propria contains few B lymphocytes but many J-chain-expressing IgA (dimers/polymers) and IgM (pentamers) plasmablasts and plasma cells. Additional features are the generation of secretory IgA (SIgA) and SIgM via pIgR membrane secretory component mediated epithelial transport. The inset (lower left corner) shows details of an M cell and its "pocket" containing various cell types (Brandtzaeg and Pabst, 2004).

1.2.2 Mucosal immunity in fish

In contrast to mammals fish do not possess organized lymphoid tissue such as Peyer's patches. In addition, M cells or secretory IgA (SIgA) have not been described in fish. However, uptake of macromolecules by intestinal epithelial cells and many diffusely distributed leucocytes including intraepithelial lymphocytes (IELs) are described in a variety of fish species, such as carp (Abelli et al., 1997; Rombout et al., 1989; Rombout et al., 1993; Temkin and McMillan, 1986), goldfish (*Carassius auratus*) (Temkin and McMillan, 1986), rainbow trout (Joosten et al., 1996), sea bass (Abelli et al., 1997; (Picchietti et al., 1997) and Atlantic salmon (Bakke-McKellep et al., 2007; Urán et al., 2008). Not all but at least most fish species studied have a special region of gut, the so-called second gut segment, that seems to be specialized in antigen uptake (Bakke-McKellep et al., 2000; Rombout et al., 1985; Rombout and van der Berg, 1989; Stroband et al., 1979). Macromolecules are taken up by epithelial cells of this gut segment and are transferred from lumen to macrophages and the blood stream, but this phenomenon may not be present in each fish species. In addition, the uptake mechanisms may vary during the development of the fish as active uptake of macromolecules is seen more often in juveniles than in adults (Nakamura et al., 2001). The physiological function of the uptake mechanism is still unclear. Except for juveniles, the amount of molecules transferred to the blood stream is only a minor part of administrated molecules and the uptake in the second segment may be more of immunological than nutritional value (Hernandez-Blazquez and Silva, 1998; Rombout and van der Berg, 1989).

Several studies have demonstrated the presence of IgM positive cells in intestine of sea bass, turbot (*Scophthalmus maximus*), carp and cod (Abelli et al., 1997; Fournier-Betz et al., 2000; Rombout et al., 1989; Rombout et al., 1993; Schrøder et al., 1998) and they were mainly present in the lamina propria. In general, fish IELs are suggested to be IgM negative lymphoid cells and hence putative T cells (Rombout et al., 2010). In Atlantic salmon, it has been reported that serum IgM was not detected in mucus and administrated serum IgM was immediately degraded in intestinal mucus (Hatten et al., 2001). These studies lead to a preconception that fish could have alternative defense mechanisms in their intestine, like the recently described IgT which is involved in the mucosal immunity in rainbow trout (Zhang et al., 2010). Zhang et al (2010) reported that IgT is preferentially expressed at the mucosal

epithelium in the trout gut, bind to local bacteria and are induced by mucosal pathogen. In addition, IgT is transported by the polymeric Ig receptor (pIgR) which is like mammalian IgA, and maintains a portion of pIgR as an associated secretory piece but in the fish secretory component has not been evident so far (Flajnik, 2010; Zhang et al., 2010). In mammals pIgR is an essential component for the mucosal immune system, particularly for transportation of IgA. The pIgR expressed on the basolateral membrane of the epithelial cells binds to polymeric Ig molecules and transport this Ig through the cell to the mucosal surface by transcytosis. The pIgR amino acid sequences of some fish species have been published (Feng et al., 2009b; Hamuro et al., 2007; Rombout et al., 2008) and mammalian pIgR contains five Ig-like domains (ILDs) whereas teleost contains two ILDs which correspond to the mammalian ILD1 and ILD5.

Oral administration of proteins and peptides is a great potential method for vaccination in aquaculture and some studies have already shown that some physiologically active proteins, such as growth hormone (Hertz et al., 1991b; Le Bail et al., 1989; McLean et al., 1990; Moriyama et al., 1990), insulin (Hertz et al., 1991a) and antibody (Nakamura et al., 1990), were successfully delivered by the oral route. Oral vaccination can reduce stress to fish and labour cost and can be applied on young fish, but special and cost-effective vaccine delivery systems are required to make it applicable for fish.

1.3 Defense mechanisms and diseases in cod

1.3.1 The immune system of cod

Cod has some special features in its immune system. Until recently it was believed that cod could not respond to immunization or has poor responses against pathogens (Pilström et al., 2005). The meager humoral responses against some pathogens have led to the presumption that cod depend more on innate immunity than adaptive immunity (Pilström et al., 2005; Rønneseth et al., 2007). However, recent studies have demonstrated high and specific antibody responses in cod after immunization with inactivated *Vibrio anguillarum*, *Aeromonas salmonicida*, or *Francisella noatunensis* (Lund et al., 2006; Schrøder et al., 2009). It also has been reported that cod i.p. immunized with *V. anguillarum* serotype O2b showed high

individual differences in their antibody responses against LPS epitopes (Lund et al., 2007). One possible hypothesis of limited humoral responses in cod is the absence of MHC class II (Pilström et al., 2005). MHC class II is crucial for antigen presentation to CD4+Th2 cells to start the antibody production (Murphy et al., 2008), and an activating signal by CD4+Th2 cells, to initiate the differentiation of B cell into antibody producing plasma cells. Rønneseth et al (2007) support this hypothesis by showing sufficient numbers of B cells in the cod leukocyte population but remarkably only a few antibody producing plasma cells. In mammals negative selection of T cells in thymus has been demonstrated and the numbers of MHC class I sequences expressed have influences on the negative selection (Parham, 1994). If one could speculate that cod have similar mechanisms of negative selection, the high number of MHC class I molecules expressed could have a profound impact on the T cell repertoire.

The extraordinary high concentration of natural antibodies in the serum (Israelsson et al., 1991; Magnadóttir et al., 1999a; Magnadóttir et al., 1999b; Solem and Stenvik, 2006) and unusually high numbers of neutrophils in peripheral blood leukocytes also support the theory that cod have a well-developed innate immune system (Rønneseth et al., 2007). The high respiratory burst activity (Nikoskelainen et al., 2006) that corresponds with the high number of neutrophils in the blood is also supportive for this hypothesis (Rønneseth et al., 2007).

Some antimicrobial peptides and proteins involved in innate immunity have been identified and characterized in cod, and include bactericidal permeability-increasing protein/ lipopolysaccharide-binding protein (BPI/LBP), g-type lysozyme, hepcidin (Solstad, 2008), cathelicidin (Maier et al., 2008) and piscidin (Fernandes et al., 2010). For instance Atlantic cod have two variants of g-type lysozyme, *codg1* and *codg2* (Larsen et al., 2009). By comparing the transcription of the two g-type lysozyme variants it was found that *codg2* was significantly more expressed than *codg1* in head kidney, whereas an opposite profile was observed in gills. The *codg2* transcript is significantly upregulated in the peritoneum and gills after i.p. injection of inactivated *Vibrio anguillarum*, indicating a role of g-type lysozyme in immunity of Atlantic cod (Larsen et al., 2009). However, mRNA levels do not necessarily correlate with the amount of protein expressed in a given cell or tissue, and studies on the protein level are required to further characterize the cellular and tissue expression and localisation of g-type lysozyme(s) in cod.

Many important immune cells such as macrophages, B cells, plasma cells, granulocytes and neutrophils are found in cod head kidney (Rønneseth et al., 2007; Sørensen et al., 1997b; Steiro et al., 1998). In addition, scavenger endothelial cells isolated from heart are also suggested to be important in the innate immune system of cod and contribute to the clearance of waste macromolecules (Sørensen et al., 1997a). It has been reported that cod scavenger endothelial cells have displayed both uptake of LPS (Seternes et al., 2001) and a response to poly I:C treatment (Martin-Armas et al., 2008).

Recently a significant amount of cod functional genomic data has been accumulated, such as microarray platforms (Booman et al., 2010; Edvardsen et al., 2011; Lie et al., 2009), full genome project (Johansen et al., 2009), and single nucleotide polymorphism (SNP) linkage map (Hubert et al., 2010). Despite the abundant genomic data currently available, applicable tools for cod immunology studies and immune-competence monitoring are still very limited. Currently only few antibodies are available for cod, namely anti-cod IgM (Aquatic Diagnostics Ltd, Institute of Aquaculture, University of Stirling), anti-cytochrome p450 (Aquatic Diagnostics Ltd, Institute of Aquaculture, University of Stirling), anti-CRP (Gisladottir et al., 2009) and anti-C3 beta chain (Lange et al., 2004). The development of new markers is crucial for advanced studies of cod immunology.

1.3.2 Diseases in cod

In cod farming, most losses occur during the larval and juvenile stages when their immune system has not been fully developed. Vibriosis has been the major bacterial disease in cod, whereas francisellosis is an increasing problem. Besides vibriosis and francisellosis atypical furunculosis caused by *Aeromonas salmonicida* is also registered as a serious disease problem in cod farming. According to our knowledge, viral diseases such as IPN (infectious pancreatic necrosis) has not yet been reported in cod farming in Norway whereas VNN (viral nerve necrosis) are reported a few times (reviewed in Samuelsen et al., 2006, Bornø et al., 2009).

