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

by

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## 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 supraphysiological 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

Abstract	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xiii
ACKNOWLEDGEMENTS	xvii
DEDICATION	xviii
CO-AUTHORSHIP STATEMENT	xix
CHAPTER ONE: INTRODUCTION	1
1.1 GENERAL INTRODUCTION	1
1.2 Research Aims	
1.3 LITERATURE REVIEW	5
1.3.1 Immune System	
1.3.1.1 INNATE IMMUNE RESPONSE	
1.3.1.1.1 Innate Humoral Immune Response	7
1.3.1.1.1 Inhibitors	7
1.3.1.1.1.2 Lysins	8
1.3.1.1.1.3 Complement	9
1.3.1.1.1.4 Interferons	10
1.3.1.1.1.5 Natural Antibodies	11
1.3.1.1.2 Innate Cell-Mediated Immune Response	
1.3.1.2 Adaptive Immune Response	
1.3.1.2.1 Adaptive Humoral Immune Response	
1.3.1.2.2 Adaptive Cell-Mediated Immune Response	
1.3.2 VACCINES	
1.3.2.1 VACCINE TYPES	
1.3.2.1.1 Whole Organism Vaccines	
1.3.2.1.1.1 Adjuvants	20
1.3.2.1.2 DNA Vaccines	
1.3.2.1.3 Polyvalent Vaccines	
1.3.2.3 VACCINE ADMINISTRATION	

1.3.2.4 VACCINE-RELATED SIDE-EFFECTS	23
1.3.3 CONCLUSION	24
1.4 TABLES	25
1.5 FIGURES	26
1.6 References	31
CHAPTER TWO: GROWTH AND PERFORMANCE OF ATLANTIC SALMON, SALMO SALAR L.,	
FOLLOWING ADMINISTRATION OF A RHABDOVIRUS DNA VACCINE ALONE OR CONCURREN	NTLY
2 1 INTRODUCTION	41
2.1 INTRODUCTION	41
2.2 THATERIALS AND METHODS	42
	42
2.2.2 VACCINATION I ROCEDORE	45
2.2.5 GROW III	45
2.2.1 SWIMMING FERIORALINE	46
2.3 RESULTS	47
2 3 1 GROWTH	47
2.3.2 Swimming Performance	48
2.4 Discussion	48
2.5 TABLES	52
2.6 FIGURES	53
2.7 References	57
CHAPTER THREE: THE ASSOCIATION BETWEEN METABOLIC RATE, IMMUNE PARAMETERS GROWTH PERFORMANCE OF RAINBOW TROUT, <i>ONCORHYNCHUS MYKISS</i> (WALBAUM),	5, AND
FOLLOWING THE INJECTION OF A DNA VACCINE ALONE AND CONCURRENTLY WITH A POLYVALENT, OIL-ADJUVANTED VACCINE,	60
3.1 INTRODUCTION	60
3.2 MATERIALS AND METHODS	61
3.2.1 Animal Care and Experimental Design	61
3.2.1.1 Experiment #1	61
3.2.1.2 Experiment #2	62
3.2.2 GENERAL EXPERIMENTAL PROCEDURES	62
3.2.2.1 VACCINATION PROCEDURE	62
3.2.2.2 Blood Sampling	63

3.2.2.3 SERUM LYSOZYME ACTIVITY	
3.2.2.4 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	
3.2.2.5 OXYGEN CONSUMPTION	
3.2.2.6 Statistical Analysis	
3.3 RESULTS	
3.3.1 Experiment #1	
3.3.1.1 WEIGHT	
3.3.1.2 Serum Lysozyme Activity	
3.3.1.3 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	
3.3.1.4 OXYGEN CONSUMPTION	
3.3.2 Experiment #2	
3.3.2.1 Weight	
3.3.2.2 Specific Growth Rate	
3.3.2.3 Daily Weight Gain	
3.3.2.4 Feed Conversion Ratio	
3.4 DISCUSSION	
3.5 TABLES	
3.6 FIGURES	
3.7 REFERENCES	
CHAPTER FOUR: CONCURRENT INJECTION OF A RHABDOVIRUS-SPECIFIC DN	A VACCINE WITH
A POLYVALENT, OIL-ADJUVANTED VACCINE DELAYS THE SPECIFIC ANTIVIRAL	L RESPONSE IN
A I LATE ODVICTION	
4.1 INTRODUCTION	
4.2 MATERIALS AND METHODS	
4.2.1 FISH STOCK AND REARING CONDITIONS	
4.2.2 VACCINATION PROCEDURE	
4.2.3 BLOOD SAMPLING	
4.2.4 SERUM LYSOZYME ACTIVITY	
4.2.5 IHINV SERUM NEUTRALIZING ANTIBODY TITRE	
4.2.0 ENZYME LINKED IMMUNOSORBENT ASSAY ANTIBODY 1 ITRE	
4.2. / STATISTICAL ANALYSIS	
<b>4.3 KESULTS</b>	
4.5.1 SERUM LYSOZYME ACTIVITY	

