THE PHYSIOLOGICAL AND IMMUNOLOGICAL EFFECTS OF VACCINATION ON FISH HEALTH, WELFARE, AND PERFORMANCE

by

LISA ANN SKINNER

B.Sc., Thompson Rivers University, 1998
M.Sc., University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Animal Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2009

ABSTRACT

To prevent the outbreak of pathogenic diseases, the salmonid aquaculture industry relies on the use of vaccines. While traditional, polyvalent, oil-adjuvanted vaccines (AV) are effective, they do not work against all types of pathogens and the vaccination process and vaccine composition can be stressful for individual fish. Continuing advances in technology have led to the development of a new type of pathogen-specific vaccine; a DNA vaccine (DV). While there are many benefits to DVs, including a physiologically less stressful vaccine formulation, a more rapid immune response, and prolonged protection compared to traditional vaccines, the impacts of DVs on the general physiology of fishes, especially when coupled with an AV, are not well understood.

To assess these impacts, growth performance, routine metabolic rate (RMR), and immunological responsiveness were examined in Atlantic salmon (*Salmo salar*, L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum) following the injection of a DV. When injected alone, the DV did not affect fish performance parameters. When injected concurrently with an AV, there were general differences in growth performance and RMR, and species-specific differences in immune responsiveness. Concurrent injection of a DV with an AV in Atlantic salmon was associated with a transient decrease in specific growth rate. As well, concurrent injection elicited an increase in lysozyme activity, an antigen-specific increase in specific antibody (Ab) production, and a delay in the production of virus-specific neutralizing antibodies (NAb). In rainbow trout, concurrent injection of a DV with an AV led to a temporary increase in RMR, an increase in lysozyme activity, and an earlier seroconversion of NAbs. To determine the impact of stress on the vaccine-induced immune response, Atlantic salmon were injected with supra-physiological levels of cortisol following concurrent vaccine injection. If cortisol was presented after initiation of the immune response, there was suppression of lysozyme activity and no effect on the production of specific Abs.

Although the current research shows that DVs are highly beneficial to the aquaculture industry, it highlights the need for species-specific studies, especially when combining the DV with traditional, polyvalent vaccines.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	antibody
ACP	alternate complement pathway
ACTH	adrenocorticotrophic hormone
Ag	antigen
Ab-Ag	antibody-antigen complex
AMP	anti-microbial peptide
ANOVA	analysis of variance
APC	antigen presenting cell
ASC	antibody secreting cell
AUP #	animal use protocol number
AV	adjuvanted vaccine
βEND	β-endorphin
BCR	B-lymphocyte/cell receptor
bl s^{-1}	body length per second
BSA	bovine serum albumin
C	constant domain (or segment)
Ca^{2+}	calcium ion
CAER	Centre for Aquaculture and Environmental Research
CCP	classical complement pathway
CDR	complementarity-determining region
C_H	constant domain – Heavy chain
C_L	constant domain – Light chain
cm	centimetre (10^{-2} m)
CMV-IEP	cytomegalovirus immediate early promoter
CRF	corticotrophin-releasing factor
CRP	C-reactive protein
C-T	carboxy-terminal coding exon
D	diversity segment
d	day
DC	dendritic cell
dd	degree days
DFO	Department of Fisheries and Oceans
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
DV	DNA vaccine
EAVR	early antiviral response
EGC	eosinophilic granular cell
ELISA	enzyme linked immunosorbent assay
Epi	epinephrine
EPC	*epithelioma papulosum cyprini*
Fab	antibody antigen-binding amino terminus
Fc	antibody carboxy-terminal effector region
FAO	Food and Agriculture Organization of the United Nations
FCR	feed conversion ratio
FFA	free fatty acid
FR	framework region

FW	freshwater
g	relative centrifuge force
g	gram
G	glycoprotein
H_c	antibody heavy chain
h	hour
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HEWL eq	hen egg white lysozyme equivalent
HPI	hypothalamo-pituitary-interrenal axis
HSW	high salt wash buffer
IFN	interferon
Ig	immunoglobulin
IgM	immune macroglobulin
IHN	infectious haematopoietic necrosis
IHNV	infectious haematopoietic necrosis virus
IL	interleukin
IM	intramuscularly
IP	intraperitonealy
J	joining segment
K	Fulton's condition factor
Kg	kilogram (10^3 g)
L	litre
L_c	antibody light chain
LAVR	long-term antiviral response
LCP	lectin complement pathway
L_F	fork length
LPC	long-lived plasma cells
LPS	lipopolysaccharide
LSW	low salt wash buffer
m	metre
M	molar (moles litre^{-1})
MAb	monoclonal antibody
MAC	membrane attack complex
MBP	mannan-binding protein
m	metre
mg	milligram (10^{-3} g)
MHC	major histocompatibility complex
min	minute
M_{O_2}	oxygen consumption
MPO	myeloperoxidase
mRNA	messenger RNA
MSH	melaophore stimulating hormone
MS222	tricaine methane sulphonate
Mx	myxovirus protein
µg	microgram (10^{-6} g)
µL	microlitre (10^{-6} L)
NAb	neutralizing antibody
NaCl	sodium chloride

NADPH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NCC	non-specific cytotoxic cell
NE	norepinephrine
ng	nanogram (10^{-9} g)
nm	nanometre (10^{-9} m)
NK	natural killer cell
NO	nitric oxide
O_2^-	superoxide anion
OD	optical density
OH^+	hydroxyl free radical
OIE	World Organization for Animal Health
PAMP	pathogen associated molecular patterns
PBS	phosphate-buffered saline
pci	post-cortisol injection
pfu	plaque forming units
PNT	plaque neutralization titre
Poly I:C	dsRNA polyinosinic polycytidylic acid
PRR	pattern recognition receptor
PVC	polyvinyl chloride
pvi	post-vaccine injection
RAG	recombination-activating gene
RMR	routine metabolic rate
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	U_{crit} recovery ratio
RSS	recombination signal sequence
RT-PCR	reverse transcriptase - polymerase chain reaction
s	second
SAP	serum amyloid protein
SAVR	specific antiviral response
SE	standard error
SGR	specific growth rate
S-S	disulphide bond
SW	sea water
t	time
Tc	cytotoxic T-cell
TCR	T-cell receptor
TD	T-dependent antigenic structure
Th	helper T-cell
TI	T-independent antigenic structure
TLR	Toll-like receptor
TNF	tumour necrosis factor
TSB	tryptic soy broth
UBC	University of British Columbia
U_{crit}	critical swimming speed
USD	United States Dollar
V	variable domain (or segment)
V_H	variable domain – Heavy chain

V_L	variable domain – Light chain
VI alpha	alphanumeric visible implant tag
VIE	visible implant elastomer tan
V_L	variable domain – Light chain
WG	daily weight gain
wt	weight
WTO-SPS	Word Trade Organization Sanitary and Phytosanitary Agreement
w/v	weight per volume
v/v	volume per volume
↑	stimulatory
↓	inhibitory
˚C	degrees Celsius
‰	parts per thousand
50% PNT	50% plaque neutralization titre

ACKNOWLEDGEMENTS

Embarking on a doctoral degree is no easy feat, and one that should never be taken lightly. Without the strong and steadfast support of co-workers, friends, and family, I'm not sure I would have survived the first year, let alone the second, third, and fourth years. Through every catastrophe, frustration, and set-back, I have learned a great deal and have so many people to thank.

First and foremost, I am indebted to Trish Schulte who has shown such kindness and support for a student who did not belong. Her readiness to answer my questions, edit my manuscripts, and guide me through difficult times will always be remembered. Trish, I may not have been an official member of the Schulte lab, but you always made sure I felt like one! Although a little farther away, Buhl, ID, USA to be exact, Scott LaPatra and the gang at Clear Springs Foods Research Division welcomed me with open arms. Scott, you taught me many wonderful things about rainbow trout and fish immunology. The time you spent encouraging me to think outside the box will never be forgotten, and without your assistance editing and re-editing my manuscripts, the written portion of this thesis would not have succeeded. Bob Devlin was my pillar at the DFO-UBC CAER. Bob, without your kindness, generosity, and open door, I'm not sure I would have survived. Thank you.

There are many others who helped me in so many ways during this thesis. A long time ago, Bill Milsom and Dave Jones showed me that science is fun and there is always a way to answer the unanswerable. Their passion for science and research is undeniable and, as I've discovered, quite contagious. Sandra Adams, Kim Thompson, and all the wonderful researchers I've met in the field of fish immunology showed me that research does not make a person succeed; it is the person who makes the research succeed.

This thesis could not have been possible without the love and support of my friends. Rav, you have stood by me since grade 8, and no matter where life has taken us, I always knew you were there. Amelia, Jodie, Manuela, Dan and Colin; I will never forget our talks over coffees, drinks, and sushi. As successful UBC graduates, you were always there to remind me of the "big picture". And last, but not least, Donald, you somehow always managed to remind me that there really is life outside of school.

It is impossible to find words to express the love and gratitude I feel towards my family. Grandma, Mom, Dad & Elaine, Twila, Kevin & Ieashia, Brianne, Mathew, and Patrick; you may not have always understood what I was doing or why, but your unconditional love and support was always felt and will be forever appreciated.

Chris, you have always reminded me that love is the best part of life. I don't think I will ever be able to find words grand enough to express my appreciation for the support you have shown me. An education is an amazing thing, but without your love, friendship, and respect, I can only go so far. To quote a very wise woman:

"Make a little room in your plans for romance. All the degrees and scholarships in the world can't make up for the lack of it"
Lucy Maud Montgomery 1874-1972

Financial support for this research was provided by an AquaNet grant awarded to Drs. Patricia M Schulte and Shannon K Balfry, and a Faculty of Land and Food Systems Elizabeth Howland fellowship to myself.

For Chris

You deserve a special degree
for putting up with me
during this entire process

CO-AUTHORSHIP STATEMENT

Chapter Two: Growth and performance of Atlantic salmon, *Salmo salar* L., following administration of a rhabdovirus DNA vaccine alone or concurrently with an oil-adjuvanted, polyvalent vaccine.

Comments: All aspects of this study were conducted by Lisa A Skinner under the supervision of Robert Scott McKinley and Patricia M Schulte. Scott E LaPatra and Shannon K Balfry provided expert advice.

Chapter Three: The association between metabolic rate, immune parameters, and growth of rainbow trout, *Oncorhynchus mykiss* (Walbaum), following the injection of a DNA vaccine alone and concurrently with a polyvalent, oil-adjuvanted vaccine.

Comments: All aspects of this study were conducted by Lisa A Skinner under the supervision of Scott E LaPatra and Robert Scott McKinley. Patricia M Schulte and Shannon K Balfry provided expert advice. Scott E LaPatra conducted the neutralizing antibody assay.

Chapter Four: Concurrent injection of a rhabdovirus-specific DNA vaccine with a polyvalent, oil-adjuvanted vaccine delays the specific antiviral immune response in Atlantic salmon, *Salmo salar* L.

Comments: All aspects of this study were conducted by Lisa A Skinner under the supervision of Patricia M Schulte and Robert Scott McKinley. Scott E LaPatra, Alexandra Adams, Kim D Thompson, and Shannon K Balfry provided expert advice. Scott E LaPatra conducted the neutralizing antibody assay. Although not listed in this thesis, Dr Hidehiro Kondo provided some technical assistance with the determination of anti-*Listonella anguillarum* antibody titres.

Chapter Five: Cortisol suppresses lysozyme activity but not the antibody response in Atlantic salmon, *Salmo salar* L., following vaccine injection.

Comments: All aspects of this study were conducted by Lisa A Skinner under the supervision of Robert Scott McKinley and Patricia M Schulte. Scott E LaPatra, Alexandra Adams, Kim D Thompson, and Shannon K Balfry provided expert advice. Scott E LaPatra conducted the neutralizing antibody assay. Although not listed in this thesis, Dr Hidehiro Kondo provided some technical assistance with the determination of anti-*Listonella anguillarum* antibody titres.

CHAPTER ONE: INTRODUCTION

1.1 GENERAL INTRODUCTION

Throughout the world, the cultivation of fish and shellfish in a controlled environment, i.e. aquaculture, is one of the fastest growing food production industries accounting for almost fifty percent of the world's food fish [1]. The wide diversity of groups and species cultivated by the aquaculture industry, both for trade and consumption, is region specific. Developing countries such as Asia and the Pacific region (East Asia, South Asia, Southeast Asia, West Asia, and Oceania) produce the bulk of the omnivorous and herbivorous fish species, while developed countries such as Norway, Chile, the United Kingdom, and Canada account for the majority of the carnivorous fish species, principally finfish (subdivision Teleostei) such as Atlantic salmon (*Salmo salar*, L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum) [1, 2]. In Canada, teleost aquaculture accounts for over seventy-five percent of the country's total aquaculture production and ninety percent of the total aquaculture values [3]. In the last fifty years, worldwide aquaculture production of marine and freshwater species has increased at an average rate of eight percent per year, jumping in production from less than one million tonnes in 1950 to over fifty-nine million tonnes in 2004, worth an estimated value of 70.3 billion USD [1]. In their most recent report, the Food and Agriculture Organization of the United Nations (FAO) [1] estimated an additional forty million tonnes of aquatic food will be required by 2030 just to maintain current per capita consumption rates.

Maintaining the health and quality of aquaculture species is important to ensuring that the industry remains economically viable now and in the future. Presently, the multi-million dollar Canadian and multi-billion dollar worldwide aquaculture industries lose millions of dollars in profit to unexpected and/or un-prevented outbreaks of disease [2]. It is estimated that ten to twenty percent of all cultured fish are lost each year due to infectious diseases, both among individuals and throughout populations [4, 5]. Currently, the international trade of aquatic animals and their products is thought to be the major underlying reason for new disease occurrences among aquaculture sites throughout the world [1, 2]. To minimize the risk of pathogen transfer and diseases associated with aquatic animal movement, a series of global instructions and codes of practices and guidelines exist including the World Trade Organization Sanitary and Phytosanitary (WTO-SPS) Agreement, the World Organization for Animal Health

(OIE) Aquatic Animal Health Standards, and the FAO Code of Conduct for Responsible Fisheries [1].

Although increases in pathogen number (viral, bacterial, and parasitic) are often considered to be the primary cause of disease outbreak among teleost fish, in many instances secondary factors such as changes in environmental factors, poor water quality, and inadequate farm-management are to blame [2, 6]. While seemingly unrelated, these factors are the foundation for subsequent changes in the normal physiological equilibrium of an organism; they cause physiological stress [7, 8]. In teleost fish, as with other vertebrates, when changes occur to the external and subsequently to the internal environment, the resulting physiological stress can cause structural and functional alterations to occur in the immune response [7, 9] such that an organism's susceptibility to disease can be increased.

As a tool to help decrease the incidence of disease outbreak at the individual and population levels, thereby increasing overall productivity, the teleost aquaculture industry employs two strategies: treatment and prevention. Treatment involves the use of anti-microbial agents (chemotherapeutics); chemical or medicinal substances that inhibit the growth or metabolic activities of bacteria and other micro-organisms by means of a chemical substance of microbial or synthetic origin. The majority of chemotherapeutics enhance the activity of a fish's innate (non-specific) immune response [10], and although commonly available for many (not all) pathogens, including numerous fungal and bacterial diseases as well as some parasites, in the context of the aquaculture industry there are no chemotherapeutics available for viral diseases [2, 11-13]. In addition, chemotherapeutics can have undesirable side-effects, not only on the fish but on the environment as well. The accumulation of chemicals and anti-microbial agents in the flesh of treated animals, along with the development of drug-resistant strains of certain pathogens, and the potential contamination of the aquatic environment are just some of the disadvantages associated with chemotherapeutic use in aquaculture [10-15].

Prevention of disease can be accomplished through direct and indirect methods. Indirect methods include strict bio-security measures (staff hygiene, proper disinfection procedures, avoidance of staff and equipment movements between areas) and good farming practices (stocking of fish with known disease status, use of sites with good water quality, fallowing sites regularly) [6, 16]. Direct methods of disease prevention involve the use of immunostimulants and vaccines. Immunostimulants, which can include chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors, and cytokines, are naturally occurring compounds that modulate and enhance the innate immune response while boosting the

adaptive (specific) immune response, thereby increasing the level of individual immunocompetency and disease resistance [8, 17-19]. One of the benefits of using immunostimulants as a preventative measure to disease in fish is that they can be used to up-regulate the innate immune responses of an individual, putting it in a more prepared state to meet and overcome an invading pathogen [19]. Because of their ability to enhance pathogen destruction through increased activity of non-specific phagocytes, immunostimulants protect fish from a variety of infectious disease agents simultaneously and can be used to promote the recovery of individuals from immunosuppressive states such as seen during times of physiological stress [8].

There is still much to be learned however, regarding the use of immunostimulants as a preventative measure in aquaculture, and care must be taken when using immunostimulants as unwanted side-effects may result. If excessive doses of immunostimulants are used for example, immunosuppression or other as yet undetermined, non-desirable effects may result [19]. Although immunostimulants provide a suitable and viable alternative to conventional chemotherapeutics, immune stimulation is short-term and not all pathogen outbreaks, bacterial or viral, are prevented [8].

It is currently thought that the ability of vaccines to manipulate immunological memory resulting in long term, specific protection, makes them the most effective and common tool for disease control and prevention in the aquaculture industry [8, 20]. In its simplest form, a vaccine is a preparation of antigens derived from pathogenic organisms rendered non-pathogenic by various means [20, 21]. Within the teleost aquaculture industry, more specifically the salmonid aquaculture industry, the majority of vaccines utilized are polyvalent (i.e. contain multiple antigens) and require the use of an adjuvant [17, 22-25]. Unpredictable interactions between the antigen(s) and adjuvant, however, can result in negative morphological and physiological side-effects [26-29]. When combined, these can result in variations in overall fish growth and immunological performance [26-39].

Recent advances in biotechnology have led to the development of DNA vaccines (DV) whereby a gene of interest, typically one that codes for a protective antigen, is inserted into a bacterial plasmid construct [40, 41]. In 1996, Andersen *et al*. [40] first described a novel fish vaccine using the glycoprotein (G) gene of the infectious haematopoietic necrosis virus (IHNV), a rhabdovirus of significant economic importance to the salmonid aquaculture industry. The mechanisms of immune stimulation following the injection of this and similar rhabdovirus-specific DVs have been studied in depth and appear to closely resemble those of a natural viral

infection [41-50]. As such, only a small amount of the DNA plasmid construct is needed with no adjuvant requirement, thereby significantly reducing the possibility of vaccine-related side-effects [41, 45]. Although there has been a substantial amount of research performed investigating the mechanisms of action and efficacy of these novel DVs [45, 51-58] very little work has been published regarding their impact on fish performance parameters such as growth or energetics. As well, because all current DVs within the aquaculture industry are virus-specific, they are typically injected concurrently with traditional polyvalent, adjuvant-based, bacterial vaccines. There are no published studies examining the immunological or physiological effects of such concurrent vaccine injections.

1.2 RESEARCH AIMS

The primary research objective of this thesis was to establish a more comprehensive understanding of the immediate effects that vaccine injection has on the overall physiology of two commercially important salmonid species; Atlantic salmon and rainbow trout. More specifically, this thesis sought to illustrate the intricate relationship that exists between physiology and immunology. By examining individuals injected with a rhabdovirus-specific DV alone and concurrently with a traditional, polyvalent, oil-adjuvanted bacterial vaccine (AV), this thesis is able to identify the variability of the immune response and associated physiological changes that occur as a result of vaccine injection.

Specific aims of the research were to examine the influence a rhabdovirus-specific DV has on: (1) fish growth and swimming performance, (2) basic immunological parameters and energetics, (3) the timing of antibody (Ab) development, and (4) the development of the innate and adaptive immune response when supra-physiological levels of cortisol are presented post-vaccine injection (pvi).

In all four studies key physiological and immunological parameters were measured following the injection of a DV alone and concurrently with a polyvalent, oil-AV. In separate studies, growth performance was measured in both Atlantic salmon and rainbow trout for a minimum of four months pvi. Swimming performance was determined for Atlantic salmon at approximately 100 degree days (dd) pvi, while the energetics of rainbow trout was calculated approximately 200, 300, and 400 dd pvi. Coinciding with the measurement of the above performance parameters, immunological variables including lysozyme activity, bacteria-specific Ab titres, and virus-specific neutralizing antibody (NAb) titres were measured at approximately

100, 200, 300, and 400 dd pvi. As a way to determine if high levels of stress and associated hyper-cortisol influence the vaccine-induced immune response, supra-physiological levels of cortisol were injected into Atlantic salmon at 53 and 212 dd pvi. Lysozyme activity, bacteria-specific Ab titres, and virus-specific NAb titres were measured 74 dd post cortisol injection (pci).

The overall goal of these studies is to gain a better understanding of the physiological and immunological responses that DVs have on individuals, especially when compared to the responses elicited by traditional AVs. In addition, this research provides important insights into how the injection of one vaccine can influence the immunological responses to a separate, but concurrently injected vaccine. Moreover, this research sheds light onto the differences that occur between closely related species with respect to the ability to respond to multiple antigens.

1.3 LITERATURE REVIEW

1.3.1 IMMUNE SYSTEM

Aquatic environments expose animals living within them, such as fish, to a plethora of infectious or disease causing pathogens including bacteria, viruses, fungi, and parasites. To prevent such pathogenic invasions from occurring, fish, like higher vertebrates, rely on their immune system, which is comprised of widely distributed cells, tissues, and organs that function to recognize, neutralize, and destroy foreign substances and micro-organisms [59, 60]. The vertebrate immune system is composed of two parts: the innate (non-specific) immune response and the adaptive (specific) immune response. The innate immune response is present from birth and acts as the body's non-specific, first line of defense to invasion. It does not target a particular pathogen, begins within minutes to hours of infection, and can be identified by the processes associated with inflammation [61-63]. The adaptive immune response is characterized by the acquired responses against specific foreign antigens and is key to immunological memory [64, 65]. Although commonly discussed as separate systems, the innate and adaptive immune responses frequently overlap in structure and function [64, 66]. It is now thought that the key to a potent and long duration adaptive response is to precede it with a strong innate response [62, 67-69].

The evolution of the immune system has been a gradual process, beginning with the innate immune response, whose origins are as old as the first multi-cellular organism [66, 67].

Insects, echinoderms, and ascidians, all invertebrates, posses some form of innate immunity, complete with anti-microbial peptides (AMP), pattern recognition receptors (PRR), and intracellular signalling pathways [67]. While the innate immune response has continued to refine its functionality over the course of vertebrate evolution, from jawless fish through to cartilaginous fish, teleost fish, amphibians, reptiles, birds, and mammals, the primary function of providing a method of early defense against pathogen attack, has remained unchanged. In doing so, certain key elements such as complement protein C3, toll-like receptors (TLR), and defensins can be identified in all living groups [66, 67].

The evolution of the adaptive immune response occurred seemingly abruptly 450 million years ago, around the time of cartilaginous fish. The acquisition of somatic gene rearrangement is thought to have allowed for the development of key adaptive immunological features such as pathogen-specific immunoglobulins (Ig), T-lymphocytes, and major histocompatibility complex (MHC) molecules [66, 69, 70]. Although teleost fish separated from the common ancestor of higher vertebrates some 300 million years ago, the functionally of the teleost immune system, both innate and adaptive immune responses, is surprisingly similar to that of extant higher vertebrates, including mammals [66, 69, 70]. The goal of this literature review is to provide readers with a brief overview of the vertebrate immune system, with the primary focus on teleost immunity. Although there are some key structural and functional variations between the teleost immune system and that of higher vertebrates, the overall functions are similar. Where significant variations or differences in structure and/or function occur, both will be discussed.

1.3.1.1 Innate Immune Response

If an infectious pathogen survives the physical (epithelial surfaces of the skin, gills, and gut), mechanical (mucus), and chemical (low pH, digestive enzymes, and mucosal enzymes such as lysozyme) barriers of a potential host organism, [65, 71] the innate immune response is activated in an attempt to eliminate the pathogen before development of disease can occur [72, 73]. The innate immune response uses germ line-encoded PRRs, such as soluble humoral components (complement protein C3, lectins) or receptors located on macrophages and dendritic cells (DC) to identify and bind to pathogen associated molecular patterns (PAMP) such as polysaccharides, lipopolysaccharides (LPS), peptidoglycans, bacterial DNA and double stranded viral RNA (dsRNA), and other molecules not normally found on the surface of multi-cellular organisms [62, 63, 74]. It is currently thought that TLRs are the principal PRRs in both

mammalian and fish innate immune responses, with each individual TLR recognizing a different PAMP [62, 63, 74]. Following recognition of PAMPs, TLRs activate both common and unique transcription factors through different signalling pathways, and initiate intracellular signal transduction resulting in the expression of genes involved in inflammation, antiviral responses, and maturation of DCs [62, 63, 74].

Although it is often considered non-specific, the innate immune response is specific in that the PRR of each of its components reacts with just one type of PAMP. However, because the PAMPs with which they react are so common, the components of the innate immune response do not influence the growth of only one micro-organism and are thus termed non-specific [75].

There are three main advantages to the innate immune response when compared with the adaptive immune response: (1) the protection is non-specific and does not depend on recognition of distinctive molecular structure of pathogens, (2) there is little or no lag time for the response, such that even inducible defenses like inflammation are quick to respond, thereby giving pathogens little time to establish themselves, and (3) it is relatively temperature independent, making it an important defense mechanism of fish and other ectothermic vertebrates [61, 76].

1.3.1.1.1 Innate Humoral Immune Response

Once a pathogen enters a host, innate humoral factors located in the serum, mucus, or ova of fish work to destroy the pathogen, prevent the attachment, invasion, or multiplication of the pathogen, and enhance specific immune responses such as phagocytosis [21, 61, 73, 77]. The humoral factors of the innate immune response include inhibitors such as transferrin, antiproteases, and lectin, as well as lysins [AMP, proteases, lysozyme, C-reactive protein (CRP), serum amyloid protein (SAP), complement] and interferons [21, 61, 75].

1.3.1.1.1.1 Inhibitors

Transferrin, a glycoprotein found in vertebrate blood, has a high affinity for iron and limits the amount of free iron available for uptake by pathogens [21, 77]. During the inflammatory response, transferrin removes iron from damaged tissue and acts as an activator of fish macrophages [62]. Antiproteases, such as α1-antiproteinase and α2-macroglobulin, are located in serum and inhibit bacterial production of proteolytic toxins which are used to digest host tissue proteins [21, 77]. Lectins (haemagglutinins) found in the mucosal tissue and serum of

many vertebrate species, are a type of PRR capable of binding to certain sugars on the glycoproteins and glycolipids of bacterial cell walls, and play an important role in neutralizing bacterial components such as exotoxins, and in immobilizing micro-organisms thereby aiding in the facilitation of phagocytosis [21, 63, 75, 77]. While the exact mechanisms of action of fish lectin are unknown, mannan-binding protein (MBP), a mammalian lectin, acts as an opsonin, enhancing the phagocytosis of bacteria, and is involved in the activation of the complement system [21]. It is thought that fish lectin functions in a similar manner [21].

1.3.1.1.1.2 Lysins

Lysin, an AMP, is a type of humoral factor that works to destroy invading pathogens by opsonisation and lysis [21]. AMPs, such as perforin, histone H1, and defensin are important families of lysin located in the skin, mucus, and serum of vertebrates. AMPs have the ability to disrupt bacterial membranes [78-80]. Proteases, another important lysin family, are also located in vertebrate mucosal tissue, and display trypsin-like activity, hydrolyzing bacterial proteins into smaller polypeptide units [21].

Lysozyme is an important lysin of the innate humoral response. Lysozyme is commonly located in mucus, serum, tissues rich in leucocytes (anterior kidney of fish), and at sites where the risk of micro-organism invasion is high (skin, nasal cavity, tear ducts, gills, alimentary tract). Lysozyme is an enzyme that hydrolyses the β (1→4) linkages between N-acetylmuramic acid and N-acetylglucosamine, two constituents of the peptidoglycan layer of bacterial cell walls [75, 81]. In mammals, lysozyme acts directly on Gram-positive bacteria, and requires complement to disrupt the outer cell wall of Gram-negative bacteria before it can hydrolyse the cell wall. In fish, lysozyme appears to be able to lyse both Gram-positive and Gram-negative bacteria without the assistance of complement [21, 81]. Contrary to what is observed in mammals, fish have two types of lysozyme, Type I and II, with Type II being three times as potent as Type I [81]. Because of this, it is thought that lysozyme plays a key role in the innate immunity of fish [61, 75, 81].

CRP and SAP are pentraxins, a type of lectin that acts as a lysin in fish [62]. In the presence of Ca^{2+}, CRP binds to the C-polysaccharide of phosphorylcholine, a surface component of bacteria, fungi, and parasites, while SAP shows affinity for phosphoryl-ethanolamine, agarose, and carbohydrate moieties and is known to bind to LPS of Gram-negative bacteria [62]. Interestingly, most fish appear to have either the CRP pentraxin (cod, *Gadus morhua*; channel

catfish, *Ictalurus punctatus*) or the SAP pentraxin (salmonids; wolf fish, *Anarhichas lupus*; halibut, *Hippoglussus hippoglossus*), whereas others have both (plaice, *Pleuronectes platessa*; rainbow trout) [62, 63, 82]. Once bound, CRP and SAP act as opsonins, activating the complement system and enhancing the phagocytic and lytic defenses of the fish [21, 62, 77]. Although low in normal, un-infected mammalian serum, CRP and SAP levels in un-infected fish serum are high (~ 50 $\mu g\ L^{-1}$) and along with body-wide distribution, located in serum, ova, and skin mucus, researchers believe that CRP and SAP play key roles in fish immunity, more so than in other vertebrates [21]. More research is needed to verify the importance of CRP and SAP in the fish innate immune response.

1.3.1.1.1.3 Complement

Complement is an essential part of both the innate and adaptive immune responses of vertebrates, including both fish and mammals, and is composed of a system with approximately thirty-five soluble and membrane-bound proteins synthesized as inactive precursors that function either as enzymes or as binding proteins [83]. When activated, inactive complement components are turned into active serine proteases that split other inactive complement components in a sequential manner, ultimately leading to the opsonisation or direct killing of pathogens through the activation of the lytic pathway or through inflammation [83, 84]. By opsonising pathogens, complement proteins stimulate phagocytosis, a process mediated by complement receptors located on the surface of phagocytic cells, including neutrophils and macrophages [83-85]. Additionally, the complement system plays an important role in immune complex clearance and participates in the inflammatory response by attracting phagocytic cells to the site of injury [83, 85].

There are three primary pathways through which complement can be initiated in vertebrates (Figure 1.1); the classical complement pathway (CCP), the alternate complement pathway (ACP) and the lectin complement pathway (LCP). The CCP, associated with adaptive immunity, is triggered by the binding of Abs to the antigen (Ag) surface, forming the Ab-Ag complex, as well as by acute phase proteins such as ligand-bound CRP, or directly by viruses, bacteria, and virus-infected cells [83, 85, 86]. The ACP, associated with the innate immune response, is Ab-independent and can be activated directly by viruses, bacteria, or fungi [61, 63, 83, 85]. Activation of the ACP occurs when a complement protein binds to the hydroxyl or amine groups of carbohydrates or proteins located on foreign cell surfaces [83]. The LCP is

similar to the CCP however instead of being antigen induced it is activated by the binding of a protein complex, consisting of mannose-binding lectins, to sugar moieties such as mannans on bacterial cell surfaces [63, 75, 83, 85]. All three complement pathways cascade to generate the central complement component, factor C3, and converge to the lytic pathway following formation of the membrane attack complex (MAC), a porous trans-membrane structure that, when inserted into the lipid membrane of a pathogen, causes opsonisation and cytolysis [63, 83]. C3 is one of the most abundant and important vertebrate complement proteins that when activated, results in enhanced phagocytosis through opsonisation, recruitment of immune cells and promotion of the inflammatory response, stimulation of B-lymphocyte proliferation, and formation of the MAC [63, 83].

Activation of the innate and adaptive immune responses via the complement pathways can be studied by measuring (1) the haemolytic activity of serum; an indication of level of activation of the lytic pathway, and (2) the phagocytic activity of monocytes and macrophages in the presence of normal and heat-inactivated serum; an indication of the involvement of opsonisation by complement [63, 83]. Recently, it was noted that the haemolytic activity of fish varies greatly within and between species, and that the ACP appears more active and has a broader optimum reaction temperature compared to the CCP. These data suggest a greater role of the ACP in the immune response of fish compared to the more temperature sensitive CCP, which has a more important role in the mammalian immune response [63, 83, 85]. As well, the variations in ACP and CCP activity between different species of fish, attributed primarily to genetic differences in C3 isoforms, could account for the observed differences in disease resistance or susceptibility [63, 85].

1.3.1.1.1.4 Interferons

Interferons (IFN) are secreted proteins or glycoproteins (cytokines) produced by a variety of host cell types in response to viral envelope proteins and viral dsRNA [61, 62, 75-77, 87]. There are three distinct families of IFN that can be distinguished on the basis of their biological and biochemical properties; type I IFN, type II IFN, and type III IFN [63, 88]. Type I IFN, which includes the classical IFNs α and β is induced by viruses in most cell types and plays a critical role in the antiviral response. By expressing a number of proteins within the host cell, including 2', 5'-oligoadenylate synthetase, protein kinase P1, and Mx proteins, type I IFN blocks viral entry into the cell, controls viral transcription, cleaves viral mRNA, and prevents translation of

the viral genome [61, 63, 87, 88]. Type II IFN, which is identical to IFN-γ, is produced by non-specific cytotoxic cells (NCC) and T-lymphocytes in response to interleukin-12 (IL12), IL18, mitogens, or antigens [63, 87]. In mammals, type II IFN functions in cell regulation, cell differentiation, intercellular communication, and activation of natural killer (NK) cells and macrophages for specific immune responses [61, 75, 76]. It is thought that type II IFN in teleost fish has a similar function. Type III IFN, IFN-λ, is a cytokine with IFN-like activities [87, 88]. At present, it is unknown if type III IFN occurs in teleost fish, and if so, what role it has with respect to the immune system.

Following viral infection, IFN production in fish occurs rapidly (i.e. within two days) and in a variety of cells, with peak production of antiviral proteins, such as Mx protein, occurring within the first 48 hours [61]. Because IFN mediated antiviral defense mechanisms are able to respond during the early days of a viral infection such that the IFN response provides a degree of protection until the specific adaptive responses are active, poly I:C, a potent synthetic stimulator of type I IFN, has the potential to be used in vaccines as a type of adjuvant [61].

Until recently, the IFN proteins and/or genes had not been identified or cloned in teleost fish and measurement of the expression of Mx gene mRNA by RT-PCR was commonly used as a method for detection of type I IFN activation, and thus the antiviral response in fish [61, 76]. IFN genes have recently been identified and cloned from zebrafish (*Danio rerio*), Atlantic salmon, Japanese pufferfish (*Fugu rubripes*), spotted green pufferfish (*Tetraodon nigroviridis*), and channel catfish [63].

1.3.1.1.1.5 Natural Antibodies

Natural antibodies recognize and bind antigenic epitopes on invading pathogens. They can lead to isotype class switching of Igs and improved Ab affinity for antigen binding [76]. Natural antibodies play a role in antigen trapping for presentation to the adaptive immune response, as well as function in the neutralization and opsonisation of pathogens, and activation of the CCP [76]. Natural antibodies may play a more significant role in fish immunity when compared to mammals, given that fish do not have an appreciable Ab affinity maturation response or class switching capabilities [76].

1.3.1.1.2 Innate Cell-Mediated Immune Response

There are a variety of leukocyte cell types involved in the innate cell-mediated immune response (i.e. inflammation) including macrophages, granulocytes (neutrophils, eosinophils, basophils), and NCCs [61, 89]. Macrophages and granulocytes are mobile phagocytic cells found in the blood and secondary lymphoid tissues with neutrophils being the predominant granulocyte in fish [72, 89-91]. NCCs, present in the blood, lymphoid tissues, and mucosal sites of fish are the functional equivalent of mammalian NK cells [89]. NCCs activate type II IFN and work to spontaneously kill foreign cells via apoptic and necrotic mechanisms [87, 89].

When a pathogen gains entry into the tissues of an organism, the acute inflammatory response ensues. Beginning with an increased supply of blood to the infected area, increased capillary permeability, and a migration of macrophages and neutrophils out of capillaries and into surrounding tissues, the acute inflammatory response is highly complex and ends with phagocytosis of the pathogen [21, 61, 62, 89]. A number of blood enzyme systems, including the clotting and the complement systems are involved in the control of inflammation and although little is known about the relative contribution in fish, most of these systems are thought to share many similarities with their mammalian counterparts [21, 89]. For example, similar to mammals, once the complement system has been activated, either directly by the ACP or indirectly by lectins or the CRP, anaphylactic complement factors C3a and C5a are produced (Figure 1.1) [61, 85, 89]. In mammals, these factors induce the release of vasoactive amines, such as histamine or 5-hydroxytryptamine, from platelets and mast cells [61]. In fish, complement factors C3a and C5a release functionally equivalent vasoactive amines from thrombocytes and eosinophilic granular cells (EGC), although histamine does not appear to be present [61]. In fish and mammals, vasoactive amines, once released, induce local vasodilatation and the extravasation of macrophages and neutrophils into the infected site, with neutrophils typically preceding the appearance of macrophages [61, 89]. The C5a component of the ACP also has chemotactic activity for phagocytes, allowing for accumulation of macrophages and neutrophils at the site of infection, an event further stimulated by cytokines, such as tumour necrosis factor-α (TNF-α), IL1, and eicosanoid, a substance with chemotactic and pro-inflammatory activity [61, 89].

Phagocytosis begins with the attachment of invading pathogens to the phagocyte membrane, a relatively passive process involving hydrophobic interactions, sugar-lectin interactions, or as is most often the case the C3 component of complement, which is bound to bacterial surface LPS directly via the ACP or indirectly via lectins or CRP [61, 89]. Once

attached, macrophages and neutrophils engulf the pathogen via endocytosis, leading to the creation of a phagosome. The phagosome membrane makes contact and fuses with one of the phagocyte's lysosomes, creating a phagolysosome [89]. Lysosomes contain numerous hydrolytic enzymes that kill the pathogen through production of reactive oxygen species (ROS) during the so-called respiratory burst [21, 89]. The primary reaction of the respiratory burst is the one electron reduction of molecular oxygen to O_2^- catalyzed by NADPH oxidase, a multi-component enzyme found in the plasma membrane of phagocytes [89]. In addition, neutrophils contain myeloperoxidase (MPO) in their cytoplasmic granules which, in the presence of halide ions and H_2O_2 kill bacteria by the halogenation of the bacterial cell walls, as well as by the production of bactericidal hypohalite ions such as iodine [21]. Following infiltration of neutrophils at the site of pathogenic infection, macrophages have been observed to contain the neutrophil-derived MPO and glycogen granules. It is thought that these substances are transferred from neutrophils as a way of enhancing the macrophage's bactericidal powers [21, 61]. Neutrophils and macrophages also contain lysozyme and other hydrolytic enzymes in their lysosomes. Macrophages can also produce nitric oxide (NO) which forms potent bactericidal agents such as peroxynitrites and OH^+ [61, 89].

Recent studies have shown a developmental relationship between vertebrate B-lymphocytes [antibody producing cells (APC) of the adaptive immune response] and macrophages, suggesting an evolutionary relationship between the two cell types [92, 93]. Subsequent studies have revealed that B-lymphocytes in teleost fish demonstrate phagocytic capabilities complete with phagolysosome formation and intracellular killing of ingested microbes [94]. These data support the idea that B-lymphocytes of higher vertebrates evolved from an ancestral phagocytic cell type.

In cases where the inflammatory stimuli (i.e. pathogens) are not eliminated during an acute inflammatory response, a chronic inflammatory response may result with granulomas being produced [89]. Granulomas are organized collections of mature mononuclear phagocytes within a fibrous tissue stroma and represent an attempt to isolate and destroy pathogens evading the acute inflammatory response [89]. Granulomas can be induced *in vivo* by a wide range of bacterial, fungal, or parasitic diseases, by diet-related diseases, or by injection with adjuvants [89].

1.3.1.2 Adaptive Immune Response

While the innate immune system is thought to be of ancient origin, as far back as the early metazoan, the origins of the adaptive immune response first appeared 450 million years ago in cartilaginous and bony fish [62, 72]. The adaptive immune response develops within days to weeks of initial infection (in teleost fish it can take up to 4 to 6 weeks to develop because it is temperature dependent) and is comprised of a complex network of specialized cells, proteins, biochemical messages (cytokines), and genes that work together to produce an inducible and specific response which requires the presence and action of antigen-specific Abs [61, 62, 64, 65 76]. Although it is the basis for immunological memory and vaccine use, and is well known for its specificity, the adaptive immune response is not well understood in fish and, at first glance, does not appear to be as crucial to fish immunocompetence as the innate immune response [72, 83]. Due to the lack of easily available fish-specific immunological techniques and cell lines, the amount of research performed on the adaptive immune response in fish pales in comparison to that of innate immunity. However, with the advent of newer technology and recent advances in mammalian adaptive immune research, scientists are beginning to better understand the interactions and importance of the fish adaptive immune response.

