Improving Aquaculture: the Impact of Bacterial Disease Treatments on Salmonid Immune Performance

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

In the case of published chapters, the numbering system of any published chapters has been updated to fit this thesis.

Chapter 2 – L. Al-Hussinee performed the initial infection trials to determine resistant/susceptible rainbow trout families. C.J. Kellendonk genotyped the MH class II β1 genes in six of the forty rainbow trout families and analyzed these results. All other experiments, analyses, and the final manuscript preparation was completed by S.L. Semple. This chapter was published in 2018 in *Aquaculture*, volume 483, pp. 131-140.

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Chapter 4 – T. Rodríguez-Ramos determined the minimum inhibitory concentration (MIC) of PACAP for *F. psychrophilum* and helped with determining bacterial survival throughout live infection of RTS11. All other experiments, analyses, and the final manuscript preparation was completed by S.L. Semple. This chapter was published in 2019 in *Frontiers in Immunology*, volume 10, pp. 1-14.

Chapter 5 – G. Heath optimized and completed the subtractive indirect ELISA assay to determine relative serum IgM levels against *Vibrio anguillarum* between the Chinook salmon crosses. C. Filice helped to complete approximately 40% of the MH class II β1 genotyping. All other experiments, analyses and the final manuscript preparation was completed by S.L. Semple. This chapter was formatted for submission to *Nature Heredity*.

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Abstract

The farming of aquatic organisms, or aquaculture, is a global multibillion-dollar industry that is continuously threatened by infectious diseases, including those of bacterial origin. Currently, the only method to combat bacterial disease outbreaks as they occur is the use of antibiotics. Though effective, this method increases both the prevalence and risk of generating antibiotic resistant bacteria. Any prophylactic treatments available, such as vaccines, provide slightly increased protection but are in need of improvements. To facilitate vaccine design and/or alternative treatment efforts, a deeper understanding of the teleost immune system is essential. When considering finfish aquaculture production in Canada, the industry is currently dominated by salmonids, which includes freshwater rainbow trout (*Oncorhynchus mykiss*) and saltwater Chinook salmon (*Oncorhynchus tshawytcha*). The contents of this thesis examine bacterial diseases that are relevant to the intensive culture of these two salmonid species. More specifically, both *in vitro* and *in vivo* analyses were used to explore the immune performance of salmonids when assessing breeding strategies, bacterial pathogenesis and alternative treatment options.

The freshwater rainbow trout model was used to study infection with *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease (BCWD). Initially, forty full-sibling families were created and assessed for resistance or susceptibility to *F. psychrophilum* following i.p. injection with the pathogen. Based on survival, the immune performance of the highest and lowest performing crosses was evaluated in regard to serum IgM production, MH class II β1 genotype and respiratory burst activity (RBA) in replicated infection trials. Though there were no significant differences observed between the high and low performers for any of the immune parameters analyzed, RBA of head kidney leukocytes was observed to significantly decrease at the time when fish were presenting clinical signs of disease. This observation led to the use of the monocyte/macrophage-like cell line, RTS11, as a model system to learn more about the pathogenesis of the organism, an area of limited understanding. Exposing RTS11 to live, heat-killed and the conditioned media of *F. psychrophilum* (*Fp*CM) revealed that the *Fp*CM significantly reduced the phagocytic activity of

RTS11. Furthermore, *Fp*CM alone was able to stimulate cytokine transcript expression in a manner that was similar to live bacteria but was short-lived comparatively. Meanwhile, heat-killed *F. psychrophilum* resulted in little to no changes of RTS11, indicating that the common use of bacterins for fish vaccines may not be optimal for all bacterial pathogens. Lastly, the efficacy of an antimicrobial peptide, PACAP as an alternative treatment option for BCWD was assessed using the RTS11 infection model system. PACAP was found to permeabilize the membrane of *F. psychrophilum* and, when RTS11 received 24 hr pre-treatment with the peptide, could stimulate the immune cells to decrease the number of viable bacteria in culture. Additionally, PACAP was able to significantly stimulate transcript expression of pro-inflammatory cytokines both in the presence or absence of *F. psychophilum*.