4.3.2 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	85
4.3.3 Enzyme Linked Immunosorbent Assay Antibody Titre	85
4.3.3.1 Anti-Aeromonas salmonicida Antibody Titre	85
4.3.3.2 Anti- <i>Listonella anguillarum</i> Antibody Titre	86
4.4 DISCUSSION	86
4.5 TABLES	90
4.6 FIGURES	91
4.7 References	97
CHAPTER FIVE: CORTISOL SUPPRESSES LYSOZYME ACTIVITY BUT NOT THE ANTIBODY RESPONSE IN ATLANTIC SALMON, <i>SALMO SALAR</i> L., FOLLOWING VACCINE INJECTION	103
5.1 INTRODUCTION	103
5.2 MATERIALS AND METHODS	104
5.2.1 FISH STOCK AND REARING CONDITIONS	104
5.2.2 VACCINATION PROCEDURE	104
5.2.3 Experimental Design	105
5.2.3.1 Experiment $\#1-53$ Degree Days Post-Vaccine Injection	105
5.2.3.2 Experiment #2 – 212 Degree Days Post-Vaccine Injection	106
5.2.4 BLOOD SAMPLING	106
5.2.5 SERUM CORTISOL	106
5.2.6 SERUM LYSOZYME ACTIVITY	107
5.2.7 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	107
5.2.8 Enzyme Linked Immunosorbent Assay Antibody Titre	107
5.2.9 Statistical Analysis	109
5.3 RESULTS	109
5.3.1 Experiment #1 – 53 Degree Days Post-Vaccine Injection	109
5.3.1.1 SERUM CORTISOL	109
5.3.1.2 SERUM LYSOZYME ACTIVITY	110
5.3.1.3 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	110
5.3.1.4 Enzyme Linked Immunosorbent Assay Antibody Titre	110
5.3.1.4.1 Anti-Aeromonas salmonicida Antibody Titre	110
5.3.1.4.2 Anti-Listonella anguillarum Antibody Titre	110
5.3.2 EXPERIMENT #2 – 212 DEGREE DAYS POST-VACCINE INJECTION	110
5.3.2.1 Serum Cortisol	110

5.3.2.2 SERUM LYSOZYME ACTIVITY	111
5.3.2.3 IHNV SERUM NEUTRALIZING ANTIBODY TITRE	111
5.3.2.4 Enzyme Linked Immunosorbent Assay Antibody Titre	111
5.3.2.4.1 Anti-Aeromonas salmonicida Antibody Titre	
5.3.2.4.2 Anti-Listonella anguillarum Antibody Titre	
5.4 DISCUSSION	
5.5 FIGURES	
5.6 References	
CHAPTER SIX: CONCLUSIONS	
6.1 References	
APPENDICES	
Appendix A	

# LIST OF TABLES

<b>Table 1.1</b> Defense mechanisms in teleost fish.    25
<b>Table 2.1</b> Type of vaccine(s) injected intraperitonealy (IP) and intramuscularly (IM) intoAtlantic salmon (Salmo salar L.) (wt: $39.1 \pm 0.4$ g, mean $\pm$ SE)
<b>Table 2.2</b> Mean Fulton condition factor $[K=100(wt \cdot L_F^{-3})] (\pm SE)$ 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)
<b>Table 2.3</b> Measures of mean swimming performance ( $U_{crit,1}$ , $U_{crit,2}$ , RR, and normalized RR values) ( $\pm$ 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)
<b>Table 3.1</b> 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
<b>Table 3.2</b> 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 ( $\pm$ SE), n = 3
<b>Table 4.1</b> 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

# LIST OF 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.

**Figure 2.2** Fork length of Atlantic salmon following injection of phosphate-buffered saline (control group;O), 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.