1.3.2.1 Vibriosis

Classical vibriosis caused by *Vibrio anguillarum* is one of the major bacterial diseases in Norwegian cod farming and is also known in a wide range of fish species such as Atlantic halibut, Atlantic salmon, common wolfish (*Anarhichas lupus*) and turbot (VESO, 2007). More than 23 serotypes of *V. anguillarum* have been described and serotypes O2a and O2b are most commonly associated with cod (Larsen et al., 1994; Pedersen et al., 1999). Lately deviating sero-subtypes other than O2a and O2b have been isolated from diseased cod (Mikkelsen et al., 2007). The feature of clinical vibriosis is the level of anaemia. Haemorrhages in the head region, especially in and around the eyes (exopthalmia) in small fish, and haemorrhages and wounds on the skin and fins in adult fish are often observed (Samuelsen et al., 2006). The mortality is high in juveniles and in acute and severe epizootics most of fish die without showing external signs. High temperature of rearing water and stress enhance the risk of disease. Currently water-based and oil-based vaccines for vibriosis are available in Norway (http://www.pharmaq.no/).

1.3.2.2 Francisellosis

Francisella noatunensis (previously named as *Francisella philomiragia* subsp. *noatunensis* and *Francisella piscicida*) is a facultative intracellular Gram-negative bacterium causing francisellosis. Francisellosis was first reported in Norway in 2004 (Nylund et al., 2006; Olsen et al., 2006) and has become the most serious disease problem in the cod farming industry (Mikalsen et al., 2007; Nylund et al., 2006; Olsen et al., 2006; Ottem et al., 2007) but no efficient vaccine has been developed against *Fracicella* yet. The fish suffering from this disease show loss of appetite reduced swimming performance and dark pigmentation. The most prominent internal clinical signs are swollen spleen and head kidney with white granuloma-like structures (nodules) but the disease can also develop without clinical signs. In the terminal stage of the disease the spleen may have grown to three times the normal size being completely covered with and penetrated with granulomas, in addition to the presence of granulomas in other organs as skin, gills and mouth cavity (Nylund et al., 2006; Olsen et al., 2006). The target cells for this pathogen seem to be cells with phagocyte functions (i.e. phagocytes). In the early stage of the disease bacteria are always present in phagocytes in the spleen and head kidney, but can also be found in

endothelial cells lining in the heart chambers and leucocytes attached to the blood vessel walls in the liver, pseudobranch and gills (Nylund et al., 2006). Recently, the intracellular lifestyle of *Francisella* has been displayed in adherent cod leucocytes (mainly monocytes and macrophages) containing numerous intracellular *F. noatunensis* following an *in vitro* infection (Furevik et al., 2011). Bacteria were initially observed grouped together and located near the nucleus, and later they were found spread within the cytoplasm.

2 Aims of study

The objectives of this study were to investigate the defense mechanisms of Atlantic cod with special emphasis on intestinal mucosal immunology. The mucosal immune system provides the first line of defense against invading pathogens but still not much is known from this system in cod. Information on anatomic functional characteristics would help the understanding of how it induces and regulates immune responses. In addition, investigation of immune responses and the components involved during bacterial infection would help to shed light on how systemic and local immunity are mounted in cod.

- Study the anatomy of cod intestine and characterize the antigen uptake capacity.
- Investigate the immune components, cells and immune gene expression that are involved in *Vibrio anguillarum* and *Francisella noatunensis* infections.
- Develop new tools for immunological research and infection studies.
- Study immunopathological changes and inflammatory cells during *Francisella noatunensis* infection.

3 Abstract of papers

Paper I

Immune gene expression in the initial phase of *Vibrio auguillarum* infection in cod (*Gadus morhua* L.)

Makoto Inami, Jan H.W.M. Rombout, Viswanath Kiron and Merete Bjørgan Schrøder

The aim of the present study was to investigate the expression of immune related genes in foregut, hindgut and spleen of cod in the first days after intraperitoneal (i.p.) injection of live *V. anguillarum*. Real-time quantitative PCR was used to measure the mRNA expression of cytokine (*IL-1β* and *IL-8*) and antimicrobial (*hepcidin* and *BPI/LBP*) genes. Significant increases of *IL-1β* expression were observed both in foregut and hindgut (approximately 3-fold and 10-fold, respectively) at day 4. *IL-8* showed similar expression patterns as *IL-1β*, although *IL-8* levels were observed to be higher than *IL-1β* levels. In spleen a higher expression of *IL-1β* and *IL-8* was observed from 1 day post infection. The foregut and hindgut showed the highest expression of *hepcidin* at day 4 (approximately 23-fold and 70-fold, respectively) whereas spleen showed the highest up-regulated expression at day 1 (approximately 55-fold). Elevated expressions of *BPI/LBP* in infected groups were observed in all organs tested. In summary, fast and elevated expression of *IL-1β*, *IL-8* and *hepcidin* was observed in spleen after infection with live *V. anguillarum*, whereas later up-regulation was observed in foregut and hindgut. This study revealed higher expression levels of immune genes in hindgut compared to foregut. Up-regulation of *BPI/LBP* was observed in all organs from day 1 post infection but with variable expression levels.

Paper II

Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.).

Makoto Inami, Anja J. Taverne-Thiele, Merete Bjørgan Schrøder, Viswanath Kiron and Jan H.W.M. Rombout

The defence system of the distal gut (hindgut and rectum) of Atlantic cod, (Gadus morhua L.) was studied using (immuno)histochemical, electron microscopical and real-time quantitative PCR techniques. The uptake and transport of macromolecules in the intestinal epithelium was also investigated. In this study we observed that cod has many and large goblet cells in its intestinal epithelium and that IgM+ cells are present in the lamina propria and their number is considerably higher in the rectum than in the intestine. Myeloperoxidase staining revealed low numbers of granulocytes in and under the epithelium of the distal intestine, whereas high numbers were found clustered in the submucosa of the rectum. Electron microscopy not only confirmed these observations, but also revealed the presence of lymphoid cells and macrophages within the intestinal epithelium. Acid phosphatase staining demonstrated more positive macrophage-like cells in the rectum than in the distal intestine. Antigen uptake studies showed a diffused absorption of horse radish peroxidase (HRP) and LTB-GFP, whereas ferritin uptake could not be detected. Basal gene expression of cytokines (*IL-1β, IL-8* and *IL-10*) and immune relevant molecules (*hepcidin* and *BPI/LPB*) were compared in both the intestine and rectum and revealed approximately 2–9 times higher expression in the rectum, of which *IL-1β* expression showed the most prominent difference. The present results clearly indicate that intestinal immunity is very prominent in the rectum of cod.

Paper III

***Francisella noatunensis* in Atlantic cod (*Gadus morhua* L.); waterborne transmission and immune responses.**

Terje Ellingsen*, Makoto Inami*, Mona Cecilie Gjessing, Koen Van Nieuwenhove, Rannveig Larsen, Marit Seppola, Vera Lund and Merete Bjørgan Schrøder

*contributed equally

This is the first report that confirms horizontal waterborne transmission of francisellosis in Atlantic cod. To investigate the transmission of disease, particle reduced water was transferred from a tank with intraperitoneally infected cod to a tank with healthy cod. Waterborne transmission was confirmed in the effluent group using immunohistochemistry and real-time quantitative PCR (RT-qPCR) detecting *Francisella noatunensis*. The bacteria were located inside of accumulated macrophage-like cells. In addition, specific and high antibody responses against live and inactivated bacteria were observed in which the live bacteria gave the highest antibody titres. Oil adjuvant had no effect on the antibody responses against inactivated *F. noatunensis*. The antigen epitope was a 20-25 kDa component suggested to be a lipopolysaccharide detected with Western blot, Coomassie blue, Sypro Ruby and Silver staining. Systemic immune reactions were investigated by measuring the expression of *IFN-γ*, *IL-1β* and *IL-10* genes with RT-qPCR. After i.p. injection of live bacteria, a significant up-regulation of *IFN-γ* and *IL-1β* expression was observed from 15 to 60 days post infection in spleen and head kidney. In intestine, *IFN-γ* was significantly up-regulated after 60 days whereas rectum showed no significant differences in expression. Elevated expression of *IL-10* was observed in all the organs tested but was only significantly up-regulated at 60 days post infection in intestine from i.p. infected fish. Up-regulation of *IL-1β* and *IFN-γ* was observed in spleen whereas intestine and rectum showed a down-regulation in cohabitant fish after 60 days. *IL-10* was up-regulated in intestine of cohabitant fish from day 30 to day 60. These results indicate that *F. noatunensis* infection provokes both specific antibody responses and long term inflammatory responses in cod. The present study provides new knowledge about infection routes and shows that both humoral and cellular defence mechanisms are triggered by *F. noatunensis* in cod.

Paper IV

Characterization of an antiserum against Atlantic cod (*Gadus morhua* L.) g-type lysozyme.