Vibrio anguillarum, the causative agent of the hemorrhagic septicaemia known as vibriosis, was used to study the immune function of the Chinook salmon saltwater model. In this system, outbreeding was used to determine if the immune function of an inbred aquaculture stock (YIAL) could be enhanced via hybrid vigor. The inbred stock was crossed with seven wild populations and, following i.p. challenge with live *V. anguillarum*, various immune parameters were compared between high and low performing crosses. More specifically, serum IgM production, MH class II β1 genotype, immune transcript expression of spleen tissue and co-infection with *Renibacterium salmoninarum* was analyzed. This revealed that the higher performing crosses had a greater percentage of individuals that were heterozygous for MH class II β1 genotypes and also presented significantly reduced inflammatory transcript expression when compared to the lower performing groups. Also, more individuals were co-infected with *R. salmoninarum* in the low performing crosses providing further evidence that crosses using less energetic resources had better outcomes when challenged with bacterial infection. To explore *V. anguillarum* infection at the cellular level, a stromal cell line was created from Chinook salmon spleen, CHST. Following the development and characterization of CHST, the cells were exposed to heat-killed *V. anguillarum* and were observed to respond with both pro- and anti- inflammatory cytokine production.

The findings presented in this thesis were derived from both *in vivo* and *in vitro* approaches to understand the salmonid immune response to bacterial pathogens. Thus, immune function was analyzed at both the whole animal level, using large-scale infection trials, as well as at the cellular level, using relevant cell lines. The resulting data was able to provide novel and functional information regarding both the immune function of salmonids and the pathologic cycle of two relevant bacterial pathogens. The knowledge presented within this thesis could aid in making some much needed improvements to vaccine design to combat both *F. psychrophilum* and *V. anguillarum* infections. Additionally, a greater understanding of the teleost immune system in response to bacterial infection was obtained and could be used to enhance current and future treatment options for aquaculture. This will lead to less bacterial disease outbreaks in aquaculture settings, translating into greater profits and more job opportunities within this growing industry. Moreover, a steady and increasing supply of fish protein from responsible aquaculture will reduce pressures on wild stocks, some of which are extensively exploited and in danger of collapse.

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When I began my graduate degree, I was probably the most excited about doing live bacterial disease trials, even though I had NO idea how. Everything that I now know about fish pathology and the execution of infection trials was learned through John Lumsden. As this has been a massive part of my thesis and a primary research interest of mine, this meant that initially he had to deal with my constant questions and lack of experience (all while being super busy as Chair of Pathobiology). Your patience and reassurance were always very much appreciated and were also critical for me to gain more

confidence. Thank you for welcoming me into your lab, for your blunt honesty and for all of your valuable recommendations regarding my data, experimental design and future career goals.

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Dedication

I dedicate this thesis to my father, James Elliot, and to my aunt, Sherry Semple.

Though our time together was cut short, you both played a massive role in shaping the person that I am today.