**Figure 3.1** Serum lysozyme activity of juvenile rainbow trout following injection of phosphatebuffered 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  $\pm$  SE (n = 10). <sup>a, b, c, d</sup> Significant differences between vaccine group; <sup>w, x, y</sup> Significant differences between sampling periods within a vaccine group; two-way ANOVA, P < 0.05.....72

**Figure 3.2** Oxygen consumption (Mo<sub>2</sub>) 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  $\pm$  SE (n = 8). <sup>a, b, c, d</sup> Significant differences between vaccine group; <sup>w, x, y</sup> Significant differences between sampling periods within a vaccine group; two-way ANOVA, *P* < 0.05..... 73

**Figure 4.1** Serum lysozyme activity of Atlantic salmon following injection of phosphatebuffered 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. <sup>a, b, c, d</sup> Significant differences between vaccine groups. <sup>w, x, y, z</sup> Significant differences between sampling periods within a vaccine group. Values are mean ± SE. (n = 7-10); 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

## 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 <sup>-1</sup>	body length per second
BSA	bovine serum albumin
C	constant domain (or segment)
$Ca^{2+}$	calcium ion
CAER	Centre for Aquaculture and Environmental Research
ССР	classical complement pathway
CDR	complementarity-determining region
C <sub>H</sub>	constant domain – Heavy chain
$C_L$	constant domain – Light chain
cm	centimetre $(10^{-2} \text{ m})$
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
Ğ	glycoprotein
H <sub>c</sub>	antibody heavy chain
h	hour
$H_2O_2$	hydrogen peroxide
$H_2SO_4$	sulphuric acid
HEWLeq	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
Κ	Fulton's condition factor
Kg	kilogram $(10^3 \text{ g})$
L	litre
L <sub>c</sub>	antibody light chain
LAVR	long-term antiviral response
LCP	lectin complement pathway
$L_{ m F}$	fork length
LPC	long-lived plasma cells
LPS	lipopolysaccharide
LSW	low salt wash buffer
m	metre
М	molar (moles litre <sup>-1</sup> )
MAb	monoclonal antibody
MAC	membrane attack complex
MBP	mannan-binding protein
m	metre
mg	milligram $(10^{-3} \text{ g})$
MHC	major histocompatibility complex
min	minute
Mo <sub>2</sub>	oxygen consumption
MPO	myeloperoxidase
mRNA	messenger RNA
MSH	melaophore stimulating hormone
MS222	tricaine methane sulphonate
Mx	myxovirus protein
μg	microgram $(10^{-6} \text{ g})$
μL	microlitre $(10^{-6} L)$
NAb	neutralizing antibody
NaCl	sodium chloride

NADPH	nicotinamide adenine dinucleotide
NaHCO <sub>3</sub>	sodium bicarbonate
NCC	non-specific cytotoxic cell
NE	norepinephrine
ng	nanogram $(10^{-9} \text{ g})$
nm	nanometre $(10^{-9} \text{ m})$
NK	natural killer cell
NO	nitric oxide
O <sub>2</sub> -	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	$U_{\rm crit}$ 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
Тс	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 <sub>crit</sub>	critical swimming speed
USD	United States Dollar
V	variable domain (or segment)
$V_{\rm H}$	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
$\downarrow$	inhibitory
°C	degrees Celsius
<b>‰</b>	parts per thousand
50% PNT	50% plaque neutralization titre

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"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

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## **CO-AUTHORSHIP STATEMENT**

Chapter Two:	Growth and performance of Atlantic salmon, <i>Salmo salar</i> 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, <i>Salmo salar</i> 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- <i>Listonella anguillarum</i> 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 fiftynine 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 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 sideeffects [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 response 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  $\alpha$ 1-antiproteinase and  $\alpha$ 2-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  $\beta$  (1 $\rightarrow$ 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<sup>-1</sup>) 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  $\alpha$  and  $\beta$  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- $\gamma$ , is produced by nonspecific 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- $\lambda$ , 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- $\alpha$  (TNF- $\alpha$ ), 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<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sup>+</sup> [61, 89].

Recent studies have shown a developmental relationship between vertebrate Blymphocytes [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 ish-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<sub>c</sub> and L<sub>c</sub>, 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<sub>c</sub> and L<sub>c</sub> (V<sub>H</sub> and V<sub>L</sub>), as well as the constant domain of the L<sub>c</sub> (C<sub>L</sub>) and the N-terminal constant domain of the H<sub>c</sub> (C<sub>H</sub>) [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<sub>c</sub> 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<sub>c</sub>, 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<sub>c</sub> 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, Blymphocytes in the peripheral immune system undergo proliferation and differentiation into short-lived (life span of days to weeks) plasma cells. During this process, memory Blymphocytes 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  $\gamma$ -IFN, TNF- $\beta$ , 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 Tlymphocyte (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- $\alpha$ , 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