1.3.1.2.1 Adaptive Humoral Immune Response

In vertebrates, the primary effector molecule of the adaptive humoral immune response is the Ab, belonging to the protein-group of Ig [95, 96]. Igs are produced by B-lymphocytes in a vast range of antigen-specificities with each B-lymphocyte producing an Ig of single specificity [67]. Upon binding to an antigen, B-lymphocytes are activated to divide and produce many identical progeny, collectively known as clones. These clones can either house the specific antigenic Ig receptor, or secrete the Ig as an Ab into the extracellular spaces of the body [67].

Structurally, each vertebrate Ig molecule is comprised of two heavy polypeptide chains (H_c) held together by a flexible, joint-like polypeptide chain. Associated with each H_c is a light polypeptide chain (L_c), interlinked to the H_c by disulphide bonds (Figure 1.2) [95]. Each H_c and L_c consist of characteristic domains approximately 110 amino acids long, with the L_c containing two domains and the H_c containing three, four, or five domains [67, 95]. There are two different types of domain; the variable (V) domain, which comprises the first 110 amino acids (i.e. the first domain) of both the H_c and L_c, and the constant (C) domain, comprising the remaining amino acid sequences (i.e. the remaining domains) [67, 95]. The V-domains are responsible for

determining antigen specificity, while the C-domains are responsible for determining Ig class [59].

Together, the H_c and L_c, along with their characteristic V- and C-domains create two distinct and functionally different regions: the antigen-binding amino terminus (Fab) and the carboxy-terminal effector region (Fc) [67, 77, 86, 96-98]. The domains of the Fab region include the V-domains of the H_c and L_c (V_H and V_L), as well as the constant domain of the L_c (C_L) and the N-terminal constant domain of the H_c (C_H) [59]. The Fab region of membrane bound Ig is similar in structure to the Ab Fab region and acts as a B-lymphocyte (cell) receptor (BCR) recognizing and binding specific antigen molecules. It is the specific binding of the antigen to the Fab region that activates B-lymphocytes, leading to clonal expansion and specific Ab production [67].

The Fc region is composed of constant domains, and functions to stimulate the effector functions of the immune system in a constant and unchanging manner [67, 86]. Membrane bound Ig does not have an effector function as the Fc region is inserted into the membrane of the B-lymphocyte [67].

The extensive diversity of Ab specificity can be related to genetic sequence variability in the V-domain of the H_c and L_c. Within the variable domains are hypervariable or complementarity-determining regions (CDR) and framework regions (FR) (Figure 1.3). It is within the CDRs that frequent and important differences in amino acid sequence occur, allowing for and contributing to the large diversity of antigen-specific Abs [95, 98]. These differences in genetic sequence can be attributed to the rearrangement of gene sequences, somatic mutations, or the generation of different codons during Ab gene splicing [59].

Structurally, the CDRs and FRs of the H_c and L_c are comprised of two or three different segments of DNA. In the H_c, these segments of DNA are termed the variable (V), the diversity (D), and the joining (J) segments, while in the L_c, only V- and J-segments are present (Figure 1.4). The rearrangement of gene segments, in a process referred to as combinatorial joining, occurs during the differentiation of B-lymphocytes and is very important to the diversity of vertebrate Abs (Figure 1.4) [59, 67, 95]. During combinatorial joining, the different segments of DNA are brought together to form the mature genes encoding the two Ig chains (H_c and L_c). The recombination is achieved by the utilisation of a conserved recombination signal sequences (RSS) and requires the presence of recombination-activating genes (RAG) [67]. Briefly, at the time of deletion, a variable length of DNA will be spliced, resulting in the joining together of a V-segment (either a CDR or FR) with a D-segment (H_c only), and a J-segment [59, 95]. When

the L_c gene is transcribed, transcription continues through the DNA region that encodes for the constant portion of the gene. RNA splicing will subsequently join the V-, J-, and C-segments to create mRNA [59]. Prior to transcription of the H_c, a second DNA splicing event occurs resulting in the joining together of the V-, D-, and J-segments with a class-specific constant region [59, 67]. Initially, all H_c constant regions have the amino acid sequence for IgM. To switch Ig class, the second DNA splice joins the VDJ region with a different constant region [59, 95]. Each Ig class is responsible for activating different immune effector mechanisms and unlike mammals where there are five, well-known Ig classes (IgA, IgD, IgE, IgG, and IgM), and cartilaginous fish where there are three Ig classes (IgM, IgW, and IgNAR), teleost fish appear to have 2 primary Ig classes (IgM and IgD) with IgM being the functionally predominant class [64, 77, 95, 96, 98]. Recently, two new classes of Ig have been identified in teleost fish, IgZ and IgT, although their function and evolutionary relevance are still unknown [99, 100]. Igs from each class can form monomeric Ab units or polymers of Ab molecules, such as the commonly observed tetrameric teleost IgM or the pentameteric mammalian IgM [86].

Abs can act as both soluble effector molecules in serum and as cell surface receptors bound to B-lymphocytes [64, 95]. As an effector molecule in serum, an Ab can destroy an antigen in a variety of ways. Abs can neutralize the antigen by blocking a critical function such as a receptor, an enzymatic active site, or toxigenic determinant [86]. Alternatively, because of the multivalent binding ability of Abs (each monomeric Ab molecule can effectively bind 2 antigens – see Figure 1.2), large macromolecular Ab-Ag complexes can be created. If sufficiently large, these macromolecular complexes will precipitate (or agglutinate if the antigen is cellular) out of solution allowing for the phagocytising of the Ab-Ag complex [86]. If a pathogen such as a bacteria, fungi, or parasite cannot be easily phagocytised, Abs will effectively coat the pathogen and allow for opsonisation. Once the pathogen has been opsonised, phagocytosis and destruction can occur [86]. The final method of antigen destruction by Abs is through the classical complement pathway mentioned previously. Briefly, the binding of the Ab to an antigen results in a conformational change in the Ab's Fc region. This change permits the binding and activation of the first component of the complement system, which in turn results in further conformational changes that facilitate the binding of other complement components [64, 83, 86]. At each step, proteolytic enzymes are produced that can opsonise or lyse the pathogens [64, 86, 95].

Knowing the type of antigenic structure is important in understanding the development of specific Abs and immunological memory. Antigenic structures can be divided into T-

independent (TI, polysaccharides) or T-dependent (TD, proteins). If an antigen is of different composition (nucleic acid, glycolipid), work with mammalian systems suggests that it is dealt with as either a TI or a TD antigen [86]. TI antigens trigger the receptive state on antigen-specific B-lymphocytes, while simultaneously inducing macrophages to produce IL1, a cytokine critical to the differentiation of B-lymphocytes into antibody-secreting cells (ASC). TD antigens require cellular cooperation to activate B-lymphocytes. In order to produce an Ab response to a TD antigen, the antigen must be processed by an accessory cell such as a macrophage, and be presented on the cell surface of a T-lymphocyte. This, in turn, stimulates the production of requisite ILs which then provides the specific signals and growth factors necessary for B-lymphocyte differentiation and Ab secretion [86].

Although it is the hallmark of the specific Ab response, immunological memory in teleost fish is not well understood. Immunological memory is a differentiated response to a secondary exposure to a specific antigen [101]. It is postulated that upon antigenic stimulation, B-lymphocytes in the peripheral immune system undergo proliferation and differentiation into short-lived (life span of days to weeks) plasma cells. During this process, memory B-lymphocytes are produced that can be re-stimulated upon subsequent exposure [101]. In mammals this re-stimulation results in the secondary Ab response and can lead to rapid induction, logarithmic increases in Ab titre and affinity, and greatly extended duration of the Ab response [86, 101]. While this classical viewpoint of immunological memory is prevalent in mammals, it either does not occur, or occurs to a much lesser degree in teleost fish [86]. In fact, Ab molecules of teleost fish appear to have a low intrinsic affinity (affinity of the individual binding site), an apparent lack of ability for serum Ab to increase in affinity over time after immunisation (affinity maturation), and a limited amount of Ab binding site heterogeneity [86, 101, 102].

Current research suggests a different form of immunological memory is present in teleost fish. It is thought that immunological memory is the result of humoral memory, referring to a persistent primary Ab response involving long-lived plasma cells (LPC) [101]. LPC are ASCs that persist for months in the central lymphoid tissue (in teleost fish, this is the anterior kidney), continuing to secrete Ab induced at a prior immunisation [101]. The origin of LPC and their regulation within the anterior kidney of fish is still being studied and this information may be of significant importance with respect to understanding fish immunological memory and may enhance the development of vaccines.

1.3.1.2.2 Adaptive Cell-Mediated Immune Response

Aside from B-lymphocytes, the primary cell-type responsible for adaptive cell-mediated immunity is the T-lymphocyte. T-lymphocytes are derived in the thymus of vertebrates and respond to antigen fragments exposed either on the surface of APC such as macrophages, DCs, and B-lymphocytes, or on the surface of altered self-cells or virus infected cells [89, 102-105].

In the case of APCs, foreign material from the blood and tissue is scavenged and digested to produce antigen fragments. These antigen fragments combine with preformed class II MHC molecules which function to deliver the antigen to the APC surface membrane [67, 102-104]. Once attached to the surface membrane, T-lymphocytes, specifically T-helper (Th) lymphocytes recognize and bind to the MHC-antigen complex in an antigen-specific manner [59]. Th cells secrete cytokines that initiate and regulate a variety of immune processes and are divided into types; Th1 and Th2, depending on the type of cytokine released [102-104]. Th1 cells release γ-IFN, TNF-β, and IL2, and activate macrophages to a microbicidal state and induce delayed type hypersensitivity reactions, while Th2 cells release cytokines IL4, IL5, IL6, IL9, IL10, IL11, and IL13 and give rise to a strong Ab response [104].

In the case of altered self-cells and virus infected cells, endogenous antigen proteins (e.g. viral RNA) are digested with the resultant short peptide antigen fragments combining with preformed class I MHC molecules. Similar to class II MHC, class I MHC molecules present antigen fragments to the cell's surface membrane where T-lymphocytes, in this case cytotoxic T (Tc) lymphocytes, recognize and bind to the MHC-antigen complex in an antigen-specific manner [67, 102-104]. Tc-lymphocytes kill altered self-cells and those cells infected with viruses [102-104].

To identify class I and class II MCH-antigen complexes, Th and Tc cells use T-lymphocyte (cell) receptors (TCR). TCRs are similar in structure to BCRs, and like BCRs use the rearrangement of gene segments to produce a wide diversity of antigen-specific receptors [67].

Important to proper functioning of cell-mediated adaptive immunity is the release of soluble, immune enhancing regulatory factors (i.e. cytokines) from activated macrophages and APCs [103]. TNF-α, and IL1 are two common and important macrophage-derived cytokines. IL1 is important to cell-mediated immunity as it serves as the starting point for a number of cascade reactions including T-lymphocyte expression of IL2, a cytokine that enhances immune functions

such as NCC, and IL4, a cytokine that stimulates the production of Ab-secreting B-lymphocytes [103].

The cell-mediated immune responses in fish are difficult to assess due to the poor understanding of teleost adaptive immune response [12]. Although very little is known about the cell-mediated immune response in fish, recent advances in the cloning of fish-specific immune genes has allowed for the identification of a number of adaptive cell-mediated components. These advances, which show striking similarity to mammalian cell-mediated immunity, will allow for the development of reagents and probes that will more precisely define immune responses post-vaccination and post-infection [104].

For a summary of innate and adaptive immune responses, refer to Table 1.1.

1.3.2 VACCINES

1.3.2.1 VACCINE TYPES

In its simplest form, a vaccine is a preparation of antigens derived from pathogenic organisms rendered non-pathogenic by various means, stimulating the immune system in such a way as to increase the resistance to disease from subsequent infection by a pathogen [20, 21]. Vaccines that are commonly used in the aquaculture industry are composed of antigens formulated from bacterins (formalin or heat inactivated whole cells), live attenuated cells, bacterial toxins, recombinant vectors, and more recently nucleic acids [14, 59, 106]. Vaccines are a form of active immunisation whereby the antigen stimulates the innate and adaptive immune responses, ultimately leading to specific humoral and cell-mediated immunity with pathogen and antigen-specific Abs and immunological memory being formed. Immunological memory can be the result of circulating “memory” cells or systemically held LPCs [86,101].

1.3.2.1.1 Whole Organism Vaccines

Of the vaccine types used within aquaculture, bacterins and live attenuated cells are the most common. They are easy to manufacture, relatively cheap, and for the most part stable, allowing for long periods of storage. That being said, whole organism vaccines such as bacterins and live attenuated cells can be problematic. Not only do these vaccines sometimes fail to adequately stimulate the cell-mediated immune responses, they usually require a booster, and

can potentially mutate in ways that restore pathogen virulence [59]. As well, because there is often a need to use an adjuvant with many bacterins, side-effects are very problematic [28].

1.3.2.1.1.1 Adjuvants

The main components of vaccines (i.e. the antigen) usually lack sufficient immunogenicity and require the assistance of adjuvants [24]. Adjuvants are substances which aid a vaccine in the stimulation of the immune response (through activation of the innate immune response) against a given vaccine antigen, and increase the pronouncement of the adaptive immune response, both humoral and cell-mediated types, through acceleration, prolongation, and enhancement [23, 25]. Adjuvants are defined by their chemistry and principal mode of action (mineral-based adjuvants [aluminum hydroxide], oil based adjuvants [mineral oil or vegetable oil], and lipo-adjuvants [liposomes]) and play a major role in determining the efficacy of the vaccine in question [23, 24]. For example, injected oil-adjuvants, which are the most commonly used adjuvants in the aquaculture industry, act as reservoirs in that they hold the antigen(s) in globules at the site of injection [17]. This facilitates the induction of the innate immune response, including the inflammatory response and the release of cytokines, which ultimately stimulates the production of antigen-specific Abs [23]. Unfortunately, adjuvants, especially oil-adjuvants, cause significant morphological and physiological side-effects and as such, care must be taken when incorporating them into vaccine formulations.

1.3.2.1.2 DNA Vaccines

A DV is a relatively new type of genetic vaccine consisting of a plasmid construct, typically an *E. coli* plasmid, with a strong viral promoter [usually human cytomegalovirus immediate early promoter (CMV-IEP)], the gene of interest (coding for a protective antigen), and a polyadenylation/transcriptional terminal sequence [40, 41, 56, 58]. Theoretically the antigen of a DV can be any gene that codes for the protein of a pathogen, however, the only efficacious licensed DVs currently developed are against viruses [12, 56, 107].

When a virus-specific DV is injected into the muscle of an individual, the resulting immune response closely resembles that of a natural viral infection with transcription, translation, and replication of the antigen occurring in a similar manner [12, 40, 41, 97, 108, 109]. Briefly, following vaccine injection, the plasmid construct is taken up by the surrounding muscle cells where it enters the cell nuclei, and expresses the appropriate antigen gene. Once the

muscle cells commence protein synthesis, the pathogenic antigen protein is produced, stimulating humoral and cell-mediated immune responses [108, 109].

During the adaptive humoral response, the newly synthesized antigen proteins are released from the muscle cells and bind to B-lymphocyte receptors. At the same time, APCs ingest the antigen proteins and display the antigen fragments on class II MHC molecules. Th cells recognize the antigen fragments and secrete cytokines which activate the antigen bound B-lymphocytes to multiply and differentiate into antigen-specific LPCs and memory cells [56, 59, 109]. Similarly, the cell-mediated immune response begins with the muscle cells displaying the antigenic proteins or protein fragments on class I MHC molecules. The DV is incorporated into APCs which synthesize and express the antigen fragments on class I MHC molecules. Tc cells recognize the signals from stimulated class I MHC molecules and are activated to multiply and attack all cells infected by the pathogen (vaccine). Some of the activated T-lymphocytes develop into memory T-lymphocytes which help LPC and memory cells protect against future infections [59, 107, 109].

DNA vaccines, like viruses, use the host cell's replication mechanisms, therefore there is no need for an adjuvant and only a very small amount of the plasmid construct is needed for protection: as little as 1-10 ng DNA vaccine per fish [45, 110]. This is different from vaccines produced with bacterins or live attenuated cells where large amounts of antigen and adjuvant are required to induce a sufficient and long-lasting immune response [37, 109]. Because of the lack of need for adjuvants, the negative side-effects commonly associated with injected whole organism and adjuvant vaccines (adhesions, granulomas, growth) have not yet been reported with DVs [12, 112, 113].

1.3.2.1.3 Polyvalent Vaccines

In the aquaculture industry, specifically salmonid aquaculture, nearly all vaccines are, or have the potential to be polyvalent (i.e. they contain multiple antigens; bacterial and viral), including DVs. Polyvalent vaccines can protect individual fish against the major relevant diseases they might encounter throughout the entire production cycle, reducing the need for re-vaccination [24, 28, 38]. Although polyvalent vaccines are beneficial in many aspects, the immune system of fish has a defined and limited capacity to respond to multiple antigens [27]. As such, this finite clonal capacity and limited protective immunity (there is an average approximate limit of 5 $x10^5$ antigens to which the fish immune system can recognize and

respond to at any one time) can lead to both positive and negative interactive effects within the fish immune response [27]. These can include cross-protection between antigens (the presence of one antigen confers protection against a different, unrelated antigen), antigenic competition (the presence of one antigen interferes with or suppresses the activity of another antigen), and immunodominance among antigens (the degree to which a subunit of an antigenic determinant is involved in binding or reacting with an antibody), all of which can affect the specificity, avidity, and level of production of Abs [27, 29]. Although commonly examined following the injection of polyvalent vaccines alone, the interactive effects of polyvalent vaccine antigens on the antigen(s) from a separate, concurrently administered vaccine is equally important although not as aggressively studied [27, 29].

1.3.2.3 Vaccine Administration

There are three primary methods for vaccine administration: oral, injection, and immersion. The method of choice is not always straightforward and is often determined by a combination of factors including the molecular form of the antigen, the favoured route of administration, the concentration of antigen required, ambient temperature, the species being vaccinated, age and size of the individual being vaccinated, and the balance between positive and negative immunity where positive immunity refers to the active immunity stimulated by NCCs and lymphocyte producing cells, and negative immunity refers to the process switching off by suppressor cells [12, 20, 116-118].

In theory, oral administration of vaccines is the most suitable for mass vaccination of fish of all sizes and can easily be accomplished by incorporating the vaccine into the daily feed [117, 119]. Although ideal for the aquaculture industry due to the ease of administration, minimal stress on the animal and apparent low relative cost, oral administration is not as immunologically efficient as injection or immersion administration of vaccines [12, 20, 120-122]. While oral administration can and often does stimulate mucosal immunity, it is difficult to regulate and monitor the vaccine dose ingested by each individual and large quantities of antigen must be used to ensure sufficient vaccination of the entire population [12, 86, 117, 122]. As well, it is thought that one of the major downfalls of effectiveness for the oral administration of vaccines is the location of antigen absorption within the intestines. While it has been shown that the intestines of teleost fish can and do absorb soluble and particulate antigens, gastric fluids in the

anterior intestine are thought to destroy or inactivate the vaccine antigen before it can be absorbed [117].

Immersion administration of vaccines involves several different methods including spray, direct immersion, hyperosmotic dip, and flush exposure (direct addition of vaccine to water in which the fish are held) [116]. Because of good antigen absorption across the skin and gills, immersion administration of vaccines typically provides better overall protection and is often more widely used in aquaculture compared to oral vaccine administration [12, 120, 122]. As well, immersion administration has many advantages over injection vaccine administration including reduced stress on the fish, the ability to vaccinate very small fish, low labour costs, time involved to vaccinate large numbers of fish, and safety for vaccinators as well as for the fish [116, 123]. Similar to oral administration however, the immersion technique typically requires large volumes of vaccine solution and can therefore be costly [116, 122].

The most effective method of vaccinating fish is by direct injection. While potentially stressful for the individual fish, it elicits a strong immune response with long-lasting immunological memory [12, 118, 120]. In Canada, the majority of vaccines used in aquaculture are injection vaccines.

1.3.2.4 Vaccine-Related Side-Effects

While very successful at disease prevention, it has been well established that the injection of adjuvanted vaccines can lead to adverse morphological and physiological side-effects. These include inflammation at the site of injection, intra-abdominal adhesions, pigmentation, and granulomas, all of which can affect the overall health and welfare, as well as the market value of the fish [28, 31, 36, 37]. Combined, these effects have also been shown to influence the overall growth of fish in a positive [30, 33], negative [26, 32, 36, 37, 39] or neutral [34] manner depending on the combination of adjuvant and antigen(s) used [28, 33, 37].

There are several possible explanations for the observed changes in growth in response to vaccine administration. The formation of adhesions and granulomas at or around the site of injection can lead to impaired peristaltic movements of the digestive tract, or to the destruction of secretory tissues such as the pancreas, thus reducing overall feed intake, nutrient absorption, and potentially decreasing overall growth [26, 28, 125]. Alternatively, Ackerman *et al*. [33] and Sørum & Damsgård [37] attribute the changes in growth to interactions between the vaccine components [adjuvant and antigen(s)] and the fish's immune system, and the resultant increases

in energy costs. Ackerman *et al*. [33], using indirect measures of metabolic rate (M_{O_2}), demonstrated that the energy consumption of vaccinated salmonids differed depending on type of adjuvant used, and that the resultant effects on growth were not necessarily predictable, suggesting that decreased growth performance could be attributed to increased catabolism, and increased growth performance could be attributed in increased anabolism.

1.3.3 Conclusion

The increased worldwide consumption of fish and fish products has led to a significant increase in the production of a variety of fish species. Jeopardizing both the productivity of the aquaculture industry and the health of the animals are aquatic pathogens. Through an increased understanding of the fish immune system, researchers have been able to develop a variety of efficacious vaccines. The impact of these vaccines on the overall health, welfare, and performance of individual fish, however, is complex and not well understood. There is an intricate relationship that exists between the immune system and the overall physiology of an individual. By better understanding how a vaccine stimulates the immune response, both innate and adaptive, and the overall impact this has on the physiology of an individual, researchers may be able to develop better and more comprehensive vaccines. In addition to examining the complex relationship between physiology and immunology, we also need to better understand how the presence of one antigen (environmental or vaccine induced) can influence the immunological response to a separate, but simultaneously presented antigen.

1.4 TABLES

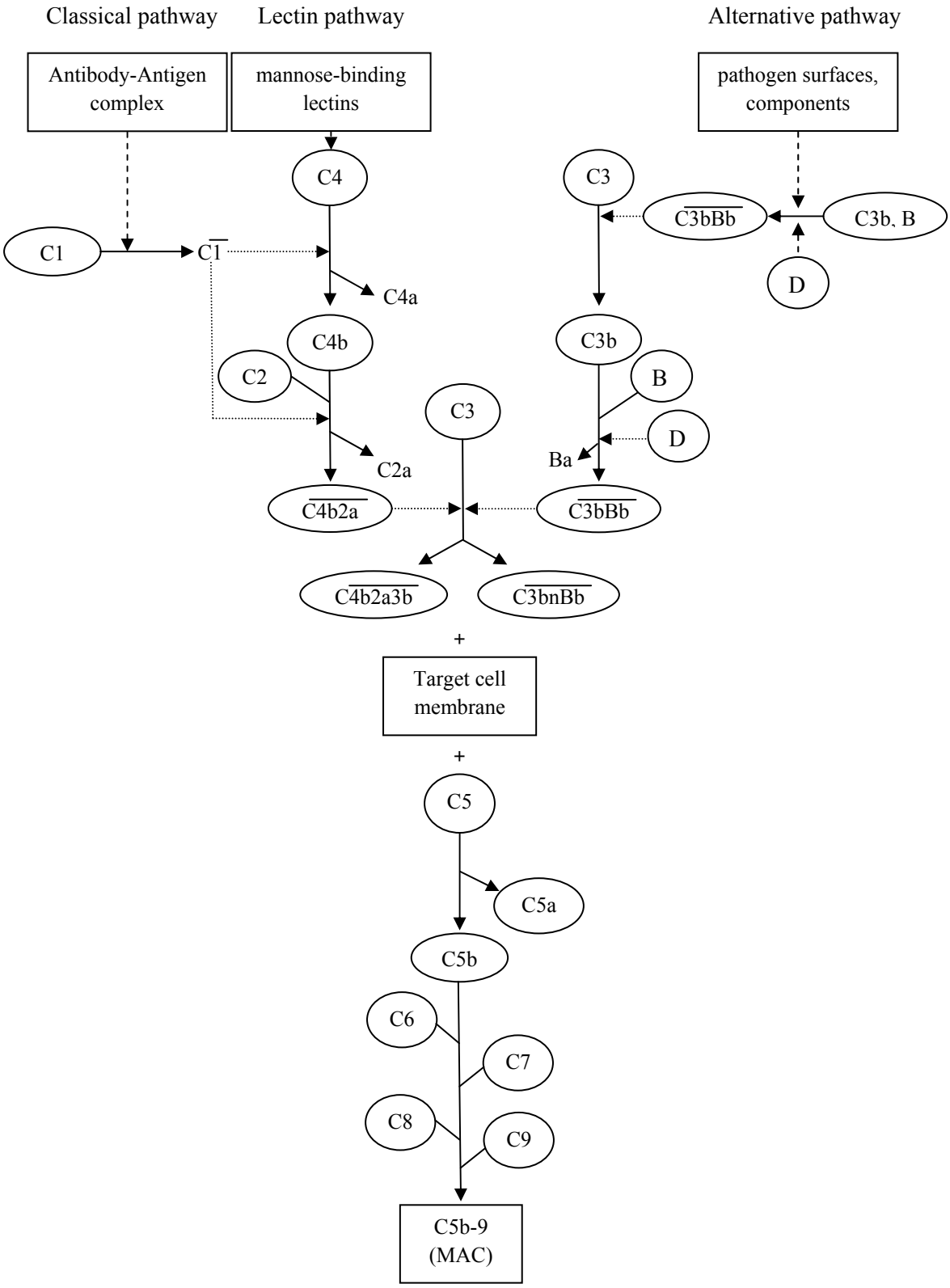
Table 1.1 Defense mechanisms in teleost fish. Adapted from Ellis [21].

Innate Humoral Immune Response	**Innate Cell-Mediated Immune Response**
(a) Inhibitors	*(a) Neutrophils*
(i) Transferrin (different genotypes)	(i) Respiratory burst → O_2^-, H_2O_2, OH^+
(ii) Antiproteases	(ii) Halide + $H_2O_2 \xrightarrow{(MPO)}$ hypohalite ions
(iii) Antibacterial peptides	(iii) Lysozyme
(iv) Lectins	
(b) Lysins	*(b) Macrophages*
(i) Proteases	(i) Hydrolytic enzymes
(ii) Lysozyme	(ii) Respiratory Burst
(iii) CRP/SAP; activates complement	(iii) NO (+ O_2^- → peroxynitrite → OH^+)
(iv) Complement (lytic, pro-inflammatory, chemotactic, opsonic, interacts with cell-mediated response	
(v) interferon	
Adaptive Humoral Immune Response	**Adaptive Cell-Mediated Immune Response**
Antibody:	Activated macrophages:
(i) Anti-adhesins	Specific T-lymphocytes and antigen
(ii) Anti-toxins	↓
(iii) Anti-invasins	Cytokines (IFNγ, TNF)
(iv) Activates classical complement pathway	↓
(v) immunological memory	Activate macrophages (enhanced RB, enhanced bactericidal activity

MPO, myeloperoxidase; IFNγ, interferon gamma; TNF, tumour necrosis factor; NO, nitric oxide; O_2^-, superoxide anion; OH^+, hydroxyl free radical

1.5 FIGURES

Figure 1.1 Complement activation pathways and functions. Activation of the complement system through any of the three existing pathways (classical, lectin, or alternative) leads to the activation of C3 into C3b and C3a. C3b covalently binds to complement activating surfaces (i.e., bacteria, fungi, viruses) and promote phagocytosis, respiratory burst, and antigen-uptake processes. C4 activated through the classical or lectin pathways can also bind to an activating surface and promote its uptake, however the number of C4 molecules binding to a surface is always many fold less than that of C3 molecules. Antigen containing covalently bound C3b or C4b molecules (or their degradation fragments) can be further processed and presented to T-lymphocytes. C3b/C4b bound to a micro-organism can lead to the formation of the membrane attack complex (MAC) which results in cell lysis. C5a and C3a anaphylatoxins generated during complement activation play a key role in inflammatory processes. Adapted from Yano [75]; Holland and Lambris [83]; Boshra *et al*. [85]


Classical pathway
Lectin pathway
Alternative pathway
Antibody-Antigen complex
mannose-binding lectins
pathogen surfaces, components
C4
C3
C3bBb
C3b, B
C1
C1
D
C4a
C4b
C3b
C2
B
C3
D
Ba
C2a
C4b2a
C3bBb
C4b2a3b
C3bnBb
+
Target cell membrane
+
C5
C5a
C5b
C6
C7
C8
C9
C5b-9 (MAC)

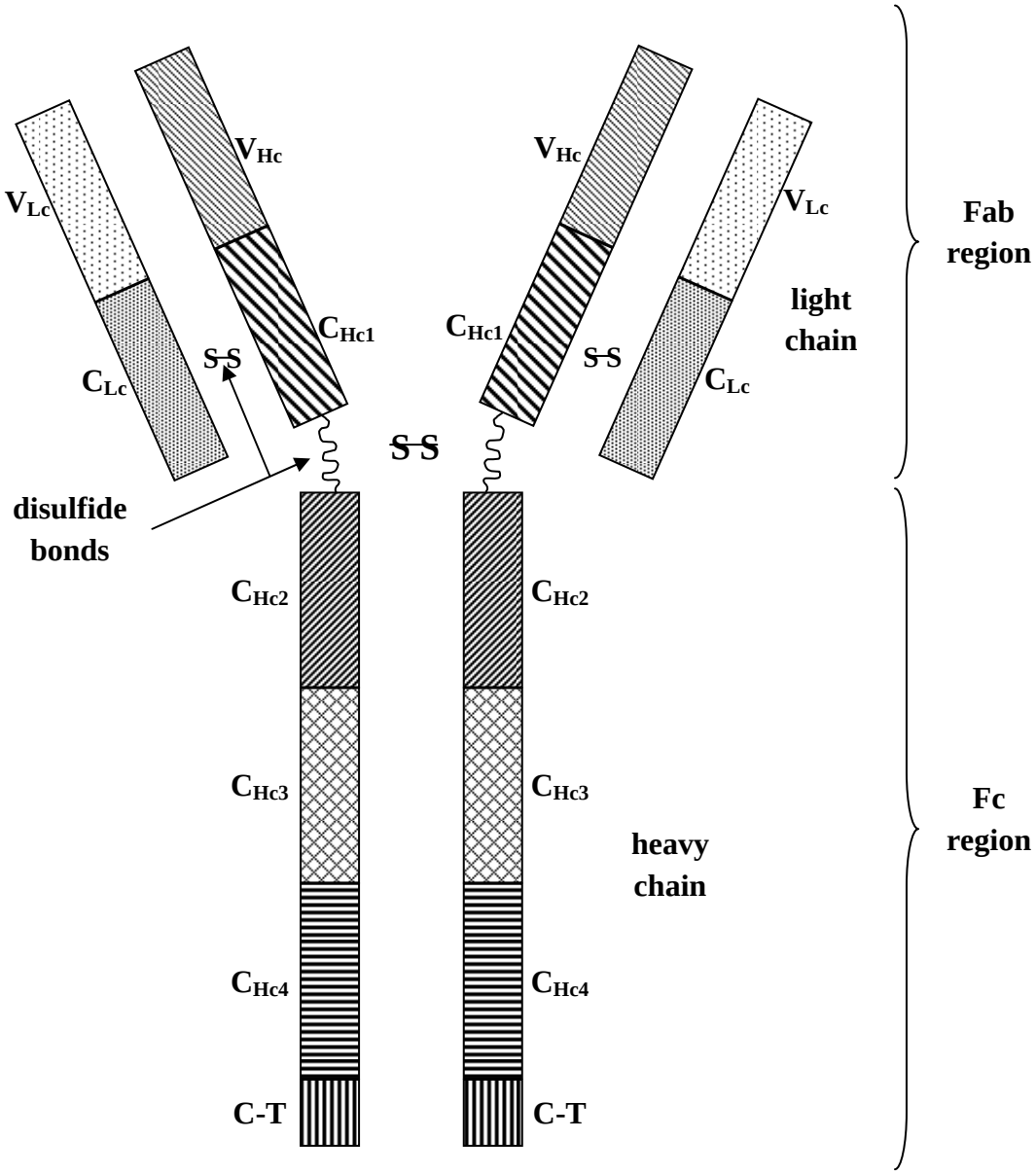


Figure 1.2 Immunoglobulin (Ig) molecules, such as the teleost IgM molecule above, are composed of two heavy chains (H_c) and two light chains (L_c) joined by disulfide bonds. Each H_c is linked to an L_c and the two H_c are linked together. The antibody antigen-binding amino terminus (Fab) region, which contains the variable (V) domain of the H_c and L_c, confers specificity. The antibody carboxy-terminal effector (Fc) region determines Ig class. Each H_c and L_c contains constant (C) and V-domains. S-S, disulfide bond; C-T, carboxy-terminal coding exon. Adapted from Janeway [67]; Kaattari and Piganelli [86].

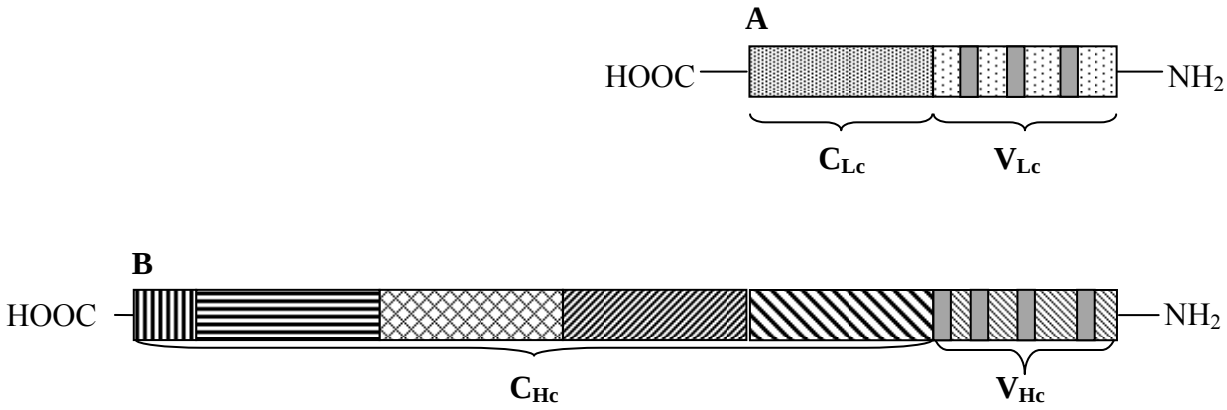


Figure 1.3 Constant (C) and variable (V) domains within A) light chains (L_c) and B) heavy chains (H_c). The solid gray bands represent hypervariable regions or complementarity-determining regions (CDR) within the variable domains. The remaining portions of the V-domain are termed the framework (FR) regions. Adapted from Prescott *et al*. [59].

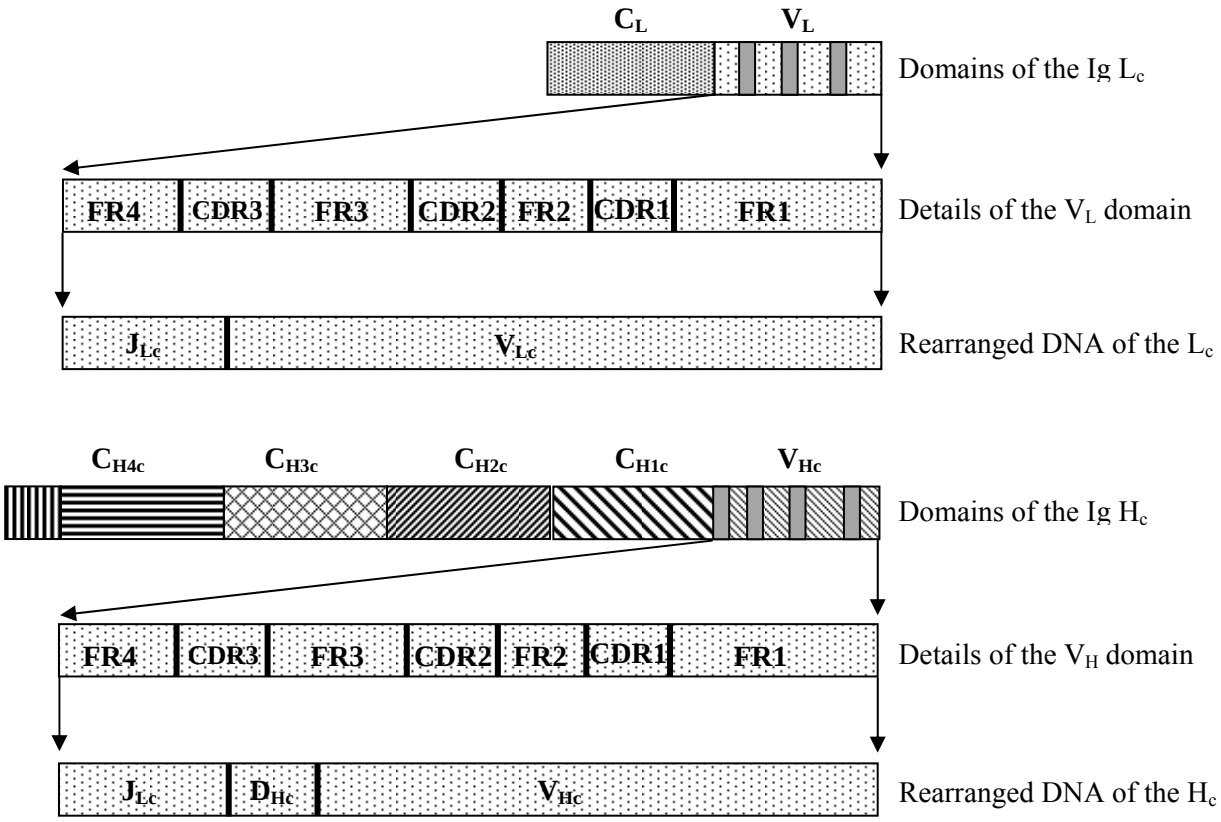


Figure 1.4 Schematic of the different domains of immunoglobulin heavy (H_c) and light (L_c) chains and the different DNA segments encoding the different parts of the variable (V) domains. CDR - complementarity diversity region; FR - framework region; C - constant domain; V - variable domain; J – joining segment; D – diversity segment. Adapted from Prescott *et al.* [59]; Pilström and Bengtén [95].

1.6 REFERENCES

[1] FAO. The state of world aquaculture. Rome: Food and Agriculture Organisation of the United Nations; 2006

[2] Hill BJ. The need for effective disease control in international aquaculture. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 3-12.

[3] Statistics Canada. Aquaculture Statistics. Catalogue no. 23-222-XIE; 2005.

[4] Leong JC, Fryer FL. Viral vaccines for aquaculture. Annual Review of Fish Diseases 1993; 3: 225-40.

[5] Thorarinsson R, Powell DB. Effects of disease risk, vaccine efficacy, and market price on the economics of fish vaccination. Aquaculture 2006; 256: 42-9.

[6] Winton J. Fish health and management. In: Wedemeyer GA, editor. Fish hatchery management, 2nd ed., Bethesda, USA: American Fisheries Society; 2001, p. 559-639.

[7] Tatner MF. Natural changes in the immune system of fish. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 255-87.

[8] Sakai M. Current research status of fish immunostimulants. Aquaculture 1999; 172: 63-92.

[9] Wedemeyer, GA, Barton BA, Mcleay DJ. Stress and acclimation. In: Schreck CB, Moyle PB, editors. Methods for fish biology, Bethseda, USA: American Fisheries Society; 1990, p. 451-89.

[10] Stoffregen DA, Bowser PR, Babish JG. Antibacterial chemotherapeutants for finfish aquaculture: A synopsis of laboratory and field efficacy and safety studies. Journal of Aquatic Animal Health 1996; 8(3): 181-207.

[11] Alderman DJ, Hastings TS. Antibiotic use in aquaculture: development of antibiotic resistance – potential for consumer health risks. International Journal of Food Science and Technology 1998; 33: 139-55.

[12] Heppell J, Davis HL. Application of DNA vaccine technology to aquaculture. Advanced Drug Delivery Reviews 2000; 43: 29-43.

[13] Shalaby AM, Khattaby YA, Abdel Rahman AM. Effects of garlic (*Allium sativum*) and chloramphenicol on growth performance, physiological parameters, and survival of Nile tilapia (*Oreochromis niloticus*). Journal of Venomous Animals and Toxins Including Tropical Diseases 2006; 12(2): 172-201.