Makoto Inami, Sein Tore Solem, Trond Ø Jørgensen and Atle N Larsen

In this study we describe the production and characterization of an antiserum against recombinant g-type lysozyme derived from Atlantic cod. This is also the first initial analyses of g-type lysozyme protein expression in tissues of Atlantic cod. Recombinant expression and purification of cod g-type lysozyme was used for immunization to rabbit and the rabbit sera were analysed for anti g-type lysozyme antibodies using enzyme-linked immunosorbent assay (ELISA), Western blot and immunohistochemistry. ELISA results showed that antibody titres were mounted between 12,800 and 25,600 as measured at an optical density corresponding to 50% of the maximal level. By Western blot analysis of both spleen and head kidney homogenates from the Atlantic cod, the rabbit immune serum detected a single ~ 23 kDa band representing the size of the injected antigen. Immunohistochemisrty detected the native folded g-type lysozyme in tissues and revealed that g-type lysozyme immunoreactive cells were observed in haematopoietic tissue of the head kidney and in red pulp of spleen. In conclusion, the rabbit anti g-type lysozyme immune sera developed can effectively utilized for ELISA, Western analysis as well as for immunohistochemistry. This has allowed us to obtain new knowledge about this protein regarding localization and distribution in cod tissue.

Paper V

Presence and interaction of inflammatory cells in the spleen of Atlantic cod infected with *Francisella noatunensis*

Mona Cecilie Gjessing, Makoto Inami*, Simon Weli*, Terje Ellingsen, Knut Falk, Erling Olaf Koppang and Agnar Kvellestad

*contributed equally

Serious infectious diseases accompanied by macrophage-dominated chronic inflammation, are common in farmed Atlantic cod. To increase knowledge relating to morphological aspects of such inflammatory responses, cod were challenged with *Francisella noatunensis,* an important bacterial pathogen of this fish species. Tissue and cell dynamics in the spleen were examined sequentially over a period of 60 days. Small clusters of mainly macrophage-like cells staining for non-specific esterase and acid phosphatase, developed with time. These foci were transiently infiltrated by pleomorphic proliferating cells of unknown nature and by granulocyte-like cells staining for peroxidase and lysozyme. The latter cell-type, which appeared to be resident in the red pulp of control fish, migrated into the inflammatory foci of infected fish. Cells expressing genes encoding IFN-γ and IL-8 increased in number during the study period. Bacteria were detected only in the macrophage-like cells and their number increased despite the extensive inflammation. Our results demonstrate an intimate spatial relationship in inflammatory foci between at least three cell types. The presence of granulocyte-like cells, together with macrophage-like cells, suggests pyogranulomatous inflammation as a more appropriate descriptive term than granulomatous inflammation.

4 Discussion

4.1 Intestinal immunity in cod

4.1.1 Second gut segment in cod?

In most teleost species investigated the intestine can be divided in three segments based on the microscopical anatomy of their mucosa (Rombout et al., 2010; Stroband et al., 1979). Among these three segments, the second gut segment is characterized by a strong antigen uptake capacity. The epithelial cells have large supranuclear vacuoles (SNV) and show high apical pinocytotic activity. Interestingly, in cod the appearance of the second gut segment is not obvious (paper II). The microscopic observation revealed a rather homogeneous morphology in the entire intestine and active pinocytosis (uptake of ferritin or horseradish peroxidase: HRP) and SNV were not observed in any part of the intestine of adult cod (paper II). Instead, the rectal part of the intestine, which is separated with a valve-like muscular structure (Fig. 2), showed a clearly different morphology, especially in terms of distribution and number of immune cells (paper II). In some studies cod rectum is also described as a hindgut (fermentation) chamber (Morrison, 1987; Ringø et al., 2006) and it has been suggested that this chamber is a part of the cod gastrointestinal tract, wherein carnobacteria (able to inhibit the growth of pathogens) are the major bacterial group (Ringø et al., 2006; Seppola et al., 2006). Whether the bacterial load and/or the invasion of microbes via the anus is the reason for the higher amount of immune cells in the rectum is still a matter of debate.

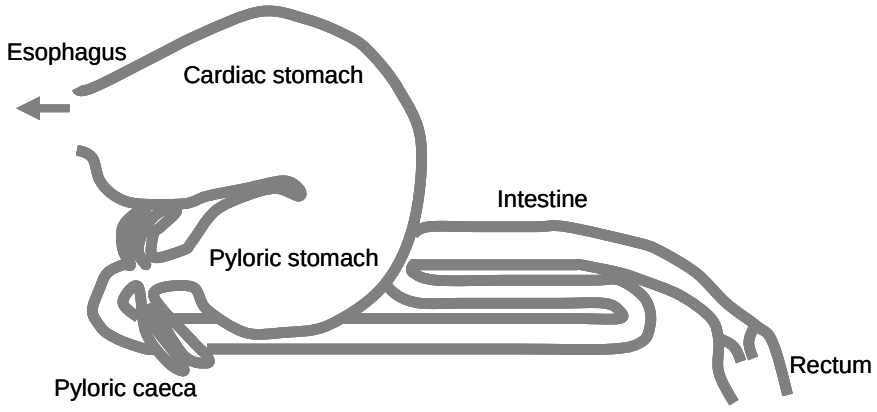


Figure 2 Schematic image of the digestive tract of cod which consists of the esophagus, stomach, pyloric caeca, intestine and rectum. The intestine enters the rectum with a musculature valve.

In paper II uptake of ferritin was not observed in the intestine or rectum neither with light microscopy nor with electron microscopy, although uptake of macromolecules, such as ferritin and HRP, were well described in species like carp, grass carp, goldfish, barbs, perch, trout and salmon. (Gauthier and Landis, 1972; Georgopoulou et al., 1988; Noaillac-Depeyre and Gas, 1973; Noaillac-Depeyre and Gas, 1976; Noaillac-Depeyre and Gas, 1979; Rombout et al., 1985; Stroband and Kroon, 1981; Stroband and van der Veen, 1981). Even though no ferritin uptake was observed after anal administration to the rectum, some grouped enterocytes in the rectal epithelium showed SNV-like structure possessing iron-containing molecules (paper II). The function and nature of these iron-containing molecules observed in rectal enterocytes are still unknown but this could be an indication of iron-withholding or an iron-regulation system as a part of defence mechanisms against microbes in rectal enterocytes. The higher basal expression of hepcidin, which has a role in iron homeostasis in the rectum, compared to the intestine is in accordance with this speculation. For cod, anal intubation of macromolecules to study uptake capacity is physically difficult because of the valve-like structure between intestine and rectum (paper II), that does not allow the passage from rectum to gut (Fig. 2). However in *ex vivo* conditions LTB-GFP seemed to be absorbed well in both intestine and rectum (paper II), and hence LTB may also be considered as a suitable intestinal delivery system in cod, as is reported for other fish species (Rombout et al., 2010). In addition, remarkably large numbers of myeloperoxidase (MPO) positive cells and granulocytes were observed in the connective tissue of rectal submucosa (paper II). In the pearl gouramy and carp a subpopulation of eosinophilic granular cells (ECGs) has been suggested to have different stages of development (Leknes, 2007; Rombout et al., 1989) and at present it cannot be excluded that these differences in maturation also exist in cod granulocytes, based on the individual variation of the granule size. The nature and function of these cells are not yet identified in cod. In Atlantic salmon, antigen-sampling cells in intestine were described in the second gut segment and suspected to be analogous to the mammalian M cells (Fuglem et al., 2010). As far as we know from the anatomical investigation of cod intestine (paper II) this type of cell was not detectable but further investigation is required to answer this issue in cod.

Overall, it can be concluded that the rectum of cod contain more immune cells than the distal intestine and hence seems to be better equipped against pathogen invasion compared to the intestine. Although the second gut segment in adult cod was

not evident active pinocytosis and SNV was observed in the rectum of juvenile cod (Fig. 3, preliminary results). Some early histological studies have reported that enteric protein uptake mainly occurs in the larval stages and disappears during further development of the digestive tract (Nakamura et al., 2001; Walford and Lam, 1993; Watanabe, 1984). Our preliminary results on cod larvae also suggest that active pinocytosis and SNV could be an age dependent phenomenon in cod. However, it may also be diet dependent as each developmental stage of cod has its own diet. It has been well demonstrated that apical pinocytosis and SNV can disappear under certain dietary conditions (Urán et al., 2008; Urán et al., 2009). Uptake of intact antigen from bacteria in the intestine of four- to six-day old cod larvae has been demonstrated and it has been suggested that this uptake may play a role in the immune development or in nutritional uptake (Olafsen and Hansen, 1992). In young fish attention has to be paid to the development of the immune system to avoid oral tolerization of the antigen, resulting in an undesirable negative memory formation.

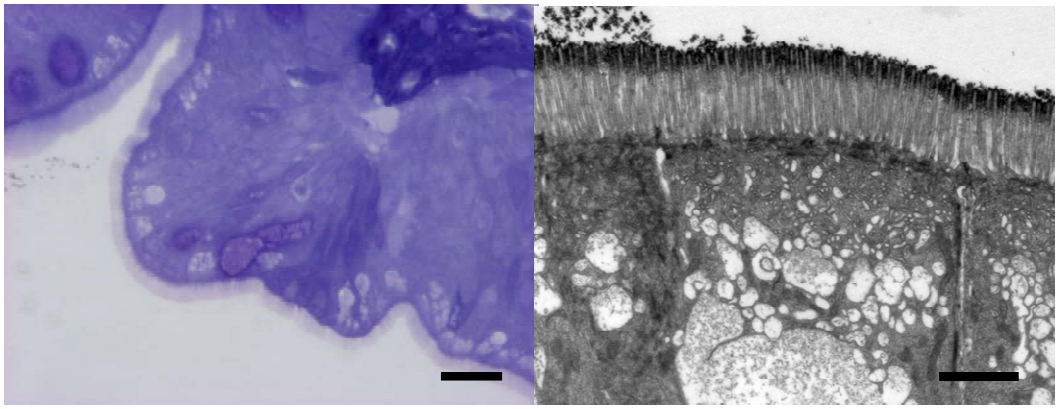


Figure 3 Microscopic pictures of cod juvenile (100 day post hatcing). **Left**: semi-thin Epon sections stained with toluidin-blue showing the clear SNV in the apex of the epithelial cells. Bar is 10 µm. **Right**: Transmission electron micrograph showing that rectal epithelial cells in 100 dpf cod show strong endocytoic capacity and large SNV in the apex of the enterocytes. Bar is 1 µm.