18

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 revaccination [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<sup>5</sup> 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 (Mo<sub>2</sub>), 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	1 Ellis [21].
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Innote Humanal Immuna Dognamaa	Innata Call Madiated Immuna Dograms
(a) Inhibitors	(a) Neutrophils
(1) Transferrin (different genotypes)	(1) Respiratory burst $\rightarrow O_2^-$ , H <sub>2</sub> O <sub>2</sub> , 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 (+ $\Omega_2^- \rightarrow$ peroxynitrite $\rightarrow OH^+$ )
(iv) Complement (lytic pro-inflammatory	
chemotactic opsonic interacts with cell-	
madiated response	
(x) interferen	
(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	$\downarrow$
(iii) Anti-invasins	Cytokines (IFNy, TNF)
(iv) Activates classical complement pathway	
(v) immunological memory	* Activate macronhages (enhanced RR enhanced
() minimuloiogical memory	hactericidal activity
	Dacterieruar activity

MPO, myeloperoxidase; IFN $\gamma$ , interferon gamma; TNF, tumour necrosis factor; NO, nitric oxide; O<sub>2</sub><sup>-</sup>, superoxide anion; OH<sup>+</sup>, 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]





**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].

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# 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.<sup>1</sup>

## **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 (Mo<sub>2</sub>), 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

<sup>&</sup>lt;sup>1</sup>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<sup>®</sup>; 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 °C) housed all 240 experimental fish (average density at time of transfer was 8.6 kg m<sup>-3</sup>) 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 °C) 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<sub>3</sub>; Sigma Aldrich, Oakville, ON, Canada) was added to the MS222 in a 1:2 ratio (100 mg L<sup>-1</sup> MS222 to 200 mg L<sup>-1</sup> NaHCO<sub>3</sub>) as a buffering agent. Fish from the control group were injected with (i) 50  $\mu$ L of phosphate-buffered saline (PBS) intramuscularly (IM), immediately anterior and lateral to the dorsal fin (i.e. in the epaxial muscle) and (ii) 100  $\mu$ L 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  $\mu$ L IM of PBS and (ii) 100  $\mu$ L 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<sup>®</sup>; Novartis Aqua Health). Fish from the DV group were injected with (i) 50  $\mu$ L IM of a rhabdovirus DV containing 10  $\mu$ g of plasmid encoding the G-protein gene from the IHN virus (APEX IHN<sup>®</sup>; Novartis Aqua Health) and (ii) 100  $\mu$ L IP of PBS. Fish from the combined vaccine group were injected with (i) 50  $\mu$ L IM of the DV and (ii) 100  $\mu$ L 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  $\mu$ g DNA in a volume of 10–50  $\mu$ L [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<sup>-1</sup> MS222 to 1000 mg L<sup>-1</sup> NaHCO<sub>3</sub>). 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<sup>-1</sup>). Because SW has a significantly greater buffering capacity than FW, NaHCO<sub>3</sub> 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:  $K = 100 \text{ (wt } L_{\text{F}}^{-3} \text{ )}$ , where wt is the individual weight of a fish to the nearest 0.1 g, and  $L_{\text{F}}$  is the fork length to the nearest 0.1 cm; SGR = 100 [(lnwt<sub>2</sub> - lnwt<sub>1</sub>) · (t<sub>2</sub> - t<sub>1</sub>)<sup>-1</sup>], where SGR is the mean growth rate achieved [% degree day<sup>-1</sup>, wt<sub>2</sub> and wt<sub>1</sub> are