[14] Munn CB. The use of recombinant DNA technology in the development of fish vaccines. Fish and Shellfish Immunology 1994; 4: 459-73.

[15] Evelyn TP. A historical review of fish vaccinology. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 3-12.

[16] Chinabut S, Puttinaowarat S. The choice of disease control strategies to secure international market access for aquaculture products. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 255-61.

[17] Anderson DP. Adjuvants and immunostimulants for enhancing vaccine potency in fish. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 257-65.

[18] Anderson DP. Immunostimulants, vaccines, and environmental stressors in aquaculture: NBT assays to show neutrophil activity by these immunomodulators. In: Cruz Suárez LE, Ricque MD, Nieto López MG, Villarreal D, Scholz UY, González M, editors. Avances en Nutrición Acuicola VII. Memorias dell VII Simposium Internacional e Nutrición Acuicola, Hermosillo, Sonora, México; 16-19 Noviembre, 2004, p. 320-8.

[19] Bricknell I, Dalmo RA. The use of immunostimulants in fish larval aquaculture. Fish and Shellfish Immunology 2005; 19: 457-72.

[20] Ellis AE. General principles of fish vaccination. In: Ellis, AE, editor. Fish Vaccination, London, UK: Academic Press; 1988, p. 1-19.

[21] Ellis AE. Immunity to bacteria in fish. Fish and Shellfish Immunology 1999; 9: 291-308.

[22] Sommerset I, Krossøy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1): 89-101

[23] Evensen Ø, Brudeseth B, Mutoloki S. The vaccine formulation and its role in inflammatory processes in fish – Effects and adverse effects. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel: Karger; 2005, 121, p.117-26.

[24] Schijns VEJC, Tangerås A. Vaccine adjuvant technology: From theoretical mechanisms to practical approaches. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 127-34.

[25] Mutoloki S, Reite OB, Brudeseth B, Tverdal A, Evensen Ø. A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. Vaccine 2006; 24: 578-88.

[26] Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 335-50.

[27] Busch RA. Polyvalent vaccines in fish: the interactive effects of multiple antigens. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology;

Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 245-56.

[28] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 371-79.

[29] Nikoskelainen S, Verho S, Järvinen S, Madetoja J, Wiklund T, Lilius E. Multiple whole bacterial antigens in polyvalent vaccine may result in inhibition of specific responses in rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology 2007; 22: 206-17.

[30] Buchmann K, Dalsgaard I, Nielsen ME, Pedersen K, Uldal A, Garcia JA, Larsen JL. Vaccination improves survival of Baltic salmon (*Salmo salar*) smolts in delayed release sea ranching (net-pen period). Aquaculture 1997; 156: 335-48

[31] Midtlyng PJ, Lillehaug A. Growth of Atlantic salmon (*Salmo salar*) after intraperitoneal administration of vaccines containing adjuvants. Diseases of Aquatic Organisms 1998; 32: 91-7.

[32] Rønsholdt B, McLean E. The effect of vaccination and vaccine components upon short-term growth and feed conversion efficiency in rainbow trout. Aquaculture 1999; 174: 213-21.

[33] Ackerman PA, Iwama GK, Thornton JC. Physiological and immunological effects of adjuvanted *Aeromonas salmonicida* vaccines on juvenile rainbow trout. Journal of Aquatic Animal Health 2000; 12: 157-64.

[34] Pylkkö P, Lyytikäinen T, Ritola O, Sinikka Pelkonen S. Vaccination influences growth of Arctic charr. Diseases of Aquatic Organisms 2000; 43: 77-80.

[35] Mutoloki S, Alexandersen S, Evensen Ø. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish and Shellfish Immunology 2004; 16: 633-44.

[36] Melingen GO, Wergeland HI. Physiological effects of an oil-adjuvanted vaccine on out-of-season Atlantic salmon (*Salmo salar* L.) smolt. Aquaculture 2002; 214: 397-409

[37] Sørum U, Damsgård B. Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). Aquaculture 2004; 232: 333-41.

[38] Berg A, Rødseth OM, Tangerås A, Hansen T. Time of vaccination influences development of adhesions, growth and spinal deformities in Atlantic salmon, *Salmo salar*. Diseases of Aquatic Organisms 2006; 69: 239-48.

[39] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[40] Anderson ED, Mourich DV, Leong JAC. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 1996; 5(2): 105-13.

[41] Anderson ED, Mourich DV, Fahrenkrug SC, LaPatra S, Shepherd J, Leong JC. Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 1996; 5(2): 114-22.

[42] Boudinot P, Blanco M, deKinkelin P, Benmansour A. Combined DNA immunisation with the glycoprotein gene of viral hemorrhagic septicaemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. Virology 1998; 249: 297-306.

[43] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish and Shellfish Immunology 1998; 8: 261-70.

[44] Heppell J, Lorenzen N, Armstrong NK, Wu T, Lorenzen E, Einer-Jensen K, Schorr J, Davis HL. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology 1998; 8: 271-86.

[45] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000; 18: 2817-24.

[46] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, Leong JC. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. Journal of Virology 2000; 75(15): 7048-54

[47] Boudinot P, Bernard D, Boubekeur S, Thoulouze MI, Bremont M, Benmansour A. The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. Journal of General Virology 2004; 85: 3099-3108.

[48] Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish and Shellfish Immunology 2004; 17: 447-62.

[49] Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, Park LK. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Molecular Immunology 2006; 43: 2089-2106.

[50] Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Köllner B, Dalmo RA, Vesely T, Ototake M, Fischer U. Cell-mediated immune response in the rainbow trout after DNA immunisation against the viral hemorrhagic septicaemia virus. Developmental and Comparative Immunology 2008; 32: 239-52.

[51] Corbeil S, Kurath G, LaPatra SE. Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routs of immunisation. Fish and Shellfish Immunology 2000; 10: 711-23

[52] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Kurath G. The dose-dependent effect on protection and humoral response to a DNA vaccine against infectious hematopoietic

necrosis (IHN) virus in subyearling rainbow trout. Journal of Aquatic Animal Health 2000; 12: 181-8.

[53] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection – early protection correlates with Mx expression. Fish and Shellfish Immunology 2003; 15: 39-50.

[54] Lorenzen E, Lorenzen N, Einer-Jensen K, Brudeseth B, Evensen Ø. Time course study of in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) fry. Fish and Shellfish Immunology 2005; 19: 27-41.

[55] Kurath G. Overview of recent DNA vaccine development for fish. In: Midtlyng PG, editor. Developments in Biological Standardization, Basel, Switzerland: Karger, 2005; vol. 121 p. 201-14.

[56] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Revue Scientifique et Technique – Office International des Epizooties 2005; 24 : 201-13.

[57] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006; 24: 345-54.

[58] Kurath G, Purcell MK, Garver KA. Fish rhabdovirus models for understanding host response to DNA vaccines. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2007; 2(48): 1-12.

[59] Prescott LM, Harley JP, Klein DA. Microbiology. 6th Edition, New York, NY: McGraw Hill; 2005, p.673-760.

[60] Silverthorn DU. Human Physiology: An integrated approach, 4th Edition, San Francisco, USA: Pearson Education Inc.; 2007.

[61] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology 2001; 25: 827-39.

[62] Magnadóttir B. Innate immunity of fish (overview). Fish and Shellfish Immunology 2006; 20: 137-51.

[63] Whyte SK. The innate immune response of finfish – A review of current knowledge. Fish and Shellfish Immunology 2007; 23: 1127-50.

[64] Bernstein RM, Schluter SF, Marchalonis JJ. Immunity. In: Evans DH, editor. The Physiology of Fishes. 2nd Edition, Boca Raton, USA: CRC Press; 1998, p. 215-42.

[65] Press CM, Evensen Ø. The morphology of the immune system in teleost fishes. Fish and Shellfish Immunology 1999; 9: 309-18.

[66] Zapata AG, Amemiya CT. Phylogeny of lower vertebrates and their immunological structures. In: Du Pasquier L, Litman GW, editors. Current Topics in Microbiology and Immunolgy: Origin and Evolution of the Vertebrate Immune System, Berlin, Germany: Springer; 2000; vol. 248, p. 67-110.

[67] Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology: The Immune System in Health and Disease, 6th Edition, New York, USA: Garland Science Publishing; 2005.

[68] Getz GS. Bridging the innate and adaptive immune systems. Journal of Lipid Research 2005; 46: 619-22.

[69] Litman GW, Cannon JP, Dishaw LJ. Reconstructing immune phylogeny: New perspecties. Nature Reviews: Immunology 2005; 5: 866-79.

[70] Chistiakov DA, Hellemans B, Volckaert FAM. Review on the immunology of European sea bass *Dicentrarchus labrax*. Veterinary Immunology and Immunopathology 2007; 117: 1-16.

[71] Evelyn RPT. Infection and Disease. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 339-67.

[72] Zapata AG, Chibá A, Varas A. Cells and tissues of the immune system of fish. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p.1-12.

[73] Dalmo RA, Ingebrigtsen K, Bøgwald J. Non-specific defense mechanisms in fish, with particular reference to the reticuloendothelial system (RES). Journal of Fish Diseases 1997; 10: 241-73.

[74] Kawai T, Akira S. Pathogen recognition with Toll-like receptors. Current Opinion in Immunology 2005; 17: 338-44.

[75] Yano T. The non-specific immune system: humoral defense. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p.106-59.

[76] Magor BG, Magor KE. Evolution of effectors and receptors of innate immunity. Developmental and Comparative Immunology 2001; 25: 651-82.

[77] van Muiswinkel WB. The piscine immune system: innate and acquired immunity. In: Woo, PT, editor. Fish Diseases and Disorders: Volume 1 Protozoan and Metazoan Infections: Wallingford, UK: CAB International; 1995, p. 729-50.

[78] Hancock REW. Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet: Infectious Diseases 2001; 1: 156-64.

[79] Ganz T. Defensins: Antimicrobial peptides of innate immunity. Nature Reviews: Immunology 2003; 3: 710-20.

[80] Zou J, Mercier C, Koussounadis A, Secombes C. Discovery of multiple beta-defensin like homologues in teleost fish. Molecular Immunology 2007; 44: 638-47.

[81] Grinde B. Lysozyme from rainbow rout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. Journal of Fish Diseases 1989; 12: 95-104.

[82] Lund V, Olafsen JA. A comparative study of pentraxin-like proteins in different fish species. Developmental and Comparative Immunology 1998; 22: 185-94.

[83] Holland MCH, Lambris JD. The complement system in teleosts. Fish and Shellfish Immunology 2002; 12: 399-420.

[84] Magnadóttir B, Lange S, Gudmundsdottir S, Bøgwald J, Dalmo RA. Ontogeny of humoral immune parameters in fish. Fish and Shellfish Immunology 2005; 19: 429-39.

[85] Boshra H, Li J, Sunyer JO. Recent advances on the complement system of teleosts fish. Fish and Shellfish Immunology 2006; 20: 239-62.

[86] Kaattari SL, Piganelli JD. The specific immune system: humoral defense. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 207-54.

[87] Robertsen B. The interferon system of teleost fish. Fish and Shellfish Immunology 2006; 20: 172-91.

[88] Ank N, West H, Barholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-λ), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections *in vivo*. Journal of Virology 2006; 80(9): 4501-9.

[89] Secombes C. (1996) The non-specific immune system: cellular defenses. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 63-105.

[90] Yasutake WT, Wales JH. Microscopic anatomy of salmonids: an atlas. Fish and Wildlife Service U.S. Department of the Interior: Resource publication 150; 1983.

[91] Rønneseth A, Pettersen EF, Wergeland HI. Neutrophils and B-cells in blood and head kidney of Atlantic salmon (*Salmo salar* L.) challenged with infectious pancreatic necrosis virus (IPNV). Fish and Shellfish Immunology 2006; 20: 610-20.

[92] Cumano A, Paige CJ, Iscove NN, Brady G. Bipotential precursors of B cells and macrophages in murine fetal liver. Nature 1992; 356: 612-15.

[93] Montecino-Rodriguez E, Leathers H, Dorshkind K. Bipotential B-macrophage progenitors are present in adult bone marrow. Nature Immunology 2001; 2: 83-8.

[94] Li J, Barreda DR, Zhang YA, Boshra H, Gelman AE, LaPatra S, Tort L, Sunyer JO. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nature Immunology 2006; 7(10): 1116-24.

[95] Pilström L, Bengtén E. Immunoglobulin in fish: genes, expression and structure. Fish and Shellfish Immunology 1996; 6: 243-62.

[96] Pilström L. Adaptive immunity in teleosts: humoral immunity. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 23.

[97] Kanellos TS, Sylvester ID, Howard CR, Russell PH. DNA is as effective as protein at inducing antibody in fish. Vaccine 1999b; 17: 965-72.

[98] Dooley H, Flajnik MF. Antibody repertoire development in cartilaginous fish. Developmental and Comparative Immunology 2006; 30: 43-56.

[99] Danilova NJ, Bussmann K, Jekosch L, Steiner A. The immunological heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nature Immunology 2005; 6(3): 295-302.

[100] Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proceedings of the National Academy of Sciences 2005; 102(19): 6919-24.

[101] Kaattari S, Bromage E, Kaattari I. Analysis of long-lived plasma cell production and regulation: implications for vaccine design for aquaculture. Aquaculture 2005; 246: 1-9.

[102] Warr GW. The adaptive immune system of fish. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 15-21.

[103] Manning M. J., T. Nakanishi. The specific immune system: cellular defenses. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 160-206.

[104] Secombes C, Bird SJ, Zou J. Adaptive immunity in teleosts: cellular immunity. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 25-32.

[105] Fischer U, Utke K, Somamoto T, Köllner B, Ototake M, Nakanishi T. Cytotoxic activity of fish leucocytes. Fish and Shellfish Immunology 2006; 20: 209-26.

[106] Håstein T, Gudding R, Evensen Ø. Bacterial vaccines for fish – An update of the current situation worldwide. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 55-74.

[107] Salonius K, Simard N, Harland R, Ulmer JB. The road to licensure of a DNA vaccine. Current Opinion in Investigational drugs 2007; 8(8): 635-41.

[108] Lorenzen N, LaPatra SE. Immunity to rhabdoviruses in rainbow trout: the antibody response. Fish and Shellfish Immunology 1999; 9: 345-60.

[109] Kurath G. Overview of recent DNA vaccine development for fish. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 201-14.

[110] Robinson HL. Nucleic acid vaccines: an overview. Vaccine 1997; 15: 785-87.

[111] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Scientific and Technical Review Office International des Epizooties 2005; 24(1): 201-13.

[112] Corbeil, S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000; 18: 2817-24.

[113] Kanellos TS, Sylvester ID, Butler VL, Ambali AG, Partidos CD. Mammalian granulocyte-macrophage colony-stimulating factor and some CpG motifs have an effect on the immunogenicity of DNA and subunit vaccines in fish. Immunology 1999a; 96: 507-10.

[114] Kanellos TS, Sylvester ID, Ambali AG, Howard CR, Russell PH. The safety and longevity of DNA vaccines for fish. Immunology 1999b; 96: 307-13.

[115] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[116] Nakanishi T, Ototake M. Antigen uptake and immune response after immersion vaccination. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 59-68.

[117] Quentel C, Vigneulle M. Antigen uptake and immune responses after oral vaccination. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 69-78.

[118] van Muiswinkel WB, Wiegertjes GF. Immune responses after injection vaccination of fish. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 55-7.

[119] Duff DCB. The oral immunisation of trout against bacterium salmonicida. Journal of Immunology 1942; 44: 87-94.

[120] Palm RC, Landolt ML, Busch RA. Route of vaccine administration: effects on the specific humoral response in rainbow trout, *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 1998; 33: 157-66.

[121] Akhlaghi M. Passive immunisation of fish against vibriosis, comparison of intraperitoneal, oral and immersion routes. Aquaculture 1999; 180: 191-205.

[122] Navot N, Kimmel E, Avtalion RR. Immunisation of fish by bath immersion using ultrasound. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 135-42.

[123] Lillehaug A. A cost-effectiveness study of three different methods of vaccination against *Vibriosis* in salmonids. Aquaculture 1989; 83: 227-36.

[124] Midtlyng PJ. A field study on intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 553-65.

[125] Poppe TT, Breck O. Pathology of Atlantic salmon *Salmo salar* intraperitonealy immunized with oil-adjuvanted vaccine. A case report. Diseases of Aquatic Organisms 1997; 29: 219-26.

[126] Lecocq-Xhonneux F, Thiry M, Dheur I, Rossius M, Vanderheijden N, Martial J, deKinkelin P. A recombinant viral haemorrhagic septicaemia virus glycoprotein

expressed in insect cells induces protective immunity in rainbow trout. Journal of General Virology 1994; 75: 1579-87.

[127] Engelking HM, Leong JA. The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. Virus Research 1989; 13: 213-30.

[128] Lorenzen N, Olesen NJ, Jorgensen PE. Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. Journal of General Virology 1990; 71: 561-7.

CHAPTER TWO: GROWTH AND PERFORMANCE OF ATLANTIC SALMON, *SALMO SALAR* L., FOLLOWING ADMINISTRATION OF A RHABDOVIRUS DNA VACCINE ALONE OR CONCURRENTLY WITH AN OIL-ADJUVANTED, POLYVALENT VACCINE.[1]

2.1 INTRODUCTION

In an effort to prevent costly disease outbreaks, the salmonid aquaculture industry relies heavily on the administration of vaccines, particularly polyvalent, oil-based adjuvant vaccines (AV) which have been proven to induce long-lasting protective immunity against a variety of diseases [1-4]. While highly effective at disease prevention, it has been well-established that the administration of these oil-AV can lead to adverse morphological and physiological side-effects such as inflammation at the injection site, intra-abdominal adhesions, pigmentation and granulomas [2]. Combined, these effects have been shown to influence the overall growth of fish in a positive [5, 6], negative [4, 7-10] or in a neutral manner [11] depending on the combination of adjuvant and antigen(s) used [2, 6, 10].

There are several possible explanations for the observed changes in growth in response to vaccine administration. The formation of adhesions and granulomas at or around the site of vaccine injection can lead to impaired peristaltic movements of the digestive tract, or to the destruction of secretory tissues such as the pancreas, thus reducing overall feed intake, nutrient absorption and potentially decreasing overall growth [1, 2, 12]. Alternatively, Ackerman *et al*. [6] and Sørum and Damsgård [10] attributed the changes in growth to interactions between the vaccine components [adjuvant and antigen(s)] and the fish's immune system, and the resultant increases in energy costs. Ackerman *et al*. [6], using indirect measures of active metabolic rate (M_{O_2}), demonstrated that the energy consumption of vaccinated salmonids differs depending on the type of adjuvant used, and that the resultant effects on growth are not necessarily predictable.

In July 2005, the Canadian government approved the production of a rhabdovirus DNA vaccine (DV) for use in farmed salmonids as a method of protection against infectious

---

[1]A version of this chapter has been published. Skinner LA, Schulte PM, LaPatra SE, Balfry SK, McKinley RS. Growth and performance of Atlantic salmon, *Salmo salar* L., following administration of a rhabdovirus DNA vaccine alone or concurrently with an oil-adjuvanted, polyvalent vaccine. Journal of Fish Diseases 2008; 31: 687-697.

haematopoietic necrosis (IHN) disease caused by the IHN virus. While very effective at eliciting a long-lasting, strong immune response, fish rhabdovirus DVs, such as the one used in the current study (APEX IHN®; Novartis Aqua Health, Charlottetown, PE, Canada), do not contain an adjuvant and therefore would not be expected to show the same adjuvant-related side-effects on growth [13, 14]. To date, there are no published reports regarding the growth-related effects of a rhabdovirus DV injected alone or concurrently with a polyvalent, oil-AV.

It is known that individual antigens can, and often do, affect a host's response to other antigens (i.e. antigenic competition), especially when presented simultaneously [15]. One might expect, therefore, that when a rhabdovirus DV is injected concurrently with a polyvalent, oil-AV, the number of antigenic interactions and the degree of antigenic competition will increase, thus changing the energy requirements for the immune system. This in turn would result in the reallocation of total energy stores and subsequent alterations in the energetic demands of the fish. In theory, this could potentially lead to a decrease in overall specific growth rate (SGR) and possibly a decrease in the overall health and welfare of individual fish.

The goal of this research was to determine what effect, if any, a rhabdovirus DV, injected alone or concurrently with a commonly used polyvalent, oil-AV has on the growth of Atlantic salmon, *Salmo salar*, L., raised in a laboratory setting. In addition, we examined the effects of concurrent vaccination on the repeat swimming performance of Atlantic salmon.

Repeat swimming performance, as indicated through consecutive measures of critical swimming speed (U_{crit}), is a non-lethal method of evaluating the overall health, welfare and physical capabilities of individual fish [16, 17]. A healthy fish is expected to swim to the same U_{crit} level following a brief recovery period while an unhealthy or physically challenged fish should not [16]. Because of the potential morphological, physiological and growth-related side-effects of oil-AVs and the unknown side-effects of injecting a rhabdovirus DV, assessment of swimming performance of concurrently vaccinated Atlantic salmon could be a sensitive measure of vaccine side-effects.

2.2 MATERIALS AND METHODS

2.2.1 FISH STOCK AND REARING CONDITIONS

Unvaccinated Atlantic salmon (approximately 30 g each), were transported from Big Tree Creek Hatchery (Marine Harvest Canada, Campbell River, BC, Canada) to the Department

of Fisheries and Oceans Canada – University of British Columbia Centre for Aquaculture and Environmental Research (DFO-UBC CAER) located in West Vancouver, BC, Canada. The fish appeared healthy at the time of transportation and had no prior history of disease.

A single, 1100 L indoor tank, filled with well water at a constant flow and temperature (10.6 ºC) housed all 240 experimental fish (average density at time of transfer was 8.6 kg m^{-3}) for the freshwater (FW) portion of the experiment [0 - 413 degree days (dd) post-vaccine injection (pvi)]. Fish were fed to satiation twice daily and held under natural photoperiod (ranging from 10:14 to 13:11, light:dark across the course of the FW portion of the experiment). At 415 dd pvi, the fish were moved to a single outdoor, 4000 L circular tank where they were gradually acclimatized to sea water (SW; 32 ‰, 9.2 ºC) over a five day period. Fish were fed to satiation twice daily and held under a natural photoperiod (ranging from 13:11 to 16:8, light:dark across the SW portion of the experiment). On local fish farms, feeding would typically be three times daily to satiation and photoperiod would be natural during this stage of development. For all fish, food was withheld 24 h prior to vaccination and sampling.

All fish were maintained in accordance with the Canadian Council on Animal Care, and experiments were carried out according to procedures approved by the University of British Columbia Animal Care Committee (AUP # A04-1018).

2.2.2 Vaccination Procedure

Following a four week acclimatization period in the 1100 L indoor tank, fish were randomly divided into four vaccine-specific groups (60 fish per group) and tagged with alphanumeric visible implant (VI alpha) tags (Northwest Marine Technology, Shaw Island, WA, USA) for individual identification and visible implant elastomer (VIE) tags (Northwest Marine Technology) for group identification. At the time of vaccination, fish were netted and transferred to small FW filled containers where they were individually anaesthetized with a non-lethal dose of aerated tricaine methane sulphonate (MS222; Syndell Laboratories, Vancouver, BC, Canada). Because MS222 is strongly acidic when mixed in FW [18], sodium bicarbonate ($NaHCO_3$; Sigma Aldrich, Oakville, ON, Canada) was added to the MS222 in a 1:2 ratio (100 mg L^{-1} MS222 to 200 mg L^{-1} $NaHCO_3$) as a buffering agent. Fish from the control group were injected with (i) 50 µL of phosphate-buffered saline (PBS) intramuscularly (IM), immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle) and (ii) 100 µL of PBS intraperitonealy (IP), one fin length ahead of the pelvic fins, along the midline of the fish. Fish from the AV

group were injected with (i) 50 μL IM of PBS and (ii) 100 μL IP of a commercially available, polyvalent, oil-AV containing formalin inactivated bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* (formally *Vibrio anguillarum*) serotypes O1 and O2, *Vibrio ordalii* and *Vibrio salmonicida* (Lipogen Forte®; Novartis Aqua Health). Fish from the DV group were injected with (i) 50 μL IM of a rhabdovirus DV containing 10 μg of plasmid encoding the G-protein gene from the IHN virus (APEX IHN®; Novartis Aqua Health) and (ii) 100 μL IP of PBS. Fish from the combined vaccine group were injected with (i) 50 μL IM of the DV and (ii) 100 μL IP of the AV (Table 2.1). Prior to government licensing, numerous studies examined the efficacy and dosage of similar DVs with typical doses for fish falling in the range of 1–50 μg DNA in a volume of 10–50 μL [14, 19]. Our vaccination protocol, including doses and timing of injection, were designed to closely mimic the procedures currently in use in Atlantic salmon farming operations in BC, Canada. Following vaccination, all fish were allowed to recover from anaesthesia in well-aerated FW, and then returned to the 1100 L indoor tank.

We chose to individually tag all fish and hold them in a single tank to avoid potentially confounding tank-effects that might result if the different treatment groups had been held separately. This design has the advantage that growth and physiological parameters can be measured on individual fish over time, and that any differences between groups must be due to vaccine treatment. However, this design potentially limits our ability to extend the obtained results to other settings (e.g. different stocking densities, feeding regimes, etc) as any observed effects could be due to an interaction of the vaccine treatment with the specific conditions in our experimental tank.

2.2.3 GROWTH

As with most vaccines, including the polyvalent, oil-adjuvanted and DNA vaccines utilized in this study, fish are thought to be fully protected (i.e. have elicited complete innate and adaptive immunity) against the antigens used in the vaccination by 400 dd pvi. Fork length (L_F) and live animal weight (wt) of individual fish were measured approximately every 100 dd following vaccination, until SW entry at which point measurements took place approximately every 300 dd (FW: 106, 201, 296, 413 dd, and SW: 443, 683, 990, 1300, 1616, 2028 dd). For the first 413 dd pvi, while fish were housed in FW, on the day of sampling 10 fish per vaccine group were netted and transferred to small FW-filled containers where they were individually anaesthetized with a lethal dose of aerated MS222, mixed with sodium bicarbonate in a 1:2 ratio

(500 mg L^{-1} MS222 to 1000 mg L^{-1} $NaHCO_3$). Individual fish were identified by VI alpha and VIE tags, and were sampled for weight (to the nearest 0.1 g) and fork length (to the nearest 0.1 cm), following which blood and tissue samples were collected for a separate study. Following SW entry, on the day of sampling fish were netted and transferred to small SW-filled containers where they were individually anaesthetized with a non-lethal dose of aerated MS222 (100 mg L^{-1}). Because SW has a significantly greater buffering capacity than FW, $NaHCO_3$ was not added to the anaesthetic bath. All fish housed in the SW tank (~ 20 fish per vaccine group) were identified by VI alpha and VIE tags, and individually sampled for weight (to the nearest 0.1 g) and fork length (to the nearest 0.1 cm) before being returned to the outdoor SW tank.

Fulton's condition factor (K) and the SGR were calculated for individual fish at all sampling periods using the following equations: $K = 100 (wt\ L_F^{-3})$, where wt is the individual weight of a fish to the nearest 0.1 g, and L_F is the fork length to the nearest 0.1 cm; $SGR = 100 [(\ln wt_2 - \ln wt_1) \cdot (t_2 - t_1)^{-1}]$, where SGR is the mean growth rate achieved [% degree day^{-1}, wt_2 and wt_1 are the weights of an individual fish to the nearest 0.1 g at sampling times t_2 and t_1 (in dd) respectively]. To minimize stress during the FW sampling of fish (106, 201, 296, and 413 dd pvi) only 10 fish per vaccine group were measured for wt and L_F. As such the SGR calculations made for this time period assume that wt_1 and t_1 are from the day of vaccination, and wt_2 and t_2 are from the specific sampling day. Once fish were placed in the SW tank and sampling occurred less frequently (once a month compared to every 100 dd), all fish were measured for wt and L_F. Therefore, while in SW, wt_1 and t_1 reflect the previous sampling period and wt_2 and t_2 reflect the specific sampling day (443, 683, 990, 1300 and 2028 dd pvi). At 1616 dd pvi, SGR of individual fish could not be calculated due to significant tissue growth over the VI alpha tags. The presence of VIE tags allowed fish to be group identified and therefore group-specific weight and length measurements are presented for this time point.

2.2.4 Swimming Performance

To determine if fish injected concurrently with the DV and the polyvalent oil-AV (i.e. the combined vaccine group) differed in swimming performance compared to unvaccinated control fish, U_{crit}, U_{crit} recovery ratios (RR) and normalized RR were determined 106 dd following vaccine injection, a time when the innate immune response to both the DV and polyvalent, oil-AV is known to be fully elicited [20, 21]. On two consecutive days, four post-absorptive Atlantic salmon, vaccinated and tagged 106 dd earlier were anaesthetized in a FW-filled container with

an aerated, non-lethal dose of MS222 mixed with sodium bicarbonate in a 1:2 ratio (100 mg L^{-1} MS222 to 200 mg L^{-1} $NaHCO_3$), measured for weight and fork length, and placed in a large (53 L) FW filled, Blazka-type swimming tube. Following recovery, and after undergoing a 'practice swim' in water velocities that were gradually increased to 1.2 m s^{-1} [22], fish were left to swim overnight (14 – 16 h) at a constant speed of 0.1 m s^{-1}. U_{crit}, RR and normalized RR were determined in a manner similar to that described by Jain *et al*. [16, 22] with two consecutive U_{crit} trials separated by a recovery period of 60 min. For each U_{crit} trial, the starting velocity was 0.1 m s^{-1} and increased in increments of 0.1 m s^{-1} every 20 min until fatigue was reached. Fatigue was defined as the point at which a fish could not maintain its position in the water column following three consecutive attempts to do so, and finally positioned itself on the screen at the posterior of the swimming tube despite the presence of gentle negative stimuli (i.e. bright light and motion). U_{crit} values in body lengths per second (bl s^{-1}) were calculated as in Brett [23] and the RR and normalized RR were determined as described in Jain *et al*. [15]. $U_{crit} = U_i + [(t_i \cdot U_{ii}) \cdot (t_{ii})^{-1}]$ where U_i is the highest velocity at which the fish swam for the entire time period (m s^{-1}), μ_{ii} is the incremental speed increase (m s^{-1}), t_i is the time the fish swam at the fatigue velocity (min), and t_{ii} is the predetermined time interval for swimming at a given velocity (min); RR = $(U_{crit,2}) \cdot (U_{crit,1})^{-1}$; normalized RR = $[(U_{crit,1}) \cdot (U_{crit,1(control)})^{-1} + (U_{crit,2}) \cdot (U_{crit,1(control)})^{-1}] \cdot 2^{-1}$. Measurements of swimming performance in the control fish were performed in a similar manner.

2.2.5 Statistical Analysis

Data are presented as mean values with standard errors of the mean values (± SE). Following a normality test, comparisons of mean weights, fork lengths, condition factors and SGR were performed across all groups using a one-way analysis of variance (ANOVA) at each sampling event. If a significant difference between groups was detected ($P < 0.05$), the Holm-Sidak method for multiple comparisons was utilized to identify groups that differed significantly ($P < 0.05$). Data that failed the normality test and could not be successfully transformed were subjected to a Kruskal–Wallis ANOVA on ranks, followed by Dunn's method of multiple comparisons ($P < 0.05$). Values of U_{crit} were compared using a two-way repeated measures ANOVA. If a significant difference between groups was detected ($P < 0.05$) the Holm–Sidak method for multiple comparisons was utilized to identify groups that differed significantly ($P < 0.05$). RR and normalized RR data were compared using a t-test. All data were analysed using Sigmastat software (version 3.5; Systat Software Inc., San Jose, CA, USA).

2.3 RESULTS

2.3.1 GROWTH

There were no weight or fork length differences among the four groups of fish at the time of vaccination (wt: 39.1 ± 0.4 g, L_F: 15.2 ± 0.1 cm; mean ± SE) and no mortalities were recorded for the duration of the experiment.

While the mean weight (Figure 2.1) of fish that received the DV alone was not significantly different from fish in the control group, fish that received the DV concurrently with the polyvalent, oil-AV (i.e. the combined vaccine group) did show significant differences (Figure 2.1). At 106 dd pvi, fish from the combined vaccine group (36.4 ± 2.1 g) were significantly smaller than fish from the unvaccinated control group (44.3 ± 2.1 g). Fish from the AV group (35.5 ± 0.8 g) also weighed significantly less than the unvaccinated control group at 106 dd pvi and, similar to the combined vaccine group, were not significantly different in mean weight compared to the DV group (41.9 ± 2.0 g). There were no differences in weight between fish from the AV group and the combined vaccine group (Figure 2.1). By 201 dd pvi there were no differences in mean weight between any of the three vaccinated groups of fish compared to the unvaccinated control group and all four groups remained of similar weight until smoltification and subsequent SW entry (443 dd pvi). At this time, the fish from the AV group weighed significantly less (44.9 ± 1.6 g) than the unvaccinated control fish (52.1 ± 2.1 g), but were similar in weight to both the DV (48.3 ± 1.6 g) and the combined vaccine (46.7 ± 1.5 g) groups (Figure 2.1). By 683 dd pvi, fish from all four groups were once again similar in weight. These data suggest that there are no long-term negative growth effects in Atlantic salmon due to the injection of a DV alone or concurrently with an AV. Also, the lack of differences between the AV and combined vaccine groups suggest that the negative growth implications observed at 106 and 443 dd pvi are due to interactions of the polyvalent, oil-AV and not the DV (Figure 2.1).

The fork lengths of fish from the DV and combined vaccine groups were not significantly different from that of the unvaccinated control group or the AV group. At 106 dd pvi, the mean L_F of fish from the AV group (14.9 ± 0.1 cm) was significantly smaller than that of the control group (15.8 ± 0.2 cm) (Figure 2.2), but was similar to fish from the DV (15.5 ± 0.2 cm) and combined vaccine (15.1 ± 0.2 cm) groups. There were no differences in mean L_F between any of the vaccine groups or the control group for the remainder of the growth trial (Figure 2.2).

There were no observed differences in Fulton's condition factor among the three groups of vaccinated fish and the unvaccinated control group (Table 2.2).

The SGR of fish in the AV and combined vaccine groups were significantly lower than in the unvaccinated control and DV groups for most of the first 296 dd pvi (Figure 2.3A). At 106 dd pvi, fish from the AV and the combined vaccine groups had significantly lower SGR than both the control and DV groups, and in fact, the SGR was negative indicating a significant decrease in growth which corresponded with the observed drop in weight at 106 dd (Figure 2.1). At 201 and 296 dd pvi, there were significant differences in SGR between the control group and the AV and combined vaccine groups, but not in the DV group (Figure 2.3A). Also, the rate of fish growth in the AV and combined vaccine groups showed a steady increase for the first 413 dd pvi while the rate of fish growth in the control and DV groups remained relatively constant (i.e. fish grew at a constant rate) (Figure 2.3A). Once fish entered SW (443 dd pvi), there was a significant increase in SGR observed in all groups of fish, consistent with the significant increases in mean weight and L_F during this time period (Figure 2.3B). By 683 dd pvi there were no observed differences in SGR between the groups (Figure 2.3B).

2.3.2 Swimming Performance

The significant difference in weight and SGR between concurrently vaccinated fish and unvaccinated control fish did not appear to affect the swimming performance or the repeat swimming ability of Atlantic salmon at 106 dd pvi. There were no differences in $U_{crit,1}$, $U_{crit,2}$, RR or normalized RR between the control and combined vaccine groups (Table 2.3). The significant difference observed between $U_{crit,1}$ and $U_{crit,2}$ in the combined vaccine group can be attributed to a higher than normal $U_{crit,1}$, not a decreased performance during $U_{crit,2}$. The concurrent injection of the polyvalent, oil-AV and the DV in the combined vaccine group did not negatively impact the normalized RR [i.e. normalized RR did not fall below unity (95% $U_{crit\ 1}$)], which would indicate the fish were healthy and physically unchallenged [17].

2.4 Discussion

Many researchers have reported decreased growth as a side-effect of a single vaccination with an AV in fish [2, 4, 7-10]. This is the first published report that indicates that there are no growth-related side-effects in Atlantic salmon due to the injection of a DV alone. This study is

also the first to report no synergistic effects with respect to negative growth-related side-effects following concurrent injection of a DV and a polyvalent, oil-AV.

When a DV is injected intramuscularly into a fish, the resulting immune response closely resembles that of a natural infection. Once the vaccine antigen (in this case the IHNV G-protein) has entered the host cell, transcription, translation and replication occur in a manner similar to that observed during a naturally occurring viral infection [24, 25]. The innate and adaptive immune responses are stimulated quickly and efficiently, and the production of neutralizing antibodies against the viral antigen allow for the establishment of long-term immunological memory [24, 25]. DNA vaccines, like viruses, use the host cell's replication mechanisms; therefore, there is no need for an adjuvant and only a very small amount of the plasmid construct is needed for protection: as little as 1–10 ng DV per fish [13, 26]. This is different from vaccines produced with bacterins, where large amounts of antigen and adjuvant are required to induce a sufficient and long-lasting immune response [10, 13]. Adjuvanted vaccines have a depot effect [27] where the adjuvant acts as a reservoir holding the antigen(s) in globules at the site of injection. This facilitates the induction of the pro-inflammatory response and the release of cytokines, which ultimately stimulates the production of antigen-specific antibodies [28]. Unfortunately adjuvants, especially the oil-based adjuvants that are commonly used in fish vaccines, cause significant morphological and physiological side-effects. Thus, we expected the AV group to show negative growth-related side-effects compared to the control group, but the effects of combined vaccination are more difficult to predict.

Ackerman *et al*. [6] and Sørum and Damsgård [10] suggested that the combined interactions among the antigens, the adjuvant, and the host immune system lead to depressed growth rate. We therefore expected the antigens from the DV to interact to a greater extent with the antigens of the polyvalent, oil-AV thus leading to greater effects on growth. Currently, there are no published reports examining the level of antibody interaction in fish concurrently vaccinated with a DV and a polyvalent, oil-AV. The lack of negative growth-related side-effects in fish injected with the DV alone combined with the similarities in weight and SGR of the concurrently vaccinated fish, suggests that the use of a DV does not influence the growth of Atlantic salmon in a negative way. Future studies are needed to determine if antibody interaction occurs, and to what extent it influences the immune response.

Rønsholdt and McLean [8] suggested that once a period of decreased growth occurs, as is seen in the AV and combined vaccine groups, fish cannot overcome the lost growth potential, and the resulting weight loss will be carried throughout the production cycle. Our data do not

support this conclusion. Atlantic salmon were able to compensate for the both the initial decrease in growth (106 dd pvi) and the decrease in growth observed at the time of SW entry (443 dd pvi). If fish are indeed able to recover from decreases in SGR and weight, the timing of measurements in our growth-related studies could be important when determining the overall growth-related effects of vaccination. In the current study, there was an initial decrease in SGR and weight at 106 dd pvi, after which the fish were able to recover quickly, such that by 201 dd pvi there were no differences between unvaccinated control fish and any of the vaccine groups. At the time of smoltification and SW entry (443 dd pvi), once again fish that received the oil-AV alone or concurrent with the DV experienced significant decreases in SGR and weight, which were quickly compensated for such that by 683 dd pvi there were no observed growth differences between the groups.

Swimming performance (U_{crit}) is often used as an indicator of a fish's ability to swim through stretches of strong current, and can be used as a physiological endpoint to assess the impact of physiological and environmental changes such as toxicant exposure and disease [16]. Repeat swimming ability provides a good approximation of the recovery ability of fish, whereby a healthy fish will easily regain its swimming ability after an initial U_{crit} test and an unhealthy or physiologically stressed fish will have lower recovery ability [17]. Vaccination of fish with AVs can lead to unwanted morphological and physiological side-effects due in part to prolonged stimulation of the immune system [2]. In this study, concurrent injection with a DV and a polyvalent, oil-AV significantly decreased the growth of Atlantic salmon, due primarily to the effects of the oil-AV. However, concurrent vaccine injection does not affect the swimming performance of fish, compared to unvaccinated controls. According to Gregory and Wood [29], swimming performance is unaffected by chronic stress and the associated increases in cortisol, and correspondingly, Tierney and Farrell [17] and Tierney *et al*. [30] demonstrated that moderate changes in health (and the associated physiological stresses) due to disease (bacterial and parasite) do not affect repeat U_{crit} values. Our data are consistent with these previous findings. Swimming is a predominant behaviour in fish and, as such, influences the ability of fish to obtain food, find a mate and avoid unfavourable environmental conditions [31]. Thus, we hypothesize that any energy reallocation required by the activation of the immune system following vaccination must come at the expense of other processes, avoiding impairment of swimming ability.