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List of Abbreviations

AID	activation-induced (Cytidine) deaminase
AIP56	apoptosis inducing protein of 56 kDa
AMP	antimicrobial peptide
aMT	alpha-methyltestosterone
ANOVA	analysis of variance
APC	antigen presenting cell
BC	British Columbia
BCR	B cell receptor
BCWD	bacterial coldwater disease
BGD	bacterial gill disease
BKD	bacterial kidney disease
BLAST	basic local alignment search tool
BMPA	broth microdilution peptide assay
BQ	Big Qualicum River
CA	cytophaga agar
Cap	Capilano River
CB	cytophaga broth
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
Chil	Chillawack River
CHSE-214	Chinook salmon embryonic cell line
CHSS	Chinook salmon spleen cell line
CHST	Chinook salmon spleen stromal cell line
Cox1	cytochrome c oxidase subunit
CSR	class switch recombination
CV	coldwater vibriosis
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1α
ELISA	enzyme linked immunosorbent assay
EUS	epizootic ulcerative syndrome
FACS	fluorescence-activated cell sorter
FAO	Food and Agriculture Organization of the United Nations
FBS	fetal bovine serum
F_{cell}	bacterial cell fluorescence intensity
FITC	fluorescein isothiocyanate
Fp	*Flavobacterium psychrophilum*
***Fp*CM**	*F. psychrophilum* conditioned media
FPG101	*F. psychrophilum* strain 101
FPG25	*F. psychrophilum* strain 25
GC	germinal centre
GCPR	G-coupled protein receptors
HDP	host defense peptides
HK	head kidney
HKL	head kidney leukocytes

HRP	horseradish peroxidase
HSP70	heat shock protein of 70 kDa
HWE	Hardy-Weinberg equilibrium
Ig	immunoglobulin
IHNV	infectious hematopoietic necrosis virus
IL	interleukin
i.p.	intraperitoneal
IPNV	infectious pancreatic necrosis virus
IPTG	isopropyl-β-D-thiogalactoside
L15	Leibovitz's media
L15/FBS	Leibovitz's media supplemented with fetal bovine serum
LB	lysogeny broth
LLPC	long-lived plasma cell
LPS	lipopolysaccharide
LSD	least significant difference
MAS	motile *Aeromonas* septicemia
MH	major histocompatibility
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MMC	melanomacrophage centre
MOI	multiplicity of infection
NADPH	nicotinamide adenine dinucleotide phosphate
Nit	Nitinat River
NK	natural killer cell
NOX	NADPH oxidase
OD	optical density
PAC1	PACAP type 1 receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
PI	propidium iodide
PIT	passive integrated transponder
PMA	phorbol 12-myristate 13-acetate
p-NPP	p-nitrophenyl phosphate
Poly I:C	polyinosinic: polycytidylic acid
PRR	pattern recognition receptor
Punt	Puntledge River
qRT-PCR	quantitative reverse transcriptase PCR
Quin	Quinsam River
RAG	recombination-activating gene
RBA	respiratory burst activity
RBC	red blood cells
RC	Robertson Creek
RLU	relative light units
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
RT	room temperature
RTS11	rainbow trout monocyte/macrophage-like cell line
RTSF	rainbow trout fry syndrome

SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SPC	standard plate count
TBS	tris-buffered saline
TBS-T	TBS with Tween 80
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TLR	toll-like receptor
TNF	tumour necrosis factor
TSA	tryptic soy agar
TSB	tryptic soy broth
TSS	rainbow trout spleen stromal cell line
TYESB	tryptone yeast extract salts broth
USD	United States dollar
UV	ultraviolet
Va	*Vibrio anguillarum*
VIP	vasoactive intestinal polypeptide
VPAC1	VIP receptor 1
VPAC2	VIP receptor 2
WBC	white blood cell
WGD	whole genome duplication
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YIAL	Yellow Island Aquaculture Ltd.

Chapter 1: General Introduction

Given that both fresh- and saltwater account for 72% of Earth's surface area, it was only a matter of time before aquatic environments became the new frontier for agriculture. Because the majority of food animals are currently raised on land, it is unsurprising that insights or advancements in aquatic animal husbandry have lagged behind that of terrestrial species. As the global population increases and with limited availability of productive land, the necessity of utilizing aquatic habitats for animal food production is clear. Additionally, due to their high polyunsaturated fatty acid content (Tasbozan & Gokce, 2017), many aquatic species provide an alternative and heart healthy protein source in an age when cardiovascular disease is the leading cause of death worldwide (WHO, 2018). For these reasons and more, global interest for fish protein is high. So high in fact that fisheries cannot meet the global demand while also adhering to the harvesting restrictions that are currently in place (Kvamsdal et al., 2016). This places a burden on wild populations because effective enforcement of these restrictions is logistically difficult.