the weights of an individual fish to the nearest 0.1 g at sampling times t<sub>2</sub> and t<sub>1</sub> (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  $L_{\text{F}}$ . As such the SGR calculations made for this time period assume that wt<sub>1</sub> and t<sub>1</sub> are from the day of vaccination, and wt<sub>2</sub> and t<sub>2</sub> 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  $L_{\text{F}}$ . Therefore, while in SW, wt<sub>1</sub> and t<sub>1</sub> reflect the previous sampling period and wt<sub>2</sub> and t<sub>2</sub> 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<sup>-1</sup> MS222 to 200 mg  $L^{-1}$  NaHCO<sub>3</sub>), 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  $1.2 \text{ m s}^{-1}$  [22], fish were left to swim overnight (14 - 16 h) at a constant speed of 0.1 m s<sup>-1</sup>.  $U_{\text{crit}}$ , 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<sup>-1</sup> and increased in increments of 0.1 m s<sup>-1</sup> 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<sup>-1</sup>) were calculated as in Brett [23] and the RR and normalized RR were determined as described in Jain *et al.* [15].  $U_{crit} = U_i + [(t_i \cdot U_{ii}) \cdot U_{ii}]$  $(t_{ii})^{-1}$  where  $U_i$  is the highest velocity at which the fish swam for the entire time period (m s<sup>-1</sup>),  $\mu_{ii}$  is the incremental speed increase (m s<sup>-1</sup>), t<sub>i</sub> 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 =  $(U_{\text{crit}2}) \cdot (U_{\text{crit}1})^{-1}$ ; normalized RR =  $[(U_{\text{crit}1}) \cdot (U_{\text{crit}1(\text{control})})^{-1} + (U_{\text{crit}2}) \cdot (U_{\text{crit}1(\text{control})})^{-1}] \cdot 2^{-1}$ . 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 ( $\pm$  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:  $39.1 \pm 0.4$  g,  $L_F$ :  $15.2 \pm 0.1$  cm; mean  $\pm$  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  $(36.4 \pm 2.1 \text{ g})$  were significantly smaller than fish from the unvaccinated control group  $(44.3 \pm 2.1 \text{ g})$ . Fish from the AV group  $(35.5 \pm 0.8 \text{ g})$  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  $(41.9 \pm 2.0 \text{ g})$ . 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  $(44.9 \pm 1.6 \text{ g})$  than the unvaccinated control fish  $(52.1 \pm 2.1 \text{ g})$ , but were similar in weight to both the DV (48.3  $\pm$  1.6 g) and the combined vaccine (46.7  $\pm$  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  $U_{\text{crit}}$  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 sideeffects, 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, 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) int	3
Atlantic salmon (Salmo salar L.) (wt: $39.1 \pm 0.4$ g, mean $\pm$ 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  $[K=100(wt \cdot L_F^{-3})] (\pm SE)$  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	n	Control	Adjuvant	<b>DNA vaccine</b>	Combined
post-vaccination		group	vaccine group	group	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) ( $\pm$  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	п	$U_{\text{crit},1}$ (bl sec <sup>-1</sup> )	$U_{\text{crit},2}$ (bl sec <sup>-1</sup> )	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  $U_{\text{crit},1}$  and  $U_{\text{crit},2}$  within the combined vaccine group; t-test, P < 0.05 $U_{\text{crit}} = U_i + [(t_i \cdot U_{ii}) \cdot (t_{ii})^{-1}]; \text{RR} = (U_{\text{crit},2}) \cdot (U_{\text{crit},1})^{-1}; \text{ normalized } \text{RR} = [(U_{\text{crit},1}) \cdot (U_{\text{crit},1(\text{control})})^{-1} + (U_{\text{crit},2}) \cdot (U_{\text{crit},1(\text{control})})^{-1}] \cdot 2^{-1}$
# **2.6 FIGURES**