In conclusion, this study clearly showed that the use of a DV alone or concurrent with a polyvalent oil-AV, under laboratory conditions, did not have a negative effect on the growth or

swimming performance of Atlantic salmon. Furthermore, this study suggests that the timing of growth measurements post-vaccination is important when determining growth-related side-effects, as changes in physiology (vaccination-related stress, smoltification) appear to influence the rate of growth. Although an effort was made to ensure that the laboratory fish were treated in a manner similar to farmed fish (i.e. fed to satiation, vaccinated as at fish farms in BC, Canada), caution must to be taken when extrapolating these data to large-scale fish farms. Studies strongly suggest that fish species, size and level of development, as well as feed intake and tank density influence both the physiological and immunological responses to vaccination [9, 10, 14, 32]. If farmed fish are vaccinated under different conditions than those studied here (younger, smaller, pre-vs-post-smolt) or held under different conditions, it is possible that significant growth and/or performance differences could occur. Future studies will address the concept of antigenic competition between a DV and a polyvalent, oil-AV. These studies should allow the assessment of potential effect on the immune response(s) of a vertebrate that is given several different antigens to respond to simultaneously.

2.5 TABLES

Table 2.1 Type of vaccine(s) injected intraperitonealy (IP) and intramuscularly (IM) into Atlantic salmon (*Salmo salar* L.) (wt: 39.1 ± 0.4 g, mean ± SE).

Group ID	IP injection (100 µL)	IM injection (50 µL)
Control Group	PBS	PBS
Adjuvant Vaccine Group	AV	PBS
DNA Vaccine Group	PBS	DV
Combined Vaccine Group	AV	DV

AV, polyvalent, oil-adjuvanted vaccine; DV, rhabdovirus DNA vaccine; PBS, phosphate-buffered saline

Table 2.2 Mean Fulton condition factor [$K=100(wt \cdot L_F^{-3})$] (± SE) of Atlantic salmon following injection of phosphate-buffered saline (control group), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group), a DNA vaccine (DNA vaccine group), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group).

Degree days post-vaccination	n	Control group	Adjuvant vaccine group	DNA vaccine group	Combined vaccine group
0	60	1.13 (0.02)	1.11 (0.01)	1.10 (0.01)	1.10 (0.01)
106	7 – 10	1.12 (0.02)	1.07 (0.02)	1.11 (0.03)	1.05 (0.06)
201	7 – 10	1.07 (0.01)	1.12 (0.02)	1.08 (0.01)	1.07 (0.02)
296	7 – 10	1.03 (0.04)	1.04 (0.02)	1.08 (0.02)	1.08 (0.02)
413	7 – 10	1.07 (0.03)	1.10 (0.01)	1.03 (0.03)	1.08 (0.03)
443	18 – 24	1.09 (0.02)	1.07 (0.01)	1.07 (0.01)	1.07 (0.01)
683	18 – 24	1.04 (0.02)	1.04 (0.01)	1.04 (0.02)	1.04 (0.02)
990	18 – 24	1.04 (0.01)	1.03 (0.01)	1.01 (0.02)	1.02 (0.01)
1300	18 – 24	0.98 (0.01)	0.95 (0.01)	0.95 (0.01)	0.97 (0.01)
1616	18 – 24	1.00 (0.02)	0.99 (0.01)	0.96 (0.01)	0.98 (0.01)
2028	18 – 24	1.04 (0.02)	1.00 (0.02)	1.00 (0.01)	1.03 (0.02)

Table 2.3 Measures of mean swimming performance ($U_{crit,1}$, $U_{crit,2}$, RR, and normalized RR values) (± SE) at 106 degree days post-vaccine injection of Atlantic salmon injected intramuscularly and intraperitonealy with phosphate-buffered saline (control group) or concurrently with a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group).

Group ID	*n*	$U_{crit,1}$ (bl sec^{-1})	$U_{crit,2}$ (bl sec^{-1})	RR	Normalized RR
Control Group	8	6.6 (0.4)	6.3 (0.5)	0.91 (0.06)	0.99 (0.06)
Combined Vaccine Group	8	*6.9 (0.2)	*6.2 (0.3)	0.86 (0.04)	1.03 (0.05)

* Significant difference between $U_{crit,1}$ and $U_{crit,2}$ within the combined vaccine group; t-test, $P < 0.05$
$U_{crit} = U_i + [(t_i \cdot U_{ii}) \cdot (t_{ii})^{-1}]$; $RR = (U_{crit,2}) \cdot (U_{crit,1})^{-1}$; normalized $RR = [(U_{crit,1}) \cdot (U_{crit,1(control)})^{-1} + (U_{crit,2}) \cdot (U_{crit,1(control)})^{-1}] \cdot 2^{-1}$

2.6 FIGURES

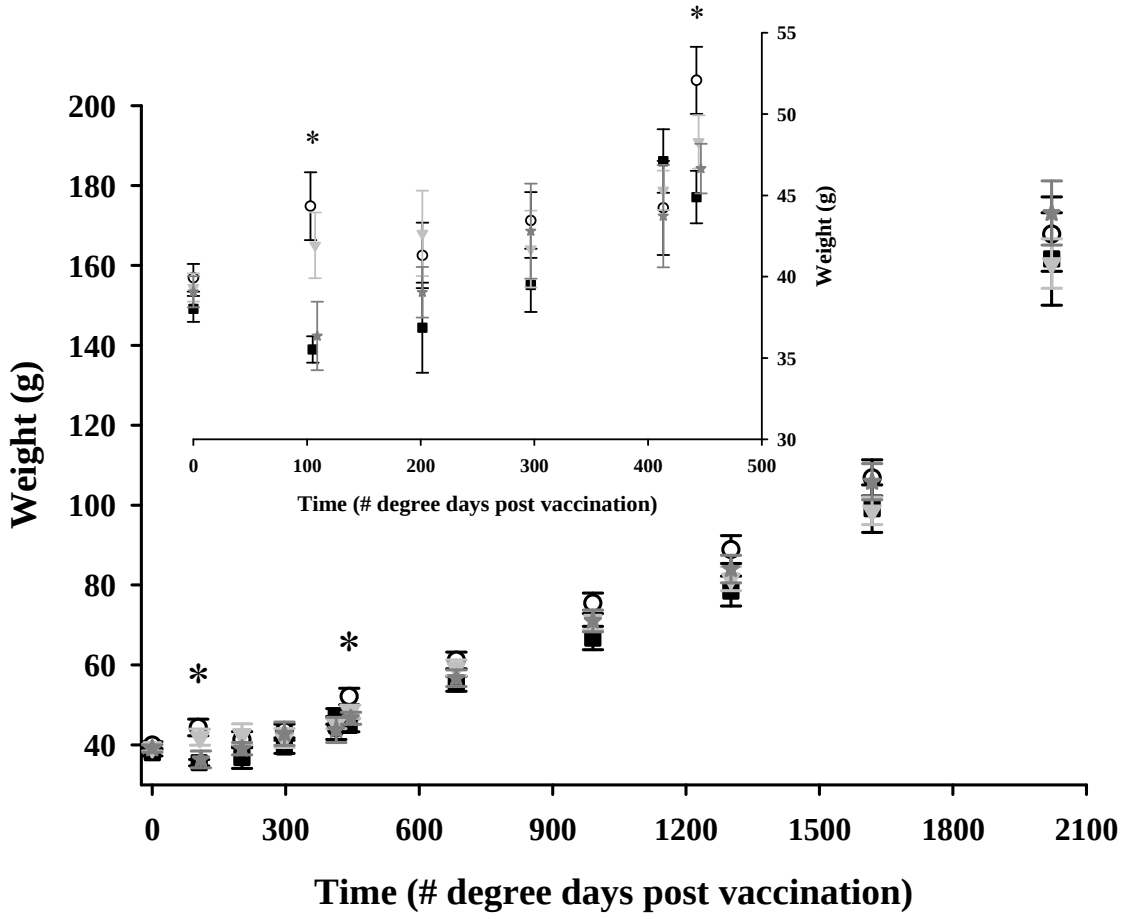


Figure 2.1 Mean weight of Atlantic salmon following injection of phosphate-buffered saline (control group;○), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;■), a DNA vaccine (DNA vaccine group;▽), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;☆). Figure insert shows the mean weight of fish for the first 443 degree days (dd) post-vaccine injection (pvi). *Significant difference between the vaccine groups (one-way ANOVA, $P < 0.05$). To assist in visualisation of statistically significant differences, data points are artificially staggered along the x-axis at 106 and 443 dd pvi. Values are mean ± SE. 0 dd, n = 60; 106, 210, 296, 413 dd pvi, n = 7-10; 443, 683, 990, 1300, 1616, 2028 dd pvi, n = 18-24.

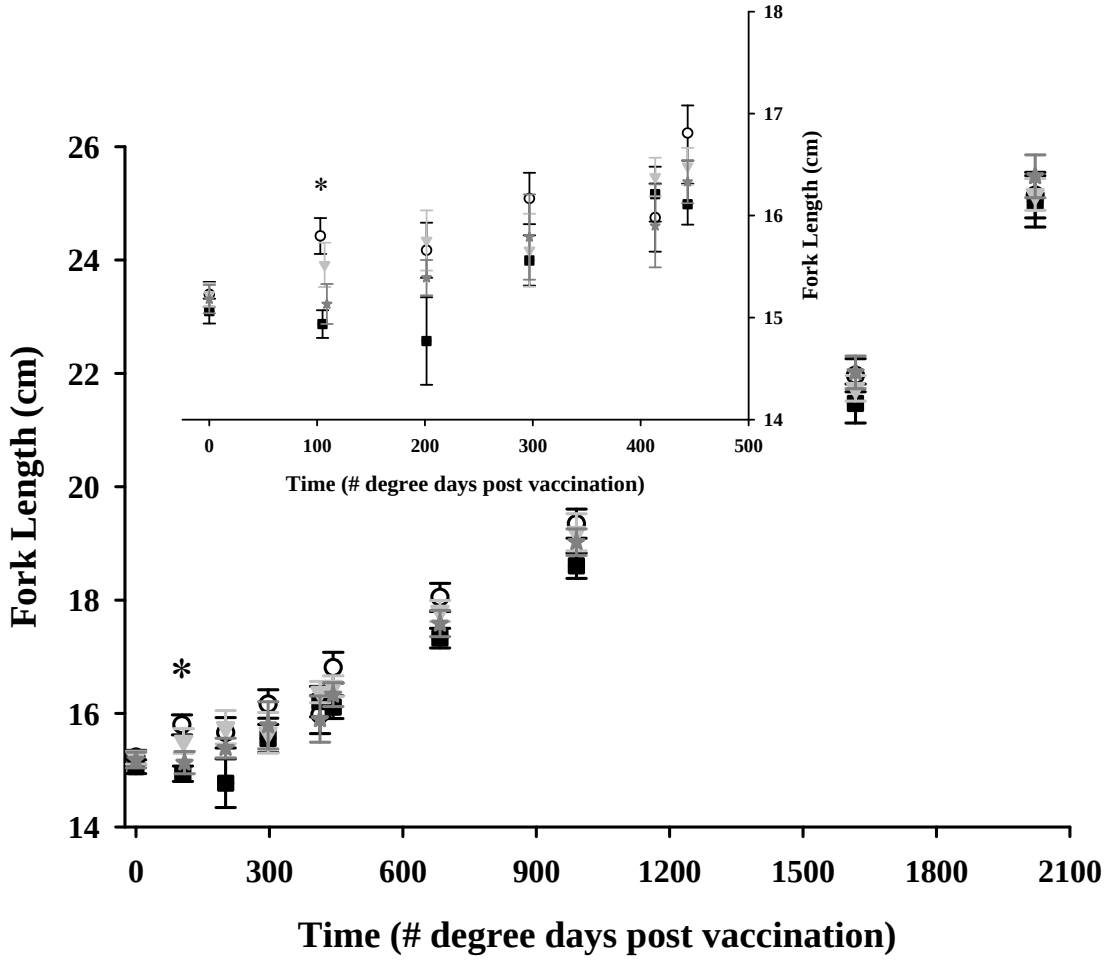
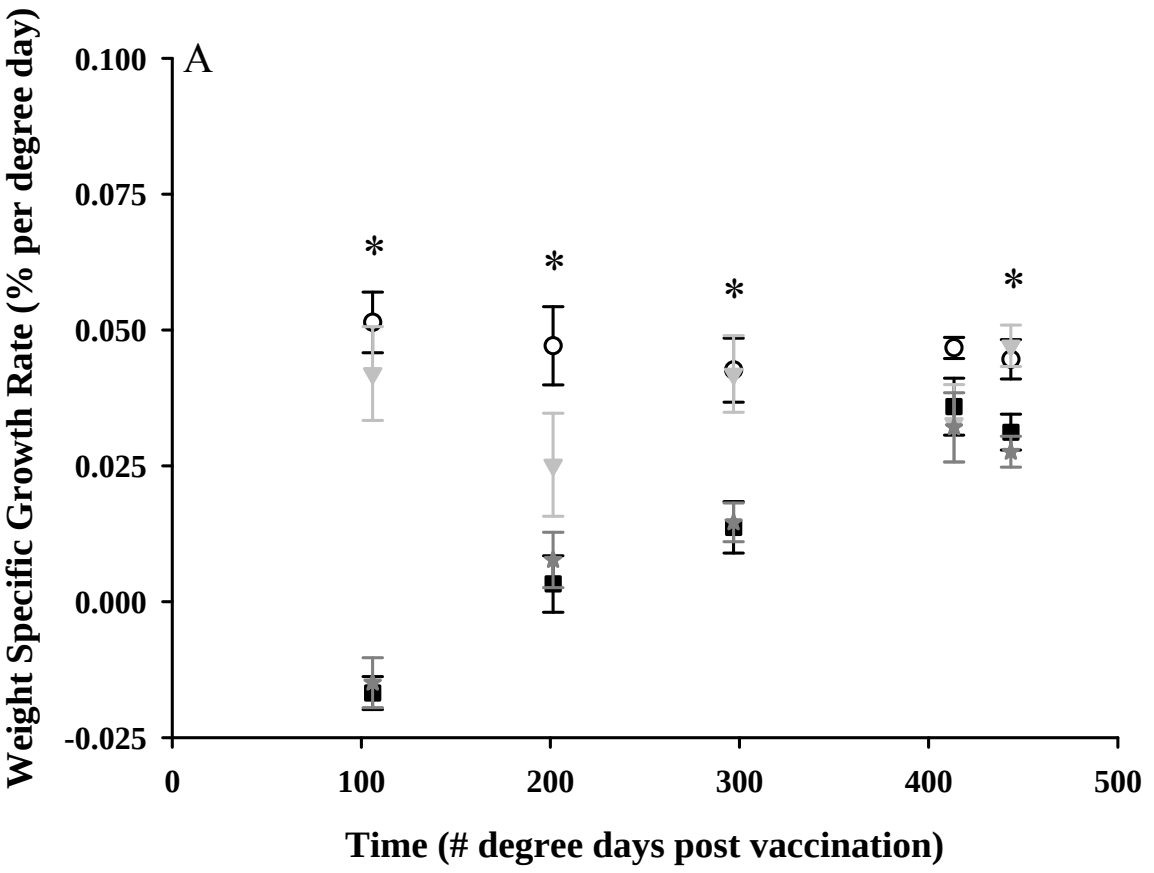
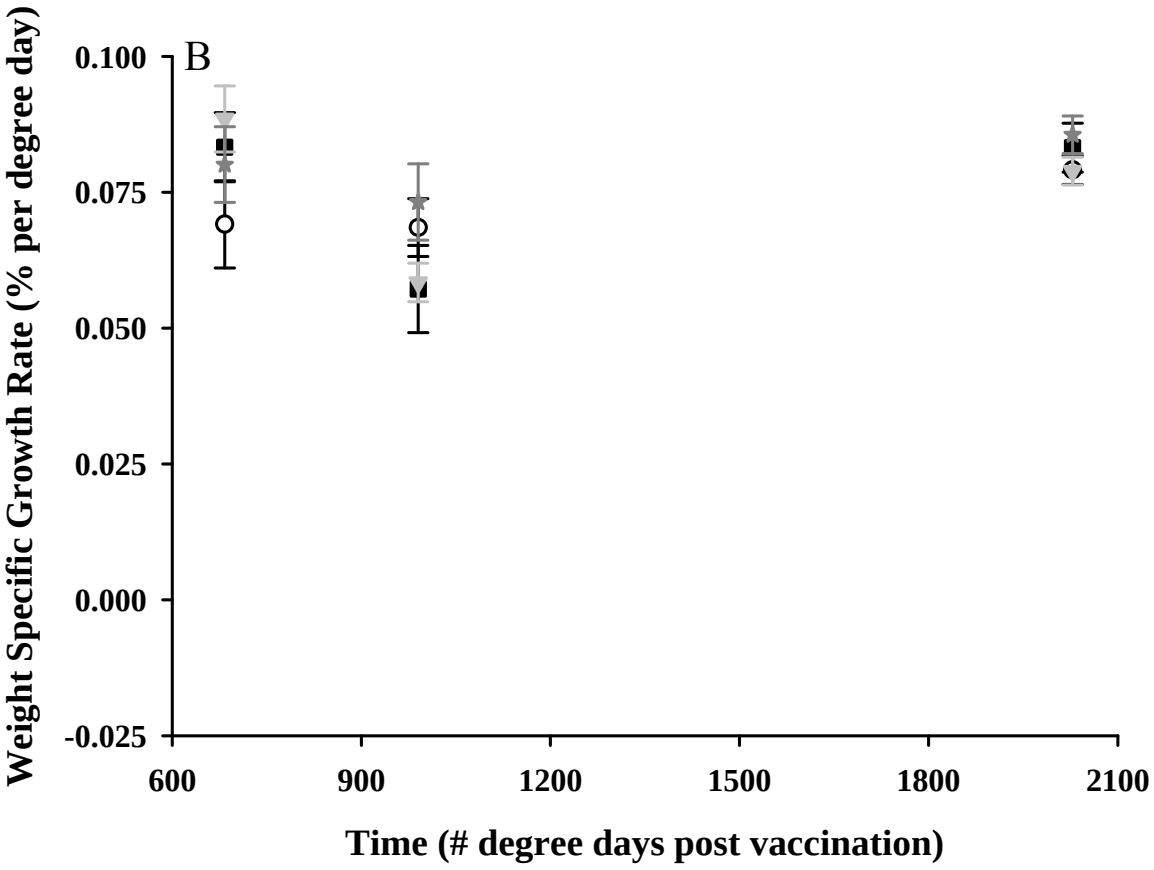


Figure 2.2 Fork length of Atlantic salmon following injection of phosphate-buffered saline (control group;○), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;■), a DNA vaccine (DNA vaccine group;▽), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;☆). Figure insert shows the mean weight of fish for the first 443 degree days (dd) post vaccine injection (pvi). *Significant difference between the vaccine groups (one-way ANOVA, $P < 0.05$). To assist in visualisation of statistically significant differences, data points are artificially staggered along the x-axis at 106 dd pvi. Values are mean ± SE. 0 dd, n = 60; 106, 210, 296, 413 dd pvi, n = 7-10; 443, 683, 990, 1300, 1616, 2028 dd vpi, n = 18-24.

Figure 2.3 Specific growth rate (SGR = 100 [$(\ln wt_2 - \ln wt_1) \cdot (t_2\text{-}t_1)^{-1}$]) of Atlantic salmon following injection of phosphate-buffered saline (control group;○), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;■), a DNA vaccine (DNA vaccine group;▽), or concurrent injection of polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;★). A) SGR for the first 443 degree days (dd) post vaccine injection (pvi) B) SGR for the sampling events at 683, 990, 1300, and 2028 dd pvi. *Significant difference between the vaccine groups (one-way ANOVA, $P < 0.05$). At 1616 dd pvi the SGR of individual fish could not be calculated due to significant tissue growth over the alphanumeric visible implant tags. The presence of visible implant elastomer tags allowed fish to be group identified and therefore group-specific weight and length measurements still exist for this time point. Values are mean ± SE. 0 dd, n = 60; 106, 210, 296, 413 dd, n = 7-10; 443, 683, 990, 1300, 1616, 2028 dd, n = 18-24.


A
0.100
0.075
0.050
0.025
0.000
-0.025
Weight Specific Growth Rate (% per degree day)
0
100
200
300
400
500
Time (# degree days post vaccination)


B
0.100
0.075
0.050
0.025
0.000
-0.025
Weight Specific Growth Rate (% per degree day)
600
900
1200
1500
1800
2100
Time (# degree days post vaccination)

2.7 REFERENCES

[1] Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 335-50.

[2] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 371-9.

[3] Schijns VEJC, Tangerås A. Vaccine adjuvant technology: From theoretical mechanisms to practical approaches. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 127-34.

[4] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[5] Buchmann K, Dalsgaard I, Nielsen ME, Pedersen K, Uldal K, Garcia JA, Larsen JL. Vaccination improves survival of Baltic salmon (*Salmo salar*) smolts in delayed release sea ranching (net-pen period). Aquaculture 1997; 156: 335-48.

[6] Ackerman PA, Iwama GK, Thornton JC. Physiological and immunological effects of adjuvanted *Aeromonas salmonicida* vaccines on juvenile rainbow trout. Journal of Aquatic Animal Health 2000; 12: 157-64.

[7] Midtlyng PJ. A field study on intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 553-65.

[8] Rønsholdt B, McLean E. The effect of vaccination and vaccine components upon short-term growth and feed conversion efficiency in rainbow trout. Aquaculture 1999; 174: 213-21.

[9] Melingen GO, Wergeland HI. Physiological effects of an oil-adjuvanted vaccine on out-of-season Atlantic salmon (*Salmo salar* L.) smolt. Aquaculture 2002; 214: 397-409.

[10] Sørum U, Damsgård B. Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). Aquaculture 2004; 232: 333-41.

[11] Pylkkö P, Lyytikäinen T, Ritola O, Sinikka Pelkonen S. Vaccination influences growth of Arctic charr. Diseases of Aquatic Organisms 2000; 43: 77-80.

[12] Poppe TT, Breck O. Pathology of Atlantic salmon *Salmo salar* intraperitonealy immunized with oil-adjuvanted vaccine. A case report. Diseases of Aquatic Organisms 1997; 29: 219-26.

[13] Robinson HL. Nucleic acid vaccines: an overview. Vaccine 1997; 15: 785-7.

[14] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Revue Scientifique et Technique – Office International des Epizooties 2005; 24(1): 201-13.

[15] Busch RA. Polyvalent vaccines in fish: the interactive effects of multiple antigens. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 245-56.

[16] Jain KE, Birtwell IK, Farrell AP. Repeat swimming performance of mature sockeye salmon following a brief recovery period: a proposed measure of fish health and water quality. Canadian Journal of Zoology 1998; 76: 1488-96.

[17] Tierney KB, Farrell AP. The relationships between fish health, metabolic rate, swimming performance, and recovery in return-run sockeye salmon, *Oncorhynchus nerka* (Walbaum). Journal of Fish Diseases 2004; 27, 663-71.

[18] Soivio A, Nyholm K, Huhti M. Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout, *Salmo gairdneri*. Journal of Fish Biology 1977; 10: 91-101.

[19] Heppell J, Davis HL. Application of DNA vaccine technology to aquaculture. Advanced Drug Delivery Reviews 2000; 42: 29-43.

[20] Ellis AE. Immunity to bacteria in fish. Fish and Shellfish Immunology 1999; 9: 291-308.

[21] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 2001; 19: 4011-19.

[22] Jain KE, Hamilton JC, Farrell AP. Use of a ramp velocity test to measure critical swimming speed in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology 1997; 117A (4): 441-4.

[23] Brett JR. The respiratory metabolism and swimming performance of young sockeye salmon. Journal of the Fisheries Research Board of Canada 1964; 21: 1183-1226.

[24] Lorenzen N, LaPatra SE. Immunity to rhabdoviruses in rainbow trout: the antibody response. Fish and Shellfish Immunology 1999; 9: 345-60.

[25] Kurath G. Overview of recent DNA vaccine development for fish. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 201-14.

[26] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000; 18: 2817-24.

[27] Anderson DP. Adjuvants and immunostimulants for enhancing vaccine potency in fish. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 257-65.

[28] Evensen Ø, Brudeseth B, Mutoloki S. The vaccine formulation and its role in inflammatory processes in fish – effects and adverse effects. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 117-25.

[29] Gregory TR, Wood CM. The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. Physiological and Biochemical Zoology 1998; 72(3): 286-95.

[30] Tierney KB, Balfry SK, Farrell AP. Subclinical *Listonella anguillarum* infection does not impair recovery of swimming performance in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 2005; 67: 81-6.

[31] Plaut I. Critical swimming speed: its ecological relevance. Comparative Biochemistry and Physiology Part A 2000; 131: 41-50.

[32] Melingen GO, Pettersen EF, Wergeland HI. Leucocyte populations and responses to immunisation and photoperiod manipulation in Atlantic salmon (*Salmo salar* L.) 0 + smolt. Aquaculture 2002; 214: 381-96.

CHAPTER THREE: THE ASSOCIATION BETWEEN METABOLIC RATE, IMMUNE PARAMETERS, AND GROWTH PERFORMANCE OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM), FOLLOWING THE INJECTION OF A DNA VACCINE ALONE AND CONCURRENTLY WITH A POLYVALENT, OIL-ADJUVANTED VACCINE.[1]

3.1 INTRODUCTION

Within the salmonid aquaculture industry, the majority of vaccines utilized are polyvalent and require the use of an adjuvant [1]. However, interactions between the antigen(s) and adjuvant can cause negative morphological and physiological side-effects [2-4], which when combined result in un-predictable variation in overall fish growth [4-14]. In 1996, Andersen *et al*. [15, 16] first described a novel fish vaccine whereby the glycoprotein (G) gene of the infectious hematopoietic necrosis virus (IHNV), a rhabdovirus with significant economic importance to the salmonid aquaculture industry, was inserted into a bacterial plasmid along with regulatory sequences that allow for expression in eukaryotic cells. The mechanisms of immune stimulation following the injection of this and similar DNA vaccines (DV) have been studied in depth and appear to closely resemble those of a natural viral infection, with the vaccinated individual producing a viral protein that is correctly folded and modified, with both cellular and humoral immune responses being elicited [16-24]. As such, unlike traditional, whole-organism vaccines, only a small amount of the plasmid construct is needed with no adjuvant requirement, thereby significantly decreasing the possibility of vaccine-related side-effects [16, 25].

Since Anderson *et al*.'s [15] initial development of the IHNV-specific DV, there has been substantial research performed investigating the mechanisms of action and efficacy of similar rhabdovirus DVs for fish including studies related to dose, durability, and efficaciousness [25-33]. Very little work however, has been published regarding the impact of these virus-specific DVs on fish performance parameters such as energetics or growth. Because feed used for

---

[1]A version of this chapter has been accepted for publication. Skinner LA, McKinley RS, Schulte PM, Balfry SK, LaPatra SE. The association between metabolic rate, immune parameters, and growth performance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), following the injection of a DNA vaccine alone and concurrently with a polyvalent, oil-adjuvanted vaccine. Fish and Shellfish Immunology.

growing fish is very expensive and accounts for 60 - 70% of the overall production costs, it is important to know the extent to which vaccination has detrimental effect on overall fish growth as this could have significant economic impacts and needs to be known. As well, there have been few published reports regarding the immunological and performance parameters of salmonids when injected concurrently with this novel DV and a commonly used polyvalent, oil-adjuvanted bacterial vaccine (AV).

The primary aim of this research therefore, was to examine the energetic costs associated with injection of a rhabdovirus-specific DV and the subsequent stimulation of the immune response in a widely cultured salmonid species, the rainbow trout (*Oncorhynchus mykiss*, Walbaum). In addition, we examined the immunological and growth performance parameters of juvenile rainbow trout following concurrent injection of a rhabdovirus-specific DV and a polyvalent, oil-AV.

3.2 MATERIALS AND METHODS

3.2.1 ANIMAL CARE AND EXPERIMENTAL DESIGN

All experiments were performed at the Clear Springs Foods Research and Development Division in Buhl, ID, USA, utilizing specific-pathogen-free fish produced on site. For the duration of the experiments, all tanks were supplied with pathogen-free, ultraviolet-light-treated spring water at a constant temperature of 14.5 °C. Photoperiod was maintained by electronic timers set for 14 h of light and 10 h of dark. Food was withheld 24 h prior to vaccine injection and prior to all sampling protocols. Fish were maintained in accordance with the guidelines approved by the Canadian Council on Animal Care and experiments were carried out according to procedures approved by the University of British Columbia Animal Care Committee (AUP # A04-1018).

3.2.1.1 EXPERIMENT #1

Fish were randomly divided into four vaccine groups and placed into separate 145 L freshwater tanks at a density of 45 fish per tank [mean individual weight 32.5 ± 0.3 g; 4 tanks per vaccine group]. Each tank of fish was treated in a similar manner and received 50 g day^{-1} of an expanded trout feed (Clear Springs Foods, Buhl, USA). Following vaccine injection, ten fish per vaccine group were randomly selected (two to three fish per tank) and sampled for blood at

approximately 203, 305, and 406 degree days (dd) post-vaccine injection (pvi). Eight fish per vaccine group were randomly selected (two fish per tank) and sampled for routine oxygen consumption (M_{O_2}) at approximately 203, 305 and 406 dd pvi. All samples were considered lethal and fish were not returned to their tanks.

3.2.1.2 Experiment #2

At the completion of experiment #1, at approximately four weeks (450 dd) pvi, the remaining fish (mean tank bulk weight: 63.3 ± 0.7 g) were divided into 145 L freshwater tanks at a density of 30 fish per tank (3 tanks per vaccine group). All fish were fed using a feeding chart that prescribed a percent body weight fed and a feed conversion ratio such that the volume of feed fed increased daily as the fish grew. Every four weeks (798 dd, 1204 dd, and 1610 dd pvi) each tank of fish was counted and bulk weighed and specific growth rate (SGR), daily weight gain (WG), and feed conversion ratio (FCR) were calculated. $SGR = 100\ [(\ln wt_2 - \ln wt_1) \cdot (t_2 - t_1)^{-1}]$ where SGR is the mean growth rate achieved [% degree day^{-1}; wt_2 and wt_1 are the bulk weights of each tank of fish to the nearest 0.1 g at sampling times t_2 and t_1 (in dd) respectively]. $WG = [(wt_2 - wt_1) \cdot (wt_1)^{-1}] \times 100$ where WG is the mean daily weight gain [% degree day^{-1}, wt_2 and wt_1 are the bulk weights of each tank of fish to the nearest 0.1 g at sampling times t_2 and t_1 (in dd) respectively]. FCR = (total feed intake) · $(\text{total weight gain})^{-1}$ for a specified period of time.

3.2.2 General Experimental Procedures

3.2.2.1 Vaccination Procedure

Following a one week acclimation period, fish were netted and transferred to small containers where they were anaesthetized with a non-lethal dose of aerated tricaine methane sulphonate (MS222; Syndell Laboratories, Vancouver, BC, Canada) (100 mg L^{-1}) buffered with sodium bicarbonate ($NaHCO_3$; Sigma Aldrich, Oakville, ON, Canada) (200 mg L^{-1}). Live animal weight (wt) of individual fish was measured to the nearest 0.1 g and fish were injected with one of the following vaccine combinations. Fish from the control group were injected with 50 µL of 0.02 M phosphate-buffered saline (PBS) intramuscularly (IM), immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle), and 100 µL of PBS intraperitonealy (IP), one fin length ahead of the pelvic fins, along the midline of the fish. Fish from the adjuvant vaccine

(AV) group were injected with 50 µL of PBS IM, and 100 µL IP of a commercially available, polyvalent, oil-adjuvanted vaccine containing formalin inactivated bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* serotypes O1 & O2, *Vibrio ordalii*, and *Vibrio salmonicida*) (Lipogen Forte®; Novartis Aqua Health, Charlottetown, PE, Canada). Fish from the DNA vaccine (DV) group were injected with 50 µL IM of a rhabdovirus DNA vaccine containing 10 µg of plasmid encoding the G-protein gene from the IHN virus (APEX IHN®; Novartis Aqua Health) and 100 µL of PBS IP. Fish from the combined vaccine group were injected with 50 µL of the DV IM and 100 µL of the AV IP. Following vaccination procedures, all fish were returned to their respective 145 L tanks and allowed to recover from anaesthesia in well aerated freshwater. Our vaccination protocol including doses and timing of injection was carried out as suggested by the vaccine manufacturer.

3.2.2.2 Blood Sampling

On the day of sampling, individual fish were netted and transferred to a small container where they were anaesthetized with a lethal dose of MS222 (500 mg L^{-1}) buffered with $NaHCO_3$ (1000 mg L^{-1}). Following weight measurements (to the nearest 0.1 g), blood samples were drawn from caudal venepuncture using a non-heparinised syringe. Whole blood was placed at 4 °C for 4 h after which it was separated into serum and red cell components by centrifugation (10 min at 4 °C, 1600 x *g*). Serum was collected and stored at -80 °C until analysed for lysozyme activity and IHNV serum neutralizing antibody (NAb) titres.

3.2.2.3 Serum Lysozyme Activity

Serum lysozyme activity was determined by a microplate modification of the method of Litwack [34-36]. Briefly, 10 µL of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250 µL of a 0.025 % w/v suspension of *Micrococcus lysodeikticus* in 0.06 M phosphate buffer (pH 6.2). The average decrease in optical density at 450 nm (OD_{450}) over a 20 min period at 25 ˚C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity (µg mL^{-1} HEWL eq), which was used as the standard.

3.2.2.4 IHNV Serum Neutralizing Antibody Titre

IHNV serum neutralizing antibody titres were determined using a complement dependent 50% plaque neutralization titre (50% PNT) assay, as described by LaPatra *et al*. [37]. Briefly, serum samples were heat-inactivated for 30 min at 45 ˚C to destroy all residual complement, and a two-fold dilution series made. As a source of complement, serum was obtained from pathogen-free rainbow trout that had not been fed for at least two weeks. Equal volumes of this complement source (1:10 dilution) and a diluted IHN virus suspension (2000 pfu mL^{-1}) were added to each serum dilution series. Samples were plaque assayed on *Epithelioma papulosum cyprini* monolayers and NAb titres were reported as the reciprocal of the highest serum dilution that resulted in a 50 % reduction in the average number of plaques detected in negative controls. A sample was considered to be positive with a titre of 20 or above, while a titre of < 20 was considered negative [30, 37].

3.2.2.5 Oxygen Consumption

Oxygen consumption (M_{O_2}) was measured as an indicator of routine metabolic rate (RMR), which can be indicative of basal metabolism in fish. To ensure individual fish were in a post-absorptive state and therefore fit the requirements for RMR measurement (i.e. calm, motionless, and post-absorptive), food was withheld from the sampling tank(s) a minimum of 24 h prior to M_{O_2} measurements. After being immobilized with a non-lethal dose of MS222 (100 mg L^{-1}) buffered with $NaHCO_3$ (200 mg L^{-1}), individual fish were placed in darkened respirometers (1625 mL volume) with pathogen-free, ultraviolet-light-treated spring water (temperature of 14.5 °C) flowing through at a constant rate. Fish were allowed to acclimate to the respirometer for up to 15 h, after which M_{O_2} measurements were performed.

The respirometer set-up consisted of a polyvinyl chloride (PVC) tube six inches in diameter and sealed at both ends with a valve assembly, allowing the system to operate in either a flow-through (acclimation) or recirculation (M_{O_2} measurement) mode. Water temperature (˚C) and dissolved oxygen concentration (mg L^{-1}) in the respirometer were measured using a Clark type dissolved oxygen electrode (Orion862A; Thermo Electron Corporation, Beverly, MA, USA) mounted in an insulated jacket. A constant flow of water from the respirometer passed over the electrode using a Preston-model Varistaltic Power Pump (Manostat; Barnant Company, Barrington, IL, USA) and gas impermeable tubing, before being returned back into the respirometer. The dissolved oxygen concentration was recorded every 30 s for a 10 min period,

and the average oxygen consumption (milligrams per kilogram fish weight per hour; mg kg^{-1} h^{-1}) was determined using the calculated decline in dissolved oxygen concentration. To account for possible endogenous oxygen consumption within the system, blank runs were made throughout the experiments. No corrections were necessary.

3.2.2.6 Statistical Analysis

Data are presented as means with standard errors of the means (± SE). Because there were no significant differences ($P < 0.05$) between fish from the same treatment tank or between replicate tanks, data from the replicate tanks were pooled. Following a normality test, comparisons of means were performed across all groups using a two-way analysis of variance (ANOVA) with vaccine group and time post-vaccination as factors. If a significant difference between groups was detected ($P < 0.05$) the Holm-Sidak method for multiple comparisons was utilized to identify groups that differed significantly ($P < 0.05$). Neutralizing antibody titres were analyzed using a Mann-Whitney rank sum test due to non-normality of the data. Differences were considered significant if $P < 0.05$. All data were analyzed using Sigmastat software (version 3.5; Systat Software Inc., San Jose, CA, USA).

3.3 Results

3.3.1 Experiment #1

3.3.1.1 Weight

There were no differences in weight among the four groups of fish at the time of vaccination (wt: 32.5 ± 0.3 g) and no mortalities were recorded for the duration of the experiment. The mean individual weight of all fish increased over time with no significant differences observed between the four vaccine groups (data not shown). At the completion of experiment #1 (406 dd pvi), the mean weight of all individually sampled fish was 63.6 ± 1.9 g.

3.3.1.2 Serum Lysozyme Activity

Lysozyme activity in all vaccine groups was determined at 203, 305, and 406 dd pvi. At 203 dd pvi, fish from the AV and combined vaccine groups had significantly higher levels of lysozyme activity compared to fish from the control and DV groups (Figure 3.1A). At 305 dd

pvi, there were no significant differences in lysozyme activity between any of the vaccine groups. This could be attributed to an increase in lysozyme activity of the control group and a decrease in lysozyme activity of the AV group compared to the levels observed at 203 dd pvi (Figure 3.1B). At 406 dd pvi, lysozyme activity of the control group was once again similar to the level observed at 203 dd pvi. This level of lysozyme activity was not different from that of the DV group and was lower than that observed in both the AV and combined vaccine groups (Figure 3.1C).

3.3.1.3 IHNV Serum Neutralizing Antibody Titre

As NAb titres are specific to the proteins produced in response to the IHNV DV, only those fish that received the DV produced measurable amounts of NAb (Table 3.1). At 203 dd pvi, the combined vaccine group showed a stronger NAb response than the DV group which showed little to no detectable neutralizing activity ($P < 0.05$). By 305 dd pvi, fish in the DV group showed no significant differences in NAb response when compared to fish in the combined vaccine group. At 406 dd pvi, fish in the DV group showed a stronger NAb response than fish in the combined vaccine group ($P < 0.05$).

3.3.1.4 Oxygen Consumption

The M_{O_2} of fish in the combined vaccine group was significantly greater than that of the control, AV, and DV groups at 203 dd pvi (Figure 3.2A). By 305 dd pvi, the M_{O_2} of the combined vaccine group returned to control levels (Figure 3.2B) and there were no significant differences between any of the vaccine groups for the remainder of the experiment (Figure 3.2C).

3.3.2 Experiment #2

3.3.2.1 Weight

There were no differences in weight among the four groups of fish at the start of experiment #2 (mean tank bulk wt: 63.3 ± 0.7 g) and no mortalities were recorded for the duration of the experiment. The mean weight of all fish increased over time with no significant differences observed between the four vaccine groups (Table 3.2). At the completion of experiment #2 (1610 dd pvi), the mean tank bulk weight of all groups was 323.8 ± 1.9 g.

3.3.2.2 SPECIFIC GROWTH RATE

At 798 dd pvi, the SGR of the AV (0.17 ± 0.001% dd^{-1}), DV (0.17 ± 0.002 % dd^{-1}) and combined vaccine (0.17 ± 0.001 % dd^{-1}) groups were similar and all significantly higher than the SGR of the control group (0.16 ± 0.002 % dd^{-1}). At 1204 dd pvi, the SGR of all groups decreased compared to that observed at 798 dd pvi, and there were no significant differences between any of the vaccine groups. As expected, there was a decrease in growth rate as fish size increased, with the SGR of all four vaccine groups lower at 1610 dd pvi than at 1204 dd pvi. Fish in the AV (0.11 ± 0.001 % dd^{-1}), DV (0.11 ± 0.001 % dd^{-1}), and combined vaccine (0.11 ± 0.002 % dd^{-1}) groups had similar SGRs which were significantly higher than the SGR of the control group (0.10 ± 0.003 % dd^{-1}).

3.3.2.3 DAILY WEIGHT GAIN

Similar to the SGR, the percent daily WG of the AV (0.24 ± 0.002 % dd^{-1}), DV (0.24 ± 0.003 % dd^{-1}), and combined vaccine (0.24 ± 0.002 % dd^{-1}) groups were significantly greater than the WG of the control group (0.23 ± 0.004 % dd^{-1}) at 798 dd pvi. By 1204 dd pvi, all four groups showed similar levels of WG. At 1610 dd pvi, the WG of the AV (0.14 ± 0.001 % dd^{-1}), DV (0.14 ± 0.002 % dd^{-1}), and combined vaccine (0.14 ± 0.001% dd^{-1}) groups was significantly greater than that of the WG of the control group (0.12 ± 0.004 % dd^{-1}).

