Analysis of immune gene expression in infected and vaccinated rainbow trout *Oncorhynchus mykiss* with a focus on cytokines of adaptive immunity

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This thesis is dedicated to:

Husband, Fazil Harun
Kids, Rayyan & Rannia
Mum, Hajjah Zaimah Ali
&
Dad, Harun Mohamad (deceased 4th December 2005)

DECLARATION

I declare that this thesis was composed by myself and that all research presented here was performed by myself between July 2006 and August 2010. This thesis has not been submitted in any previous application for a higher degree. All sources of information have been acknowledged in the text.

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PAPERS PRODUCED AND CONFERENCES ATTENDED THAT INCORPORATE RESULTS FROM THIS THESIS

[1]**Harun**, N.O. Wang, T. & Secombes, C.J. 2011**a**. Gene expression profiling in naïve and vaccinated rainbow trout after *Yersinia ruckeri* infection: Insights into the mechanisms of protection seen in vaccinated fish. *Vaccine* 29: 4388-4399.

[2]**Harun**, N.O. Costa, M.M. Secombes, C.J & Wang, T. 2011**b**. Sequencing of a second interleukin-10 gene in rainbow trout *Oncorhynchus mykiss* and comparative investigation of the expression and modulation of the paralogues *in vitro* and *in vivo*. *Fish & Shellfish Immunology* 31: 107-117.

[3]**Harun**, N.O., Wang, T. & Secombes, C.J. 2009 (Poster). Expression Profiling Of Key Cytokines In Vaccinated Rainbow Trout After *Yersinia Ruckeri* Challenge. *11th International Congress of the ISDCI Prague, Czech Republic*. June 28th – July 4th.

[4]**Harun**, N.O., Wang, T. & Secombes, C.J. 2009 (Oral Presentation). Analysis of Markers of disease resistance in vaccinated fish. UMT-MSD PostGraduate Seminar, Leeds 12-13th May 2009. Institute for Transport Studies, University of Leeds, United Kingdom. Proceedings p. 87.

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1 I did sample the fish, perform all qPCR. Did the data analysis and help write the paper

2 I did the gene sequence analysis. The *in vitro* and *in vivo* stimulation work and sampling with the help from Costa, M.M and Wang, T.W. I did the qPCR and data analysis with the help from Wang, T.W.

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4 I did prepare for the presentation with the help from Wang, T.W and Secombes, C.J

LIST OF ABBREVIATIONS

AEC	: 3-Amino-9-Ethyl Carbazole
µM	: Micro Molar
AMPs	: Antimicrobial Peptides
ANOVA	: One-Way Analysis of Variance
APC	: Antigen Presenting Cell
BB	: Bacterial Burden
BCR	: B-Cell Receptor
CATH	: Cathelicidin
CCP	: Classical Complement Pathway
cDNA	: Complementary DNA
CFU	: Colony Forming Unit
Con A	: Concanavalin A
CRP	: C-Reactive Protein
CSF	: Colony Stimulating Factor
CTL	: Cytotoxic T cell
DEPC	: Diethyl Pyrocarbonate
DNA	: Deoxyribonucleic Acid
EF-1α	: Elongation Factor 1-Alpha
ELISA	: Enzyme linked immunosorbent assay
ELISPOT	: Enzyme linked Immunospot assay
ERM	: Enteric Red Mouth
FasL	: Fas Ligand
FCA	: Freund's Complete Adjuvant
FCS	: Foetal Calf Serum
GAS	: Gamma Activation Site
GC	: Germinal centres
HIV	: Human Immunodeficiency Virus
HRP	: Horseradish peroxidise
HSC	: Hematopoietic Stem Cells
I.P	: Intraperitoneal
ICAM	: Inter-Cellular Adhesion Molecule
IFN	: Interferon