**Figure 2.1** Mean weight of Atlantic salmon following injection of phosphate-buffered saline (control group;O), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group; $\blacksquare$ ), a DNA vaccine (DNA vaccine group; $\nabla$ ), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group; $\bigstar$ ). 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  $\pm$  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;O), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group; $\blacksquare$ ), a DNA vaccine (DNA vaccine group; $\nabla$ ), or concurrent injection of a polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group; $\bigstar$ ). 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  $\pm$  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 =  $100 [(\ln wt_2 - \ln wt_1) \cdot (t_2 - t_1)^{-1}])$  of Atlantic salmon following injection of phosphate-buffered saline (control group;O), a polyvalent, oil-adjuvanted vaccine (adjuvant vaccine group; $\blacksquare$ ), a DNA vaccine (DNA vaccine group; $\bigtriangledown$ ), or concurrent injection of polyvalent, oil-adjuvanted vaccine and a DNA vaccine (combined vaccine group; $\bigstar$ ). 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  $\pm$  SE. 0 dd, n = 60; 106, 210, 296, 413 dd, n = 7-10; 443, 683, 990, 1300, 1616, 2028 dd, n = 18-24.



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# 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.<sup>1</sup>

# **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

<sup>&</sup>lt;sup>1</sup>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  $32.5 \pm 0.3$  g; 4 tanks per vaccine group]. Each tank of fish was treated in a similar manner and received 50 g day<sup>-1</sup> 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 (Mo<sub>2</sub>) 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:  $63.3 \pm 0.7$  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 [(lnwt<sub>2</sub> – lnwt<sub>1</sub>) · (t<sub>2</sub>-t<sub>1</sub>)<sup>-1</sup>] where SGR is the mean growth rate achieved [% degree day<sup>-1</sup>; wt<sub>2</sub> and wt<sub>1</sub> are the bulk weights of each tank of fish to the nearest 0.1 g at sampling times t<sub>2</sub> and t<sub>1</sub> (in dd) respectively]. WG = [(wt<sub>2</sub> – wt<sub>1</sub>) · (wt<sub>1</sub>)<sup>-1</sup>] x 100 where WG is the mean daily weight gain [% degree day<sup>-1</sup>, wt<sub>2</sub> and wt<sub>1</sub> are the bulk weights of each tank of fish to the nearest 0.1 g at sampling times t<sub>2</sub> and t<sub>1</sub> (in dd) respectively]. FCR = (total feed intake) · (total weight gain)<sup>-1</sup> 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<sup>-1</sup>) buffered with sodium bicarbonate (NaHCO<sub>3</sub>; Sigma Aldrich, Oakville, ON, Canada) (200 mg L<sup>-1</sup>). 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L of PBS IM, and 100  $\mu$ L 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<sup>®</sup>; Novartis Aqua Health, Charlottetown, PE, Canada). Fish from the DNA vaccine (DV) group were injected with 50  $\mu$ L IM of a rhabdovirus DNA vaccine containing 10  $\mu$ g of plasmid encoding the G-protein gene from the IHN virus (APEX IHN<sup>®</sup>; Novartis Aqua Health) and 100  $\mu$ L of PBS IP. Fish from the combined vaccine group were injected with 50  $\mu$ L of the DV IM and 100  $\mu$ L 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<sup>-1</sup>) buffered with NaHCO<sub>3</sub> (1000 mg L<sup>-1</sup>). 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  $\mu$ L of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250  $\mu$ L 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<sub>450</sub>) over a 20 min period at 25 °C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity ( $\mu$ g mL<sup>-1</sup> 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<sup>-1</sup>) 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 (Mo<sub>2</sub>) 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 Mo<sub>2</sub> measurements. After being immobilized with a non-lethal dose of MS222 (100 mg L<sup>-1</sup>) buffered with NaHCO<sub>3</sub> (200 mg L<sup>-1</sup>), 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 Mo<sub>2</sub> 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 (Mo<sub>2</sub> measurement) mode. Water temperature (°C) and dissolved oxygen concentration (mg L<sup>-1</sup>) 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<sup>-1</sup> h<sup>-1</sup>) 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 ( $\pm$  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:  $32.5 \pm 0.3$  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  $63.6 \pm 1.9$  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 Mo<sub>2</sub> 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 Mo<sub>2</sub> 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:  $63.3 \pm 0.7$  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  $323.8 \pm 1.9$  g.

#### 3.3.2.2 SPECIFIC GROWTH RATE

At 798 dd pvi, the SGR of the AV  $(0.17 \pm 0.001\% \text{ dd}^{-1})$ , DV  $(0.17 \pm 0.002\% \text{ dd}^{-1})$  and combined vaccine  $(0.17 \pm 0.001\% \text{ dd}^{-1})$  groups were similar and all significantly higher than the SGR of the control group  $(0.16 \pm 0.002\% \text{ dd}^{-1})$ . 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  $(0.11 \pm 0.001\% \text{ dd}^{-1})$ , DV  $(0.11 \pm 0.001\% \text{ dd}^{-1})$ , and combined vaccine  $(0.11 \pm 0.002\% \text{ dd}^{-1})$  groups had similar SGRs which were significantly higher than the SGR of the control group  $(0.10 \pm 0.003\% \text{ dd}^{-1})$ .

# 3.3.2.3 DAILY WEIGHT GAIN

Similar to the SGR, the percent daily WG of the AV  $(0.24 \pm 0.002 \% \text{ dd}^{-1})$ , DV  $(0.24 \pm 0.003 \% \text{ dd}^{-1})$ , and combined vaccine  $(0.24 \pm 0.002 \% \text{ dd}^{-1})$  groups were significantly greater than the WG of the control group  $(0.23 \pm 0.004 \% \text{ dd}^{-1})$  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  $(0.14 \pm 0.001 \% \text{ dd}^{-1})$ , DV  $(0.14 \pm 0.002 \% \text{ dd}^{-1})$ , and combined vaccine  $(0.14 \pm 0.001\% \text{ dd}^{-1})$  groups was significantly greater than that of the WG of the control group  $(0.12 \pm 0.004 \% \text{ dd}^{-1})$ .

### 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 ( $0.80 \pm 0.03$ ), DV ( $0.81 \pm 0.02$ ), and combined vaccine ( $0.81 \pm 0.01$ ) groups were similar and significantly lower than the FCR of the control group ( $0.87 \pm 0.01$ ).

# **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 antibacterial 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.