3.3.2.4 FEED CONVERSION RATIO

There were no statistically significant differences in FCR between any of the four vaccine groups at 798 or 1204 dd pvi. At 1610 dd pvi, the FCR of the AV (0.80 ± 0.03), DV (0.81 ± 0.02), and combined vaccine (0.81 ± 0.01) groups were similar and significantly lower than the FCR of the control group (0.87 ± 0.01).

3.4 DISCUSSION

In vertebrates, it is known that maintaining a functioning immune system and mounting an immune response (innate or adaptive) can be energetically costly with individuals forced to down-regulate some physiological activities in order to up-regulate others [38, 39]. Pilorz *et al*. [39] suggest that an increase in immune response can have a negative effect on a variety of biological functions and may be evident by an increase in metabolic rate. While there have been

many studies examining the effects of immune stimulation on the metabolic rate of fish [40-47], these studies have been based on diseased individuals infected primarily with parasites. To date, only one other study has examined the metabolic impact of immune stimulation on healthy fish via vaccination. In 2000, Ackerman *et al*. [8] reported an increase in the metabolic rate of rainbow trout in response to the administration of a monovalent oil-AV. Ours is the first study to measure routine metabolic rate in a fish species following injection of a DV alone, or concurrently with a bacterial polyvalent, oil-AV.

In the current study, RMR was unchanged in individuals injected either with a single DV or with a polyvalent, oil-AV. However, when these two vaccines were injected concurrently, a situation encountered in the salmonid aquaculture industry, a significant and transient increase in RMR was observed. Associated with the increase in RMR, there was an increase in lysozyme activity and in the seroconversion of NAbs. Because there were no apparent differences in lysozyme activity between the AV and the combined vaccine groups, we suggest that the increase in RMR of the combined vaccine group was due, in part, to the earlier seroconversion of NAbs, compared to the DV group.

In three unrelated studies, Kim *et al*. [20], LaPatra *et al*. [48], and Kurath *et al*. [30] showed that fish injected with a rhabdovirus DV elicit early, specific, and long-term antiviral responses. It is thought that the early antiviral response (EAVR), which begins as early as 4-7 d pvi and lasts at least 14 d (in rainbow trout held at 15 ˚C), is a non-specific antiviral state mediated by the up-regulation of type I interferon-like (IFN) factors [17, 20, 28, 48, 51]. The EAVR offers strong protection with relatively low specificity and is associated with the ability to cross-protect individuals against related rhabdoviruses [30, 49]. The specific antiviral response (SAVR), which typically occurs 3 to 4 weeks pvi (in rainbow trout held at 15 ˚C), appears to be mediated by more specific adaptive immune factors including NAbs and other cellular immune factors, and is the response most often studied in vaccine trials [30, 48]. The exact timing of the shift from EAVR to SAVR varies with temperature and vaccine dose and correlates with the development of the NAbs [30]. The long-term antiviral response (LAVR), characterized by a reduced yet significant protective immunity, occurs from six to 25 months post-vaccine injection [30] and is beyond the scope of the current experiments.

It has been hypothesized that the non-specific EAVR observed following injection of DVs, and especially the up-regulation of type I IFN related genes, may be important for the stimulation of the specific adaptive immune response and the subsequent transition to the SAVR [17, 49-51]. Although we did not measure the expression of type I IFN factors in the current

study, previous studies have confirmed that rhabdoviruses and rhabdovirus DVs induce type I IFN factors in rainbow trout for at least 14 d pvi [22, 51]. As well, there is evidence that the lipopolysaccharide (LPS) and DNA of the bacteria *Listonella anguillarum*, and an oil-type adjuvant also induce type I IFN factors in salmonids [50, 52]. If this is the case, fish from the combined vaccine group have two key stimuli for the induction and up-regulation of type I IFN genes: the LPS and DNA of *Listonella anguillarum* (a key component of the polyvalent, oil-AV), and the DV. Fish from the combined vaccine group, therefore, may have increased levels of IFN proteins compared to the DV group and this may be a plausible explanation for the earlier seroconversion of NAbs. Future studies should examine this possibility.

The lysozyme activity of the combined vaccine group, a measure of the non-specific anti-bacterial immune response, was significantly higher than that of the control or DV groups. If we add on the energetic costs associated with the increase in antiviral immune response including the up-regulation and induction of type I IFN related genes, and the earlier seroconversion of NAbs compared to the DV group, we might be able to explain the observed increase in RMR of the combined vaccine group at 203 dd pvi. As well, it is important to remember that fish from the combined vaccine group were injected with a DV as well as a polyvalent oil-AV. Antibodies against all bacterins present in the polyvalent vaccine will have begun production by 200 dd pvi [1, 53, 54]. Although antibody titres were not measured in this experiment, data from Atlantic salmon (*Salmo salar* L.) injected with similar vaccine combinations showed an increase in antibody titre above control levels at 201 dd pvi [Chapter 4].

The development and maintenance of an immune response relies on energy and protein, particularly for the production of antibodies [38, 39]. Because energy and protein can be limited resources in rapidly growing animals such as juvenile rainbow trout, individuals may be forced to down regulate some physiological activities in order to up-regulate the immune response [39]. The increased immune activity within the combined vaccine group at 203 dd pvi could result in a significant up-regulation of the immune system and perhaps a reallocation of the energy costs resulting in a down-regulation of other activities. Unfortunately, in the current study, SGR, WG, and FCR parameters were not measured until 798, 1204, and 1610 dd pvi. Although there were significant increases in SGR and WG at 798 and 1610 dd pvi in the AV, DV, and combined vaccine groups, possibly an indication of fish compensating for any lost growth during the initial 400 dd pvi, we did not find any significant differences in overall weight of the fish. To fully understand the energetic interactions between the immune response and growth, future studies

should examine the timing of changes to SGR and WG with respect to immune stimulation following vaccine injection.

The key findings of this study were the increased RMR in the combined vaccine group and the corresponding increase/change in immune response. Knowing that concurrent administration of a DV and a polyvalent, oil-AV can change the manner in which the immune response occurs, can be very useful when designing new vaccines and when implementing vaccination protocols in an aquaculture setting. This study also suggests that, regardless of the increased immune response and resultant transient increase in RMR, overall growth performance of salmonids is not significantly affected. Care must be taken however, when extrapolating these data to large-scale fish farms and real-world situations as studies strongly suggest that fish species, size and level of development, as well as feed intake and tank density influence both the physiological and immunological responses to vaccination [10, 11, 32, 55]. To better understand the relationships between immunological parameters and RMR, and allow for extrapolation to the aquaculture industry, future studies should examine key these immunological and physiological parameters at the production level.

3.5 TABLES

Table 3.1 Neutralizing antibody titres of individual rainbow trout are determined by plaque assay and are reported as the reciprocal of the highest dilution that resulted in a 50 % reduction in the average number of plaques detected in the negative control wells. Samples were considered positive with a titre of 20 or above, while samples with a titre of < 20 were considered negative.

Fish #	203 degree days post-vaccine injection		305 degree days post-vaccine injection		406 degree days post-vaccine injection	
	DNA vaccine group	Combined vaccine group*	DNA vaccine group	Combined vaccine group	DNA vaccine group*	Combined vaccine group
1	< 20	< 20	< 20	< 20	< 20	< 20
2	< 20	< 20	< 20	< 20	40	< 20
3	< 20	< 20	< 20	< 20	80	< 20
4	< 20	< 20	< 20	< 20	80	20
5	< 20	< 20	40	20	80	20
6	< 20	40	80	40	≥ 160	20
7	< 20	40	≥ 160	40	≥ 160	20
8	< 20	80	≥ 160	≥ 160	≥ 160	40
9	20	80	≥ 160	≥ 160	≥ 160	80
10		≥ 160	≥ 160	≥ 160		≥ 160

*Significant difference between vaccine groups; Mann-Whitney rank sum test, $P < 0.05$

Table 3.2 Average weight (g) of juvenile rainbow trout following injection of phosphate-buffered saline (control group), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group), a DNA vaccine (DNA vaccine group), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group). Each tank of fish was counted and bulk weighed at 798, 1024, and 1610 degree days (dd) post-vaccine injection (pvi). Values are mean (± SE), n = 3.

	Control group	Adjuvant vaccine group	DNA vaccine group	Combined vaccine group
798 dd pvi	125.7 (1.2)^{a,w}	121.8 (0.3)^{a,w}	121.6 (1.6)^{a,w}	120.2 (2.8)^{a,w}
1204 dd pvi	214.2 (4.4)^{a,x}	209.6 (1.1)^{ax}	209.6 (2.8)^{a,x}	208.1 (3.9)$^{a.x}$
1610 dd pvi	320.2 (4.5)^{a,y}	325.4 (2.6)^{a,y}	324.8 (5.6)^{a,y}	324.8 (3.5)^{a,y}

$^{a, b, c, d}$ Significant differences between vaccine groups at each sampling period;
$^{w, x, y}$ Significant differences between sampling periods within a vaccine group;
two-way ANOVA, $P < 0.05$

3.6 FIGURES

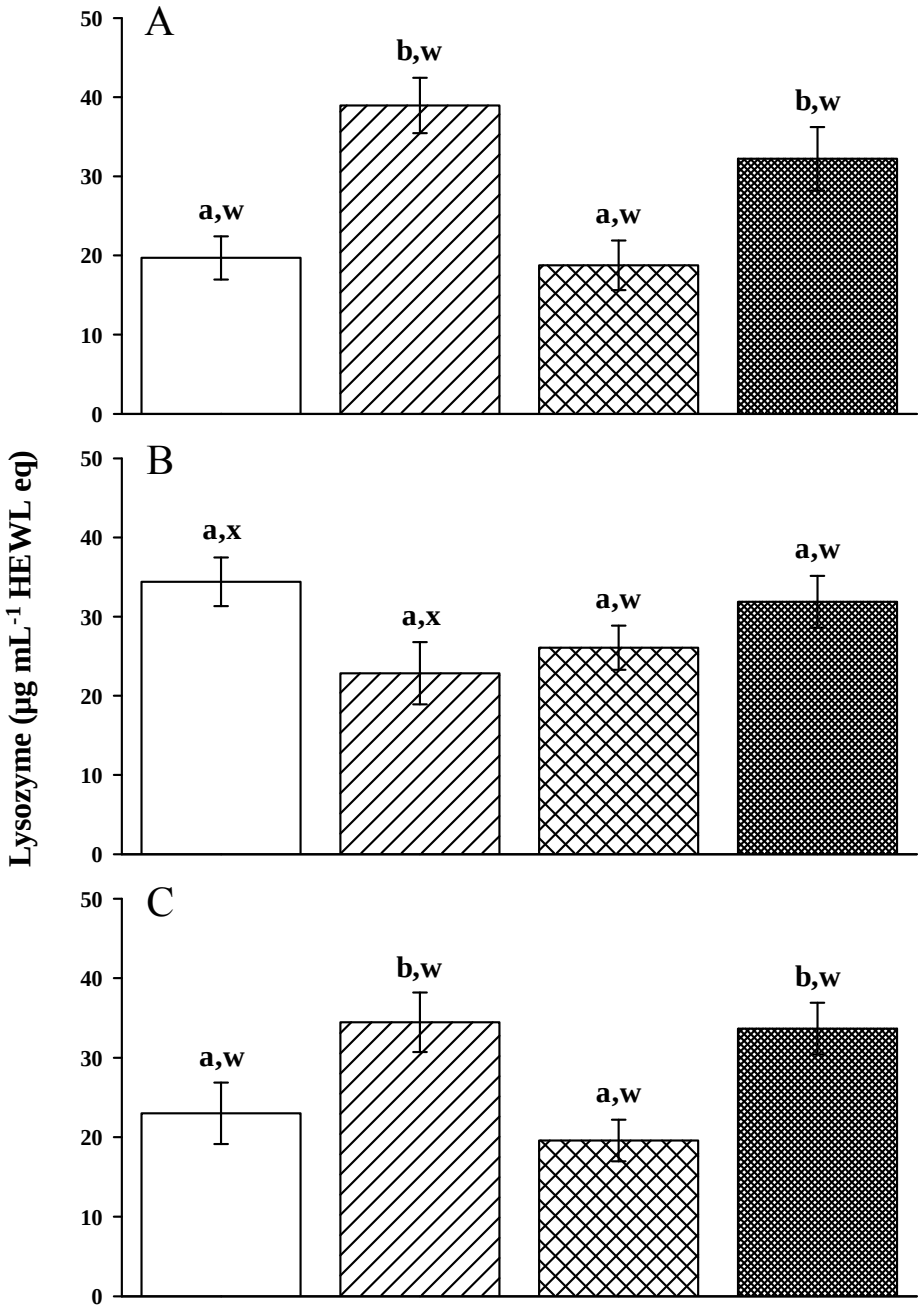


Figure 3.1 Serum lysozyme activity of juvenile rainbow trout following injection of phosphate-buffered saline (control group; ▭), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group; ▨), a DNA vaccine (DNA vaccine group; ▩), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group; ▩). Fish were sampled at A) 203, B) 305, and C) 406 degree days post-vaccine injection. Values are mean ± SE (n = 10). [a, b, c, d] Significant differences between vaccine groups; [w, x, y] Significant differences between sampling periods within a vaccine group; two-way ANOVA, $P < 0.05$.

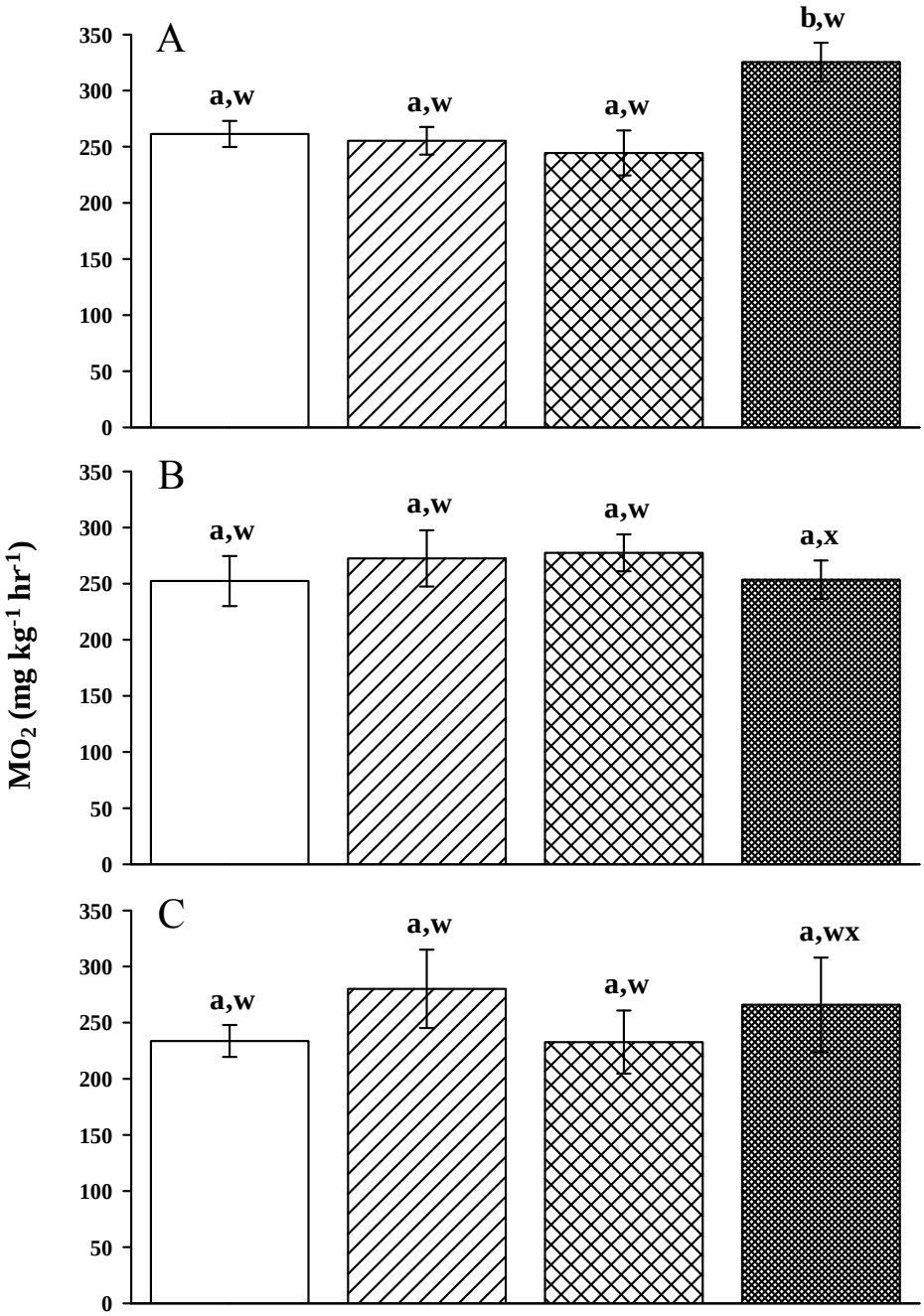


Figure 3.2 Oxygen consumption (M_{O_2}) of juvenile rainbow trout following injection of phosphate-buffered saline (control group; ▭), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group; ▨), a DNA vaccine (DNA vaccine group; ▩), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group; ▩). Fish were sampled at A) 203, B) 305, and C) 406 degree days post-vaccine injection. Values are mean ± SE (n = 8). [a, b, c, d] Significant differences between vaccine groups; [w, x, y] Significant differences between sampling periods within a vaccine group; two-way ANOVA, $P < 0.05$.

3.7 REFERENCES

[1] Sommerset I, Krossøy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1): 89-101.

[2] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 371-9.

[3] Poppe TT, Breck O. Pathology of Atlantic salmon *Salmo salar* intraperitonealy immunized with oil-adjuvanted vaccine. A case report. Diseases of Aquatic Organisms 1997; 29: 219-26.

[4] Mutoloki S, Alexandersen S, Evensen Ø. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish and Shellfish Immunology 2004; 16: 633-44.

[5] Buchmann K, Dalsgaard I, Nielsen ME, Pedersen K, Uldal A, Garcia JA, Larsen JL. Vaccination improves survival of Baltic salmon (*Salmo salar*) smolts in delayed release sea ranching (net-pen period). Aquaculture 1997; 156: 335-48.

[6] Midtlyng PJ, Lillehaug A. Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants. Diseases of Aquatic Organisms 1998; 32: 91-7.

[7] Rønsholdt, B, McLean E. The effect of vaccination and vaccine components upon short-term growth and feed conversion efficiency in rainbow trout. Aquaculture 1999; 174: 213-21.

[8] Ackerman PA, Iwama GK, Thornton JC. Physiological and immunological effects of adjuvanted *Aeromonas salmonicida* vaccines on juvenile rainbow trout. Journal of Aquatic Animal Health 2000; 12: 157-64.

[9] Pylkkö P, Lyytikäinen T, Ritola O, Sinikka Pelkonen S. Vaccination influences growth of Arctic charr. Diseases of Aquatic Organisms 2000; 43: 77-80.

[10] Melingen GO, Wergeland HI. Physiological effects of an oil-adjuvanted vaccine on out-of-season Atlantic salmon (*Salmo salar* L.) smolt. Aquaculture 2002; 214: 397-409.

[11] Sørum U, Damsgård B. Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). Aquaculture 2004; 232: 333-41.

[12] Berg A, Rødseth OM, Tangerås A, Hansen T. Time of vaccination influences development of adhesions, growth and spinal deformities in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 2006; 69: 239-48.

[13] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[14] Skinner LA, Schulte PM, LaPatra SE, Balfry SK, McKinley RS. Growth and performance of Atlantic salmon, *Salmo salar* L., following administration of a

rhabdovirus DNA vaccine alone or concurrently with an oil-adjuvanted, polyvalent vaccine. Journal of Fish Diseases 2008; 31: 687-97.

[15] Anderson ED, Mourich DV, Fahrenkrug S, LaPatra S, Shepherd J, Leong JAC. Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 1996; 5(2): 114-22.

[16] Anderson ED, Mourich DV, Leong JAC. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 1996; 5(2): 105-13.

[17] Boudinot P, Blanco M, de Kinkelin P, Benmansour A. Combined DNA immunisation with the glycoprotein gene of viral hemorrhagic septicaemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific responses in rainbow trout. Virology 1998; 249: 297-306

[18] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish and Shellfish Immunology 1998; 8: 261-70.

[19] Heppell J, Lorenzen N, Armstrong NK, Wu T, Lorenzen E, Einer-Jensen K, Schorr J, Davis HL. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology 1998; 8: 271-86.

[20] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, Leong JC. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. Journal of Virology 2000; 75(15): 7048-54

[21] Boudinot P, Bernard D, Boubekeur S, Thoulouze MI, Bremont M, Benmansour A. The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. Journal of General Virology 2004; 85: 3099-3=108.

[22] Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish and Shellfish Immunology 2004; 17: 447-62.

[23] Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, Park LK. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Molecular Immunology 2006; 43: 2089-106.

[24] Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Köllner B, Dalmo RA, Vesely T, Ototake M, Fischer U. Cell-mediated immune response in the rainbow trout after DNA immunisation against the viral hemorrhagic septicaemia virus. Developmental and Comparative Immunology 2008; 32: 239-52.

[25] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000; 18: 2817-24.

[26] Corbeil S, Kurath G, LaPatra SE. Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routs of immunisation. Fish and Shellfish Immunology 2000; 10: 711-23

[27] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Kurath G. The dose-dependent effect on protection and humoral response to a DNA vaccine against infectious hematopoietic necrosis (IHN) virus in sub-yearling rainbow trout. Journal of Aquatic Animal Health 2000; 12: 181-8.

[28] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection – early protection correlates with Mx expression. Fish and Shellfish Immunology 2003; 15: 39-50.

[29] Lorenzen E, Lorenzen N, Einer-Jensen K, Brudeseth B, Evensen Ø. Time course study of in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) fry. Fish and Shellfish Immunology 2005; 19: 27-41

[30] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006; 24: 345-54.

[31] Kurath G. Overview of recent DNA vaccine development for fish. In: Midtlyng PG, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 201-14.

[32] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Revue Scientifique et Technique – Office International des Epizooties 2005; 24: 201-13.

[33] Kurath G, Purcell MK, Garver KA. Fish rhabdovirus models for understanding host response to DNA vaccines. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2007; 2(48): 1-12.

[34] Litwack G. Photometric determination of lysozyme activity. Proceedings for the Society for Experimental Biology and Medicine 1955; 89: 401-3.

[35] Maule AG, Schrock R, Slater C, Fitzpatrick MS, Schreck CB. Immune and endocrine responses of adult Chinook salmon during fresh-water immigration and sexual maturation. Fish and Shellfish Immunology 1996; 6: 221-33.

[36] Ackerman PA, Iwama GK. Physiological and cellular responses of juvenile rainbow trout to vibriosis. Journal of Aquatic Animal Health 2001; 13: 173-80.

[37] LaPatra SE, Turner T, Lauda KA, Jones GR, Walker S. Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. Journal of Aquatic Animal Health 1993; 5: 165-71.

[38] Fair, JM, Hansen ES, Ricklefs RE. Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). Proceedings of the Royal Society of London B 1999; 266: 1735-42.

[39] Pilorz V, Jäckel M, Knudsen K, Trillmich F. The cost of a specific immune response in young guinea pigs. Physiology and Behaviour 2005; 85: 205-11.

[40] Kumaraguru AK, Beamish FWH, Woo PTK. Impact of a pathogenic haemoflagellate, *Cryptobia salmositica Katz*, on the metabolism and swimming performance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 1995; 18: 297-305.

[41] Powell MD, Fisk D, Nowak BF. Effects of graded hypoxia on Atlantic salmon infected with amoebic gill disease. Journal of Fish Biology 2000; 57: 1047-57.

[42] Fisk DM, Powell MD, Nowak BF. The effects of amoebic gill disease and hypoxia on survival and metabolic rate of Atlantic salmon (*Salmo salar*). Bulletin of the European Association of Fish Pathologists 2002; 22: 190-4

[43] Tierney KB, Farrell AP. The relationship between fish health, metabolic rate, swimming performance and recovery in return-run sockeye salmon, *Oncorhynchus nerka* (Walbaum). Journal of Fish Diseases 2004; 27: 663-71.

[44] Wagner GN, Hinch SG, Kuchel LF, Lotto A, Jones SRM, Patterson DA, Macdonald JS, Van Der Kraak G, Shrimpton M, English KK, Larsson S, Cooke SJ, Healey MC, Farrell AP. Metabolic rates and swimming performance of adult Fraser River sockeye salmon (*Oncorhynchus nerka*) after a controlled infection with *Parvicapsula minibicornis*. Canadian Journal of Fisheries and Aquatic Sciences 2005; 62: 2124-33

[45] Powell MD, Speare DJ, Daley J, Lovy J. Differences in metabolic response to *Loma salmonae* infection in juvenile rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis*. Diseases of Aquatic Organisms 2005; 67: 233-7.

[46] Jones MA, Powell MD, Becker JA, Carter CG. Effect of an acute necrotic bacterial gill infection and feed deprivation on the metabolic rate of Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 2007; 78: 29-36.

[47] Leef MJ, Harris JO, Powell MD. Metabolic effects of amoebic gill disease (AGD) and chloramine-T exposure in seawater-acclimated Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 2007; 78: 37-44.

[48] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 2001; 19: 4011-19.

[49] Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Developmental and Comparative Immunology 2002; 26: 173-79.

[50] Acosta F, Lockhart K, Gahlawat SK, Real F, Ellis AE. Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. Fish and Shellfish Immunology 2004; 255-63.

[51] Robertsen B. Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. Fish and Shellfish Immunology 2008; 25: 351-7.

[52] Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous

virus but not against bacterial pathogens. Developmental and Comparative Immunology 2002; 26: 173-9.

[53] Haugland Ø, Torgersen J, Syed M, Evensen Ø. Expression profiles of inflammatory and immune-related genes in Atlantic salmon (*Salmo salar* L.) at early time post vaccination. Vaccine 2005; 23: 5488-99.

[54] Chiller JM, Hodgins HO, Weiser RS. Antibody response in rainbow trout (*Salmo gairdneri*) II. Studies on the kinetics of development of antibody-producing cells and on complement and natural hemolysin. Journal of Immunology 1969; 102(5): 1202-7.

[55] Van Muiswinkel WB, Wiegertjes GF. Immune Responses after injection vaccination of fish. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 55-7.

[56] Melingen GO, Pettersen EF, Wergeland HI. Leucocyte populations and responses to immunisation and photoperiod manipulation in Atlantic salmon (*Salmo salar* L.) 0 + smolt. Aquaculture 2002; 214: 381-396.

CHAPTER FOUR: CONCURRENT INJECTION OF A RHABDOVIRUS-SPECIFIC DNA VACCINE WITH A POLYVALENT, OIL-ADJUVANTED VACCINE DELAYS THE SPECIFIC ANTIVIRAL RESPONSE IN ATLANTIC SALMON, *SALMO SALAR* L.[1]

4.1 INTRODUCTION

Aquaculture is a multi-billion dollar industry [1]. While the health and quality of aquaculture species is integral to its success, an estimated ten to twenty percent of all cultured fish are lost each year due to infectious disease [2, 3]. To that end, the aquaculture industry has employed the use of a variety of efficacious vaccines targeting a multitude of bacterial and viral pathogens. Worldwide, there are vaccine formulations available for approximately half of the total number of bacterial fish pathogens, with even fewer formulations available for viral fish pathogens [4, 5]. Aside from the antigen in question, one of the key components of most modern, injectable fish vaccines is an oil-based adjuvant. Adjuvants aid a vaccine in the stimulation of the overall immune response against a given vaccine antigen, and increase the prominence of the adaptive immune response, both humoral and cell-mediated types through acceleration, enhancement and prolonged stimulation [6-9]. Unfortunately, adjuvants have been shown to cause morphological and physiological side-effects [10-14].

Recent advances in biotechnology have allowed for the development of DNA vaccines (DV) whereby a gene of interest, typically one that codes for an antigen known or hypothesized to elicit a protective immune response, is inserted into a plasmid construct, along with a strong viral promoter [15, 16]. The mechanisms of immune stimulation following the injection of DVs have been studied in depth and appear to closely resemble those of a natural viral infection, with the vaccinated individual producing a viral protein that is correctly folded and modified, and both cellular and humoral immune responses being elicited [17-24]. As such, DVs do not require the aid of an adjuvant and thus significantly reduce the possibility of vaccine-related side-effects

---

[1]A version of this chapter has been submitted for publication. Skinner LA, McKinley RS, LaPatra SE, Adams A, Thompson KD, Balfry SK, Schulte PM. Concurrent injection of a rhabdovirus-specific DNA vaccine with a polyvalent, oil-adjuvanted vaccine delays the specific antiviral response in Atlantic salmon, *Salmo salar* L.

[16]. In Canada, a DV against infectious haematopoietic necrosis virus (IHNV) is commercially available (APEX IHN®; Novartis Aqua Health, Charlottetown, PE, Canada).

As a way to minimize the need for re-vaccination and protect individual fish against the major relevant diseases they might encounter throughout the entire production cycle, many vaccines currently used in salmonid aquaculture are polyvalent, i.e. they contain multiple antigens [8, 11, 14, 25]. Although polyvalent vaccines are beneficial in many respects, the immune system of fish appears to have a defined and limited capacity to respond to multiple antigens [25]. This finite clonal capacity and limited protective immunity can lead to positive and negative interactive effects such as cross-protection between antigens, antigenic competition, and antigen immunodominance, all of which can affect the specificity, avidity, and level of production of specific antibodies [25-28]. As well, it is thought that fish, in response to an excess of specific antibody, may suppress key elements of the immune response in a manner similar to higher vertebrates, in particular phagocytosis and the activation of antibody-dependent complement [26, 29].

Although there is a substantial amount of published research regarding the immunological and physiological effects following the injection of different polyvalent vaccines [10, 25-28, 30, 31] and DVs [17, 20, 22, 23, 32-35], there are no published reports examining the physiological and immunological effects of concurrent vaccine injection, which is the situation generally encountered in aquaculture. Using key immunological parameters such as lysozyme activity and specific antibody (Ab) titres we examined the short-term activation of the immune response of cultured Atlantic salmon (*Salmo salar* L.), following concurrent injection with a traditional, polyvalent, oil-adjuvanted vaccine (AV) and an IHNV-specific DV.

4.2 MATERIALS AND METHODS

4.2.1 FISH STOCK AND REARING CONDITIONS

Juvenile Atlantic salmon (approximately 30 g each) were obtained from Big Tree Creek Hatchery (Marine Harvest Canada, Campbell River, BC, Canada) and maintained at the Department of Fisheries and Oceans - University of British Columbia Centre for Aquaculture and Environmental Research (DFO-UBC CAER). Two-hundred and forty unvaccinated individuals were placed in a single 1100 L indoor tank that continuously received well water at a constant temperature (10.6 ºC). Fish were maintained under natural photoperiod ranging from

10:14 to 13:11, light:dark over the course of the experiment. With the exception of the 24 hr period preceding tagging, vaccination and sampling protocols, fish were fed to satiation twice daily with a commercially available pellet-food (Bio-Olympic Fry®; Bio-Oregon, Vancouver, BC, Canada). Fish were acclimated to these conditions for 28 days prior to tagging and vaccination protocols.

4.2.2 VACCINATION PROCEDURE

At the time of vaccination, fish were netted and transferred to a small fresh-water filled container where they were individually anaesthetized with a non-lethal dose of aerated tricaine methane sulphonate (MS222; Syndell Laboratories, Vancouver, BC, Canada). Sodium bicarbonate ($NaHCO_3$; Sigma Aldrich, Oakville, ON, Canada) was added to the MS222 in a 1:2 ratio (100 mg L^{-1} MS222 to 200 mg L^{-1} $NaHCO_3$) as a buffering agent. Fish were randomly divided into one of four vaccine groups (60 fish per group) and injected accordingly. Fish from the control group were injected with 50 µL of 0.02 M phosphate-buffered saline (PBS) intramuscularly (IM), immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle) and 100 µL of PBS intraperitonealy (IP), one fin length ahead of the pelvic fins, along the midline of the fish. Fish from the adjuvant vaccine (AV) group were injected with 50 µL of PBS IM and 100 µL IP of a commercially available, polyvalent, oil-adjuvanted vaccine containing formalin inactivated, whole-cell bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* serotype O1 and O2, *Vibrio ordalii*, and *Vibrio salmonicida* (Lipogen Forte®; Novartis Aqua Health). Fish from the DNA vaccine (DV) group were injected with 50 µL IM of a rhabdovirus DNA vaccine containing 10 µg of plasmid encoding the glycoprotein (G) gene from an endemic strain of IHN virus (APEX IHN®) and 100 µL of PBS IP. Fish from the combined vaccine group were injected with 50 µL of the DV IM and 100 µL of the oil-AV IP. Our vaccination protocol, including doses and timing of injection, was carried out as suggested by the vaccine manufacturer.

Concurrent with the vaccination procedure, all fish were tagged with alphanumeric visible implant (VI alpha) tags (Northwest Marine Technology, Shaw Island, WA, USA) for individual identification and visible implant elastomer (VIE) tags (Northwest Marine Technology) for vaccine group identification. At completion of the vaccination and tagging procedures, all fish were returned to the 1100 L holding tank and allowed to recover from anaesthesia in well aerated fresh-water.

4.2.3 BLOOD SAMPLING

At 106, 201, 296, and 413 degree days (dd) post-vaccine injection (pvi), 7 – 10 randomly selected fish per vaccine group were sampled for blood. All samples were considered lethal and fish were not returned to the holding tank. On the day of sampling, individual fish were netted and transferred to a small fresh water filled container where they were anaesthetized with a lethal dose of MS222 buffered with sodium bicarbonate (500 mg L^{-1} MS222 to 1000 mg L^{-1} $NaHCO_3$). Following weight (wt) measurements (to the nearest 0.1 g), blood samples were drawn from caudal venepuncture using a non-heparinised syringe. Whole blood was placed at 4 ºC for 4 h after which it was separated into serum and red cell components by centrifugation (10 min at 4 ºC, 1600 x *g*). Serum was collected and stored at -80 ºC until analysed for serum lysozyme activity, IHNV neutralizing antibody (NAb) titres, and Ab titres against *Aeromonas salmonicid*a and *Listonella anguillarum* serotype O1.

4.2.4 SERUM LYSOZYME ACTIVITY

Serum lysozyme activity was determined by a microplate modification of the method of Litwack [36-38]. Briefly, 10 µL of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250 µL of a 0.025% w/v suspension of *Micrococcus lysodeikticus* in 0.06 M phosphate buffer (pH 6.2). The average decrease in optical density at 450 nm (OD_{450}) over a 20 min period at 25 ˚C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity (µg mL^{-1} HEWL eq), which was used as the standard.

4.2.5 IHNV SERUM NEUTRALIZING ANTIBODY TITRE

IHNV serum neutralizing antibody titres were determined using a complement dependent 50% plaque neutralization titre (50% PNT) assay, as described previously by LaPatra *et al*. [39]. Briefly, serum samples were heat-inactivated for 30 min at 45 ˚C to destroy all residual complement, and a two-fold dilution series made. As a source of complement, serum was obtained from pathogen-free rainbow trout (*Oncorhynchus mykiss*, Walbaum) that had not been fed for at least two weeks. Equal volumes of this complement source (1:10 dilution) and a diluted IHN virus suspension (2000 pfu mL^{-1}) were added to each serum dilution series. Samples were plaque assayed on *Epithelioma papulosum cyprini* monolayers and NAb titres were reported as the reciprocal of the highest serum dilution that resulted in a 50 % reduction in the

average number of plaques detected in negative controls. A sample was considered to be positive with a titre of 20 or above, while a titre of < 20 was considered negative [35, 39].

4.2.6 Enzyme Linked Immunosorbent Assay Antibody Titre

An enzyme-linked immunosorbent assay (ELISA) was used to measure the specific Ab response of Atlantic salmon sera against heat-killed, whole cells of *Aeromonas salmonicida* and *Listonella anguillarum* serotype O1. Antibody titres were determined using a modification of the method previously outlined by Adams *et al*. [41] and as suggested by the monoclonal antibody (MAb) manufacturer (Aquatic Diagnostics, Stirling, Scotland). Unless stated otherwise, all chemicals were purchased from Sigma Aldrich. Briefly, 96-well microtitre plates (Immulon 4HBX; ThermoFisher Scientific, Nepean, ON, Canada) were coated with 0.05 % w/v poly-L-lysine in a carbonate-bicarbonate buffer and allowed to incubate for 60 min at 21 °C. Plates were then washed twice with a low salt wash buffer (LSW; 0.02 M Tris, 0.38 M NaCl, 0.05 % Tween 20). Heat killed bacteria (*Aeromonas salmonicida* or *Listonella anguillarum* serotype O1) were added to each well (100 µL well^{-1}) and plates were incubated overnight at 4 ºC. Virulent strains of *Aeromonas salmonicida* (strain # 2004-118) and *Listonella anguillarum* serotype O1 (strain # 2004-124) were graciously donated by Dr. SR Jones (DFO Pacific Biological Station, Nanaimo, BC, Canada). The *Aeromonas salmonicida* isolate was cultured at 22 ºC for 72 h in tryptic soy broth (TSB), while *Listonella anguillarum* serotype O1 was cultured at 25 ºC for 24 h in TSB with the addition of 2 % sodium chloride (NaCl). Bacteria were washed three times with PBS (8 min at 5000 x *g*) and the bacterial concentration was adjusted to an approximate absorbance of OD_{610} = 1.0. A 0.05 % v/v solution of glutaraldehyde in PBS was added to the bacteria and plates were incubated at 21 °C for 20 min before washing three times with LSW. Non-specific binding sites were blocked by incubating plates with 3 % w/v skimmed milk powder in water (Safeway Foods, Calgary, AB, Canada) at 21 °C for 120 min. After washing plates three times with LSW, 100 µL of serially diluted fish serum (from 1:40 to 1:5122 in doubling dilutions in 3% w/v skimmed milk) was added to each well and allowed to incubate overnight at 4 ºC. Fish serum was diluted in 3 % w/v skimmed milk to further block non-specific binding and decrease the high background OD often observed in fish immunoglobulin detection ELISA [42]. Plates were washed five times with high salt wash buffer (HSW; 0.02 M Tris, 0.5 M NaCl, 0.1 % Tween 20) with a five minute soak on the last wash to remove unbound antibodies. 100 µL well^{-1} anti-rainbow trout/Atlantic salmon MAb (Aquatic Diagnostics Ltd) was added and plates were

incubated at 21 °C for 60 minutes. Following the subsequent washing of the plates with HSW as previously described, goat anti-mouse immunoglobulin-G labelled with horseradish peroxidise, diluted 1:1000 in conjugate buffer [1% w/v bovine serum albumin (BSA) in LSW] was added to the wells and incubated for 60 min at 21 °C. Plates were once again washed with HSW as previously described. The assay was developed with 100 µL $well^{-1}$ of chromogen in substrate buffer [150 µL chromogen (42 mM 3,3'5,5'-Tetramethylbenzidine hydrate dihydrochloride) in 2 M acetic acid to 15 mL of substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4 containing 0.33 % v/v H_2O_2)]. Following a 10 min incubation at 21 °C, the reaction was terminated with the addition of 50 µL $well^{-1}$ of 2 M H_2SO_4 and the absorbance was measured at OD_{450}. The ELISA Ab titre was defined as the reciprocal of the highest dilution showing an OD_{450} at least three times greater than the negative control.

Both positive and negative controls were added to each plate. For the determination of anti-*Aeromonas salmonicida* Ab titres, positive controls consisted of serially diluted Atlantic salmon anti-sera and negative controls were normal, unvaccinated Atlantic salmon sera. For the determination of anti-*Listonella anguillarum* serotype O1 Ab titres, positive controls consisted of serially diluted rabbit anti-sera and negative controls were normal rabbit sera. All positive and negative controls were graciously donated by Dr. RJF Markham (University of Prince Edward Island Atlantic Veterinary College, Charlottetown, PE, Canada). The anti-rainbow trout/Atlantic salmon MAb was not added to those wells that contained the rabbit sera. In its place, 100 µL $well^{-1}$ antibody buffer (1% BSA in PBS) was added. Following the HSW procedure, goat anti-rabbit immunoglobulin-G labelled with horseradish peroxidise, diluted 1:1000 in conjugate buffer was added to these wells. The remainder of the protocol was carried out as above.

4.2.7 Statistical Analysis

Data are presented as means with standard error of the means (± SE). Following a normality test, data were analyzed using a two-way analysis of variance (ANOVA) with vaccine group and time post-vaccine injection as factors. If a significant difference between groups was detected ($P < 0.05$) the Student-Newman-Keuls method of pairwise multiple comparisons was utilized to identify groups that differed significantly ($P < 0.05$). Neutralizing antibody titres were analyzed using a Mann-Whitney rank sum test due to non-normality of the data. Differences were considered significant if $P < 0.05$. All data were analyzed using Sigmastat software (version 3.5; Systat Software Inc., San Jose, CA, USA).