Ig	: Immunoglobulin
IgA	: Immunoglobulin A
IgE	: Immunoglobulin E
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IHNV	: Infectious Haematopoietic Necrosis Virus
IL	: Interleukin
iNOS	: Nitric Oxide Synthases
IROMPS	: Iron regulated Outer Membrane Proteins
LD	: Lethal Dose
L.T	: Long Ton
LIF	: Leukemia Inhibitory Factor
LPS	: Lipopolysaccharides
LTα	: Lymphotoxin Alpha
MAF	: Macrophage Activating Factor
MCSF	: Macrophage Colony Stimulating Factor
MCSFR	: Macrophage Colony Stimulating Factor Receptor
MDP	: Muramyl Dipeptide
MHC	: Major Histocompatibility Complex
mM	: Milli Molar
MMP	: Matrix Metalloproteinase
MuMLV RNase H	: Moloney Murine Leukemia Virus
MΦ	: Macrophage
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NCC	: Non-Specific Cytotoxic Cells
NK	: Natural Killer Cells
NK	: Natural Killer
NLRs	: Nucleotide-Binding Oligomerization Domain-Like Receptors
NO	: Nitric Oxide
NOD	: Nucleotide-Binding Oligomerization Domain
OMPs	: Outer Membrane Proteins
PAMPs	: Pathogen Associated Molecular Patterns
PBS	: Phosphate Buffer Saline
pCDNA	: Plasmid DNA

PHA	: Phytohemagglutinin
PKR	: Protein Kinase R
PMA	: Phorbol Myristate Acetate
PRR	: Pattern Recognition Receptors
RIG-I	: Retinoic Acid-Inducible Gene I
RLRs	: Retinoic Acid-Inducible Gene I-Like Receptors
RNA	: Ribonucleic Acid
ROS	: Reactive Oxygen Species
RTFS	: Rainbow Trout Fry Syndrome
RT-PCR	: Reverse Transcript Polymerase Chain Reaction
S.T	: Short Ton
SAA	: Serum Amyloid
SI	: Spleen Index
SPSS	: Statistical Package for the Social Sciences
SRBC	: Sheep Red Blood Cells
Tc	: Cytotoxic T cells
TCR	: T cell Receptor
Th	: Helper T cells
TLR	: Toll-Like Receptors
TNF-α	: Tumour Necrosis Factor
TSA	: Tryptic Soy Agar
TSB	: Tryptic Soy Broth
UK	: United Kingdom
VHSV	: Viral Haemorrhagic Septicaemia Virus

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ABSTRACT

Analysis of immune gene expression in infected and vaccinated rainbow trout, Oncorhynchus mykiss with a focus on cytokines of adaptive immunity

Abstract

The aquaculture sector is currently thriving, and has expanded to meet the demand for fish and shellfish as an alternative protein source to meat. This is especially true for high value products such as Atlantic salmon, where in Scotland salmon farming is reported to be worth > £1 billion to the national economy. Currently around 40% of farmed fish and shellfish destined for human consumption are derived from aquaculture. Therefore, a great deal of attention is paid to problems that the industry faces, with fish diseases of paramount importance. A variety of species of bacteria, viruses and parasites are common in the aquatic environment, which can result in serious diseases amongst fish stocks. As a result, ways to improve disease resistance have been the focus of much attention, with the use of vaccines considered a desirable way forward. However, other approaches are also followed, such as the use of immunostimulants to improve fish health in a more limited, non-specific way, or the use of genetic markers to allow selective breeding of important disease resistance traits. For all of these approaches more information is needed on the pathways that give rise to disease resistance in fish in different situations, to allow their manipulation or monitoring, and the studies in this thesis are directed towards this goal. Fish has been used as a model to study the evolution of vertebrate immunolity for some deacades, especially work on humoral immune responses where knowledge on antibody production has dominated much of the literature on fish immunology. In contrast, little known about specific cell-mediated immunity in fish, even though it also likely plays an important role in the immune system and disease resistance. Therefore, this thesis has been focused on analysing such responses, taking advantage of the recently discovered cytokines of adaptive immune responses in fish, which allow transcriptomic studies in particular to look at the molecules turned on during infection and after vaccination. Thus the goal of this thesis was to take advantage of some successful vaccines that exist for rainbow trout, and examine the gene expression changes that occur in vaccinated trout post-challenge with the homologus pathogen, and to try to dissect pathways that may correlate with disease resistance in this species.