4.1.2 Gene expression in cod intestine

As discussed above, the features of the second gut segment reported for some fish species were not clearly noticeable in adult cod, but instead the rectum appeared to contain more immune related components compared to hindgut (paper II). In paper I-III immune gene expression in different regions of the gut were investigated and even though cod has no morphological changes throughout the intestine, the immune genes seem to have a differential expression in the intestine. The difference of basal expression of *IL-1β, IL-8, IL-10, hepcidin* and bactericidal/permeability increasing protein/ LPS-binding protein (*BPI/LBP*) between hindgut and rectum was obvious probably due to the high numbers of immune cells in the rectum (paper II). Relatively low expressions of immune genes in the gut were observed when compared to the spleen (paper I and III), but this may also be explained by the strong difference in the number of immune cells observed in these organs. A tendency that the hindgut showed higher expression level of cytokines and antibacterial genes compared to the foregut after a Vibrio infection was observed (paper I). The immune gene expression study in trout second gut segment after *A. salmonicida* bath challenge was reported and the expression of *IL-1β, IL-8, TNF-α* and *IFN-γ* was increased in the proximal intestine whereas *TGF-β* was significantly decreased in the distal intestine (Mulder et al., 2007). In Atlantic salmon (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000; Urán et al., 2008; van den Ingh et al., 1991) and carp (Urán et al., 2008) induction of enteritis in the distal intestine was observed and in carp up-regulation of cytokine genes, such as *IL-1β*, *TNF-α*, *TGF-β* and *IL-10* were observed after soy bean meal (SBM) feeding. The higher expression of these cytokines is probably related to the SBM-induced eneritis process observed. On the other hand, it has been reported that cod do not show patho-morphological changes in its digestive tract after SBM feeding, and some peptides such as cytochrome P450 3A40 (oxidative metabolism of drugs, environmental chemicals and endogenous compounds) and ras-related nuclear protein (regulation of cell cycle) showed the increased expressions after SBM feeding, but no transcriptional study regarding immune related genes has been reported yet (Lilleeng et al., 2007). In paper I, III and V i.p. injection of inactivated or live bacteria was used as the antigen delivery route and mainly the systemic immune system was studied. However, the waterborne transmission of francisellosis in paper III may occur via the mucosal route (intestine, skin, mouth and/or gills). Even though i.p.

injection was not the most suitable method to study local immunity up- and/or down-regulation of immune genes were observed in both intestine and rectum (paper I and III). For instance in paper III significant down-regulation of *IL-10* was observed in infected fish in cohabitant and effluent groups. In mammals IL-10 has been reported to be active in mucosal surfaces to counterbalance inflammatory cytokines and protect the tissue from inflammatory reactions. One could speculate that the significant changes of *IL-10* gene expression in the rectum of cod might indicate the chronical infection caused by *F. noatunensis*. In conclusion, the responses to *V. anguillarum* and *F. noatunensis* in the intestine of cod are probably the result of differential expression of numerous cytokines and this could indicate an involvement of local immunity in cod gut provoked by the bacterial challenge.

4.2 Immune responses in cod during infection and immunization

During bacterial challenge, immune gene expression in spleen (paper I and III) and head kidney (paper III) were monitored. High expression of *IL-1β* and *IL-8* was observed in spleen at 1 day post injection (d.p.i.) of live *V. anguillarum* (paper I). This result is in accordance with the previous gene expression study after immunization with inactivated *V. anguillarum,* where quick and high up-regulation of certain cytokines genes (*IL-1β, IL-8* and *IL-10*) were reported in spleen and head kidney (Seppola et al., 2008). Seppola et al (2008) concluded that cod display comparable inflammatory responses as described for other fish species (Chai et al., 2006; Chen et al., 2005; Corripio-Miyar et al., 2007; Inoue et al., 2005; Lee et al., 2001; López-Castejón et al., 2007; Pinto et al., 2007; Rojo et al., 2007; Scapigliati et al., 2001; Seppola, 2008). Up-regulation of *IFN-γ, IL-1β* and other cytokines genes were described after 3 h in gill epithelial cells infected with *V. anguillarum* and *A. salmonicida* (Caipang et al., 2010). Compared to this fast response, the elevation of gene expression was observed rather late (after day 15 to 60) in spleen, head kidney and gut (paper III) and after 1-4 days in foregut and hind gut (paper I). This could be due to the different administration routes, but also to the condition (inactivated and live) and dose of the pathogen used. In paper III high expression of *IFN-γ* was observed at 60 days post infection and in paper V many cells expressing genes encoding *IFN-γ* were observed at day 60. Taken together, the increasing number of bacteria, despite increased number of *IFN-γ* expressing cells in spleen (paper V),

suggest predominate macrophage phagocytosis as has been described in mammals (Parsa et al., 2008) and thus an unsuccessful elimination of the pathogen. Recently, the intracellular lifestyle of *F. noatunensis* was reported and the bacteria were localized within adherent leucocytes, consisting mainly of monocytes and macrophages (Furevik et al., 2011). It may be hypothesised that bacteria somehow avoid the potent intracellular killing mechanisms in cod phagocytes and escape to cytoplasm. In addition, an approximately 100-fold increase of *BPI/LBP* expression was observed in spleen after live *V. anguillarum* injection (paper I). Solstad et al (2007) reported an approximately 4-fold increase of *BPI/LBP* expression in head kidney after inactivated *V. anguillarum* injection (Solstad et al., 2007). They also displayed high expression of *BPI/LBP* using *in situ* hybridization in spleen and head kidney, where the difference between stimulated fish and control fish was more drastic in spleen than in head kidney tissue. This could indicate that the spleen may play an important role in the early phase of inflammatory responses by this pathogen.

In paper III antibody responses during infection and immunization with *F. noatunensis* were investigated. Although Atlantic cod is known to show low responsiveness against *Vibrio salmonicida* (Schrøder et al., 1992), *V. anguillarum* (Espelid et al., 1991) and protein antigens or hapten-carriers (Magnadóttir et al., 2001; Pilström and Petersson, 1991), *F. noatunensis* antigen seem to provoke constant and specific antibody responses with less individual differences (Schrøder et al., 2009). This is confirmed in paper III and lead to the conclusions that 1) cod can mount a comparable amount of antibody against this pathogen as other fish species, 2) oil adjuvant is not the key factor to provoke the immune responses in the case of this pathogen, and 3) low individual differences in immune responses are evoked (paper III). Interestingly, the injection of live bacteria gives higher antibody titre than inactivated bacteria (paper III). This may be due to the fact that the bacteria continuously grow in the fish body and keep the immune response going. The increase of IFN-γ observed in paper III and V may be produced by CD8+ T cells that binds with the protein antigen present on MHC class I molecules. This may result in the strong antibody responses against live *F. noatunensis*. This hypothesis may be in accordance with the speculation that cod lack the MHC class II molecules that are essential for CD4+ T cell activation and hence cod might not rely on T cell dependent antibody responses. On the other hand, some reports have shown weak responses against the A-layer protein of *A. salmonicida* (Lund et al., 2008; Lund et al., 2006).

The A-layer is composed of regularly arranged surface subunit proteins, making it possible to activate the antibody response in a T-cell independent manner. The mammalian pathogen *Francisella tularensis,* which is related to *F. noatunensis*, escapes acidification and degradation by disrupting the phagosomes of the phagocytes, and then reaches to the cytoplasm (Clemens et al., 2004; Golovliov et al., 2003). Phagocytes and epithelial cells have also been suggested to be the host cells for *F. noatunensis* in cod (Olsen 2006, Nylund 2006), which is also supported by the results in paper III and a recent study that confirms the migration of *F. noatunensis* from phagosomes to the cytoplasm in cod leukocytes (Furevik et al., 2011). Taken together, the observations in paper III and V indicate that both the cellular and humoral immune system seem to be stimulated during *F. noatunensis* infection and should be taken into account when vaccines are developed against this pathogen.