4.3 RESULTS

4.3.1 SERUM LYSOZYME ACTIVITY

Lysozyme activity was present in all fish at 106 dd pvi, with the combined vaccine group having significantly greater levels of lysozyme activity than the control group, and levels of lysozyme activity in the AV and DV groups intermediate (Figure 4.1A). At 201 dd pvi, the lysozyme activity of the DV group decreased somewhat and was similar to that of the control group (Figure 4.1B). Lysozyme activity remained elevated for both the AV and combined vaccine groups, with the combined vaccine group once again showing a significantly higher level of lysozyme activity than the control group (Figure 4.1B). At 297 dd pvi, the AV and combined vaccine groups both had lysozyme activity that was significantly elevated compared to the control group, while the lysozyme activity of the DV group was not different from that of the control or the AV group (Figure 4.1C). By 413 dd pvi, there were no significant differences in lysozyme activity between any of the vaccine groups (Figure 4.1D).

4.3.2 IHNV SERUM NEUTRALIZING ANTIBODY TITRE

As NAb titres are specific to the proteins produced in response to the IHNV DNA vaccine, only those fish that received the DV produced measurable amounts of NAb. Sera from all time points were tested, however NAb were not detected in any vaccine group until 413 dd pvi (Table 4.1). At this time, fish from the DV group had significantly greater neutralizing activity than fish from the combined vaccine group, where only one out of eight fish tested showed any significant NAb activity.

4.3.3 ENZYME LINKED IMMUNOSORBENT ASSAY ANTIBODY TITRE

4.3.3.1 ANTI-*AEROMONAS SALMONICIDA* ANTIBODY TITRE

At 106 dd pvi, all vaccine groups had similar low levels of anti-*Aeromonas salmonicida* Ab present (Figure 4.2A). By 201 dd pvi, the level of Ab titre in the AV and combined vaccine groups increased significantly such that the values were greater than the control and DV groups (Figure 4.2B). At 297 dd pvi, the Ab titre of the combined vaccine group was significantly greater than all other vaccine groups, including the AV group, whose Ab titre decreased slightly from that observed at 201 dd pvi (Figure 4.2C). There were no differences in Ab titre for the

control and DV groups. At 413 dd pvi, the Ab titre of the AV group increased such that there were no significant differences when compared to the combined vaccine group, with both groups having significantly higher levels of Ab compared to the control and DV groups (Figure 4.2D). As with all previous sampling times, control and DV groups maintained baseline levels of Ab.

4.3.3.2 ANTI-*LISTONELLA ANGUILLARUM* ANTIBODY TITRE

At 106 dd pvi, the control, AV, and DV groups all had similar low levels of anti-*Listonella anguillarum* serotype O1 Ab while the combined vaccine group showed significantly elevated levels of Ab (Figure 4.3A). By 201 dd pvi, the Ab titre of the AV group increased significantly and was similar to that of the combined vaccine group (Figure 4.3B). Ab titres of the control and DV groups were not different and remained at baseline levels. By 297 dd pvi, the anti-*Listonella anguillarum* Ab level of the AV and combined vaccine groups decreased and were similar to values for the control and DV groups (Figure 4.3C). For the remainder of the experiment, all vaccine groups had similar low levels of anti-*Listonella anguillarum* Ab present (Figure 4.3D).

4.4 DISCUSSION

In this study we examined the effects of concurrent vaccine injection on parameters of the innate and adaptive immune responses in cultured Atlantic salmon. More specifically, we were interested in the immunological impact of concurrent injection of a polyvalent, oil-AV with a novel IHNV-specific DV. Our results indicate that different aspects of the innate and adaptive immune responses are influenced in either a positive or negative manner. While concurrent vaccine injection elicited an almost synergistic-like effect on lysozyme activity, changes in Ab titre were antigen specific. The production of anti-*Aeromonas salmonicida* Abs was significantly greater in the combined vaccine group at 296 dd pvi, while the production of anti-*Listonella anguillarum* serotype O1 Abs was significantly greater at 106 dd pvi in the combined vaccine group. Interestingly, the production of IHNV-specific NAbs was delayed when the DV was injected concurrently with the polyvalent oil-AV.

The innate immune response is often thought of as the first line of defense in vertebrates, preventing the attachment, invasion, or multiplication of infectious pathogens on or in the tissues [44]. One key aspect of the innate immune response is that of lysozyme activity. Lysozyme is present in the serum and mucus of fish, as well as tissues rich in leucocytes such as monocytes,

macrophages, and polymorphonuclear granulocytes [44-49]. Lysozyme is known to be an opsonin and plays a key role in the inflammatory response through activation of the complement system and phagocytosis [46, 49]. During the inflammation process, macrophages and polymorphonuclear granulocytes engulf and destroy suspected pathogens partially through the actions of lysozyme [43, 46, 49, 50]. When an oil-AV is injected into a fish, the adjuvant forms a depot of antigen at the site of inoculation, thereby prolonging stimulation of the inflammatory response [6, 7].

In the current study, a polyvalent oil-AV protecting against five different Gram-negative bacteria was injected into cultured Atlantic salmon. At 106, 201, and 297 dd pvi, the level of lysozyme activity in the combined vaccine group was significantly greater than that of the control group. Although we did not measure innate antiviral parameters in this study, it is well known that levels of Mx protein, an interferon-like (IFN) molecule that stimulates inflammation and inhibits intracellular viral replication [54], increase very early on following the injection of an IHNV-specific DV [17, 20, 51-55]. Data also suggest that the injection of *Listonella anguillarum* antigens into Atlantic salmon induce Mx protein production and the IFN response [56]. It is possible therefore, that the significant increase in lysozyme activity observed in the combined vaccine group is associated with an increase in the inflammatory response brought about as a direct result of the DV and the *Listonella anguillarum* antigens present in the oil-AV. Future studies are needed to fully describe and understand the relationship between DV and *Listonella anguillarum* induced Mx protein levels and the inflammatory response of concurrently vaccinated salmonids.

In the current study, fish injected with a polyvalent, oil-AV vaccine alone and concurrently with a DV showed low levels of anti-*Aeromonas salmonicida* and anti-*Listonella anguillarum* serotype O1 Abs at 106 dd pvi (Figures 4.2A and 4.3A). This observation is surprising as antibody production is temperature dependent and in Atlantic salmon held within their thermoneutral range (10 – 12 ºC), specific antibodies are typically produced between 200 and 300 dd pvi, depending on the antigen/adjuvant combination [4, 57-59]. Our data can be explained through the presence of natural antibodies. Natural antibodies are present even in immunologically naïve fish [60, 61]. While little is known about these antibodies, they can be antigen-specific and are thought to arise either as a result of adoptive transfer from mother to embryo, are developed in the host following exposure to environmental antigens, or are germline-encoded products [61, 62].

The combined vaccine group displayed significant increases in anti-*Listonella anguillarum* serotype O1 Ab titre at 106 dd pvi and in anti-*Aeromonas salmonicida* Ab titre at 297 dd pvi. Both observations could be a result of antigenic interactions such as cross reactions between antigens present in the polyvalent oil-AV. The AV used in this study contains formalin-inactivated cells (i.e. bacterins) of five different antigens, each of which is known to cross react in a synergistic or adjuvant-like manner. For example, *Vibrio salmonicida* antigens, especially the lipopolysaccharide of the cell wall, cross react with *Aeromonas salmonicida* antigens significantly enhancing the production of anti-*Aeromonas salmonicida* Abs [10, 27, 28, 64]. It is also known that there is a serological cross reaction between *Listonella anguillarum* serotype O1 and O2, as well as a cross reaction between *Listonella anguillarum* serotype O2 and *Vibrio ordalii* antigens [5, 40, 65]. Thus, it is highly plausible that the significant increases in anti-*Listonella anguillarum* Abs observed at 106 dd pvi and in anti-*Aeromonas salmonicida* Abs observed at 297 dd pvi were due to antigenic cross reactions. The significantly higher levels of anti-*Aeromonas salmonicida* Ab titres compared to anti-*Listonella anguillarum* serotype O1 Ab titres can also be explained by the above cross reactions. Unfortunately, because we were unable to measure the anti-*Listonella anguillarum* serotype O2, anti-*Vibrio salmonicida*, and anti-*Vibrio ordalii* Ab responses due to the limited volume of serum that was available, we are unable to determine the source of the cross reaction.

There are currently no published reports examining the immunological interactions between a polyvalent, bacterin-based vaccine and a DV. While we have shown that there are positive interactions between the polyvalent, oil-AV and the DV with respect to specific Ab production, our data indicate that the seroconversion of IHNV-specific NAb is negatively affected. Fish that received concurrent injection of both the polyvalent oil-AV and the DV did not show the same level of NAb as those that received the DV alone. Individuals in the combined vaccine group appeared to be unable to seroconvert the IHNV-specific NAb within the same time-frame as the DV group. This is in contrast to an earlier study involving rainbow trout where individuals in the combined vaccine group were able to seroconvert the NAb at an earlier time point than those that received the DV alone [Chapter 3]. According to Mutoloki *et al.* [9] rainbow trout and Atlantic salmon, while similar in a variety of physiological traits, respond immunologically to antigens and adjuvants in a very different manner. Rainbow trout typically respond with a rapid onset of the inflammatory response, whereas Atlantic salmon produce a slower and more persistent response [9]. These differences could impact the manner in which multiple antigens are handled in concurrently injected fish.

It is important to remember that Ab titre is not always correlated with protection and can vary with vaccine formulation, species, and environment [66-70]. While our data do show significant differences in both anti-bacterial Abs and antiviral NAbs, we did not measure relative percent survival following vaccine injection and therefore cannot predict if our observed differences in Ab titre between vaccine groups correlate to differences in protective value for the fish.

Vaccines are commonly used in salmonid aquaculture as a method of disease prevention. Due to unpredictable interactive effects of an antigen(s) and adjuvant(s), it is impossible to formulate a vaccine that encompasses all infectious pathogens an individual fish may encounter. Farm managers must decide which infectious pathogens are important, and vaccinate their stock accordingly. Unfortunately, it is equally impossible to predict the immunological impact of concurrent vaccine injection. The results of these studies indicate that concurrent injection of a polyvalent oil-AV and a DV can be beneficial to the production of specific antibodies; however the specific antiviral response may be delayed.

4.5 TABLES

Table 4.1 Neutralizing antibody (NAb) titres of individual Atlantic salmon are determined by plaque assay and are reported as the reciprocal of the highest dilution that resulted in a 50 % reduction in the average number of plaques detected in the negative control wells. Samples were considered positive with a titre of 20 or above, while samples with a titre of < 20 were considered negative. Although sera from all time points were tested, NAb were not detected until 413 degree days post-vaccine injection.

Fish #	**413 degree days post-vaccine injection**	
	DNA vaccine group*	Combined vaccine group
1	20	40
2	40	< 20
3	40	< 20
4	40	< 20
5	80	< 20
6	≥ 160	< 20
7	< 20	< 20
8	< 20	< 20

*Significant difference between vaccine groups; Mann-Whitney rank sum test, $P < 0.05$

4.6 FIGURES

Figure 4.1 Serum lysozyme activity of Atlantic salmon following injection of phosphate-buffered saline (control group;), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;), a DNA vaccine (DNA vaccine group;), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;). Fish were sampled at A) 106, B) 201, C) 297, and D) 413 degree days post-vaccine injection. [a, b, c, d] Significant differences between vaccine groups. [w, x, y, z] Significant differences between sampling periods within a vaccine group. Values are mean ± SE. (n = 7-10); Two-way ANOVA, $P < 0.05$.

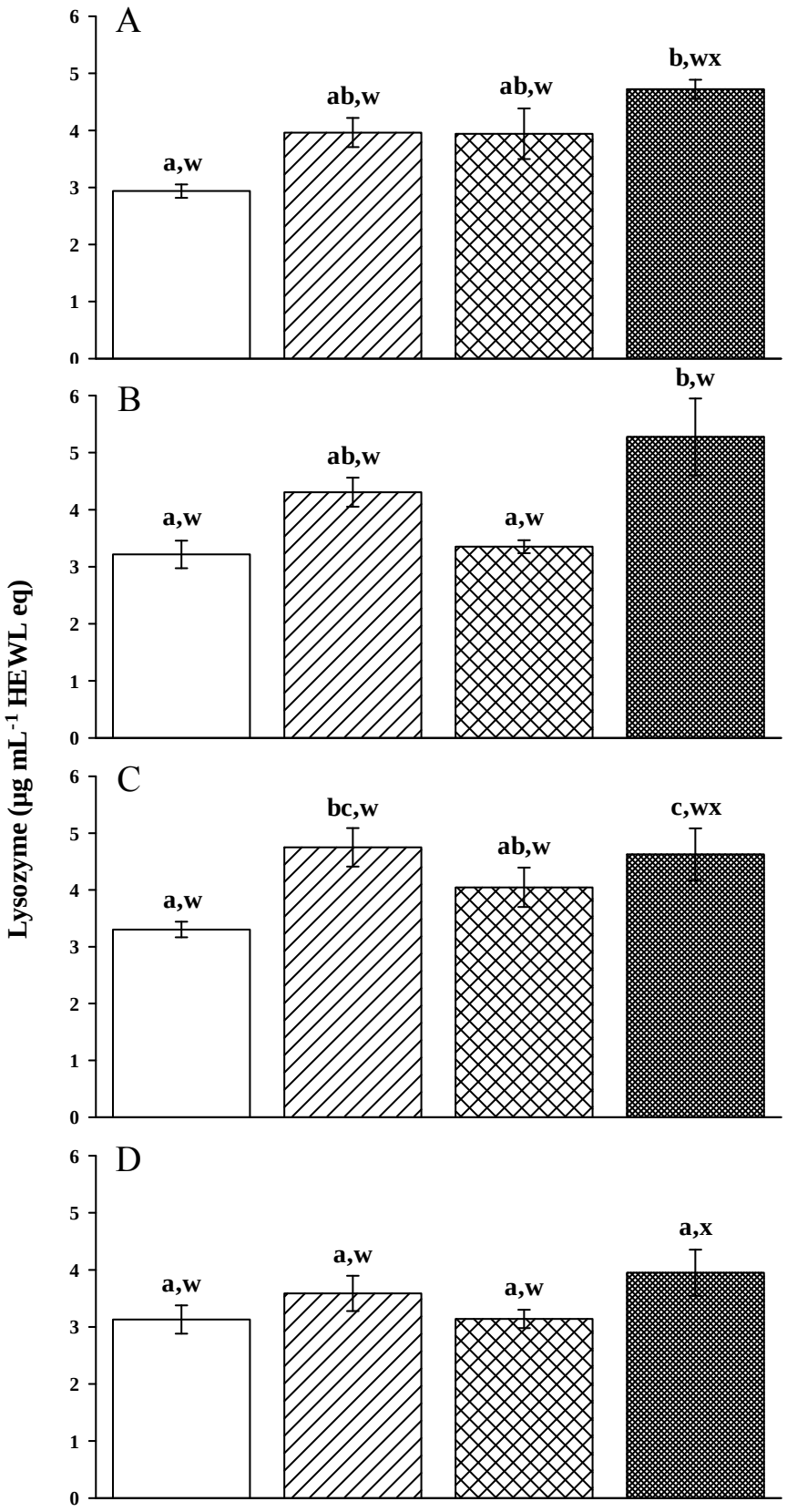

A
a,w
ab,w
ab,w
b,wx
B
a,w
ab,w
a,w
b,w
C
a,w
bc,w
ab,w
c,wx
D
a,w
a,w
a,w
a,x
Lysozyme (µg mL-1 HEWL eq)
0
1
2
3
4
5
6

Figure 4.2 Anti-*Aeromonas salmonicida* antibody (Ab) titres of Atlantic salmon following injection of phosphate-buffered saline (control group;), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;), a DNA vaccine (DNA vaccine group;), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;). Ab titres were determined by an enzyme-linked immunosorbent assay and the Ab titre is reported as the reciprocal of the highest dilution showing an optical density (OD_{450}) at least three times greater than the negative control. Fish were sampled at A) 106, B) 201, C) 297, and D) 413 degree days post-vaccine injection. [a, b, c, d] Significant differences between vaccine groups. [w, x, y, z] Significant differences between sampling periods within a vaccine group. Values are mean ± SE. (n = 7-10); Two-way ANOVA, $P < 0.05$.

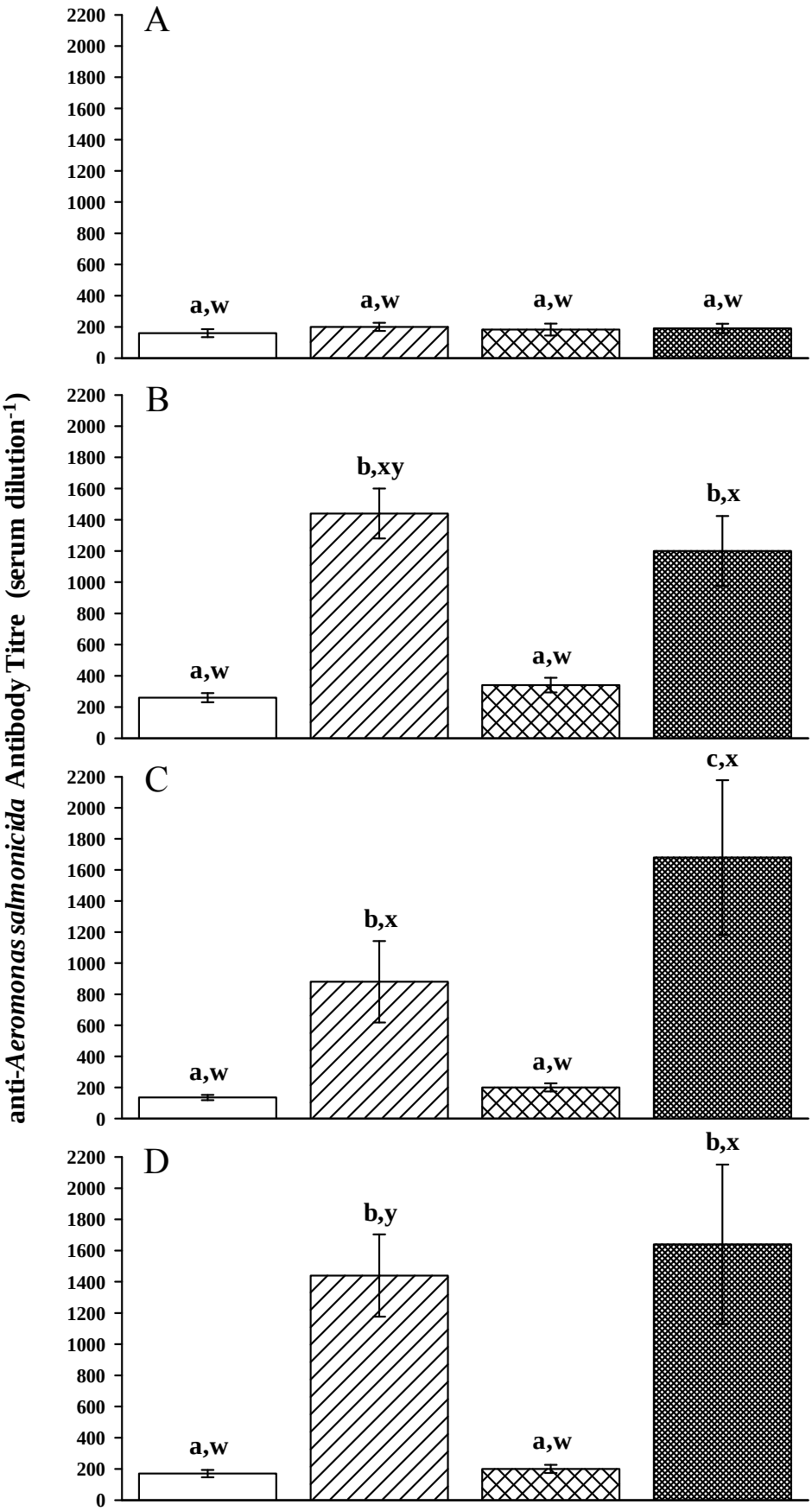
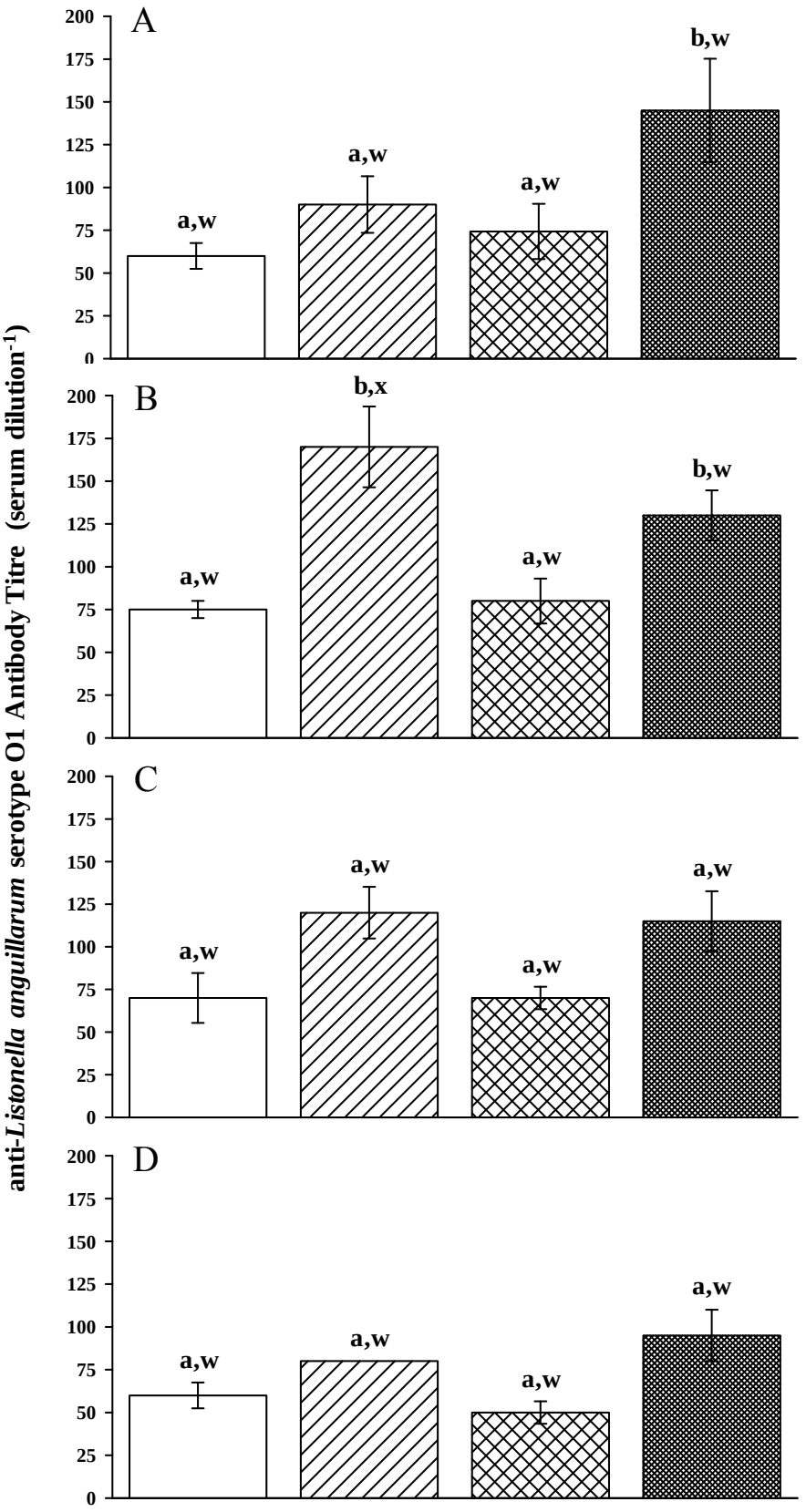

A
B
C
D
anti-Aeromonas salmonicida Antibody Titre (serum dilution-1)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a,w
a,w
a,w
a,w
a,w
b,xy
a,w
b,x
a,w
b,x
c,x
a,w
b,y
a,w
b,x

Figure 4.3 Anti-*Listonella anguillarum* serotype O1 antibody (Ab) titres of Atlantic salmon following injection of phosphate-buffered saline (control group;), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group;), a DNA vaccine (DNA vaccine group;), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group;). Ab titres were determined by enzyme-linked immunosorbent assays and the Ab titre is reported as the reciprocal of the highest dilution showing an optical density (OD_{450}) at least three times greater than the negative control. Fish were sampled at A) 106, B) 201, C) 297, and D) 413 degree days post-vaccine injection. [a, b, c, d] Significant differences between vaccine groups. [w, x, y, z] Significant differences between sampling periods within a vaccine group. Values are mean ± SE. (n = 7-10); Two-way ANOVA, $P < 0.05$.


anti-Listonella anguillarum serotype O1 Antibody Titre (serum dilution-1)
A
B
C
D
200
175
150
125
100
75
50
25
0
a,w
b,w
b,x

4.7 REFERENCES

[1] FAO: The state of world aquaculture (2006). Food and Agriculture Organisation of the United Nations, Rome.

[2] Leong JC, Fryer FL. Viral vaccines for aquaculture. Annual Review of Fish Diseases 1993; 3: 225-40.

[3] Thorarinsson R, Powell DB. Effects of disease risk, vaccine efficacy, and market price on the economics of fish vaccination. Aquaculture 2006; 256: 42-9.

[4] Sommerset I, Krossøy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1): 89-101.

[5] Austin B, Austin DA. Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. 4th ed. Chichester, UK: Praxis Publishing Ltd.; 2007.

[6] Anderson DP. Adjuvants and immunostimulants for enhancing vaccine potency in fish. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 257-65.

[7] Evensen Ø, Brudeseth B, Mutoloki S. The vaccine formulation and its role in inflammatory processes in fish – Effects and adverse effects. In: Midtlyng PG, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 117-26.

[8] Schijns VEJC, Tangerås A. Vaccine adjuvant technology: From theoretical mechanisms to practical approaches. In: Midtlyng PG, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 127-34.

[9] Mutoloki S, Reite OB, Brudeseth B, Tverdal A, Evensen Ø. A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. Vaccine 2006; 24: 578-88.

[10] Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 335-50.

[11] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 371-9.

[12] Poppe TT, Breck O. Pathology of Atlantic salmon *Salmo salar* intraperitonealy immunized with oil-adjuvanted vaccine. A case report. Diseases of Aquatic Organisms 1997; 29: 219-26.

[13] Berg A, Rødseth T, Tangerås A, Hansen TJ. Time of fish vaccination influences development of adherences, growth and spinal deformities in Atlantic salmon (*Salmo salar* L). Diseases of Aquatic Organisms 2006; 69: 239-48.

[14] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[15] Anderson ED, Mourich DV, Fahrenkrug S, LaPatra S, Shepherd J, Leong JAC. Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 1996; 5(2): 114-22.

[16] Anderson ED, Mourich DV, Leong JAC. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 1996; 5(2): 105-13.

[17] Boudinot P, Blanco M, de Kinkelin P, Benmansour A. Combined DNA immunisation with the glycoprotein gene of viral hemorrhagic septicaemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific responses in rainbow trout. Virology 1998; 249: 297-306

[18] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish and Shellfish Immunology 1998; 8: 261-70.

[19] Heppell J, Lorenzen N, Armstrong NK, Wu T, Lorenzen E, Einer-Jensen K, Schorr J, Davis HL. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology 1998; 8: 271-86.

[20] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, Leong JC. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. Journal of Virology 2000; 75(15): 7048-54.

[21] Boudinot P, Bernard D, Boubekeur S, Thoulouze MI, Bremont M, Benmansour A. The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. Journal of General Virology 2004; 85: 3099-108.

[22] Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish and Shellfish Immunology 2004; 17: 447-62.

[23] Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, Park LK. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Molecular Immunology 2006; 43: 2089-106.

[24] Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Köllner B, Dalmo RA, Vesely T, Ototake M, Fischer U. Cell-mediated immune response in the rainbow trout after DNA immunisation against the viral hemorrhagic septicaemia virus. Developmental and Comparative Immunology 2008; 32: 239-52.

[25] Busch RA. Polyvalent vaccines in fish: the interactive effects of multiple antigens. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 245-56.

[26] Nikoskelainen S, Verho S, Järvinen S, Madetoja J, Wiklund T, Lilius EM. Multiple whole bacterial antigens in polyvalent vaccine may result in inhibition of specific responses in rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology 2007; 22: 206-17.

[27] Hoel K, Salonius K, Lillehaug A. *Vibrio* antigens of polyvalent vaccines enhance the humoral immune response to *Aeromonas salmonicida* antigens in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1997; 7: 71-80.

[28] Hoel K, Reitan LJ, Lillehaug A. Immunological cross reactions between *Aeromonas salmonicida* and *Vibrio salmonicida* in Atlantic salmon (*Salmo salar* L.) and rabbit. Fish and Shellfish Immunology 1998; 8: 171-82.

[29] Taborda CP, Rivera J, Zaragoza O, Casadevall A. More is not necessarily better: Prozone-like effects in passive immunisation with IgG. The Journal of Immunology 2003; 170: 3621-30.

[30] Amend DF, Johnson KA. Evidence for lack of antigenic competition among various combinations of *Vibrio anguillarum*, *Yersinia ruckeri*, *Aeromonas salmonicida* and *Renibacterium salmnoinarum* bacterins when administered to salmonid fish. Journal of Fish Immunology 1984; 7: 293-9.

[31] Rørstad G, Aasjord PM, Robertson B. Adjuvant effect of a yeast glucans in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1993; 3: 179-90.

[32] Corbeil S, LaPatra SE, Anderson ED, Kurath G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. Vaccine 2000; 18: 2817-24.

[33] Corbeil S, Kurath G, LaPatra SE. Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routs of immunisation. Fish and Shellfish Immunology 2000; 10: 711-23.

[34] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Kurath G. The dose-dependent effect on protection and humoral response to a DNA vaccine against infectious hematopoietic necrosis (IHN) virus in sub-yearling rainbow trout. Journal of Aquatic Animal Health 2000; 12: 181-8.

[35] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006; 24: 345-54.

[36] Litwack G. Photometric determination of lysozyme activity. Proceedings for the Society for Experimental Biology and Medicine 1955; 89: 401-3.

[37] Maule AG, Schrock R, Slater C, Fitzpatrick MS, Schreck CB. Immune and endocrine responses of adult Chinook salmon during fresh-water immigration and sexual maturation. Fish and Shellfish Immunology 1996; 6: 221-33.

[38] Ackerman PA, Iwama GK. Physiological and cellular responses of juvenile rainbow trout to vibriosis. Journal of Aquatic Animal Health 2001; 13: 173-80.

[39] LaPatra SE, Turner T, Lauda KA, Jones GR, Walker S. Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. Journal of Aquatic Animal Health 1993; 5: 165-71.

[40] Toranzo AE, Magariños B, Romalde JL. A review of the main bacterial fish diseases in mariculture systems. Aquaculture; 2005: 37-61.

[41] Adams A, Thompson KD, Morris D, Farias C, Chen SC. Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. Fish and Shellfish Immunology 1995; 5: 537-47.

[42] Kim WS, Nishizawa T, Yoshimizu M. Non-specific adsorption of fish immunoglobulin (IgM) to blocking reagents on ELISA plates. Diseases of Aquatic Organisms 2007; 78: 55-9.

[43] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology 2001; 25: 827-39.

[44] Yano T. The non-specific immune system: humoral defense. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 106-59.

[45] Grinde B. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. Journal of Fish Diseases 1989; 12: 95-104.

[46] Ellis AE. Immunity to bacteria in fish. Fish & Shellfish Immunology 1999; 9: 291-308.

[47] Lie Ø, Evensen Ø, Sørensen A, Frøysadal E. Study on lysozyme activity in some fish species. Diseases of Aquatic Organisms 1989; 6: 1-5.

[48] Alcorn SW, Murray AL, Pascho RJ. Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). Fish and Shellfish Immunology 2002; 12: 303-34.

[49] Magnadóttir B. Innate immunity of fish (overview). Fish and Shellfish Immunology 2006; 20: 137-51.

[50] Secombes C. The non-specific immune system: cellular defenses. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment, San Diego, USA: Academic Press; 1996, p. 63-105.

[51] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection – early protection correlates with Mx expression. Fish and Shellfish Immunology 2003; 15: 39-50.

[52] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 2001; 19: 4011-19.

[53] Robertsen B. Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. Fish and Shellfish Immunology 2008; 25: 351-7.

[54] Trobridge GD, Chiou PP, Kim CH, Leong JC. Induction of the Mx protein of rainbow trout *Oncorhynchus mykiss in vitro* and *in vivo* with poly I:C dsRNA and infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms 1997; 30: 91-8.

[55] Robertsen B, Trobridge G, Leong JC. Molecular cloning of double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar* L.). Developmental and Comparative Immunology 1997; 21(5): 397-412.

[56] Acosta F, Lockhart K, Gahlawat SK, Real F, Ellis AE. Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. Fish and Shellfish Immunology 2004; 17: 255-63.

[57] Chiller JM, Hodgins HO, Weiser RS. Antibody response in rainbow trout (*Salmo gairdneri*) II. Studies on the kinetics of development of antibody-producing cells and on complement and natural haemolysin. Journal of Immunology 1969; 102(5): 1202-7.

[58] Van Muiswinkel WB, Wiegertjes GF. Immune responses after injection vaccination of fish. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997; vol. 90, p. 55-7.

[59] Marsden MJ, Secombes CJ. The influence of vaccine preparations on the induction of antigen specific responsiveness in rainbow trout *Oncorhynchus mykiss*. Fish and Shellfish Immunology 1997; 7: 455-69.

[60] Magor BG, Magor KE. Evolution of effectors and receptors of innate immunity. Developmental and Comparative Immunology 2001; 25: 651-82.

[61] Sinyakov MS, Dror M, Zhevelev H, Margel S, Avtalion RR. Natural antibodies and their significance in active immunisation and protection against a defined pathogen in fish. Vaccine 2002; 20: 3668-74.

[62] Mor A, Avtalion RR. Transfer of antibody activity from immunized mother to embryo in tilapias. Journal of fish Biology 1990; 37: 249-55.

[63] Midtlyng PJ, Reitan, and L. Speilberg. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 335-50

[64] Lund V, Børdal S, Kjellsen O, Mikkelsen H, Schrøder MB. Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Aeromonas salmonicida* and *Vibrio anguillarum*. Developmental and Comparative Immunology 2006; 30: 1145-55.

[65] Muthiara LW, Raymond BT, Dekievit TR, Stevenson RHW. Antibody specificities of polyclonal rabbit and rainbow trout antisera against *Vibrio ordalii* and serotype O2 strains of *Vibrio anguillarum*. Canadian Journal of Microbiology 1993; 39: 492-9.

[66] Lund T, Chiayvareesajja J, Larsen HSJ, Røed KH. Antibody response after immunisation as a potential indirect marker for improved resistance against furunculosis. Fish and Shellfish Immunology 1995; 5: 109-19.

[67] Fjalestad KT, Jørgen H, Larsen S, Røed KH. Antibody response in Atlantic salmon (*Salmo salar*) against *Vibrio anguillarum* and *Vibrio salmonicida* O-antigens:

Heritabilities, genetic correlations and correlations with survival. Aquaculture 1996; 145: 77-89.

[68] Gudmundsdóttir BK, Magnadóttir B. Protection of Atlantic salmon (*Salmo salar* L) against an experimental infection of *Aeromonas salmonicida* ssp. *Achromogenes*. Fish and Shellfish Immunology 1997; 7: 55-69.

[69] Gudmundsdóttir BK, Jónsdóttir H, Steinthórsdóttir V, Magnadóttir B, Gudmundsdóttir S. Survival and humoral antibody response of Atlantic salmon, *Salmo salar* L., vaccinated against *Aeromonas salmonicida* ssp. *Achromogenes*. Journal of Fish Diseases 1997; 20: 351-60.

[70] Hedrick RP. Relationships of host, pathogen, and environment: Implications for diseases of cultured and wild fish populations. Journal of Aquatic Animal Health 1998; 10: 107-11.

CHAPTER FIVE: CORTISOL SUPPRESSES LYSOZYME ACTIVITY BUT NOT THE ANTIBODY RESPONSE IN ATLANTIC SALMON, *SALMO SALAR* L., FOLLOWING VACCINE INJECTION.[1]

5.1 INTRODUCTION

One of the primary indicators of both acute and chronic physiological stresses in fish is the increased presence of cortisol in the plasma [1]. Studies in salmonid fish have shown that increased amounts of cortisol, both injected and naturally induced, can and do affect immune responsiveness and ultimately disease susceptibility [2-10]. Recent studies have suggested that the observed differences in immune responsiveness and disease susceptibility in relation to elevated cortisol levels are specific to species, strain, antigen type, and possibly to the timing of the stressor [10-16]. The purpose of this study, therefore, was to examine the effects of cortisol administration on the immune response of Atlantic salmon (*Salmo salar* L.) following vaccine injection.

Farmed salmonids are routinely vaccinated prior to seawater entry as a way to ensure they are protected against the various bacterial and viral diseases they might encounter in a net pen environment. The exact timing of vaccine injection is important as it ensures that pathogen-specific antibodies (Ab) are present before the pathogen is encountered. Intraperitoneal injection with a polyvalent, oil-adjuvanted bacterial vaccine (AV) used in aquaculture has been shown to elicit a strong physiological stress response in salmonids similar to that produced by a chronic stressor, including elevated levels of plasma cortisol, a temporary suppression of the immune response, and a short-term increase in disease susceptibility [12, 15, 17, 18].

Recent studies have suggested that if plasma cortisol levels are elevated after initiation of the innate and adaptive immune responses, overall disease susceptibility and the production of pathogen-specific Abs are unaffected [11, 12, 15, 16]. These findings have important implications in aquaculture with respect to the timing of vaccine injections. The specific objective of this study was, therefore, to examine vaccine-induced innate and adaptive immune parameters in farmed Atlantic salmon following the injection of supra-physiological levels of

---

[1]A version of this chapter has been submitted for publication. Skinner LA, Schulte PM, LaPatra SE, Adams A, Thompson KD, Balfry SK, McKinley RS. Cortisol suppresses lysozyme activity but not the antibody response in Atlantic salmon, *Salmo salar* L., following vaccine injection.

cortisol. Specifically, this study sought to determine if lysozyme activity and specific Ab production were negatively impacted when cortisol was injected 53 and 212 degree days (dd) after individuals were injected with a polyvalent, oil-AV alone and/or concurrently with a newly licensed rhabdovirus DNA vaccine (DV) specific to the infectious haematopoietic necrosis (IHN) virus.

5.2 MATERIALS AND METHODS

5.2.1 FISH STOCK AND REARING CONDITIONS

Juvenile Atlantic salmon (approximately 30 g each) were obtained from Big Tree Creek Hatchery (Marine Harvest Canada, Campbell River, BC, Canada) and maintained at the Department of Fisheries and Oceans - University of British Columbia Centre for Aquaculture and Environmental Research (DFO-UBC CAER). Unvaccinated individuals (640 fish) were randomly divided into 20 x 200 L indoor tanks (32 fish per tank) that continuously received well water at a constant temperature (10.6 ºC). Fish were maintained under natural photoperiod ranging from 10:14 to 13:11, light:dark over the course of the experiment. With the exception of the 24 h period preceding tagging, vaccination, and sampling protocols, fish were fed to satiation twice daily with a commercially available pellet-food (Bio-Olympic Fry®; Bio-Oregon, Vancouver, Canada). Fish were acclimated to these conditions for 28 days prior to tagging and vaccination protocols.

5.2.2 VACCINATION PROCEDURE

At the time of vaccination, fish were netted and transferred to a small fresh-water filled container where they were individually anaesthetized with a non-lethal dose of aerated tricaine methane sulphonate (MS222; Syndell Laboratories, Vancouver, BC, Canada), buffered with sodium bicarbonate ($NaHCO_3$; Sigma Aldrich, Oakville, ON, Canada) in a 1:2 ratio (100 mg L^{-1} MS222 to 200 mg L^{-1} $NaHCO_3$). Fish were randomly divided into four vaccine groups (8 fish per vaccine group in each tank) and injected both intramuscularly (IM) and intraperitonealy (IP). The IM injection was placed immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle), while the IP injection was one fin length ahead of the pelvic fins, along the midline of the fish as follows. Fish from the control group were injected with 50 µL of 0.02 M phosphate-buffered saline (PBS) IM, and 100 µL of PBS IP. Fish from the adjuvant vaccine (AV) group

were injected with 50 µL of PBS IM, and 100 µL IP of a commercially available, polyvalent, oil-adjuvanted vaccine containing formalin inactivated, whole-cell bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* serotype O1 and O2, *Vibrio ordalii*, and *Vibrio salmonicida* (Lipogen Forte®; Novartis Aqua Health, Charlottetown, PE, Canada). Fish from the DNA vaccine (DV) group were injected with 50 µL IM of a rhabdovirus DNA vaccine containing 10 µg of plasmid encoding the glycoprotein (G) gene from the IHN virus (APEX IHN®; Novartis Aqua Health), and 100 µL of PBS IP. Fish from the combined group were injected with 50 µL of the DV IM, and 100 µL of the oil-AV IP. Our vaccination protocol including doses and timing of injection was carried out as suggested by the vaccine manufacturers.