In **Chapter Two**, a commercial (Furovac 5) and suboptimal (formalin killed strain MT004) vaccine against furunculosis were used to investigate their modulatory effects on localised and systemic responses following vaccination and subsequently following challenge (by injection) of the causative agent, *Aeromonas salmonicida*. Two immune tissues, head kidney and spleen, were used to study the expression of a major cytokine known to modulate innate response to bacterial infection, interferon-γ (IFN-γ). The results showed that soon after vaccination a significantly elevated IFN-γ expression could be detected in head kidney leucocytes from fish given the commercial vaccine but that at later timings no significant differences were apparent, and that the spleen showed no clear IFN-γ induction at any of the timings sampled post-injection. Post-challenge of the vaccinated and control fish no further increases in IFN-γ expression were found, although at 48h post-challenge there was a large induction seen in the head kidney of the fish given the commercial vaccine. This may reflect poor memory induction for IFN-γ secreting cells, although it may also be that Th1-type responses are not the most important for protection against this pathogen.

In **Chapter Three** the disease model was changed to *Yersinia ruckeri*, the causative agent of Enteric Redmoth Disease (ERM), for which a commercial vaccine is also available. Sixty days post-vaccination the fish were challenged and 6, 24, 48 and 72 h later the gills and spleen were sampled for gene expression analysis. These studies showed that pro-inflammatory cytokines were up-regulated post-infection in the spleen of both naïve and vaccinated fish after *Y. ruckeri* challenge, although the pro-inflammatory cytokine expression was much lower in vaccinated fish. A correlated expression between pro-inflammatory cytokines and anti-inflammatory cytokines was only seen in the spleen of vaccinated fish, where a Th1-like response was indicated. In contrast, in the gills, the inflammatory gene response was enhanced in vaccinated fish compared to naïve fish, but intriguingly there was a strong up-regulation of IL-22. Taken together these results suggest that different types of adaptive responses can possibly occur at different sites in vaccinated fish during infection with *Y. ruckeri*, and in this case a Th1 type response may be triggered in systemic tissues (spleen) but a Th17 type response in mucosal sites (gills).

In **Chapter Four** a different approach was taken, very much focussed on innate resistance to bacterial infection. In contrast to the known protection afforded by vaccination against *Y. ruckeri*, this experiment utilised a predicted variation in resistance to generate a population of fish that showed differential spleen size (SI)/bacterial burden (BB) indices in response to infection, as a means to separate fish that may live or die post-challenge. Many pro-inflammatory genes and antimicrobial peptides were up-regulated in fish with a high SI/BB relative to those with a small SI/BB. However, in addition, these fish also had a number of molecules associated with Th1 responses elevated, as well as various down-regulators of inflammation (IL-10, nIL-1F, SOCS molecules). In contrast the fish with a small SI/BB had elevated MHC class II molecules and CD80/86 molecules, as well as many lymphocyte markers. Thus lymphocyte activation appears to be an important component of the responses in resistant fish.

Finally, in **Chapter Five** a second interleukin IL-10 gene was described in rainbow trout. It was successfully cloned and its expression studied in the context of *Y. ruckeri* infection and after stimulation with various PAMPS and cytokines. This molecule, termed IL-10b, is highly similar to IL-10a, however some interesting differences in expression were found. For example, IL-10a had higher expression levels in tissues from healthy, control fish. However, during *Y. ruckeri* infection, whilst IL-10b was not induced in the spleen, in contrast to IL-10a, the opposite was apparent in the gills. A number of PAMPS were also able to induce both paralogues, although different kinetics were again apparent. Such differences probably reflect divergence of the promoters of the two genes.