4.3 Immunopathology in cod

In paper V the development of inflammatory foci and the morphology of infected spleen was studied after intraperitoneal injection of live *F. noatunensis*. Fifteen days after injection, very small foci-like structures in spleen were visually observable, and necrotic damage was confirmed by microscopy. At a later stage of the infection granuloma-like inflammatory foci developed mostly in the spleen, head kidney and heart. The dominant cell types located in the foci were macrophage-like cells (MLCs), pleomorphic proliferating cells and granulocyte-like cells. Immunohistochemical studies using anti-*F. noatunensis* antibodies revealed that the bacteria were located in accumulated MLCs. This finding is in accordance with a recent study on the intracellular lifestyle of *F. noatunensis* (Furevik 2011). Both studies showed that the bacteria were present in the cytoplasm of macrophages/monocytes. The human pathogen *F. tularensis* was also present in the phagosome of macrophages during the infection and appear to replicate in the cytosol (Santic et al., 2008). These findings lead to the theory that *F. noatunensis* also possess cytoplasmic replication mechanisms and that antigen presentation should be through the MHC class I pathway. Lymphocyte-like cells expressing IFN-γ were observed in the red pulp of the spleen and the numbers of IFN-γ expressing cells increased after infection (paper V). IFN-γ is a powerful activator of microbicidal functions of phagocytes, but the *Francisella* bacterium somehow suppresses downstream events initiated by IFN-γ, at least in

mammalian francisellosis (Parsa et al., 2008). It is tempting to speculate that *F. noatunensis* would have similar strategies to escape from the cod immune system. Due to the lack of the specific cell markers for cod leukocytes, functional studies on cod macrophages and granulocytes *in vitro* or *in vivo* are scarce and not straightforward. For mammals non-specific esterase (NSE) is considered as a reliable macrophage marker and NSE has also been described in cod lymphocytes (Sørensen et al., 1997b). However, NSE is more generally detected in monocytes and macrophages of other teleosts (Afonso et al., 1997; Grove et al., 2006; Press et al., 1994; Tavares-Dias, 2006). In paper II and V MLCs were detected by acid phosphatase and NSE staining, whereas granulocyte-like cells were stained by MPO and anti-cod g-type lysozyme antiserum. The combination of different staining procedures may contribute to a better identification of leukocyte subpopulations, but needs further investigation to determine nature and function of these immune cells.

We have developed an anti-cod g-type lysozyme antiserum that is useful for immunohistochemistry, ELISA and western blot analysis (paper IV). For the development of anti-cod g-type lysozyme antiserum in paper IV the antigen was produced recombinantly in *Escherichia coli* (Larsen et al., 2009). In paper V this antibody was used to characterize the cell types which are involved in *F. noatunensis* infection. The cells showed positive staining for g-type lysozyme and seemed to be granulocyte-like cells, although electron microscopy is required to determine the exact nature of the g-type lysozyme-immunoreactive cells. For some fish species commercial antibodies against human CD3ε (DAKO) is reactive and useful, but it did not reliably react with cod T cells (unpublished data). In conclusion, for a better understanding of the function of cod immune cells and molecules, much more and reliable markers are essential than presently available.

5 Main conclusions

- In the intestine of adult cod a second gut segment was not detectable by light and electron microscopy, whereas active pinocytosis and supranuclear vacuoles were observed in the juvenile rectum. The uptake capacity of the cod gut may be dependent on age and/or diet.
- The distribution of immune cells in cod intestine and rectum was clearly different; higher numbers of IgM positive cells and myeloperoxidase positive cells were found in rectum compared to intestine. This indicates that cod rectum seem to be better equipped for defence than the intestine.
- HRP and plant derived LTB-GFP absorptions (probably receptor-mediated) were observed in the cod gut. The use of HRP or LTB-GFP as antigen delivery molecules may be interesting for intestinal immunization.
- The immune genes, *IL-1β*, *IL-10* and *IFN-γ*, were expressed and regulated differently in the distinct regions of cod gut and may be up- and/or down-regulated after *V. anguillarum* and *F. noatunensis* challenge, indicating an involvement of intestinal immunity.
- Polyclonal antibodies developed against recombinant cod g-type lysozyme are able to detect the granulocyte-like cells in inflammatory foci after *F. noatunensis* infection.
- During *F. noatunensis* infection chronic inflammatory responses were observed by RT-qPCR, histology, immunohistochemistry and *in situ* hybridization. Both systemic infections by i.p. injection of pathogen and water transmission provoke the diseases. The waterborne transmission of francisellosis implies that the infection may occur via the mucosal route.
- Inflammatory foci in spleen were found after i.p.-injection of live *F. noatunensis*. Within the foci macrophage-like cells, granulocyte-like cells and pleomorphic cells with lysozyme and peroxidase were observed and bacteria were localized in macrophage-like cells.
- High and specific antibody responses were measured after i.p. injection of live or inactivated *F. noatunensis.* Live bacteria resulted in higher antibody titres than

inactivated bacteria. The stimulation of both cellular and humoral responses could be very important in developing efficient vaccines against this pathogen.

6 References

Abelli L., Picchietti S., Romano N., Mastrolia L. and Scapigliati G. (1997) Immunohistochemistry of gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). *Fish Shellfish Immunol* 7, 235-245.

Afonso A., Ellis A. E. and Silva M. T. (1997) The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol* 7, 335-348.

Baeverfjord G. and Krogdahl Å. (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. *J Fish Dis* 19, 375-387.

Bakke-McKellep A. M., Frøystad M. K., Lilleeng E., Dapra F., Refstie S., Krogdahl A. and Landsverk T. (2007) Response to soy: T-cell-like reactivity in the intestine of Atlantic salmon, *Salmo salar* L. *J Fish Dis* 30, 13-25.

Bakke-McKellep A. M., Press C. M., Baeverfjord G., Krogdahl Å. and Landsverk T. (2000) Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. *J Fish Dis* 23, 115-127.

Bang J. D., Kim J. W., Lee S. D., Park S. I., Chun S. G., Jeong C. S. and Park J. W. (1996) Humoral immune response of flounder to *Edwardsiella tarda*: the presence of various sizes of immunoglobulins in flounder. *Dis Aquat Organ* 26, 197-203.

Bengtén E., Clem L. W., Miller N. W., Warr G. W. and Wilson M. (2006) Channel catfish immunoglobulins: Repertoire and expression. *Dev Comp Immunol* 30, 77-92.

Bly J. E. and Clem L. W. (1992) Temperature and teleost immune functions. *Fish Shellfish Immunol* 2, 159-171.

Boman H. G. (2003) Antibacterial peptides: basic facts and emerging concepts. *J Intern Med* 254, 197-215.

Booman M., Borza T., Feng C., Hori T., Higgins B., Culf A., Léger D., Chute I., Belkaid A., Rise M., Gamperl A., Hubert S., Kimball J., Ouellette R., Johnson S., Bowman S. and Rise M. (2010) Development and experimental validation of a 20K Atlantic cod (*Gadus morhua*) oligonucleotide microarray based on a collection of over 150,000 ESTs. *Mar Biotechnol*, 1-18.

Bornø G., Sviland C., Jensen B. B., Tarpai A., Garseth Å. H., Skjelstad H. R., Johansen R., Dale O. B., Fritsvold C., Nilsen H., Vaagnes Ø., Flesjå K., Aune S., Colquhoun D., Ørpetveit I., Hansen H., Heuch P. A., Hjeltnes B., Hellberg H., Nilsen H. K., Colquhoun D. and Jensen B. B. (2009) Fiskehelserapporten 2009 (Edited by Veterinærinstituttet), Oslo.

Borza T., Stone C., Rise M. L., Bowman S. and Johnson S. C. (2010) Atlantic cod (*Gadus morhua*) CC chemokines: Diversity and expression analysis. *Dev Comp Immunol* 34, 904-913.

Brandtzaeg P. and Pabst R. (2004) Let's go mucosal: communication on slippery ground. *Trends Immunol* 25, 570-577.

Caipang C. M. A., Lazado C. C., Brinchmann M. F. and Kiron V. (2010) Infection-induced changes in expression of antibacterial and cytokine genes in the gill epithelial cells of Atlantic cod, *Gadus morhua* during incubation with bacterial pathogens. *Comp Biochem Physiol B Biochem Mol Biol* 156, 319-325.

Chai Y., Huang X., Cong B., Liu S., Chen K., Li G. and Gaisano H. Y. (2006) Involvement of VAMP-2 in exocytosis of IL-1 beta in turbot (*Scophthalmus maximus*) leukocytes after *Vibrio anguillarum* infection. *Biochem Biophys Res Commun* 342, 509-13.

Chen L., He C., Baoprasertkul P., Xu P., Li P., Serapion J., Waldbieser G., Wolters W. and Liu Z. (2005) Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with *Edwardsiella ictaluri*. *Dev Comp Immunol* 29, 135-142.

Clemens D. L., Lee B. Y. and Horwitz M. A. (2004) Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. *Infect Immun* 72, 3204-17.

Corripio-Miyar Y., Bird S., Tsamopoulos K. and Secombes C. J. (2007) Cloning and expression analysis of two pro-inflammatory cytokines, IL-1β and IL-8, in haddock (*Melanogrammus aeglefinus*). *Mol Immunol* 44, 1361-1373.

Danilova N., Bussmann J., Jekosch K. and Steiner L. A. (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat Immunol* 6, 295-302.

Djaldetti M., Salman H., Bergman M., Djaldetti R. and Bessler H. (2002) Phagocytosis—The mighty weapon of the silent warriors. *Microsc Res Techniq* 57, 421-431.

Edvardsen R. B., Malde K., Mittelholzer C., Taranger G. L. and Nilsen F. (2011) EST resources and establishment and validation of a 16 k cDNA microarray from Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol D Genomics Proteomics* 6, 23-30.

Ellis A. E. (1977) The leucocytes of fish: A review. *J Fish Biol* 11, 453-491.

Espelid S., Rødseth O. M. and Jørgensen T. Ø. (1991) Vaccination experiments and studies of the humoral immune responses in cod, *Gadus morhua* L., to four strains of monoclonal-defined *Vibrio anguillarum*. *J Fish Dis* 14, 185-197.