	203 degree days post-		305 degree days post-		406 degree days post-	
Fish #	vaccine injection		vaccine injection		vaccine injection	
	DNA	Combined	DNA	Combined	DNA	Combined
π	vaccine	vaccine	vaccine	vaccine	vaccine	vaccine
	group	group*	group	group	group*	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	$\geq 160$	20
7	< 20	40	$\geq 160$	40	$\geq 160$	20
8	< 20	80	$\geq 160$	$\geq 160$	$\geq 160$	40
9	20	80	$\geq 160$	$\geq 160$	$\geq 160$	80
10		$\geq 160$	$\geq 160$	$\geq 160$		≥ 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 phosphatebuffered 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 ( $\pm$  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) <sup>a,w</sup>	$120.2(2.8)^{a,w}$
1204 dd pvi	$214.2 (4.4)^{a,x}$	209.6 (1.1) <sup>ax</sup>	$209.6 (2.8)^{a,x}$	$208.1(3.9)^{a.x}$
1610 dd pvi	320.2 (4.5) <sup>a,y</sup>	325.4 (2.6) <sup>a,y</sup>	324.8 (5.6) <sup>a,y</sup>	324.8 (3.5) <sup>a,y</sup>

<sup>a, b, c, d</sup> Significant differences between vaccine groups at each sampling period;

<sup>w, x, y</sup> 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 phosphatebuffered 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  $\pm$  SE (n = 10). <sup>a, b, c, d</sup> Significant differences between vaccine group; <sup>w, x, y</sup> Significant differences between sampling periods within a vaccine group; two-way ANOVA, P < 0.05.



**Figure 3.2** Oxygen consumption (Mo<sub>2</sub>) 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  $\pm$  SE (n = 8). <sup>a, b, c, d</sup> Significant differences between vaccine group; <sup>w, x, y</sup> Significant differences between sampling periods within a vaccine group; two-way ANOVA, P < 0.05.

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# 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.<sup>1</sup>

# **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 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

<sup>&</sup>lt;sup>1</sup>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<sup>®</sup>; 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 °C). 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<sup>®</sup>; 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: Svndell Laboratories, Vancouver, BC, Canada). Sodium bicarbonate (NaHCO<sub>3</sub>; 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<sub>3</sub>) 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  $\mu$ L 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  $\mu$ L 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<sup>®</sup>; 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<sup>®</sup>) 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<sup>-1</sup> MS222 to 1000 mg L<sup>-1</sup> NaHCO<sub>3</sub>). 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 °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 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  $\mu$ L of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250  $\mu$ L 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<sub>450</sub>) over a 20 min period at 25 °C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity ( $\mu$ g mL<sup>-1</sup> 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<sup>-1</sup>) 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 polv-Llysine 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<sup>-1</sup>) and plates were incubated overnight at 4 °C. 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 °C for 72 h in tryptic soy broth (TSB), while Listonella anguillarum serotype O1 was cultured at 25 °C 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 °C. 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<sup>-</sup> <sup>1</sup> 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  $\mu$ L well<sup>-1</sup> of chromogen in substrate buffer [150  $\mu$ L 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<sub>2</sub>O<sub>2</sub>)]. Following a 10 min incubation at 21 °C, the reaction was terminated with the addition of 50  $\mu$ L well<sup>-1</sup> of 2 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was measured at OD<sub>450</sub>. The ELISA Ab titre was defined as the reciprocal of the highest dilution showing an OD<sub>450</sub> 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,  $100 \,\mu$ L well<sup>-1</sup> 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 ( $\pm$  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  $(10 - 12 \,^{\circ}C)$ , 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 formalininactivated 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	$\geq 160$	< 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 phosphatebuffered 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. <sup>a, b, c, d</sup> Significant differences between vaccine groups. <sup>w, x, y, z</sup> Significant differences between sampling periods within a vaccine group. Values are mean ± SE. (n = 7-10); Two-way ANOVA, P < 0.05.



**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<sub>450</sub>) 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. <sup>a, b, c, d</sup> Significant differences between vaccine groups. <sup>w, x, y, z</sup> Significant differences between sampling periods within a vaccine group. Values are mean  $\pm$  SE. (n = 7-10); Two-way ANOVA, P < 0.05.



**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<sub>450</sub>) 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. <sup>a, b, c, d</sup> Significant differences between vaccine groups. <sup>w, x, y, z</sup> Significant differences between sampling periods within a vaccine group. Values are mean  $\pm$  SE. (n = 7-10); Two-way ANOVA, *P* < 0.05.



anti-Listonella anguillarum serotype O1 Antibody Titre (serum dilution-<sup>1</sup>)

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# CHAPTER FIVE: CORTISOL SUPPRESSES LYSOZYME ACTIVITY BUT NOT THE ANTIBODY RESPONSE IN ATLANTIC SALMON, *SALMO SALAR* L., FOLLOWING VACCINE INJECTION.<sup>1</sup>

# **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 pathogenspecific 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