The culture of aquatic species, or aquaculture, can provide an alternative to alleviate some of the pressure on wild populations. For many aquatic species, this culture production is in its nascent form, meaning that time will be required to understand and optimize these industrial practises. One manifestation of the issues faced by aquaculture is the increased prevalence of infectious disease, including bacterial pathogens. Bacteria are able to take advantage of novel, high density farm environments and thrive. This results in many of these prevalent microorganisms becoming opportunistic pathogens in aquaculture settings. Obtaining a deeper understanding of bacterial diseases that impact aquaculture, as well as what constitutes an effective immune response in relevant hosts, is invaluable for the improvement of this industry.

1.1 Canadian Aquaculture

As the second largest country in the world (Statistics Canada, 2011), Canada is rich in a variety of natural resources including water. The extensive access of this country to both fresh- and saltwater coastlines means that Canada has the potential to play a significant role in the future of productive

aquaculture enterprises. Aquaculture is the farming of aquatic organisms such as fish, molluscs, crustaceans and aquatic plants (de Silva & Anderson, 1994). Though known to have been practised for over 2000 years in China (Ling, 1977), the first record of planned aquaculture activity in Canada occurred in 1857 when the Superintendent of Fisheries studied the incubation and hatching of brook trout eggs (MacCrimmon et al., 1974). Today, industrial aquaculture operations can be found in all Canadian provinces as well as the Yukon which encompass over 45 species of finfish, shellfish and marine plants (Statistics Canada, 2017). This continually growing industry has contributed significantly to the Canadian economy by providing over 25,000 stable employment opportunities (CAIA, 2017) as well as stimulating economic growth in remote, rural and coastal communities. Currently, the value of aquaculture production in Canada is $1.39 billion, with salmonid production accounting for an impressive 80% (Statistics Canada, 2017). Of the aquaculture species being produced, salmonids are the most valuable worldwide with Canada ranking fourth among global salmon producers (DFO, 2016). Given that salmonids can be produced in both fresh- and saltwater environments, Canada has ample opportunity to make its mark in this expanding industry.

1.1.1 Freshwater Culture - Trout

Canada possesses roughly 20% of global surface freshwater (Statistics Canada, 2011), which enables the culture production of trout in every province. Rainbow trout (*Oncorhynchus mykiss*) is currently the third most valuable aquatic species farmed in Canada (Statistics Canada, 2017), however they were not always found across the country. This highly versatile species was originally native to the Western drainages of North America but due to its popularity as a sporting fish, was introduced to water bodies all across Canada and the rest of the world (Ward & Post, 2014). Today, rainbow trout is the most common species of trout in North America and can be found on every continent with the exception of Antarctica (Ward & Post, 2014). The now broad geographical distribution of the species highlights the variety of environments and water temperatures (ranging from 0°C to 29°C) that rainbow trout can survive in (Currie et al., 1998). Because of their significance in global aquaculture and

resilience in laboratory settings, rainbow trout have become the gold standard research model for salmonid immunity.

1.1.2 Saltwater Culture - Salmon

The majority of Canadian aquaculture facilities can be found on the East and West coasts as saltwater operations. This reflects the high demand and monetary value of marine aquatic species, particularly Atlantic salmon (*Salmo salar*). Depending on the coast of Canada, native salmon species will vary. On the East coast, Atlantic salmon are the only salmon species found and are also the predominant and most valuable finfish exported from Canada (DFO, 2017). In comparison, on the West coast there are five main species of Pacific salmon: Coho salmon (*Oncorhynchus kisutch*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*Oncorhynchus keta*), Chinook salmon (*Oncorhynchus tshawytcha*) and sockeye salmon (*Oncorhynchus nerka*). Of these Pacific species, solely Coho and Chinook salmon are actively being farmed in Canada and this only occurs on the West coast (BCSFA, 2003). However, British Columbia is responsible for 71% of Canadian salmon production (Statistics Canada, 2017), which means that Atlantic salmon are primarily farmed on the Pacific coast rather than their native coast. Though this seems counterintuitive, Atlantic salmon are able to be grown at much higher densities and have greater feed conversion rates when compared to their Pacific counterparts (DFO, 1991). Despite these commercial benefits, there is great public concern regarding Atlantic salmon as an invasive species on the West coast and the novel diseases the native species may be exposed to. As such, this has made the development of high-performing Pacific salmon stocks a priority for salmon culture in British Columbia.