Feng C. Y., Johnson S. C., Hori T. S., Rise M., Hall J. R., Gamperl A. K., Hubert S., Kimball J., Bowman S. and Rise M. L. (2009a) Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*. *Physiol Genomics* 37, 149-63.

Feng L. N., Lu D. Q., Bei J. X., Chen J. L., Liu Y., Zhang Y., Liu X. C., Meng Z. N., Wang L. and Lin H. R. (2009b) Molecular cloning and functional analysis of polymeric immunoglobulin receptor gene in orange-spotted grouper (*Epinephelus coioides*). *Comp Biochem Physiol B Biochem Mol Biol* 154, 282-9.

Fernandes J. M. O., Ruangsri J. and Kiron V. (2010) Atlantic Cod Piscidin and Its Diversification through Positive Selection. *PLoS ONE* 5, e9501.

Flajnik M. F. (2002) Comparative analyses of immunoglobulin genes: surprises and portents. *Nat Rev Immunol* 2, 688-98.

Flajnik M. F. (2005) The last flag unfurled? A new immunoglobulin isotype in fish expressed in early development. *Nat Immunol* 6, 229-30.

Flajnik M. F. (2010) All GOD's creatures got dedicated mucosal immunity. *Nat Immunol* 11, 777-779.

Flajnik M. F., Ohta Y., Namikawa-Yamada C. and Nonaka M. (1999) Insight into the primordial MHC from studies in ectothermic vertebrates. *Immunol Rev* 167, 59-67.

Fournier-Betz V., Quentel C., Lamour F. and Leven A. (2000) Immunocytochemical detection of Ig-positive cells in blood, lymphoid organs and the gut associated lymphoid tissue of the turbot (*Scophthalmus maximus*). *Fish Shellfish Immunol* 10, 187-202.

Fuglem B., Jirillo E., Bjerkas I., Kiyono H., Nochi T., Yuki Y., Raida M., Fischer U. and Koppang E. O. (2010) Antigen-sampling cells in the salmonid intestinal epithelium. *Dev Comp Immunol* 34, 768-74.

Furevik A., Pettersen E. F., Colquhoun D. and Wergeland H. I. (2011) The intracellular lifestyle of *Francisella noatunensis* in Atlantic cod (*Gadus morhua* L.) leucocytes. *Fish Shellfish Immunol* 30, 488-494.

Furnes C., Seppola M. and Robertsen B. (2009) Molecular characterisation and expression analysis of interferon gamma in Atlantic cod (*Gadus morhua*). *Fish Shellfish Immunol* 26, 285-92.

Gauthier G. F. and Landis S. C. (1972) The relationship of ultrastructural and cytochemical features to absorptive activity in the goldfish intestine. *Anat Rec* 172, 675-701.

Georgopoulou U., Dabrowski K., Sire M. F. and Vernier J. M. (1988) Absorption of intact proteins by the intestinal epithelium of trout, *Salmo gairdneri*. A luminescence enzyme immunoassay and cytochemical study. *Cell Tissue Res* 251, 145-52.

Gisladottir B., Gudmundsdottir S., Brown L., Jonsson Z. O. and Magnadottir B. (2009) Isolation of two C-reactive protein homologues from cod (*Gadus morhua* L.) serum. *Fish Shellfish Immunol* 26, 210-219.

Golovliov I., Baranov V., Krocova Z., Kovarova H. and Sjostedt A. (2003) An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. *Infect Immun* 71, 5940-50.

Grove S., Johansen R., Reitan L. J. and Press C. M. (2006) Immune- and enzyme histochemical characterisation of leukocyte populations within lymphoid and mucosal tissues of Atlantic halibut (*Hippoglossus hippoglossus*). *Fish Shellfish Immunol* 20, 693-708.

Gudmundsdóttir B. K., Björnsdóttir B., Gudmundsdóttir S. and Bambir S. H. (2006) A comparative study of susceptibility and induced pathology of cod, *Gadus morhua* (L.), and halibut, *Hippoglossus hippoglossus* (L.), following experimental infection with *Moritella viscosa*. *J Fish Dis* 29, 481-487.

Hamuro K., Suetake H., Saha N. R., Kikuchi K. and Suzuki Y. (2007) A teleost polymeric Ig receptor exhibiting two Ig-like domains transports tetrameric IgM into the skin. *J Immunol* 178, 5682-9.

Hancock R. E. and Diamond G. (2000) The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 8, 402-10.

Hansen J. D., Landis E. D. and Phillips R. B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *PNAS* 102, 6919-6924.

Hatten F., Fredriksen Å., Hordvik I. and Endresen C. (2001) Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. *Fish Shellfish Immunol* 11, 257-268.

Hernandez-Blazquez F. J. and Silva J. R. M. C. d. (1998) Absorption of macromolecular proteins by the rectal epithelium of the Antarctic fish *Notothenia neglecta*. *Can J Zool* 76, 1247-1253.

Hertz Y., Shechter Y., Madar Z. and Gertler A. (1991a) Oral absorptionof biologically active insulin in common carp. *Comp Biochem Physiol A* 101A, 19-22.

Hertz Y., Tchelet A., Madar Z. and Gertler A. (1991b) Absorption of bioactive human growth hormone after oral administration in the common carp (*Cyprinus carpio*) and its enhancement by deoxycholate. *J Comp Physiol B* 161, 159-63.

Holmgren J. and Czerkinsky C. (2005) Mucosal immunity and vaccines. *Nat Med*.

Hubert S., Higgins B., Borza T. and Bowman S. (2010) Development of a SNP resource and a genetic linkage map for Atlantic cod (*Gadus morhua*). *BMC Genomics* 11, 191.

Inoue Y., Kamota S., Ito K., Yoshiura Y., Ototake M., Moritomo T. and Nakanishi T. (2005) Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. *Fish Shellfish Immunol* 18, 335-344.

Israelsson O., Petersson A., Bengtén E., Wiersma E. J., Andersson J., Gezelius G. and Pilström L. (1991) Immunoglobulin concentration in Atlantic cod, *Gadus morhua* L., serum and cross-reactivity between anti-cod-antibodies and immunoglobulins from other species. *J Fish Biol* 39, 265-278.

Jakobsen S. E. and Lie E. (2009) Genetic secrets of cod revealed. The Research Council of Norway.

Janeway C. A., Jr. and Medzhitov R. (2002) Innate immune recognition. *Annu Rev Immunol* 20, 197-216.

Johansen S. D., Coucheron D. H., Andreassen M., Karlsen B. O., Furmanek T., Jørgensen T. E., Emblem Å., Breines R., Nordeide J. T., Moum T., Nederbragt A. J., Stenseth N. C. and Jakobsen K. S. (2009) Large-scale sequence analyses of Atlantic cod. *New Biotechnology* 25, 263-271.

Jolles P. and Jolles J. (1984) What's new in lysozyme research? Always a model system, today as yesterday. *Mol Cell Biochem* 63, 165-89.

Joosten P. H. M., Kruijer W. J. and Rombout J. H. W. M. (1996) Anal immunisation of carp and rainbow trout with different fractions of a *Vibrio anguillarum* bacterin. *Fish Shellfish Immunol* 6, 541-551.

Kaattari S. L. (1994) Development of a piscine paradigm of immunological memory. *Fish Shellfish Immunol* 4, 447-457.

Kapetanovic R. and Cavaillon J. M. (2007) Early events in innate immunity in the recognition of microbial pathogens. *Expert Opin Biol Ther* 7, 907-18.

Lange S., Dodds A. W. and Magnadóttir B. (2004) Isolation and characterization of complement component C3 from Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish Shellfish Immunol* 16, 227-239.

Larsen A. N., Solstad T., Svineng G., Seppola M. and Jorgensen T. Ø. (2009) Molecular characterisation of a goose-type lysozyme gene in Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 26, 122-32.

Larsen J. L., Pedersen K. and Dalsgaard I. (1994) *Vibrio anguillarum* serovars associated with vibriosis in fish. *J Fish Dis* 17, 259-267.

Le Bail P.-Y., Sire M.-F. and Vernier J.-M. (1989) Intestinal transfer of growth hormone into the circulatory system of the rainbow trout, *Salmo gairdneri*: Interference by granule cells. *J Exp Zool* 251, 101-107.

Lee E.-Y., Park H.-H., Kim Y.-T., Chung J.-K. and Choi T.-J. (2001) Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceous*). *Gene* 274, 237-243.

Leknes I. L. (2007) Eosinophilic granule cells and endocytic cells in intestinal wall of pearl gouramy (Anabantidae: Teleostei). *Fish Shellfish Immunol* 23, 897-900.

Li J., Barreda D. R., Zhang Y.-A., Boshra H., Gelman A. E., LaPatra S., Tort L. and Sunyer J. O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol* 7, 1116-1124.

Lie K. K., Lanzen A., Breilid H. and Olsvik P. A. (2009) Gene expression profiling in atlantic cod (*Gadus Morhua* L.) from two contaminated sites using a custom-made cDNA microarray. *Environ Toxicol Chem* 28, 1711-1721.

Lilleeng E., Frøystad M. K., Vekterud K., Valen E. C. and Krogdahl Å. (2007) Comparison of intestinal gene expression in Atlantic cod (*Gadus morhua*) fed standard fish meal or soybean meal by means of suppression subtractive hybridization and real-time PCR. *Aquaculture* 267, 269-283.