<sup>&</sup>lt;sup>1</sup>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 °C). 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<sup>®</sup>; 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<sub>3</sub>; Sigma Aldrich, Oakville, ON, Canada) in a 1:2 ratio (100 mg L<sup>-1</sup> MS222 to 200 mg L<sup>-1</sup> NaHCO<sub>3</sub>). 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  $\mu$ L of 0.02 M phosphate-buffered saline (PBS) IM, and 100  $\mu$ L of PBS IP. Fish from the adjuvant vaccine (AV) group

were injected with 50  $\mu$ L of PBS IM, and 100  $\mu$ L IP of a commercially available, polyvalent, oiladjuvanted vaccine containing formalin inactivated, whole-cell bacterins for *Aeromonas salmonicida*, *Listonella anguillarum* serotype O1 and O2, *Vibrio ordalii*, and *Vibrio salmonicida* (Lipogen Forte<sup>®</sup>; Novartis Aqua Health, Charlottetown, PE, Canada). Fish from the DNA vaccine (DV) group were injected with 50  $\mu$ L IM of a rhabdovirus DNA vaccine containing 10  $\mu$ g of plasmid encoding the glycoprotein (G) gene from the IHN virus (APEX IHN<sup>®</sup>; Novartis Aqua Health), and 100  $\mu$ L of PBS IP. Fish from the combined group were injected with 50  $\mu$ L of the DV IM, and 100  $\mu$ L 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<sup>-1</sup> body weight in a 1:1 vegetable oil:vegetable shortening vehicle (Crisco<sup>®</sup>, 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<sup>-1</sup> MS222 to 1000 mg L<sup>-1</sup> NaHCO<sub>3</sub>). 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 °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. 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 enzymelinked 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  $\mu$ L of serum (or hen egg white lysozyme standard) was incubated in triplicate with 250  $\mu$ L 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<sub>450</sub>) over a 20 min period at 25 °C was reported in micrograms per millilitre equivalent of hen egg white lysozyme activity ( $\mu$ g mL<sup>-1</sup> 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<sup>-1</sup>) 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  $\mu$ L well<sup>-1</sup>) and plates were incubated overnight at 4 °C. 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 °C for 72 h in tryptic soy broth (TSB), while Listonella anguillarum serotype O1 was cultured at 25 °C for 24 h in TSB with the addition of 2 % sodium chloride (NaCl). Bacteria were washed three times with PBS (8 min at  $5000 \times g$ ) 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 °C. 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<sup>-1</sup> anti-rainbow trout/Atlantic salmon MAb (Aquatic Diagnostics Ltd) was added and plates were incubated at 21 °C 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<sup>-1</sup> 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<sub>2</sub>O<sub>2</sub>)]. Following a 10 min incubation at 21 °C, the reaction was terminated with the addition of 50 µL well<sup>-1</sup> of 2 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was measured at OD<sub>450</sub>. 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  $\mu$ L well<sup>-1</sup> 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 ( $\pm$  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  $109.2 \pm 7.6$  ng mL<sup>-1</sup> and the combined mean serum cortisol level of the cortisol treatment was  $1512.1 \pm 139.8$  ng mL<sup>-1</sup>.

#### 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  $133.8 \pm 12.13$  ng mL<sup>-1</sup> and the combined mean serum cortisol level of the cortisol treatment was  $1060.1 \pm 138.4$  ng mL<sup>-1</sup>.

#### 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 shortterm 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 antiimmunoglobulin Abs [11, 31]. Recent studies have shown that the overall impact of elevated plasma cortisol on individual immuno-responsiveness and disease susceptibility is speciesspecific, 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 vaccineinduced 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 °C), 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<sup>-1</sup> [6, 65, 66], with average levels in farmed Atlantic salmon more variable at 8 ng mL<sup>-1</sup> [29], 16 ng mL<sup>-1</sup> [67], 76 ng mL<sup>-1</sup> [10], and 82 ng mL<sup>-1</sup> [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 ( $67 \pm 28.8$  and  $265.6 \pm 66.3$  ng mL<sup>-1</sup>) 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 ( $\square$ ) and cortisol ( $\blacksquare$ ) treatments. Fish in the cortisol treatment were injected intraperitonealy with a cortisol implant (50 µg cortisol g<sup>-1</sup> 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  $\pm$  SE. (n = 5) two-way ANOVA, P < 0.05.







**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  $\pm$  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  $\pm$  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 ( $\Box$ ) and cortisol ( $\blacksquare$ ) 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  $\pm$  SE. (n = 5) two-way ANOVA, P < 0.05.





# **5.6 REFERENCES**

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## **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-tosmolt 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 oiladjuvant, 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

131

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.

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# **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