1.2 Sources of economic loss in aquaculture

The utilization of aquatic environments means that some of the difficulties confronted by fish farmers are very different when compared to their terrestrial counterparts. Common sources of financial losses include environmental/husbandry (algal blooms, temperature oscillations, hypoxia, supersaturation, etc.), chemical (nitrogen fluctuations, pH variation, etc.), predation, escapees and infectious disease (Anrooy et al., 2006). Many of these problems can result in devastating financial

losses, but few compare to the consistent annual losses derived from infectious disease. In 2014, of the $70 billion dollars of aquaculture product that was destined for human consumption, 10% of this was lost due to infectious disease (World Bank, 2014; FAO, 2016). Though there are many different types of infectious agents that contribute to these significant financial losses, this thesis will focus on bacterial diseases of salmonid culture.

1.3 Bacterial pathogens of salmonid aquaculture

Cultured salmonids are susceptible to many bacterial pathogens (**Table 1.1**). The stress induced by conditions such as overcrowding, temperature fluctuations and excessive handling can result in normally benign microorganisms becoming opportunistic pathogens (Meyer, 1991). The aquatic environments in which these animals reside are known to support the growth of bacteria for long periods of time. Though not immediately causing infection, these opportunistic bacterial pathogens can survive independently of their hosts (Wedemeyer & Nelson, 1977; Hoff, 1989; Madetoja et al., 2003). When animals are stressed these microorganisms are well situated to become major impediments for aquaculture. The poikilothermic nature of fish means that these animals have no control over their body temperature as it is simply a representation of their surrounding environment (Lawrence, 2008). As a result, there are different opportunistic bacterial pathogens that have taken advantage of the variety of temperature niches. For fish farmers, this has made management and prevention strategies difficult as there is a large degree of variability in route of entry, virulence factors, disease presentations and pathologic cycle between the various bacterial pathogens (reviewed by Ringo et al., 2007; Defoirdt, 2013; Bentson-Tilla et al., 2016). The significance of bacterial pathogens in salmonid aquaculture combined with the extensive gaps in knowledge regarding their pathogenesis means that further investigation of these microorganisms could prove invaluable.

Table 1.1. Common bacterial pathogens in salmonid aquaculture. A list of bacterial pathogens that affect aquaculture production of salmonids, their gram reaction and the diseases that they cause. The organisms that are outlined in red will be the focus of this thesis.

Bacterial Species	Gram Reaction	Disease
Yersinia ruckeri	-	Enteric Redmouth Disease
Flavobacterium columnare	-	Columnaris Disease
Flavobacterium psychrophilum	-	Bacterial Coldwater Disease (BCWD)
Flavobacterium branchiophila	-	Bacterial Gill Disease (BGD)
Moritella viscosa	-	Winter Ulcer
Edwardsiella tarda	-	Edwardsiellosis
Piscirickettsia salmonis	-	Piscirickettsiosis
Aeromonas salmonicida	-	Furunculosis
Aeromonas hydrophila	-	Motile Aeromonas Septicemia (MAS)
Tenacibaculum maritimum	-	Mouth Rot
Vibrio salmonicida	-	Hitra Disease, Coldwater Vibriosis (CV)
Vibrio veronii	-	Epizootic Ulcerative Syndrome (EUS)
Vibrio anguillarum	-	Vibriosis
Renibacterium salmoninarum	+	Bacterial Kidney Disease (BKD)
Mycobacterium marinum	+	Mycobacteriosis
Streptococcus phocae	+	Streptococcosis