Lobb C. J. and Clem L. W. (1981) Phylogeny of immunoglobulin structure and function-X. Humoral immunoglobulins of the sheepshead. *Dev Comp Immunol* 5, 271-282.

López-Castejón G., Sepulcre M. P., Roca F. J., Castellana B., Planas J. V., Meseguer J. and Mulero V. (2007) The type II interleukin-1 receptor (IL-1RII) of the bony fish gilthead seabream *Sparus aurata* is strongly induced after infection and tightly regulated at transcriptional and post-transcriptional levels. *Mol Immunol* 44, 2772-2780.

Lund V., Arnesen J. A., Mikkelsen H., Gravningen K., Brown L. and Schrøder M. B. (2008) Atypical furunculosis vaccines for Atlantic cod (*Gadus morhua*); vaccine efficacy and antibody responses. *Vaccine* 26, 6791-6799.

Lund V., Børdal S., Kjellsen O., Mikkelsen H. and Schrøder M. B. (2006) Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Aeromonas salmonicida* and *Vibrio anguillarum*. *Dev Comp Immunol* 30, 1145-55.

Lund V., Børdal S. and Schrøder M. B. (2007) Specificity and durability of antibody responses in Atlantic cod (*Gadus morhua* L.) immunised with *Vibrio anguillarum* O2b. *Fish Shellfish Immunol* 23, 906-10.

Magnadóttir B., Jónsdóttir H., Helgason S., Björnsson B., Jørgensen T. Ø. and Pilström L. (1999a) Humoral immune parameters in Atlantic cod (*Gadus morhua* L.): I. The effects of environmental temperature. *Comp Biochem Physiol B Biochem Mol Biol* 122, 173-180.

Magnadóttir B., Jónsdóttir H., Helgason S., Björnsson B., Jørgensen T. Ø. and Pilström L. (1999b) Humoral immune parameters in Atlantic cod (*Gadus morhua* L.): II. The effects of size and gender under different environmental conditions. *Comp Biochem Physiol B Biochem Mol Biol* 122, 181-188.

Magnadóttir B., Jónsdóttir H., Helgason S., Björnsson B., Solem S. T. and Pilström L. (2001) Immune parameters of immunised cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 11, 75-89.

Magor B. G., Ross D. A., Pilstrom L. and Warr G. W. (1999) Transcriptional enhancers and the evolution of the IgH locus. *Immunol Today* 20, 13-7.

Maier V. H., Dorn K. V., Gudmundsdottir B. K. and Gudmundsson G. H. (2008) Characterisation of cathelicidin gene family members in divergent fish species. *Mol Immunol* 45, 3723-3730.

Martin-Armas M., Zykova S. and Smedsrød B. (2008) Effects of CpG-oligonucleotides, poly I:C and LPS on Atlantic cod scavenger endothelial cells (SEC). *Dev Comp Immunol* 32, 100-107.

McLean E., Donaldson E. M., Dye H. M. and Souza L. M. (1990) Growth acceleration of coho salmon (*Oncorhynchus kisutch*) following oral administration of recombinant bovine somatotropin. *Aquaculture* 91, 197-203.

Mikalsen J., Olsen A. B., Tengs T. and Colquhoun D. J. (2007) *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). *Int J Syst Evol Microbiol* 57, 1960-5.

Mikkelsen H., Lund V., Martinsen L.-C., Gravningen K. and Schrøder M. B. (2007) Variability among *Vibrio anguillarum* O2 isolates from Atlantic cod (*Gadus morhua* L.): Characterisation and vaccination studies. *Aquaculture* 266, 16-25.

Moriyama S., Takahashi A., Hirano T. and Kawauchi H. (1990) Salmon growth hormone is transported into the circulation of rainbow trout, *Oncorhynchus mykiss*, after intestinal administration. *J Comp Physiol B Biochem Syst Environ Physiol* 160, 251-257.

Morrison C. M. (1987) *Histology of the Atlantic Cod, Gadus morhua: An Atlas Part One. Digestive Tract and Associated Organs*. Canadian Special Publication of Fisheries and Aquatic Sciences 98, Ottawa.

Mulder I. E., Wadsworth S. and Secombes C. J. (2007) Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. *Fish Shellfish Immunol* 23, 747-759.

Murphy K., Travers P. and Walport M. (2008) *Janeway's immunobiology seventh edition*. Garland Science, Taylor & Francus Froup, New York.

Nakamura O., Kobayashi M., Suzuki Y., Aida K. and Hanyu I. (1990) Transport of orally administered rabbit IgG into blood circulation of goldfish. *Nippon Suisan Gakk* 56, 1749-1753.

Nakamura O., Suzuki Y., Aida K. and Hatta H. (2001) Decreased transport of orally administered protein into the blood circulation of developing juveniles of Japanese eel *Anguilla japonica*. *Fisheries Sci* 67, 863-869.

Nikoskelainen S., Kjellsen O., Lilius E.-M. and Schrøder M. B. (2006) Respiratory burst activity of Atlantic cod (*Gadus morhua* L.) blood phagocytes differs markedly from that of rainbow trout. *Fish Shellfish Immunol* 21, 199-208.

Noaillac-Depeyre J. and Gas N. (1973) Absorption of protein macromolecules by the enterocytes of the carp (*Cyprinus carpio* L.). Ultrastructural and cytochemical study. *Z Zellforsch Mikrosk Anat* 146, 525-41.

Noaillac-Depeyre J. and Gas N. (1976) Electron microscopic study on gut epithelium of the tench (*Tinca tinca* L.) with respect to its absorptive functions. *Tissue Cell* 8, 511-30.

Noaillac-Depeyre J. and Gas N. (1979) Structure and function of the intestinal epithelial cells in the perch (*Perca fluviatillis* L.). *Anat Rec* 195, 621-7.

Nylund A., Ottem K. F., Watanabe K., Karlsbakk E. and Krossøy B. (2006) *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. *Arch Microbiol* 185, 383-92.

Olafsen J. A. and Hansen G. H. (1992) Intact antigen uptake in intestinal epithelial cells of marine fish larvae. *J Fish Biol* 40, 141-156.

Olsen A. B., Mikalsen J., Rode M., Alfjorden A., Hoel E., Straum-Lie K., Haldorsen R. and Colquhoun D. J. (2006) A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. *J Fish Dis* 29, 307-11.

Ottem K. F., Nylund A., Karlsbakk E., Friis-Moller A., Krossoy B. and Knappskog D. (2007) New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Arch Microbiol* 188, 547-50.

Øverland H. S., Pettersen E. F., Ronneseth A. and Wergeland H. I. (2010) Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 28, 193-204.

Parham P. (1994) The rise and fall of great class I genes. *Semin Immunol* 6, 373-82.

Parsa K. V. L., Butchar J. P., Rajaram M. V. S., Cremer T. J., Gunn J. S., Schlesinger L. S. and Tridandapani S. (2008) *Francisella* gains a survival advantage within mononuclear phagocytes by suppressing the host IFNγ response. *Mol Immunol* 45, 3428-3437.

Pedersen K., Grisez L., van Houdt R., Tiainen T., Ollevier F. and Larsen J. L. (1999) Extended serotyping scheme for *Vibrio anguillarum* with the definition and characterization of seven provisional O-serogroups. *Curr Microbiol* 38, 183-9.

Persson A.-C., Stet R. J. M. and Pilström L. (1999) Characterization of MHC class I and β_2-microglobulin sequences in Atlantic cod reveals an unusually high number of expressed class I genes. *Immunogenetics* 50, 49-59.

Picchietti S., Terribili F. R., Mastrolia L., Scapigliati G. and Abelli L. (1997) Expression of lymphocyte antigenic determinants in developing gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). *Anat Embryol (Berl)* 196, 457-63.

Pillay T. V. R. and Kutty M. N. (2005) *Aquaculture: Principles and Practices, 2nd Edition*. Blackwell Publishing Ltd.

Pilström L. and Petersson A. (1991) Isolation and partial characterization of immunoglobulin from cod (*Gadus morhua* L.). *Dev Comp Immunol* 15, 143-152.

Pilström L., Warr G. W. and Strömberg S. (2005) Why is the antibody response of Atlantic cod so poor? The search for a genetic explanation. *Fisheries Sci* 71, 961-971.

Pinto R. D., Nascimento D. S., Reis M. I. R., do Vale A. and dos Santos N. M. S. (2007) Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10. *Mol Immunol* 44, 2056-2065.

Plouffe D. A., Hanington P. C., Walsh J. G., Wilson E. C. and Belosevic M. (2005) Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation* 12, 266-77.

Press C. M., Dannevig B. H. and Landsverk T. (1994) Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol* 4, 79-93.

Rebl A., Goldammer T. and Seyfert H. M. (2010) Toll-like receptor signaling in bony fish. *Vet Immunol Immunopathol* 134, 139-50.

Ringø E., Sperstad S., Myklebust R., Refstie S. and Krogdahl Å. (2006) Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 261, 829-841.

Roberts R. J. (2001) *Fish Pathology*. W.B. Saunders.

Robertsen B. (2006) The interferon system of teleost fish. *Fish Shellfish Immunol* 20, 172-191.

Rojo I., de Ilárduya Ó. M., Estonba A. and Pardo M. Á. (2007) Innate immune gene expression in individual zebrafish after *Listonella anguillarum* inoculation. *Fish Shellfish Immunol* 23, 1285-1293.