Concurrent with the vaccination procedure, all fish were tagged with alphanumeric visible implant tags (Northwest Marine Technology, Shaw Island, WA, USA) for individual identification and visible implant elastomer tags (Northwest Marine Technology) for vaccine group identification. At completion of the vaccination and tagging procedures, all fish were returned to their respective 200 L holding tanks and allowed to recover from anaesthesia in well aerated fresh-water.

5.2.3 Experimental Design

5.2.3.1 Experiment #1 – 53 Degree Days Post-Vaccine Injection

To determine the effect of chronic, supra-physiological levels of cortisol on the innate immune response post-vaccine injection (pvi), ten 200 L holding tanks were divided into two experimental treatments: control and cortisol injected (five replicate tanks in each treatment). Fifty-three degree days (dd) pvi, once the innate immune response had been fully established, fish from the cortisol treatment were individually anaesthetized with a non-lethal dose of aerated MS222 buffered with sodium bicarbonate, as above, and injected IP with a cortisol implant [50 µg cortisol (Sigma Aldrich) g^{-1} body weight in a 1:1 vegetable oil:vegetable shortening vehicle (Crisco®, Smucker Foods of Canada Co., Markham, ON, Canada)] as described previously [14, 19]. Cortisol implants have been shown to produce a slow release of cortisol into the circulation of teleosts thereby simulating a chronic stressor [5, 19-21]. Fish were returned to their holding tank and allowed to recover from anaesthesia in well aerated fresh-water. At 74 dd post-cortisol injection (pci) (127 dd pvi) all fish were lethally sampled as described below.

5.2.3.2 Experiment #2 – 212 Degree Days Post-Vaccine Injection

To determine the effect of chronic, supra-physiological levels of cortisol on the adaptive immune response pvi, ten 200 L holding tanks were divided into two experimental treatments: control and cortisol injected (five replicated tanks in each treatment). Two-hundred-twelve dd pvi, following initiation of antibody production and the adaptive immune response, fish from the cortisol treatment were individually anaesthetized with a non-lethal dose of aerated MS222 buffered with sodium bicarbonate, as above, and injected IP with the cortisol implant described above. Fish were returned to their holding tank and allowed to recover from anaesthesia in well aerated fresh-water. At 74 dd pci (286 dd pvi) all fish were lethally sampled as described below.

5.2.4 Blood Sampling

On the day of sampling, fish were netted and transferred to a small fresh-water filled container where they were anaesthetized with a lethal dose of MS222 buffered with sodium bicarbonate (500 mg L^{-1} MS222 to 1000 mg L^{-1} $NaHCO_3$). Following weight (wt) measurements (to the nearest 0.1 g), blood samples were drawn from caudal venepuncture using a non-heparinised syringe. Whole blood was placed at 4 ºC for 4 h after which it was separated into serum and red cell components by centrifugation (10 min at 4 ºC, 1600 x *g*). Serum was collected and stored at -80 ºC. Prior to analysis, serum from each vaccine group in a treatment tank (n = 8) was pooled (n = 5) and subsequently analysed for serum cortisol levels, serum lysozyme activity, IHNV-specific neutralizing antibody (NAb) titres, and Ab titres against *Aeromonas salmonicid*a and *Listonella anguillarum* serotype O1.

5.2.5 Serum Cortisol

Serum cortisol levels were determined using a commercially available cortisol enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corporation, Lexington, KY, USA) from duplicate samples. If needed, serum was diluted as necessary to ensure that levels fell within the range of the standard curve.

5.2.6 Serum Lysozyme Activity

Serum lysozyme activity was determined by a microplate modification of the method of Litwack [22-24]. Briefly, 10 µL of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250 µL of a 0.025% w/v suspension of *Micrococcus lysodeikticus* in 0.06 M phosphate buffer (pH 6.2). The average decrease in optical density at 450 nm (OD_{450}) over a 20 min period at 25 ˚C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity (µg mL^{-1} HEWL eq), which was used as the standard.

5.2.7 IHNV Serum Neutralizing Antibody Titre

IHNV serum neutralizing antibody titres were determined using a complement dependent 50% plaque neutralization titre (50% PNT) assay, as described previously by LaPatra *et al*. [25]. Briefly, serum samples were heat-inactivated for 30 min at 45 ˚C to destroy all residual complement, and a two-fold dilution series made. As a source of complement, serum was obtained from pathogen-free rainbow trout (*Oncorhynchus mykiss*, Walbaum) that had not been fed for at least two weeks. Equal volumes of this complement source (1:10 dilution) and a diluted IHN virus suspension (2000 pfu mL^{-1}) were added to each serum dilution series. Samples were plaque reduction assayed on *Epithelioma papulosum cyprini* monolayers and NAb titres were reported as the reciprocal of the highest serum dilution that resulted in a 50 % reduction in the average number of plaques detected in negative controls. A sample was considered to be positive with a titre of 20 or above, while a titre of < 20 was considered negative [25-26].

5.2.8 Enzyme Linked Immunosorbent Assay Antibody Titre

An indirect enzyme linked immunosorbent assay (ELISA) was used to measure the specific Ab response of Atlantic salmon sera against heat-killed, whole cells of *Aeromonas salmonicida* and *Listonella anguillarum* serotype O1. Ab titres were determined using a modification of the method previously outlined by Adams *et al*. [27] and as suggested by the monoclonal antibody (MAb) manufacturer (Aquatic Diagnostics, Stirling, Scotland). Unless stated otherwise, all chemicals were purchased from Sigma Aldrich. Briefly, 96-well microtitre plates (Immulon 4HBX; ThermoFisher Scientific, Nepean, ON, Canada) were coated with 0.05 % w/v poly-L-lysine in a carbonate-bicarbonate buffer and allowed to incubate for 60 min at room temperature (21 °C). Plates were then washed twice with a low salt wash buffer (LSW;

0.02 M Tris, 0.38 M NaCl, 0.05 % Tween 20). Heat killed bacteria (*Aeromonas salmonicida* or *Listonella anguillarum* serotype O1) were added to each well (100 µL well^{-1}) and plates were incubated overnight at 4 ºC. Virulent strains of *Aeromonas salmonicida* (strain # 2004-118) and *Listonella anguillarum* serotype O1 (strain # 2004-124) were graciously donated by Dr. SR Jones (DFO Pacific Biological Station, Nanaimo, BC, Canada). The *Aeromonas salmonicida* isolate was cultured at 22 ºC for 72 h in tryptic soy broth (TSB), while *Listonella anguillarum* serotype O1 was cultured at 25 ºC for 24 h in TSB with the addition of 2 % sodium chloride (NaCl). Bacteria were washed three times with PBS (8 min at 5000 x *g*) and the bacterial concentration was adjusted to an approximate absorbance of OD_{610} = 1.0. A 0.05 % v/v solution of glutaraldehyde in PBS was added to the bacteria and plates were incubated at 21 °C for 20 min before washing three times with LSW. Non-specific binding sites were blocked by incubating plates with 3 % w/v skimmed milk powder in water (Safeway Foods, Calgary, AB, Canada) at 21 °C for 120 min. After washing plates three times with LSW, 100 µL serially diluted fish serum (from 1:40 to 1:5122 in doubling dilutions in 3% w/v skimmed milk) was added to each well and allowed to incubate overnight at 4 ºC. Fish serum was diluted in 3 % w/v skimmed milk to further block non-specific binding and decrease the high background OD often observed in fish immunoglobulin detection ELISA [28]. Plates were washed five times with high salt wash buffer (HSW; 0.02 M Tris, 0.5 M NaCl, 0.1 % Tween 20) with a 5 min soak on the last wash to remove unbound antibodies. 100 µL well^{-1} anti-rainbow trout/Atlantic salmon MAb (Aquatic Diagnostics Ltd) was added and plates were incubated at 21 ºC for 60 min. Following the subsequent washing of the plates with HSW as previously described, goat anti-mouse immunoglobulin-G labelled with horseradish peroxidise, diluted 1:1000 in conjugate buffer [1% w/v bovine serum albumin (BSA) in LSW] was added to the wells and incubated for 60 min at 21 °C. Plates were once again washed with HSW as previously described. The assay was developed with 100 µL well^{-1} of chromogen in substrate buffer [150 µL chromogen (42 mM 3,3'5,5'-Tetramethylbenzidine hydrate dihydrochloride) in 2 M acetic acid to 15 mL of substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4 containing 0.33 % v/v H_2O_2)]. Following a 10 min incubation at 21 °C, the reaction was terminated with the addition of 50 µL well^{-1} of 2 M H_2SO_4 and the absorbance was measured at OD_{450}. The ELISA Ab titre was defined as the reciprocal of the highest dilution showing an OD_{450} at least three times greater than the negative control.

Both positive and negative controls were added to each plate. For the determination of anti-*Aeromonas salmonicida* Ab titres, positive controls consisted of serially diluted Atlantic

salmon anti-sera and negative controls were normal, unvaccinated Atlantic salmon sera. For the determination of anti-*Listonella anguillarum* serotype O1 Ab titres, positive controls consisted of serially diluted rabbit anti-sera and negative controls were normal rabbit sera. All positive and negative controls were kindly donated by Dr. RJF Markham (University of Prince Edward Island Atlantic Veterinary College, Charlottetown, PE, Canada). The anti-rainbow trout/Atlantic salmon MAb was not added to those wells that contained the rabbit sera. In its place, 100 μL $well^{-1}$ antibody buffer (1% BSA in PBS) was added. Following the HSW procedure, goat anti-rabbit immunoglobulin-G labelled with horseradish peroxidise, diluted 1:1000 in conjugate buffer was added to these wells. The remainder of the protocol was carried out as above.

5.2.9 Statistical Analysis

Data are presented as the means with standard error of the means (± SE). Following tests for normality and homogeneity of variance, data were analyzed using a two-way analysis of variance (ANOVA) with vaccine group and cortisol treatment as factors. If a significant difference between groups was detected ($P < 0.05$) the Student-Newman-Keuls method of pairwise multiple comparisons was utilized to identify groups that differed significantly ($P < 0.05$). All data were analyzed using Sigmastat software (version 3.5; Systat Software Inc., San Jose, CA, USA).

5.3 Results

5.3.1 Experiment #1 – 53 Degree Days Post-Vaccine Injection

5.3.1.1 Serum Cortisol

Two-way ANOVA detected a significant effect of cortisol treatment on cortisol level across all vaccine groups ($P = < 0.001$), and there was no significant effect of vaccine group on cortisol levels ($P = 0.876$) (Figure 5.1A). The combined mean serum cortisol level of the control treatment was 109.2 ± 7.6 ng mL^{-1} and the combined mean serum cortisol level of the cortisol treatment was 1512.1 ± 139.8 ng mL^{-1}.

5.3.1.2 Serum Lysozyme Activity

Two-way ANOVA revealed a significant effect of cortisol treatment on lysozyme activity (P = <0.001) and a significant difference between vaccine groups (P = < 0.001) but no interaction between cortisol treatment and vaccine group (P = 0.223). In the control treatment, the AV and combined groups had significantly higher levels of lysozyme activity than the control or DV groups (Figure 5.2). In the cortisol treatment, there were no differences in lysozyme activity between the control group and the AV, DV, or combined groups (Figure 5.2). The AV group however, had lysozyme activity levels that were significantly higher than the DV group.

While the AV, DV, and combined groups all showed significant differences in lysozyme activity between treatments, there was no apparent difference in the control group (Figure 5.2).

5.3.1.3 IHNV Serum Neutralizing Antibody Titre

At the time of sampling, there were no IHNV-specific NAbs present in Atlantic salmon injected with the DV (i.e. the DV and combined groups).

5.3.1.4 Enzyme Linked Immunosorbent Assay Antibody Titre

5.3.1.4.1 Anti-*Aeromonas salmonicida* Antibody Titre

There were no differences in anti-*Aeromonas salmonicida* Ab titres between vaccine groups or treatments (data not shown).

5.3.1.4.2 Anti-*Listonella anguillarum* Antibody Titre

There were no differences in anti-*Listonella anguillarum* Ab titres between vaccine groups or treatments (data not shown).

5.3.2 Experiment #2 – 212 Degree Days Post-Vaccine Injection

5.3.2.1 Serum Cortisol

Although two-way ANOVA detected significant differences in cortisol level between treatments within all vaccine groups (P = < 0.001), there were no differences between vaccine

groups regardless of treatment ($P = 0.684$) and no interactions between cortisol treatment and vaccine group ($P = 0.567$) (Figure 5.1B). The combined mean serum cortisol level of the control treatment was 133.8 ± 12.13 ng mL^{-1} and the combined mean serum cortisol level of the cortisol treatment was 1060.1 ± 138.4 ng mL^{-1}.

5.3.2.2 Serum Lysozyme Activity

Two-way ANOVA detected a significant effect of cortisol treatment on lysozyme activity ($P = <0.001$) and a significant difference between vaccine groups ($P = < 0.001$) but no interaction between cortisol treatment and vaccine group ($P = 0.076$). In the control treatment, the AV and combined groups had significantly higher levels of lysozyme activity than the control or DV groups (Figure 5.3). In the cortisol treatment, there were no differences in lysozyme activity between any of the vaccine groups (Figure 5.3).

The AV, DV, and combined groups showed significantly lower levels of lysozyme activity in the cortisol treatment compared to the control treatment. There was no difference in lysozyme activity between treatments in the control group (Figure 5.3).

5.3.2.3 IHNV Serum Neutralizing Antibody Titre

At the time of sampling, there were no IHNV-specific NAbs present in Atlantic salmon injected with the DV (i.e. the DV and combined groups).

5.3.2.4 Enzyme Linked Immunosorbent Assay Antibody Titre

5.3.2.4.1 Anti-*Aeromonas salmonicida* Antibody Titre

Although two-way ANOVA revealed a significant effect of vaccine group on anti-*Aeromonas salmonicida* Ab titre ($P < 0.001$), there was no difference between cortisol treatments ($P = 0.936$) and no interaction between vaccine group and cortisol treatment ($P = 0.355$). While there were no differences between control and DV groups, the anti-*Aeromonas salmonicida* Ab titres of the AV group was greater than the titres observed in both the control and DV groups. The Ab titres of the combined group was also greater than that observed in the control and DV groups, and was significantly greater than that observed in the AV group (Figure 5.4A).

5.3.2.4.2 Anti-*Listonella anguillarum* Antibody Titre

Although two-way ANOVA detected a significant effect of vaccine group on anti-*Listonella anguillarum* serotype O1Ab titre ($P < 0.001$), there was no difference between cortisol treatments ($P = 0.062$) and no interaction between vaccine group and cortisol treatment ($P = 0.596$). There were no differences in Ab titre between control and DV groups, or between AV and combined vaccine groups, however the Ab titre of the AV and combined groups was significantly higher than that of the control and DV groups (Figure 5.4B).

5.4 DISCUSSION

To maintain the health and welfare of farmed Atlantic salmon, individual fish are vaccinated against relevant pathogens prior to the parr-to-smolt transformation and sea water entry. The injection of these vaccines leads to unavoidable stress and is associated with short-term increases in plasma cortisol [11, 15]. It has been well established that increased levels of cortisol can increase disease susceptibility and affect immune responsiveness by reducing the number of circulating lymphocytes, affecting their ability to generate plaque-forming cells, and/or suppressing mitogenic responses [5, 6, 10, 20, 29, 30]. As well, there appears to be a transient decrease in immunoglobulin and total serum protein, and a possible impairment in Ab production, likely due to a decreased ability of antigen binding ligands to bind anti-immunoglobulin Abs [11, 31]. Recent studies have shown that the overall impact of elevated plasma cortisol on individual immuno-responsiveness and disease susceptibility is species-specific, antigen-specific, and possibly related to the timing of the stressor [10, 11, 13-16]. The data presented in this study indicate that if Atlantic salmon are exposed to significant elevations in plasma cortisol concentration at 53 and 212 dd post vaccine injection (pvi), the vaccine-induced innate immune response at 127 or 286 dd pvi (74 dd post cortisol injection) is suppressed while the adaptive immune response at 286 dd pvi remains unchanged.

Lysozyme is an essential element of the innate immune response with both anti-bacterial and antiviral activity [17, 32, 33-38]. During vaccination, oil-adjuvants act as reservoirs holding the antigen(s) in globules at the site of injection [39]. By slowly releasing the antigen(s), adjuvants can continuously activate the innate immune response while focusing the adaptive immune response [12, 40-42]. Our data from the control treatment group indicate that lysozyme activity was induced by the polyvalent, oil-AV vaccine at 127 dd pvi, and that this induction was maintained at 286 dd pvi. Our data also indicate that, contrary to the known antiviral capabilities

of lysozyme [32, 33, 38], the injection of a rhabdovirus-specific DV did not cause a significant increase in lysozyme activity at 127 or 286 dd pvi, nor was there a synergistic effect in lysozyme activity when an oil-AV was injected concurrently with a DV. It is possible that an increase in lysozyme activity does occur following injection of a DV, however due to the timing of our sampling protocol, we were unable to detect this.

It has previously been shown that lysozyme activity following vaccination and/or pathogen exposure changes when plasma cortisol levels are elevated and that these changes can be correlated to genetic differences [17, 43-46]. Although some studies have detected an immediate short-term enhancement in immune-related activity following an acute stressor [5, 17, 43], chronic stressors are associated with immunosuppression including significant decreases in lysozyme activity [44, 47, 48]. In our study, Atlantic salmon pre-smolts were injected with a cortisol implant at either 53 dd or 212 dd pvi. When sampled 74 dd after cortisol injection (127 dd or 286 dd pvi, respectively), individuals that received the cortisol implant (and thus had chronically elevated levels of cortisol) showed a significant suppression of the increased lysozyme activity induced by the oil-AV, regardless of when the rise in cortisol levels occurred (53 or 212 dd pvi). In the current study, the cortisol treated Atlantic salmon were exposed to chronic, supra-physiological levels of cortisol, and this treatment caused a suppression of the vaccine-induced lysozyme activity similar to that observed by others [44, 47, 48]. It is possible however, that a less pronounced response would occur under physiologically relevant conditions.

Bacteria-specific Ab production is temperature dependent. In Atlantic salmon held within their thermoneutral range (10 – 12 ºC), specific Abs are produced in response to vaccine injection between 200 and 300 dd pvi, depending on the antigen/adjuvant combination [49-52]. Unpublished data from a related experiment indicate that Atlantic salmon produce measurable titres of Ab against *Aeromonas salmonicida* and *Listonella anguillarum* serotype O1 within 200 dd pvi (Chapter 4). In the current study, Ab titres were measured at 127 and 286 dd pvi. As expected, at 127 dd pvi we did not measure any significant differences in Ab titre between the vaccine groups. Any amount of Ab measured was considered to be due to natural antibodies. Natural antibodies are present even in immunologically naïve fish [53, 54]. While little is known about these Abs, they can be antigen-specific and are thought to arise either as a result of adoptive transfer from mother to embryo, are developed in the host following exposure to environmental antigens, or are a germline-encoded product [54, 55]. Artificial elevation of plasma cortisol by the cortisol implants did not appear to affect the titre of natural antibodies specific to either bacteria tested.

At 286 dd pvi, there were significant differences in anti-*Aeromonas salmonicida* and anti-*Listonella anguillarum* serotype O1 Ab titres between vaccine groups. Individuals that received the polyvalent, oil-AV (the AV and combined groups) had significantly higher levels of Ab compared to both the control and DV groups. Interestingly, fish in the combined group had higher titres of anti-*Aeromonas salmonicida* Ab than fish in the AV group suggesting a possible cross-reaction of the antigens from both the polyvalent oil-AV and the DV. In contrast, we did not observe measurable titres of virus-specific neutralizing antibodies (NAb) at any time point during this experiment. Virus-specific NAbs are produced in response to virus-specific DVs [26, 57]. Unpublished data with Atlantic salmon indicate that NAbs are not produced until approximately 413 dd pvi (Chapter 4), suggesting that the lack of detectable NAb titres in the DV or combined groups of the present study is the result of the timing of sampling.

It has been demonstrated that chronic elevation of plasma cortisol in salmonids reduces the number of antibody-secreting cells and the number of Abs, as well decreases overall protection [5, 6, 30, 31, 57-60]. In one of the initial studies by Maule *et al*. [20], fish were injected with a cortisol implant seven days prior to the presentation of antigens. Recent studies have suggested that if an antigen is presented to the immune system prior to the elevation in cortisol such that development of the adaptive immune response has already begun, Ab production is unchanged [11, 12, 16]. Our data support this observation. When plasma cortisol levels were elevated to supra-physiological levels as a result of the cortisol implant, there were no significant differences in anti-*Aeromonas salmonicida* or anti-*Listonella anguillarum* serotype O1 Ab titres compared to the 'unstressed' control treatment. It is likely, therefore, that protection against these pathogens would be unaffected, although this was not directly assessed.

Melingen *et al*. [31, 59] and Eggset *et al*. [12] examined the timing of vaccine injection with relation to the parr-to-smolt transformation in farmed Atlantic salmon, a period where plasma cortisol is known to be significantly elevated. The findings of Melingen *et al*. [31, 59] suggested that timing of vaccine injection with relation to the parr-to-smolt transformation was important to both Ab production and overall protection. Alternatively, the findings of Eggset *et al*. [12] indicated that regardless of when vaccine injection took place (six weeks prior to, at the onset of, or during the parr-to-smolt transformation) Ab production and protection were unaffected. Thus, at present it is unknown how the timing of the elevation of plasma cortisol affects the production of Abs and overall protection in Atlantic salmon. Future studies should examine the relationship between protection and Ab titres following the injection of both

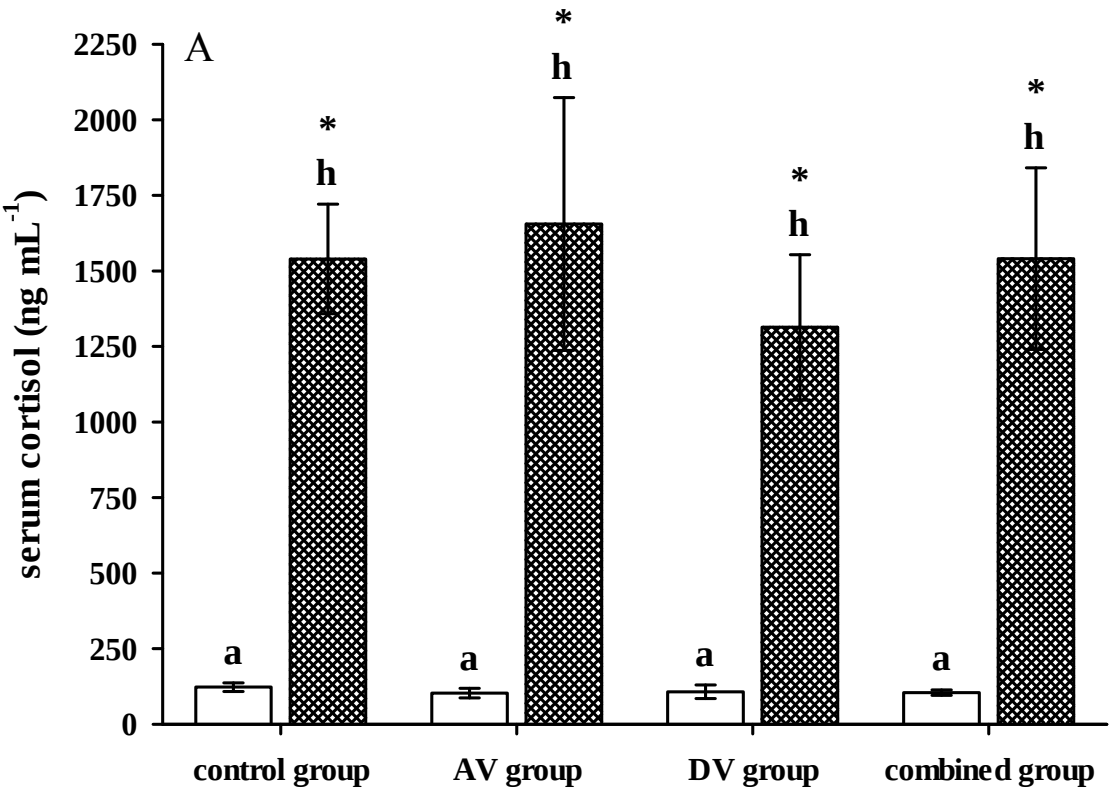
physiological and supra-physiological levels of cortisol at various time-points post-vaccine injection.

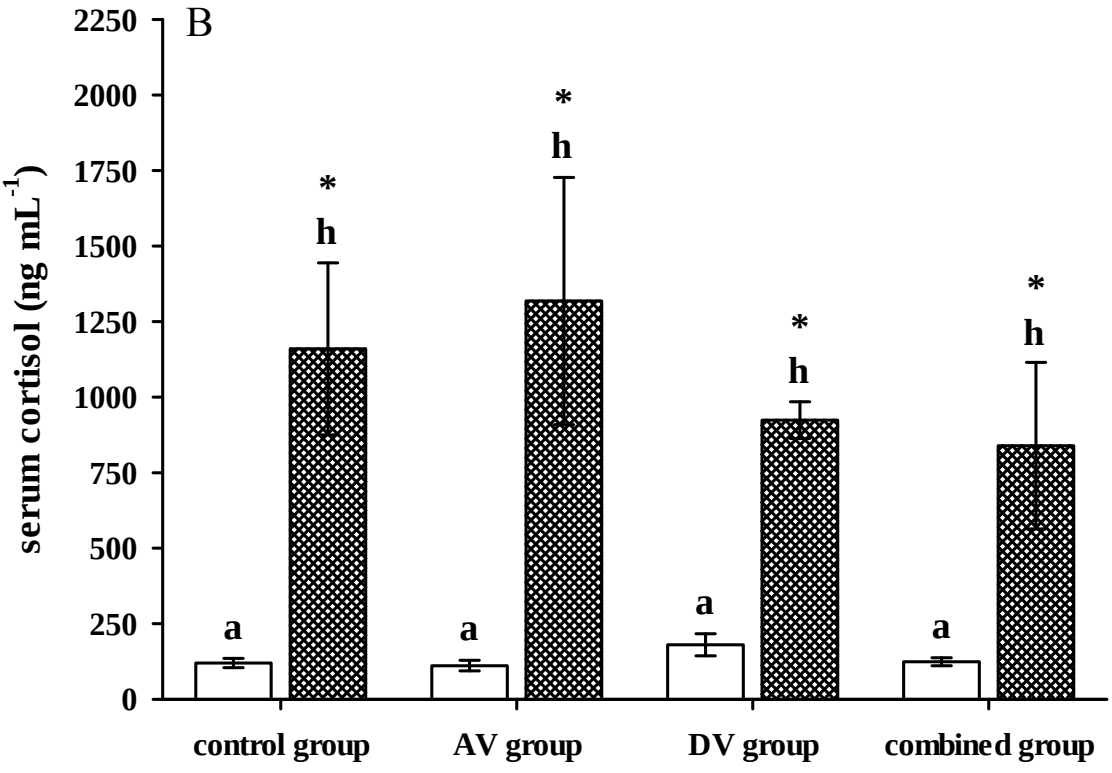
Seasonality, diel pattern, temperature, species, strain, developmental stage and husbandry are just some of the many factors that can account for variation in salmonid cortisol levels under basal conditions [14, 61-64]. Current estimates of physiologically relevant cortisol levels in unstressed salmonid fish range from 0 - 25 ng mL^{-1} [6, 65, 66], with average levels in farmed Atlantic salmon more variable at 8 ng mL^{-1} [29], 16 ng mL^{-1} [67], 76 ng mL^{-1} [10], and 82 ng mL^{-1} [11. Carey and McCormick [61] noted that developmental stage has a substantial impact on the plasma cortisol levels of stressed Atlantic salmon, with smolts being more responsive to stressors than parr. Singer *et al*. [14] reported relatively high plasma cortisol levels in two different strains of farmed Atlantic salmon (67 ± 28.8 and 265.6 ± 66.3 ng mL^{-1}) that were sham injected with a vegetable oil:vegetable shortening cortisol implant vehicle. Thus, the levels of cortisol measured in the current study (control and cortisol treatments) are high relative to other studies [6, 11, 14, 29, 61, 65-67]. This could be attributed to a variety of parameters including the transient stress of vaccine injection and the sampling protocol, as well as developmental and strain differences.

As a result of the potential for wide variability in plasma cortisol content in both unstressed and stressed salmonids [14, 61] it is important to understand the range of immunological and physiological changes that can occur. Although attempts are made to ensure stress-free husbandry practices, aquaculture sites are fraught with uncontrollable stressors such as handling, confinement, vaccination, and transport [11, 12, 15, 68, 69]. Our data indicate that chronically elevated plasma cortisol suppresses the innate immune response of Atlantic salmon, but does not affect overall Ab production, but future studies should examine the impact of these results on overall protection, and determine whether similar trends are observed with physiologically relevant levels of cortisol.

5.5 FIGURES

Figure 5.1 Serum cortisol levels of vaccinated Atlantic salmon. Fish in the control group were injected with phosphate-buffered saline, fish in the AV group were injected with a polyvalent, oil-adjuvanted vaccine, fish in the DV group were injected with a DNA vaccine, and fish in the combined group were injected with both a polyvalent, oil-adjuvanted vaccine and a DNA vaccine. A) 53 degree days (dd) post-vaccine injection (pvi) and B) 212 dd pvi, ten 200 L tanks were split into control (▭) and cortisol (▩) treatments. Fish in the cortisol treatment were injected intraperitonealy with a cortisol implant (50 µg cortisol g^{-1} body weight in a 1:1 vegetable oil:vegetable shortening vehicle). 74 dd post-cortisol injection (127 and 286 dd pvi), fish from both control and cortisol treatments were lethally sampled. Serum from each vaccine group was pooled in a tank-specific manner. Different letters indicate significant differences between vaccine groups within a treatment; *Significant difference between treatments within a vaccine group. Values are mean ± SE. (n = 5) two-way ANOVA, $P < 0.05$.


A
serum cortisol (ng mL-1)
2250
2000
1750
1500
1250
1000
750
500
250
0
a
*
h
control group
AV group
DV group
combined group


B
serum cortisol (ng mL-1)
2250
2000
1750
1500
1250
1000
750
500
250
0
a
*
h
control group
AV group
DV group
combined group

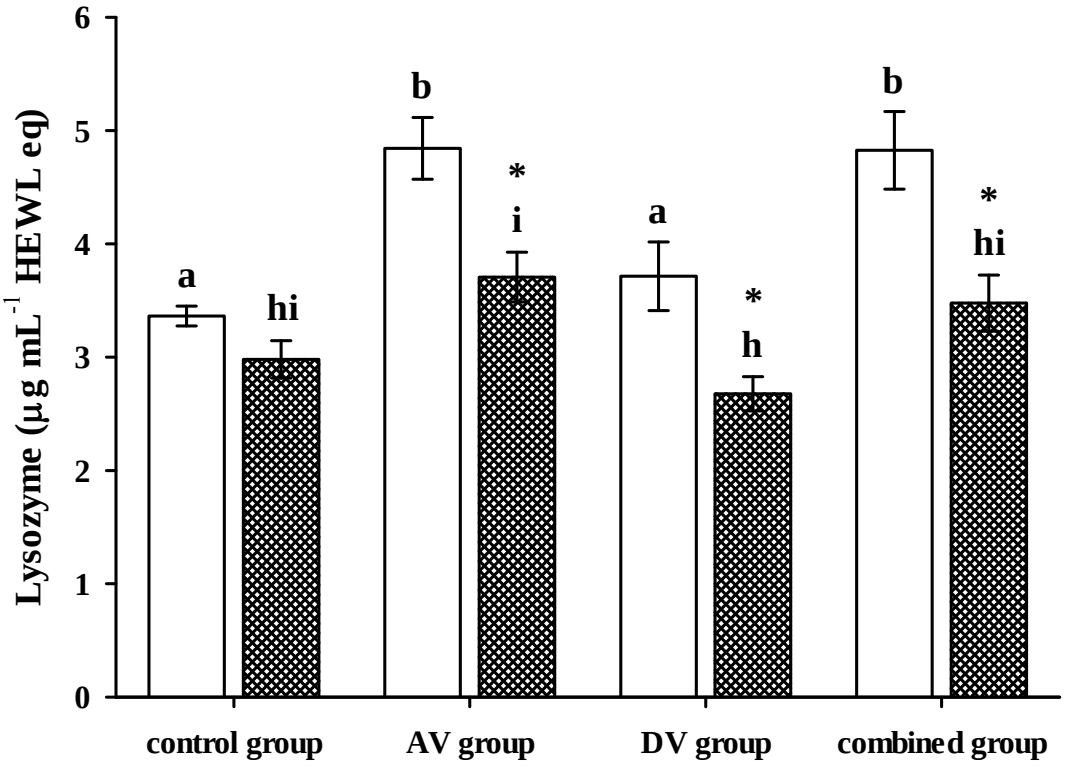


Figure 5.2 Serum lysozyme activity of vaccinated Atlantic salmon. Fish in the control group were injected with phosphate-buffered saline, fish in the AV group were injected with a polyvalent, oil-adjuvanted vaccine, fish in the DV group were injected with a DNA vaccine, and fish in the combined group were injected with both a polyvalent, oil-adjuvanted vaccine and a DNA vaccine. 53 degree days (dd) post-vaccine injection (pvi), ten 200 L tanks were split into control (□) and cortisol (▩) treatments. 74 dd post-cortisol injection (127 dd pvi) control and cortisol treatment fish were lethally sampled. Serum from each vaccine group was pooled in a tank-specific manner. Different letters indicate significant differences between vaccine groups within a treatment; *Significant difference between treatments within a vaccine group. Values are mean ± SE. (n = 5) two-way ANOVA, $P < 0.05$.

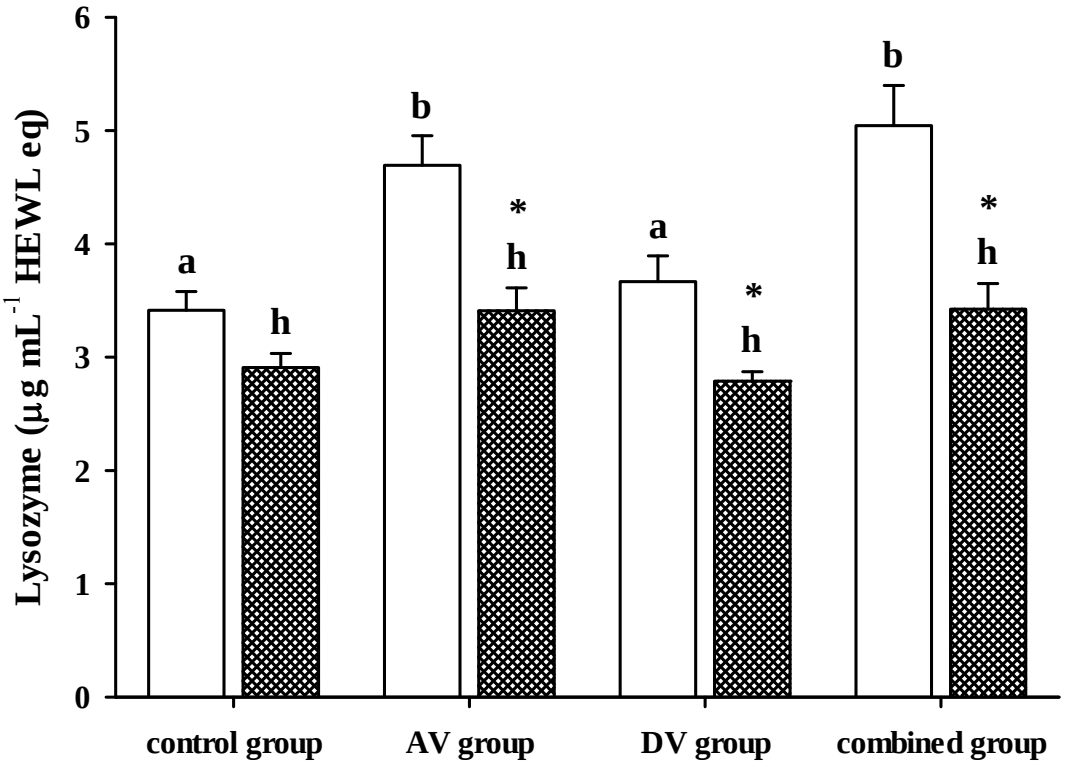
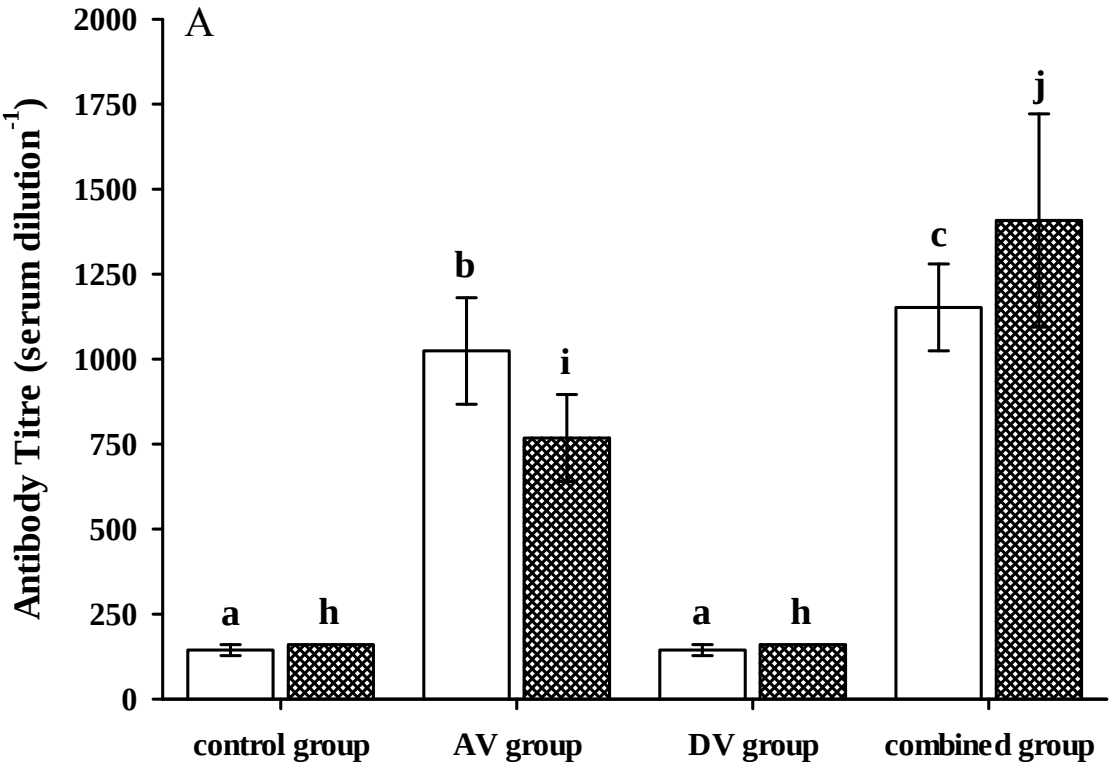
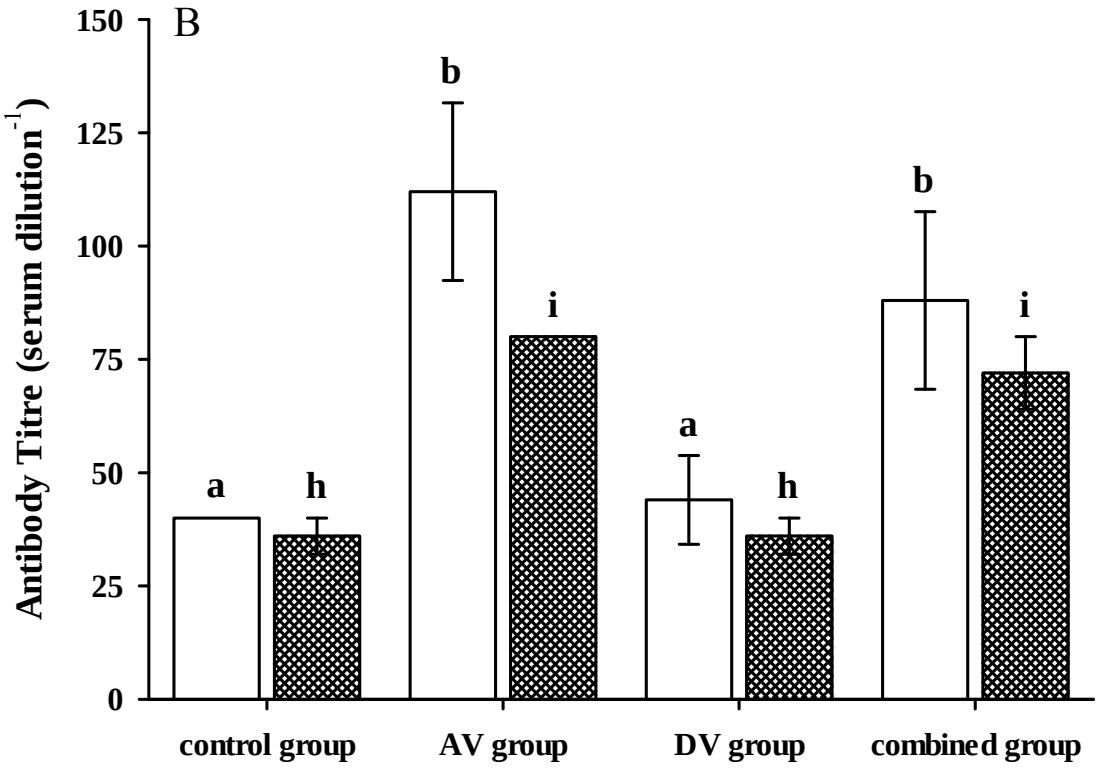


Figure 5.3 Serum lysozyme activity of vaccinated Atlantic salmon. Fish in the control group were injected with phosphate-buffered saline, fish in the AV group were injected with a polyvalent, oil-adjuvanted vaccine, fish in the DV group were injected with a DNA vaccine, and fish in the combined group were injected with both a polyvalent, oil-adjuvanted vaccine and a DNA vaccine. 212 degree days (dd) post-vaccine injection (pvi), ten 200 L tanks were split into control (▭) and cortisol (▩) treatments. 74 dd post-cortisol injection (286 dd pvi) control and cortisol treatment fish were lethally sampled. Serum from each vaccine group was pooled in a tank-specific manner. Different letters indicate significant differences between vaccine groups within a treatment; *Significant difference between treatments within a vaccine group. Values are mean ± SE. (n = 5) two-way ANOVA, $P < 0.05$.