1.4 Methods to combat bacterial infection

Currently, fish farmers are limited in treatment options during disease outbreaks of any kind. For bacterial pathogens, once an outbreak has occurred the main method of combat is the use of antibiotics. This is usually administered to fish through medicated feed but is often expensive and can be ineffective as sick fish often show reduced appetite. This use of antibiotics, many of which kill a wide spectrum of bacteria, can have a negative impact on native microbial diversity. Additionally, antibiotics may exacerbate difficulties with the disease by increasing the incidence of antibiotic resistance in the pathogen of concern (reviewed by Watts et al., 2017). As a result, antibiotics for aquaculture are tightly regulated in Canada with only four products (oxytetracycline, florfenicol, trimethoprim/sulfadiazine or ormetoprim/sulfadimethoxine) registered for practise (DFO, 2018). Use of antibiotics in aquaculture requires a prescription from a veterinarian, thereby limiting their use further. Fortunately, with the development of vaccines for some aquatic bacterial diseases, the administration of antibiotics has dramatically decreased (reviewed by Pridgeon & Klesius, 2012; Assefa & Abunna, 2018).

Though unable to control outbreaks once they have occurred, prophylactic treatments have become standard for dealing with bacterial pathogens in aquaculture. Historically, vaccines have been used to prevent many different aquatic infectious diseases, including those caused by bacterial pathogens. There are seven main types of vaccines: killed, attenuated, subunit, recombinant, synthetic peptide, genetically modified and DNA vaccines (reviewed by Assefa & Abunna, 2018). The main forms used to combat bacterial infections are killed or attenuated (live avirulent) vaccines (reviewed by Pridgeon & Klesius, 2012). Presently, there are commercial vaccines available for some bacterial diseases influencing aquaculture such as vibriosis, furunculosis and columnaris (reviewed by Pridgeon & Klesius, 2012; Assefa & Abunna, 2018). Although their use has helped to reduce bacterial infection, efficacy is not high, particularly when compared to terrestrial vaccines (reviewed by Adams, 2019). This has resulted in relatively recent endeavors for other prophylactic treatments to supplement vaccine use, such as probiotics (reviewed by Pandiyan et al., 2013), determining effective adjuvants (reviewed by Adams, 2019) and antimicrobial peptides (reviewed by Keymanesh et al., 2009; Rajanbabu & Chen, 2011). However, ensuring appropriate stimulation of the fish immune system can be difficult due to lack of knowledge regarding teleostean immune defenses. Thus, a deeper understanding of teleostean immunity would be invaluable for making some much needed improvements to the current treatment options for bacterial infections.

1.5 Teleost immune system

Bony fishes are divided into the Sarcopterygii (the lobe-finned fish) and the Actinopterygii (ray-finned fish) to which Teleostei (Greek for “complete bone”) belongs (reviewed by Ravi & Venkatesh, 2018). Salmonids, the focus of this thesis, are members of Teleostei. The teleosts comprise 95% of surviving fish species, which represents approximately half of all extant vertebrate species (Helfman et al., 1997). The striking success of this class, along with their ability to thrive in a wide range of environments, suggests that teleosts developed an impressive immune arsenal to counter pathogen challenge. Much like the highly studied mammalian model, the immune system of teleosts can be

separated into two main branches: the innate and adaptive immune systems. The following information represents a summary of teleostean immune defenses that are related to the work described in this thesis.