Rombout J. H., Abelli L., Picchietti S., Scapigliati G. and Kiron V. (2010) Teleost intestinal immunology. *Fish Shellfish Immunol*.

Rombout J. H., van der Tuin S. J., Yang G., Schopman N., Mroczek A., Hermsen T. and Taverne-Thiele J. J. (2008) Expression of the polymeric Immunoglobulin Receptor (pIgR) in mucosal tissues of common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol* 24, 620-8.

Rombout J. H. W. M., Bot H. E. and Taverne-Thiele J. J. (1989) Immunological importance of the second gut segment of carp. *J Fish Biol* 35, 167-178.

Rombout J. H. W. M., Lamers C. H., Helfrich M. H., Dekker A. and Taverne-Thiele J. J. (1985) Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.) and the possible immunological implications. *Cell Tissue Res.* 239, 519-30.

Rombout J. H. W. M., Taverne-Thiele A. J. and Villena M. I. (1993) The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical analysis. *Dev Comp Immunol* 17, 55-66.

Rombout J. H. W. M. and van der Berg A. A. (1989) Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. *J Fish Biol* 35, 13-22.

Rønneseth A., Wergeland H. I. and Pettersen E. F. (2007) Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol* 23, 493-503.

Samuelsen O. B., Nerland A. H., Jørgensen T., Schrøder M. B., Svasand T. and Bergh O. (2006) Viral and bacterial diseases of Atlantic cod *Gadus morhua*, their prophylaxis and treatment: a review. *Dis Aquat Organ* 71, 239-54.

Santic M., Asare R., Skrobonja I., Jones S. and Abu Kwaik Y. (2008) Acquisition of the vacuolar ATPase proton pump and phagosome acidification are essential for escape of *Francisella tularensis* into the macrophage cytosol. *Infect Immun* 76, 2671-7.

Saurabh S. and Sahoo P. K. (2008) Lysozyme: an important defence molecule of fish innate immune system. *Aquac Res* 39, 223-239.

Savan R., Aman A., Nakao M., Watanuki H. and Sakai M. (2005a) Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L.). *Immunogenetics* 57, 458-463.

Savan R., Aman A., Sato K., Yamaguchi R. and Sakai M. (2005b) Discovery of a new class of immunoglobulin heavy chain from fugu. *Eur J Immunol* 35, 3320-3331.

Scapigliati G., Bird S. and Secombes C. J. (2000) Invertebrate and fish cytokines. *Eur Cytokine Netw* 11, 354-61.

Scapigliati G., Buonocore F., Bird S., Zou J., Pelegrin P., Falasca C., Prugnoli D. and Secombes C. J. (2001) Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1[beta]. *Fish Shellfish Immunol* 11, 711-726.

Schluter S. F., Bernstein R. M., Bernstein H. and Marchalonis J. J. (1999) 'Big Bang' emergence of the combinatorial immune system. *Dev Comp Immunol* 23, 107-11.

Schrøder M. B., Ellingsen T., Mikkelsen H., Norderhus E. A. and Lund V. (2009) Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Vibrio anguillarum*, *Aeromonas salmonicida* and *Francisella* sp. *Fish Shellfish Immunol* 27, 112-119.

Schrøder M. B., Espelid S. and Jørgensen T. Ø. (1992) Two serotypes of *Vibrio salmonicida* isolated from diseased cod (*Gadus morhua* L.); virulence,

immunological studies and vaccination experiments. *Fish Shellfish Immunol* 2, 211-221.

Schrøder M. B., Flaño E., Pilström L. and Jørgensen T. Ø. (1998) Localisation of Ig heavy chain mRNA positive cells in Atlantic cod (*Gadus morhua* L.) tissues; identified byin situhybridisation. *Fish Shellfish Immunol* 8, 565-576.

Secombes C. J. and Fletcher T. C. (1992) The role of phagocytes in the protective mechanisms of fish. *Annu Rev Fish Dis* 2, 53-71.

Secombes C. J., Hardie L. J. and Daniels G. (1996) Cytokines in fish: an update. *Fish Shellfish Immunol* 6, 291-304.

Seppola M. (2008) *Genes involved in the innate immunity of Atlantic cod*. Ph.D. thesis, University of Tromsø.

Seppola M., Larsen A. N., Steiro K., Robertsen B. and Jensen I. (2008) Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). *Mol Immunol* 45, 887-97.

Seppola M., Olsen R. E., Sandaker E., Kanapathippillai P., Holzapfel W. and Ringo E. (2006) Random amplification of polymorphic DNA (RAPD) typing of carnobacteria isolated from hindgut chamber and large intestine of Atlantic cod (*Gadus morhua* l.). *Syst Appl Microbiol* 29, 131-7.

Seppola M., Stenvik J., Steiro K., Solstad T., Robertsen B. and Jensen I. (2007) Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (*Gadus morhua* L.). *Dev Comp Immunol* 31, 156-71.

Seternes T., Dalmo R. A., Hoffman J., Bogwald J., Zykova S. and Smedsrod B. (2001) Scavenger-receptor-mediated endocytosis of lipopolysaccharide in Atlantic cod (*Gadus morhua* L.). *J Exp Biol* 204, 4055-4064.

Solem S. T. and Stenvik J. (2006) Antibody repertoire development in teleosts--a review with emphasis on salmonids and *Gadus morhua* L. *Dev Comp Immunol* 30, 57-76.

Solstad T. (2008) *Innate defense molecules of the Atlantic cod (Gadus morhua L.) - BPI/LBP, lysozyme and hepcidin*. Ph.D. thesis, University of Tromsø.

Solstad T., Stenvik J. and Jørgensen T. Ø. (2007) mRNA expression patterns of the BPI/LBP molecule in the Atlantic cod (*Gadus morhua* L.). *Fish Shellfish Immunol.* 23, 260-71.

Sørensen K. K., Dahl L. B. and Smedsrød B. (1997a) Role of endocardial endothelial cells in the turnover of hyaluronan in Atlantic cod (*Gadus morhua*). *Cell Tissue Res* 290, 101-109.

Sørensen K. K., Sveinbjørnsson B., Dalmo R. A., Smedsrød B. and Bertheussen K. (1997b) Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *J Fish Dis* 20, 93-107.

Steiro K., Johansen A., Gildberg A. and Bøgwald J. (1998) Optimising of culture conditions and stimulation of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *J Fish Dis* 21, 335-344.

Stroband H., Meer H. and Timmermans L. (1979) Regional functional differentiation in the gut of the grasscarp, *Ctenopharyngodon idella* (Val.). *Histochem Cell Biol* 64, 235-249.

Stroband H. W. and Kroon A. G. (1981) The development of the stomach in clarias lazera and the intestinal absorption of protein macromolecules. *Cell Tissue Res* 215, 397-415.

Stroband H. W. J. and van der Veen F. H. (1981) Localization of protein absorption during transport of food in the intestine of the grasscarp, *Ctenopharyngodon idella* (Val.). *J Exp Zool* 218, 149-156.

Szalai A. J., Bly J. E. and Clem L. W. (1994) Changes in serum concentrations of channel catfish (*Ictalurus punctatus Rafinesque*) phosphorylcholine-reactive protein (*PRP*) in response to inflammatory agents, low temperature-shock and infection by the fungus *Saprolegnia* sp. *Fish Shellfish Immunol* 4, 323-336.

Tavares-Dias M. (2006) A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. *J Fish Biol* 68, 1822-1833.

Temkin R. J. and McMillan D. B. (1986) Gut-associated lymphoid tissue (GALT) of the goldfish, *Carassius auratus*. *J Morphol* 190, 9-26.

Urán P. A., Gonçalves A. A., Taverne-Thiele J. J., Schrama J. W., Verreth J. A. J. and Rombout J. H. W. M. (2008) Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol* 25, 751-760.

Urán P. A., Schrama J. W., Rombout J. H. W. M., Taverne-Thiele J. J., Obach A., Koppe W. and Verreth J. A. J. (2009) Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo salar* L., at two different soybean meal inclusion levels. *J Fish Dis* 32, 733-744.

van den Ingh T. S. G. A. M., Krogdahl Å., Olli J. J., Hendriks H. G. C. J. M. and Koninkx J. G. J. F. (1991) Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. *Aquaculture* 94, 297-305.

VESO. (2007) *Review of disease interactions and pathogen exchange between farmed and wild finfish and shellfish in Europe*. (Edited by Raynard R., Wahli T., Vatsos I. and Mortensen S.), p. 459.

Walford J. and Lam T. J. (1993) Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. *Aquaculture* 109, 187-205.

Watanabe Y. (1984) Morphological and functional changes in rectal epithelium cells of pond smelt during postembryonic development. *Nippon Suisan Gakk* 50, 805-814.

Watts M., Munday B. L. and Burke C. M. (2001) Immune responses of teleost fish. *Aust Vet J* 79, 570-4.

Wishkovsky A. and Avtalion R. R. (1987) Induction of immunosuppression to bacterial antigens in carp, *Cyprinus carpio*. *J Fish Biol* 31, 101-106.

Zasloff M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415, 389-95.

Zhang Y.-A., Salinas I., Li J., Parra D., Bjork S., Xu Z., LaPatra S. E., Bartholomew J. and Sunyer J. O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol* 11, 827-835.

Paper I

Paper II

Paper III

Paper IV

Paper V

ISBN 978-82-8266-010-5