Figure 5.4 A) Anti-*Aeromonas salmonicida* antibody (Ab) titres and B) Anti-*Listonella anguillarum* Ab titres of vaccinated Atlantic salmon. Fish in the control group were injected with phosphate-buffered saline, fish in the AV group were injected with a polyvalent, oil-adjuvanted vaccine, fish in the DV group were injected with a DNA vaccine, and fish in the combined group were injected with both a polyvalent, oil-adjuvanted vaccine and a DNA vaccine. 212 degree days (dd) post-vaccine injection (pvi), ten 200 L tanks were split into control (□) and cortisol (▩) treatments. 74 dd post-cortisol injection (286 dd pvi) control and cortisol treatment fish were lethally sampled. Serum from each vaccine group was pooled in a tank-specific manner. Different letters indicate significant differences between vaccine groups within a treatment; *Significant difference between treatments within a vaccine group. Values are mean ± SE. (n = 5) two-way ANOVA, $P < 0.05$.


A
Antibody Titre (serum dilution -1)
2000
1750
1500
1250
1000
750
500
250
0
a
h
b
i
a
h
c
j
control group
AV group
DV group
combined group


B
Antibody Titre (serum dilution -1)
150
125
100
75
50
25
0
a
h
b
i
a
h
b
i
control group
AV group
DV group
combined group

5.6 REFERENCES

[1] Wendelaar Bonga SE. The stress response in fish. Physiological Reviews 1997; 77(3): 591-625.

[2] Pickering AD, Dustin J. Administration of cortisol to brown trout, *Salmo trutta* L., and its effects on the susceptibility to *Saprolegnia* infection and furunculosis. Journal of Fish Biology 1983; 23: 163-75.

[3] Pickering AD, Pottinger TG. Cortisol can increase the susceptibility of brown trout, *Salmo trutta* L., to disease without reducing the white blood cell count. Journal of Fish Biology 1985; 27: 611-9.

[4] Angelidis P, Baudin-Laurencin F, Youiniou P. Stress in rainbow trout, *Salmo gairdneri*: effects upon phagocyte chemiluminescence, circulating leucocytes and susceptibility to *Aeromonas salmonicida*. Journal of Fish Biology 1987; 31(Supplement A): 113-22.

[5] Maule AG, Tripp RA, Kaattari SL, Schreck CB. Stress alters immune function and disease resistance in Chinook salmon (*Oncorhynchus tshawytscha*). Journal of Endocrinology 1989; 120: 135-42.

[6] Pickering AD, Pottinger TG. Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. Fish Physiology and Biochemistry 1989; 7: 253-8.

[7] Salonius K, Iwama GK. Effects of early rearing environment on stress response, immune function, and disease resistance in juvenile Coho (*Oncorhynchus kisutch*) and Chinook salmon (*O. tshaawytscha*). Canadian Journal of Fisheries and Aquatic Sciences 1993; 50: 759-66.

[8] Thompson I, White A, Fletcher TC, Houlihan DF, Secombes CJ. The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C. Aquaculture 1993; 114: 1-18.

[9] Ruane NM, Wendelaar Bonga SE, Balm PHM. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance and confinement. General and Comparative Endocrinology 1999; 115: 210-9.

[10] Wiik R, Andersen K, Uglenes I, Egidius E. Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with effects of the blood cell pattern. Aquaculture 1989; 83: 201-15.

[11] Espelid S, Løkken GB, Steiro K, Bøgwald J. Effects of cortisol and stress on the immune system of Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1996; 6: 95-110.

[12] Eggset G, Mortensen A, Løken S. Vaccination of Atlantic salmon (*Salmo salar* L.) before and during smoltification and immunological protection. Aquaculture 1999; 170: 101-12.

[13] Engelsma MY, Hougee S, Nap D, Hofenk M, Rombout JHWM, van Muiswinkel WB, Verburg-van Kemenade BML. Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio* L. Fish and Shellfish Immunology 2003; 15: 397-410.

[14] Singer TD, Finstad B, McCormick SD, Wiseman SB, Schulte PM, McKinley RS. Interactive effects of cortisol treatment and ambient seawater challenge on gill Na^+, K^+-ATPase and CFTR expression in two strains of Atlantic salmon smolts. Aquaculture 2003; 222: 15-28.

[15] Funk VA, Jones SRM, Kim E, Kreiberg H, Taylor K, Wu S, Young C. The effect of vaccination and sea water entry on immunocompetence and susceptibility to *Kudoa thyrsites* in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 2004; 17: 375-87.

[16] Lovy J, Speare DF, Stryhn H, Wright GM. Effects of dexamethasone on host innate and adaptive immune response and parasite development in rainbow trout *Oncorhynchus mykiss* infected with *Loma salmonae*. Fish and Shellfish Immunology 2008; 6: 649-58.

[17] Fevolden SE, Røed KH, Gjerde B. Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. Fish and Shellfish Immunology 1994; 4: 507-519.

[18] Wedemeyer GA. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama GK, Pickering AD, Sumpter JP, Schreck, editors. Fish stress and health in aquaculture. Society for Experimental Biology Seminar Series. Cambridge, UK: University Press; 1997, vol. 62, p. 35-71.

[19] Specker JL, Portesi DM, Cornell SC, Veillette PA. Methodology for implanting cortisol in Atlantic salmon and effects of chronically elevated cortisol on osmoregulatory physiology. Aquaculture 1994; 121: 181-93.

[20] Maule AG, Schreck CB. Changes in the immune system of Coho salmon (*Oncorhynchus kisutch*) during parr-to-smolt transformation and after implantation of cortisol. Canadian Journal of Fisheries and Aquatic Sciences 1987; 44: 161-6.

[21] Vijayan MM, Reddy PK, Leatherland JF, Moon TW. The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. General and Comparative Immunology 1994; 96: 75-84.

[22] Maule AG, Schreck R, Slater C, Fitzpatrick MS, Schreck CB. Immune and endocrine responses of adult Chinook salmon during fresh-water immigration and sexual maturation. Fish and Shellfish Immunology 1996; 6: 221-33.

[23] Litwack G. Photometric determination of lysozyme activity. Proceedings for the Society for Experimental Biology and Medicine 1955; 89: 401-3.

[24] Ackerman PA, Iwama GK. Physiological and cellular responses of juvenile rainbow trout to vibriosis. Journal of Aquatic Animal Health 2001; 13: 173-80.

[25] LaPatra SE, Turner T, Lauda KA, Jones GR, Walker S. Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. Journal of Aquatic Animal Health 1993; 5: 165-71.

[26] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006; 24: 345-54.

[27] Adams A, Thompson KD, Morris D, Farias C, Chen SC. Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. Fish and Shellfish Immunology 1995; 5: 537-47.

[28] Kim WS, Nishizawa T, Yoshimizu M. Non-specific adsorption of fish immunoglobulin (IgM) to blocking reagents on ELISA plates. Diseases of Aquatic Organisms 2007; 78: 55-9.

[29] Mazur CF, Iwama GK. Handling and crowding stress reduces number of plaque-forming cells in Atlantic salmon. Journal of Aquatic Animal Health 1993;5: 98-101

[30] Tripp RA, Maule A, Schreck CB, Kaattari SL. Cortisol mediated suppression of salmonid lymphocyte responses *in vitro*. Developmental and Comparative Immunology 1987; 11: 565-76.

[31] Melingen GO, Stefansson SO, Berg A, Wergeland HI. Changes in serum protein and IgM concentration during smolting and early post-smolt period in vaccinated and unvaccinated Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1995; 5: 211-21.

[32] Desvignes L, Quentel C, Lamour F, Le Ven A. Pathogenesis and immune response in Atlantic salmon (*Salmo salar* L) parr experimentally infected with salmon pancreas disease virus (SPDV). Fish and Shellfish Immunology 2002; 12: 77-95.

[33] Ellis A. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology 2001; 25: 827-39.

[34] Fletcher TC, White A. Lysozyme activity in the plaice (*Pleuronectes platessa* L.). Experientia 1973; 29: 1283-5.

[35] Ingram GA. Substances involved in the natural resistance of fish to infection – a review. Journal of Fish Biology 1980; 16: 23-60.

[36] Jollès P and Jollès J. What's new in lysozyme research? Always a model system, today as yesterday. Molecular biochemistry 1984; 63: 165-89.

[37] Lie Ø, Evensen Ø, Sørensen A, Frøysadal E. Study on lysozyme activity in some fish species. Diseases of Aquatic Organisms 1989; 6: 1-5.

[38] Siwicki AK, Morand M, Klein P, Kiczka W. Treatment of infectious pancreatic necrosis virus (IPNV) disease using dimerized lysozyme (KLP-602). Journal of Applied Ichthyology 1998; 14: 229-32.

[39] Anderson DP. Adjuvants and immunostimulants for enhancing vaccine potency in fish. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology;

Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 257-65.

[40] Evensen Ø, Brudeseth B, Mutoloki S. The vaccine formulation and its role in inflammatory processes in fish – Effects and adverse effects. In: Midtlyng PG, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 117-26.

[41] Fevolden SE, Røed KH, Fjalestad. Selection response of cortisol and lysozyme in rainbow trout and correlation to growth. Aquaculture 2002; 205: 61-75.

[42] Mutoloki S, Reite OB, Brudeseth B, Tverdal A, Evensen Ø. A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. Vaccine 2006; 24: 578-88.

[43] Demers NE, Bayne CJ. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Developmental and Comparative Immunology 1997; 21: 363-73.

[44] Moeck A, Peters G. Lysozyme activity in rainbow trout, Oncorhynchus mykiss (Walbaum), stressed by handling, transport and water pollution. Journal of Fish Biology 1990; 37: 873-85.

[45] Røed KH, Fjalestad KT, Strømsheim A. Genetic variation in lysozyme activity and spontaneous haemolytic activity in Atlantic salmon (*Salmo salar*). Aquaculture 1993; 114; 19-31.

[46] Røed KH, Fevolden SE, Fjalestad KT. Disease resistance and immune characteristics in rainbow trout (*Oncorhynchus mykiss*) selected for lysozyme activity. Aquaculture 2002; 209: 91-101.

[47] Fevolden SE, Røed KH, Fjalestad KT, Stein J. Post-stress levels of lysozyme and cortisol in adult rainbow trout: Heritabilities and genetic correlations. Journal of Fish Biology 1999; 54: 900-10.

[48] Yin Z, Lam TJ, Sin YM. The effects of crowding stress on the non-specific immune response of the fancy carp (*Cyprinus carpio*). Fish and Shellfish Immunology 1995; 5: 519-29.

[49] Chiller JM, Hodgins HO, Weiser RS. Antibody response in rainbow trout (*Salmo gairdneri*) II. Studies on the kinetics of development of antibody-producing cells and on complement and natural haemolysin. Journal of Immunology 1969; 102(5): 1202-7.

[50] Marsden MJ, Secombes CJ. The influence of vaccine preparations on the induction of antigen specific responsiveness in rainbow trout *Oncorhynchus mykiss*. Fish and Shellfish Immunology 1997; 7: 455-69.

[51] Van Muiswinkel WB, Wiegertjes GF. Immune Responses after injection vaccination of fish. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology; Developments in Biological Standardization: Basel, Switzerland; Karger, 1997, vol. 90, p. 55-7.

[52] Sommerset I, Krossøy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1): 89-101.

[53] Magor BG, Magor KE. Evolution of effectors and receptors of innate immunity. Developmental and Comparative Immunology 2001; 25: 651-82.

[54] Sinyakov MS, Dror M, Zhevelev H, Margel S, Avtalion RR. Natural antibodies and their significance in active immunisation and protection against a defined pathogen in fish. Vaccine 2002; 20: 3668-74.

[55] Mor A, Avtalion RR. Transfer of antibody activity from immunized mother to embryo in tilapias. Journal of fish Biology 1990; 37: 249-55.

[56] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 2001; 19: 4011-19.

[57] Kaattari SL, Tripp RA. Cellular mechanisms of glucocorticoid immunosuppression in salmon. Journal of Fish Biology 1987; 31A: 129-32.

[58] Maule AG, Schreck CB, Kaattari SL. Changes in the immune system of coho salmon (*Oncorhynchus kisutch*) during parr-to-smolt transformation and after implantation of cortisol. Canadian Journal of Fisheries and Aquatic Sciences 1987; 44: 161-6

[59] Melingen GO, Nilsen F, Wergeland HI. The serum antibody levels in Atlantic salmon (*Salmo salar* L) after vaccination with *Vibrio salmonicida* at different times during the smolting and early post-smolt period. Fish and Shellfish Immunology 1995b; 5: 223-35.

[60] Sunyer JO, Gómez E, Navarro V, Quesada J, Tort L. Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata)* following daily acute stress. Canadian Journal of Fisheries and Aquatic Sciences 1995; 52: 2339-46.

[61] Carey JB, McCormick SD. Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. Aquaculture 1998; 168: 237-53.

[62] Ebbesson LOE, Björnsson, Ekström P, Stefansson SO. Daily endocrine profiles in parr and smolt Atlantic salmon. Comparative Biochemistry and Physiology, Part A 2008: 151: 698-704.

[63] Pickering AD, Pottinger TG. Seasonal and diel changes in plasma cortisol levels of the brown trout, Salmo trutta L. General and Comparative Endocrinology 1983; 49: 232-9.

[64] Thorpe JE, McConway MG, Miles MS, Muir JS. Diel and seasonal changes in resting plasma cortisol levels in juvenile Atlantic salmon, *Salmo salar* L. General and Comparative Endocrinology 1987; 65: 19-22.

[65] Barry TP, Lapp AAF, Kayes TB, Malison JA. Validation of a microtitre plate ELISA for measuring cortisol in fish and comparison of stress responses of rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). Aquaculture 1983; 351-63.

[66] Pottinger TG, Moran TA, Morgan JAW. Primary and secondary indices of stress in the progeny of rainbow trout (*Oncorhynchus mykiss*) selected for high and low responsiveness to stress. Journal of Fish Biology 1994; 44: 149-63.

[67] Waring CP, Stagg RM, Poxton MG. The effects of handling on flounder (*Platichthys flesus* L.) and Atlantic salmon (*Salmo salar* L.). Journal of Fish Biology 1992; 41: 131-44.

[68] Esteban MÁ, Rodríguez A, Ayala AG, Meseguer J. Effects of high doses of cortisol on innate cellular immune responses of sea bream (*Sparus aurata* L.). General and Comparative Endocrinology 2004; 137: 89-98.

[69] Yada T, Nakanishi T. Interaction between endocrine and immune systems in fish. In: Jeon KW, editor. International Review of Cytology, London, UK: Academic Press; 2002, vol. 220, p. 35-92.

CHAPTER SIX: CONCLUSIONS

Fish vaccination is a standard protocol in aquaculture. Not only does it reduce the need for chemotherapeutics [1, 2] it significantly decreases the frequency of disease outbreaks amongst individuals and throughout populations [3-7]. Within the salmonid aquaculture industry, the majority of vaccines are polyvalent and require the use of an adjuvant [7-13]. Although very effective at inducing long-lasting protective immunity, intraperitoneal administration of oil-adjuvanted vaccines can lead to adverse morphological and physiological side-effects. These include inflammation at the site of injection, intra-abdominal adhesions, pigmentation, and granulomas [10, 14-19]. When combined, the above physiological attributes have been shown to influence overall growth in a positive, negative, or neutral manner depending on the species studied and the combination of antigen(s) and adjuvant used [9, 10, 13, 20-25].

In 1996, Anderson *et al*. [26, 27] first described a novel vaccine whereby the glycoprotein (G) gene of the infectious haematopoietic necrosis virus (IHNV) was inserted into a bacterial plasmid along with regulatory sequences that allow for expression in eukaryotic cells. The mechanisms of action of the resulting immune response for this, and similar rhabdovirus-specific DNA vaccines (DV) have been shown to closely resemble those of a natural viral infection, with the vaccinated individual producing a viral protein that is correctly folded and modified, and both cellular and humoral immune responses being elicited [27-35]. Because rhabdovirus-specific DVs do not require an adjuvant, the potential for vaccine-related morphological and physiological side-effects is significantly reduced. In Chapters 2 and 3, I demonstrated that there were no growth-related side-effects in Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*) following the injection of a rhabdovirus-specific DV. Furthermore, I demonstrated that the concurrent injection of a DV with a traditional, polyvalent oil-adjuvanted, bacterial vaccine (AV) did not lead to significant changes in overall growth.

In Chapter 2, Atlantic salmon that received the polyvalent oil-AV alone and concurrently with the DV showed an initial decrease in specific growth rate (SGR) and weight at 106 degree days (dd) post-vaccine injection (pvi). These same groups also displayed significant decreases in growth at the time of sea water entry, 443 dd pvi. Because there were no observed differences between fish that received the oil-AV alone and those that received it concurrently with the DV, the negative growth was most likely due to the oil-AV and not the concurrent injection of vaccines. Although lost growth was quickly compensated for in both instances, it raised the question of the initial driving forces behind the apparent oil-AV-induced negative growth.

Atlantic salmon are anadromous fish with significant physiological changes occurring prior to sea water entry. As such, the parr-to-smolt transformation can be very influential with respect to the overall energetics of an individual fish [36]. Maintaining a functioning immune system and mounting an immune response is also energetically costly to fish with individuals forced to down-regulate some physiological activities in order to up-regulate others [37, 38]. To determine if the decreased growth observed in Atlantic salmon at 106 and 443 dd pvi was a result of vaccine-induced immunological changes or the parr-to-smolt transformation, I measured the routine metabolic rate (RMR) of rainbow trout following the injection of a DV and an AV individually, as well as concurrently. Because rainbow trout do not undergo the parr-to-smolt transformation, any energetic changes we observed should be due to the vaccine-induced immunological stimulation and not the physiological changes associated with the parr-to-smolt transformation. In Chapter 3, I observed a significant and transient increase in RMR at 203 dd pvi in rainbow trout that received concurrent injection of a DV and a polyvalent, oil-AV. Corresponding with the increased RMR were changes in the innate and adaptive immune responses, suggesting that any negative growth immediately following concurrent vaccine injection could be a result of the antigenic interactions, and the resultant stimulation of the immune response, both within the polyvalent, oil-AV and between the oil-AV and the DV.

Unfortunately I was unable to measure SGR of rainbow trout for the first 406 dd pvi and therefore am unable to state the exact relationship between RMR and growth following individual or concurrent vaccine injection. It is plausible, however, that the elevated RMR observed in rainbow trout at 203 dd pvi affected growth in a transient and negative manner, similar to that observed at 106 dd pvi in the Atlantic salmon. The negative growth observed in Atlantic salmon at 443 dd pvi was probably associated with the parr-to-smolt transformation. Thus, from Chapters 2 and 3, I can conclude that concurrent injection of a rhabdovirus-specific DV and a traditional, polyvalent, oil-AV significantly alters the allocation of energy in salmonids. Furthermore, while this can result in decreased growth of individuals, the effects are transient.

The use of polyvalent vaccines in aquaculture reduces the need for re-vaccination and allows fish to be protected against the majority of pathogenic diseases they might encounter throughout the production cycle [8, 11, 12, 39, 40]. Although beneficial in many aspects, the use of multiple antigens, either through polyvalent vaccines or the concurrent injection of multiple vaccines, increases the potential for interactive effects. Antigenic cross-protection, competition, and immunodominance, for example, can affect the specificity, avidity, and level of production

of specific antibodies [39-42]. In Chapters 3, 4, and 5, I examined key parameters of the innate and adaptive immune responses following concurrent injection of a rhabdovirus-specific DV and a polyvalent, bacterial, oil-AV.

In Chapter 3, I discovered that when concurrently injected with a DV and a polyvalent AV, rainbow trout exhibited an earlier seroconversion of virus-specific neutralizing antibodies (NAbs). It has been well established that when injected with a DV, salmonids respond with an early and a specific antiviral response [31, 43, 44]. The early antiviral response (EAVR) is a non-specific state mediated by the up-regulation of type I interferon-like (IFN) factors [28, 31, 43, 45], while the specific antiviral response (SAVR) is mediated by more specific adaptive immune factors including NAbs and other cellular immune factors [43, 44]. The non-specific EAVR, in particular the up-regulation of type I IFN related genes, is thought to be important for the stimulation of the specific adaptive immune response and the subsequent transition to the SAVR [28, 45-47]. While I did not measure the expression of type I IFN factors in Chapter 3, previous studies have confirmed that rhabdoviruses and rhabdovirus-specific DVs induce type I IFN factors for at least 200 dd pvi [33, 45]. There is also evidence that type I IFN factors can be induced by oil-type adjuvants and by the lipopolysaccharide (LPS) and DNA of the bacteria *Listonella anguillarum* [46, 48]. Fish that received both the DV and the polyvalent, oil-AV therefore, had three key stimuli for the induction and up-regulation of type I IFN genes: an oil-adjuvant, the LPS and DNA of *Listonella anguillarum* (a key component of the oil-AV), and the DV. This may have increased the amount of IFN proteins to a significant level, allowing for the earlier seroconversion of the NAbs. If this is the case, it might be beneficial to the aquaculture industry to include the LPS and DNA from *Listonella anguillarum* in rhabdovirus-specific DVs.

Caution must be used, however, when extrapolating the rainbow trout data to other salmonid species. Mutoloki *et al*. [17] made the observation that, while they are similar in a variety of physiological traits, rainbow trout and Atlantic salmon respond very differently to antigens and adjuvants. Rainbow trout appear to respond with a rapid onset of the inflammatory response (as indicated by INF factors), whereas Atlantic salmon produce a slower and more persistent response. In Chapter 3, I measured rhabdovirus-specific NAbs in rainbow trout as early as 203 dd pvi in fish that were concurrently injected with the DV and the AV, and as late as 305 dd pvi in fish that were injected with the DV alone. In Chapter 4, I was unable to detect any NAbs in Atlantic salmon until 413 dd pvi. Contrary to what we observed in rainbow trout, concurrent injection of a DV and an oil-AV in Atlantic salmon delayed the seroconversion of the rhabdovirus-specific NAbs. These data seem to support the idea of Mutoloki *et al*. [17] with

respect to differences in the development of the immune response between rainbow trout and Atlantic salmon.

In Chapter 4, I examined the interactive effects of multiple antigens on antigen-specific antibody (Ab) production in Atlantic salmon. It is well known that the immune system of vertebrates has a defined and limited capacity to respond to multiple antigens [40]. Polyvalent vaccine formulations therefore, are specially designed to maximize immune responsiveness and overall protection. Until now, however, there have been no published reports examining the impact concurrent vaccine injection has on the innate and adaptive immune responses of salmonids. Furthermore, there have been no published reports examining the impact of a DV on the innate and adaptive immune response of salmonids when injected concurrently with a polyvalent oil-AV. The results in Chapter 4 indicate that when injected with a DV and an AV, the innate immune response of Atlantic salmon increases in an almost synergistic-like manner. At 106, 201, and 297 dd pvi, the lysozyme activity of concurrently vaccinated Atlantic salmon was significantly greater than fish injected with phosphate-buffered saline (PBS), suggesting an increase in the inflammatory response. Unfortunately, because I was unable to measure other key innate immune parameters, such as alternative complement activity, or IFN-like factors, I cannot speculate as to the importance of this result with respect to overall immune responsiveness and protection.

Although I was only able to measure antigen-specific Ab production for two of the five antigens present in the polyvalent oil-AV, the results in Chapter 4 suggest that concurrent injection of a DV with a polyvalent AV has a positive effect on the production of antigen-specific Abs. Anti-*Aeromonas salmonicida* and anti-*Listonella anguillarum* Ab titres were significantly greater in individuals that were concurrently injected with the DV and the AV. It is important to remember that Ab titre is not always correlated with protection and can vary with vaccine formulation, species, and environment [49-53]. Due to permit limitations it was not possible to measure relative percent survival following vaccine injection and therefore I cannot predict if the observed differences in Ab titre correlate to differences in protective value for the fish.

In aquaculture, individual fish are exposed to a variety of stressors, including high densities, handling, and transportation [54]. It is well known that increased levels of plasma cortisol significantly increase disease susceptibility and affect immune responsiveness in Atlantic salmon [56-61]. Recent studies, however, have shown that the overall impact of elevated plasma cortisol on individual immunoresponsiveness and disease susceptibility is

species-specific, antigen-specific, and possibly related to the timing of the stressor [60, 62-65]. In Chapter 5, I demonstrate that if Atlantic salmon are vaccinated prior to supra-physiological elevations in plasma cortisol, the vaccine induced innate immune response is suppressed, while the adaptive immune response is unchanged. Although I could not correlate these findings with overall protection, these data are promising with respect to the salmonid aquaculture industry where individual fish are vaccinated 400 dd prior to sea water entry, an event that is known to significantly increase plasma cortisol levels [36, 55, 57, 61]. If Ab production is unchanged at the time of sea water entry, regardless of plasma cortisol levels, individual fish should maintain significant protection against the pathogens for which they were vaccinated. Although I observed no change in the adaptive immune response, lysozyme activity was significantly suppressed following the injection of supra-physiological levels of cortisol. Lysozyme activity is known to be suppressed following physiologically relevant elevations in cortisol [66-68]. It is unknown, however, if the level of cortisol affects overall protection and disease susceptibility.

To summarize, this thesis has demonstrated that DVs stimulate the immune response of salmonids with no negative growth-related side-effects. Concurrent injection of a DV with a traditional, polyvalent oil-AV, however, can influence the immune responsiveness of rainbow trout and Atlantic salmon in a species-specific manner. Because of the species-specific differences we found with relation to lysozyme activity, NAb titres, and antigen-specific Ab titres in individuals concurrently injected with a DV and a polyvalent AV, future studies needed to correlate these finding with overall protectiveness.

6.1 REFERENCES

[1] Stoffregen DA, Bowser PR, Babish JG. Antibacterial chemotherapeutants for finfish aquaculture: A synopsis of laboratory and field efficacy and safety studies. Journal of Aquatic Animal Health 1996; 8(3): 181-207.

[2] Chinabut S, Puttinaowarat S. The choice of disease control strategies to secure international market access for aquaculture products. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 255-61.

[3] Ellis AE. General principles of fish vaccination. In: Ellis, AE, editor. Fish Vaccination, London, UK: Academic Press; 1988, p. 1-19.

[4] Evelyn TP. A historical review of fish vaccinology. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 3-12.

[5] Ellis AE. Immunity to bacteria in fish. Fish and Shellfish Immunology 1999; 9: 291-308.

[6] Håstein T, Gudding R, Evensen Ø. Bacterial vaccines for fish – An update of the current situation worldwide. In: Midtlyng, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 55-74.

[7] Sommerset I, Krossøy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Review of Vaccines 2005; 4(1): 89-101.

[8] Midtlyng PJ, Reitan LJ, Speilberg L. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 335-50.

[9] Buchmann K, Dalsgaard I, Nielsen ME, Pedersen K, Uldal K, Garcia JA, Larsen JL. Vaccination improves survival of Baltic salmon (*Salmo salar*) smolts in delayed release sea ranching (net-pen period). Aquaculture 1997; 156: 335-348.

[10] Midtlyng PJ. Vaccinated fish welfare: Protection versus side-effects. In: Gudding R, Lillehaug A, Midtlyng PJ, and Brown F, editors. Fish Vaccinology: Developments in Biological Standardization. Basel, Karger; 1997, vol. 90 p. 371-79.

[11] Schijns VEJC, Tangerås A. Vaccine adjuvant technology: From theoretical mechanisms to practical approaches. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 127-34.

[12] Berg A, Rødseth OM, Tangerås A, Hansen T. Time of vaccination influences development of adhesions, growth and spinal deformities in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 2006; 69: 239-48.

[13] Berg A, Rødseth OM, Hansen T. Fish size at vaccination influences the development of side-effects in Atlantic salmon (*Salmo salar* L.). Aquaculture 2007; 265: 9-15.

[14] Poppe TT, Breck O. Pathology of Atlantic salmon *Salmo salar* intraperitonealy immunized with oil-adjuvanted vaccine. A case report. Diseases of Aquatic Organisms 1997; 29: 219-26.

[15] Mutoloki S, Alexandersen S, Evensen Ø. Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. Fish and Shellfish Immunology 2004; 16: 633-44.

[16] Evensen Ø, Brudeseth B, Mutoloki S. The vaccine formulation and its role in inflammatory processes in fish – Effects and adverse effects. In: Midtlyng PJ, editor. Progress in Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 2005, vol. 121, p. 117-126.

[17] Mutoloki S, Reite OB, Brudeseth B, Tverdal A, Evensen Ø. A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. Vaccine 2006; 24: 578-88.

[18] Mutoloki S, Brudeseth B, Reite O, Evensen Ø. The contribution of Aeromonas salmonicida extracellular products to the induction of inflammation in Atlantic salmon (*Salmo salar* L.) following vaccination with oil-based vaccines. Fish and Shellfish Immunology 2006; 20: 1-11.

[19] Mutoloki S, Alexandersen S, Gravningen K, Evensen Ø. Time-course study of injection site inflammatory reactions following intraperitoneal injection of Atlantic cod (*Gadus morhua* L.) with oil-adjuvanted vaccines. Fish and Shellfish Immunology 2008; 24: 386-93.

[20] Midtlyng PJ. A field study on intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. Fish and Shellfish Immunology 1996; 6: 553-65.

[21] Rønsholdt B, McLean E. The effect of vaccination and vaccine components upon short-term growth and feed conversion efficiency in rainbow trout. Aquaculture 1999; 174: 213-221.

[22] Ackerman PA, Iwama GK, Thornton JC. Physiological and immunological effects of adjuvanted *Aeromonas salmonicida* vaccines on juvenile rainbow trout. Journal of Aquatic Animal Health 2000; 12: 157-164.

[23] Pylkkö P, Lyytikäinen T, Ritola O, Sinikka Pelkonen S. Vaccination influences growth of Arctic charr. Diseases of Aquatic Organisms 2000; 43: 77-80.

[24] Melingen GO, Wergeland HI. Physiological effects of an oil-adjuvanted vaccine on out-of-season Atlantic salmon (*Salmo salar* L.) smolt. Aquaculture 2002; 214: 397-409.

[25] Sørum U, Damsgård B. Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). Aquaculture 2004; 232: 333-341.

[26] Anderson ED, Mourich DV, Leong JAC. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 1996; 5(2): 105-13.

[27] Anderson ED, Mourich DV, Fahrenkrug S, LaPatra S, Shepherd J, Leong JAC. Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 1996; 5(2): 114-22.

[28] Boudinot P, Blanco M, de Kinkelin P, Benmansour A. Combined DNA immunisation with the glycoprotein gene of viral hemorrhagic septicaemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific responses in rainbow trout. Virology 1998; 249: 297-306.

[29] Heppell J, Lorenzen N, Armstrong NK, Wu T, Lorenzen E, Einer-Jensen K, Schorr J, Davis HL. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology 1998; 8: 271-86.

[30] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish and Shellfish Immunology 1998; 8: 261-70.

[31] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, Leong JC. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. Journal of Virology 2000; 75(15): 7048-54

[32] Boudinot P, Bernard D, Boubekeur S, Thoulouze MI, Bremont M, Benmansour A. The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. Journal of General Virology 2004; 85: 3099-3108.

[33] Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish and Shellfish Immunology 2004; 17: 447-62.

[34] Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, Park LK. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Molecular Immunology 2006; 43: 2089-2106.

[35] Utke K, Kock H, Schuetze H, Bergmann SM, Lorenzen N, Einer-Jensen K, Köllner B, Dalmo RA, Vesely T, Ototake M, Fischer U. Cell-mediated immune response in the rainbow trout after DNA immunisation against the viral hemorrhagic septicaemia virus. Developmental and Comparative Immunology 2008; 32: 239-52.

[36] Hoar, WS. The physiology of smolting salmonids. In: Hoar WS, Randall DJ, editors. The Physiology of Developing Fish, Part B: Viviparity and post-hatching juveniles. Fish Physiology, Volume XI, London, UK: Academic Press; 1988, p. 275-343.

[37] Fair, JM, Hansen ES, Ricklefs RE. Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). Proceedings of the Royal Society of London B 1999; 266: 1735-42.

[38] Pilorz V, Jäckel M, Knudsen K, Trillmich F. The cost of a specific immune response in young guinea pigs. Physiology and Behaviour 2005; 85: 205-11.

[39] Nikoskelainen S, Verho S, Järvinen S, Madetoja J, Wiklund T, Lilius EM. Multiple whole bacterial antigens in polyvalent vaccine may result in inhibition of specific responses in rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology 2007; 22: 206-17.

[40] Busch RA. Polyvalent vaccines in fish: the interactive effects of multiple antigens. In: Gudding R, Lillehaug PJ, Midtlyng PJ, Brown F, editors. Fish Vaccinology; Developments in Biological Standardization, Basel, Switzerland: Karger; 1997, vol. 90, p. 245-56.

[41] Hoel K, Salonius K, Lillehaug A. *Vibrio* antigens of polyvalent vaccines enhance the humoral immune response to *Aeromonas salmonicida* antigens in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1997; 7: 71-80.

[42] Hoel K, Reitan LJ, Lillehaug A. Immunological cross reactions between *Aeromonas salmonicida* and *Vibrio salmonicida* in Atlantic salmon (*Salmo salar* L.) and rabbit. Fish and Shellfish Immunology 1998; 8: 171-82.

[43] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. Vaccine 2001; 19: 4011-19.

[44] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006; 24: 345-54.

[45] Robertsen B. Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. Fish and Shellfish Immunology 2008; 25: 351-7.

[46] Acosta F, Lockhart K, Gahlawat SK, Real F, Ellis AE. Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. Fish and Shellfish Immunology 2004; 255-63.

[47] Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Developmental and Comparative Immunology 2002; 26: 173-9.

[48] Haugland Ø, Torgersen J, Syed M, Evensen Ø. Expression profiles of inflammatory and immune-related genes in Atlantic salmon (*Salmo salar* L.) at early time post vaccination. Vaccine 2005; 23: 5488-99.

[49] Lund T, Chiayvareesajja J, Larsen HSJ, Røed KH. Antibody response after immunisation as a potential indirect marker for improved resistance against furunculosis. Fish and Shellfish Immunology 1995; 5: 109-119.

[50] Fjalestad KT, Jørgen H, Larsen S, Røed KH. Antibody response in Atlantic salmon (*Salmo salar*) against *Vibrio anguillarum* and *Vibrio salmonicida* O-antigens: Heritabilities, genetic correlations and correlations with survival. Aquaculture 1996; 145: 77-89.

[51] Gudmundsdóttir BK, Magnadóttir B. Protection of Atlantic salmon (*Salmo salar* L) against an experimental infection of *Aeromonas salmonicida* ssp. *Achromogenes*. Fish and Shellfish Immunology 1997; 7: 55-69.

[52] Gudmundsdóttir BK, Jónsdóttir H, Steinthórsdóttir V, Magnadóttir B, Gudmundsdóttir S. Survival and humoral antibody response of Atlantic salmon, *Salmo salar* L., vaccinated against *Aeromonas salmonicida* ssp. *Achromogenes*. Journal of Fish Diseases 1997; 20: 351-60.

[53] Hedrick RP. Relationships of host, pathogen, and environment: Implications for diseases of cultured and wild fish populations. Journal of Aquatic Animal Health 1998; 10: 107-11

[54] Wendelaar Bonga SE. The stress response in fish. Physiological Reviews 1997; 77(3): 591-625.

[55] Maule AG, Schreck CB. Changes in the immune system of Coho salmon (*Oncorhynchus kisutch*) during parr-to-smolt transformation and after implantation of cortisol. Canadian Journal of Fisheries and Aquatic Sciences 1987; 44: 161-6.

[56] Tripp RA, Maule A, Schreck CB, Kaattari SL. Cortisol mediated suppression of salmonid lymphocyte responses *in vitro*. Developmental and Comparative Immunology 1987; 11: 565-76.

[57] Maule AG, Tripp RA, Kaattari SL, Schreck CB. Stress alters immune function and disease resistance in Chinook salmon (*Oncorhynchus tshawytscha*). Journal of Endocrinology 1989; 120: 135-42.

[58] Pickering AD, Pottinger TG. Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. Fish Physiology and Biochemistry 1989; 7: 253-8

[59] Wiik R, Andersen K, Uglenes I, Egidius E. Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with effects of the blood cell pattern. Aquaculture 1989; 83: 201-15.

[60] Mazur CF, Iwama GK. Handling and crowding stress reduces number of plaque-forming cells in Atlantic salmon. Journal of Aquatic Animal Health 1993;5: 98-101.

[61] Espelid S, Løkken GB, Steiro K, Bøgwald J. Effects of cortisol and stress on the immune systemof Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 1996; 6: 95-110.

[62] Engelsma MY, Hougee S, Nap D, Hofenk M, Rombout JHWM, van Muiswinkel WB, Verburg-van Kemenade BML. Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio* L. Fish and Shellfish Immunology 2003; 15: 397-410.

[63] Singer TD, Finstad B, McCormick SD, Wiseman SB, Schulte PM, McKinley RS. Interactive effects of cortisol treatment and ambient seawater challenge on gill Na^+, K^+-ATPase and CFTR expression in two strains of Atlantic salmon smolts. Aquaculture 2003; 222: 15-28.

[64] Funk VA, Jones SRM, Kim E, Kreiberg H, Taylor K, Wu S, Young C. The effect of vaccination and sea water entry on immunocompetence and susceptibility to *Kudoa thyrsites* in Atlantic salmon (*Salmo salar* L.). Fish and Shellfish Immunology 2004; 17: 375-87.

[65] Lovy J, Speare DF, Stryhn H, Wright GM. Effects of dexamethasone on host innate and adaptive immune response and parasite development in rainbow trout O*ncorhynchus mykiss* infected with *Loma salmonae*. Fish and Shellfish Immunology 2008; 6: 649-58.

[66] Moeck A, Peters G. Lysozyme activity in rainbow trout, Oncorhynchus mykiss (Walbaum), stressed by handling, transport and water pollution. Journal of Fish Biology 1990; 37: 873-85.

[67] Yin Z, Lam TJ, Sin YM. The effects of crowding stress on the non-specific immune response of the fancy carp (*Cyprinus carpio*). Fish and Shellfish Immunology 1995; 5: 519-29.

[68] Fevolden SE, Røed KH, Fjalestad KT, Stein J. Post-stress levels of lysozyme and cortisol in adult rainbow trout: Heritabilities and genetic correlations. Journal of Fish Biology 1999; 54: 900-10.

APPENDICES

APPENDIX A

Animal care certificate for vaccine-related experimental studies

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A04-1018

Investigator or Course Director: Patricia M. Schulte

Department: Zoology

Animals:

Salmon Salmo salar 20000

Start Date: **December 13, 2004** **Approval Date:** **January 18, 2007**

Funding Sources:

Funding Agency:

Funding Title: The effect of a novel vaccine against infectious hematopoietic necrosis virus on the health and welfare of farmed Atlantic Salmon

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3 Phone: 604-827-5111 Fax: 604-822-5093