1.6 Innate immunity of fish

Due to the limitations of teleost adaptive immunity (i.e., slow initiation, limited antibody repertoire, etc.), the burden of preventing and combatting infectious agents falls heavily to the innate immune system. Fish have been shown to have all of the mammalian aspects generally associated with innate immunity including physical barriers (skin and mucous membranes), humoral parameters (complement, natural antibody, TLRs, etc.) and cellular components (phagocytosis, NK cells, etc.). As the first line of defense, it is not surprising that the majority of the broad-spectrum parameters of innate immunity are highly conserved across species and taxa. In all jawed vertebrates, the innate immune system features a rapid defensive response towards invading pathogens and tissue damage. However, it cannot provide well-directed, specific protection from individual pathogens or long-term immunological memory.

1.6.1 Cells of innate immunity

All of the innate immune cells that are observed in mammalian blood are also present in the blood of teleosts (monocytes, neutrophils, basophils and eosinophils), albeit at very different circulating concentrations. Neutrophils, basophils and eosinophils are together referred to as granulocytes and are aptly named due to the presence of cytoplasmic granules. These are filled with enzymes and host defense peptides that can support immune responses during infections and/or allergic reactions (reviewed by Shamri et al., 2010; Sheshachalam et al., 2014; Voehringer et al., 2017; Varricchi et al., 2018). Monocytes patrol the blood contributing to inflammation, immune defenses and homeostasis by clearing pathogens and cellular debris. Additionally, monocytes can enter tissues and differentiate into macrophages or dendritic cells (DCs) to replenish these important immune cells (reviewed by Jakubzick et al., 2017). Of these peripheral blood leukocytes, only monocytes, eosinophils and neutrophils are phagocytic. When it comes to cell numbers, the granulocytes are the most prevalent circulating WBC in mammals and represent 45-65% of this population, 92% of which are neutrophils. Meanwhile,

monocytes make up only 8% of total mammalian WBCs (reviewed by Houwen, 2001; Mathur et al., 2013). In comparison, granulocytes comprise just 2-3% of teleostean WBCs while monocytes are merely 0.1% (Blaxhall & Daisley, 1973; Niimi & Lowe-Jinde, 1984). Despite differences in circulating concentrations, the function of these WBCs appears to be conserved between the two taxa.

As the principal phagocytic cell in fish, macrophages are considered one of the most important contributors to the innate immune defenses of these animals. Though macrophages can derive from monocytes (reviewed by Jakubzick et al., 2017), this happens relatively infrequently (Hashimoto et al., 2013). Instead, recent evidence in mammals has shown that these cells are present in embryonic tissues (yolk sac and fetal liver) prior to hematopoiesis and can then persist as self-maintaining populations to perform organ specific functions (reviewed by Gordon & Pluddemann, 2017). Though further investigation is required, recent work with zebrafish has shown that tissue macrophages are present throughout adulthood even when adult hematopoiesis is absent (Soza-Reid et al., 2010). This indicates that fish may also have tissue resident, self-maintaining macrophages. Functionally, macrophages are armed with many pattern recognition receptors (PRRs) enabling these cells to detect a multitude of pathogen associated molecular patterns (PAMPs) wherein strong binding will initiate phagocytosis of the foreign entity (reviewed by Mogensen et al., 2009). Once ingested, macrophages can rapidly kill foreign invaders through the production of toxic reactive intermediates and phagolysosomal acidification (reviewed by Grayfer et al., 2018). Besides their antimicrobial function, these cells are also able to present antigens to T cells and, depending on the surrounding stimuli, can orchestrate the appropriate immune response via cytokine secretion (reviewed by Grayfer et al., 2018). Finally, once an immune reaction has ceased, the phagocytic function of macrophages is critical for maintaining tissue homeostasis by clearing cellular debris (reviewed by Wynn et al., 2013). The dynamic and heterogeneous nature of macrophages means that these cells can be distinguished depending on the source of activation and the resultant differences in cellular function, referred to as polarization (reviewed by Zhou et al., 2014; Murray, 2017). In fish, the M1 macrophage polarization state is the

best characterized and appears to serve a vital role in host protection against bacterial pathogens (reviewed by Hodgkinson et al., 2015; Grayfer et al., 2018).

1.6.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are a diverse class of highly conserved molecules that are produced as a first line of defense in all multicellular organisms, including teleosts. These small peptides (12-50 amino acids) are essential components of innate immunity capable of antimicrobial activity against a broad range of microbial pathogens (reviewed by Zhang & Gallo, 2016), which notably includes multi-drug resistant isolates (Lai & Gallo 2009; Lee et al., 2018). Quite often, AMPs are produced at the constitutive level but are able to be induced upon exposure to pathogens or other trauma (O'Neil et al., 1999; Schittek et al., 2001; reviewed by Katzenback, 2015). Most AMPs are cationic amphipathic peptides that function by attacking the negatively charged membranes of microorganisms (reviewed by Mahlapuu et al., 2016). Based on their secondary structures, AMPs can be characterized as one of four types, β-sheet, α-helix, extended and loop. Of these four types, β-sheet and α-helix are the most common (reviewed by Bahar and Ren, 2013). Functionally, they can be characterized as either membrane disruptive AMPs, causing membrane permeabilization, or nonmembrane disruptive AMPs, which directly passage into cells and act on intracellular targets (reviewed by Kang et al., 2017). Besides direct destruction of pathogens, AMPS can perform immunomodulatory functions in higher vertebrates (reviewed by Otvos, 2016) and as a result are also called "host defense peptides" (HDPs) to emphasize these additional activities. The potential immunomodulatory effects are diverse including stimulation of chemotaxis, immune cell differentiation, initiation of adaptive immunity and stimulation of both pro- and anti- inflammatory cytokines (Elssner et al, 2004; Yu et al, 2007; Mookherjee et al, 2006; Di Nardo et al, 2007). As many AMPs have multiple functions that can be both bactericidal and immunostimulatory in nature, there has been growing interest regarding their use in aquaculture as an alternative for antibiotics.

1.6.3 Respiratory burst activity

An essential immunological response to eliminate bacterial pathogens is the respiratory burst activity (RBA) of phagocytes. Following ingestion of foreign particles, these immune cells can kill most bacteria by producing reactive oxygen intermediates (ROI). The production of ROI requires NADPH oxidase (NOX), which catalyzes the conversion of molecular oxygen into superoxide anions (reviewed by Panday et al., 2015; Nguyen et al., 2017). Upon formation, the superoxide anion will then transform into further ROIs such as hydrogen peroxide, hydroxyl radical, and hypochlorous acid, all of which efficiently kill the phagocytosed microorganisms (DeLeo & Quinn, 1996). Inactive NOX consists of six subunits wherein $gp91^{phox}$ and $p22^{phox}$ are membrane proteins (**Figure 1.1A**) that together are known as flavocytochrome b_{558} (cyt b_{558}, Nauseef, 2018). The remaining four regulatory subunits, ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac2) normally exist in the cytosol (**Figure 1.1A)** but upon the activation of leukocytes by particulate stimuli, will translocate to the membrane and associate with cyt b_{558} (**Figure 1.1B**) to form the active oxidase (reviewed by Panday et al., 2015; Nauseef, 2018). It is well established that fish phagocytes possess all of these NADAPH oxidase components as well as an RBA response comparable to that of mammals (Stafford et al., 2002; Sepulcre et al., 2007a; Boltana et al., 2009). Additionally, this immune defense has been extensively studied in relation to bacterial pathogens of fish (Sharp et al., 1993; Ardo et al., 2010; Hodgkinson et al., 2012; Havixbeck et al., 2017). Given that the RBA in fish is not markedly influenced by temperature (reviewed by Le Morvan et al., 1998; Collazos et al., 1994a; Collazos et al., 1994b; Nikoskelainen et al., 2004), this innate immune response is an essential defensive mechanism for these poikilothermic organisms.

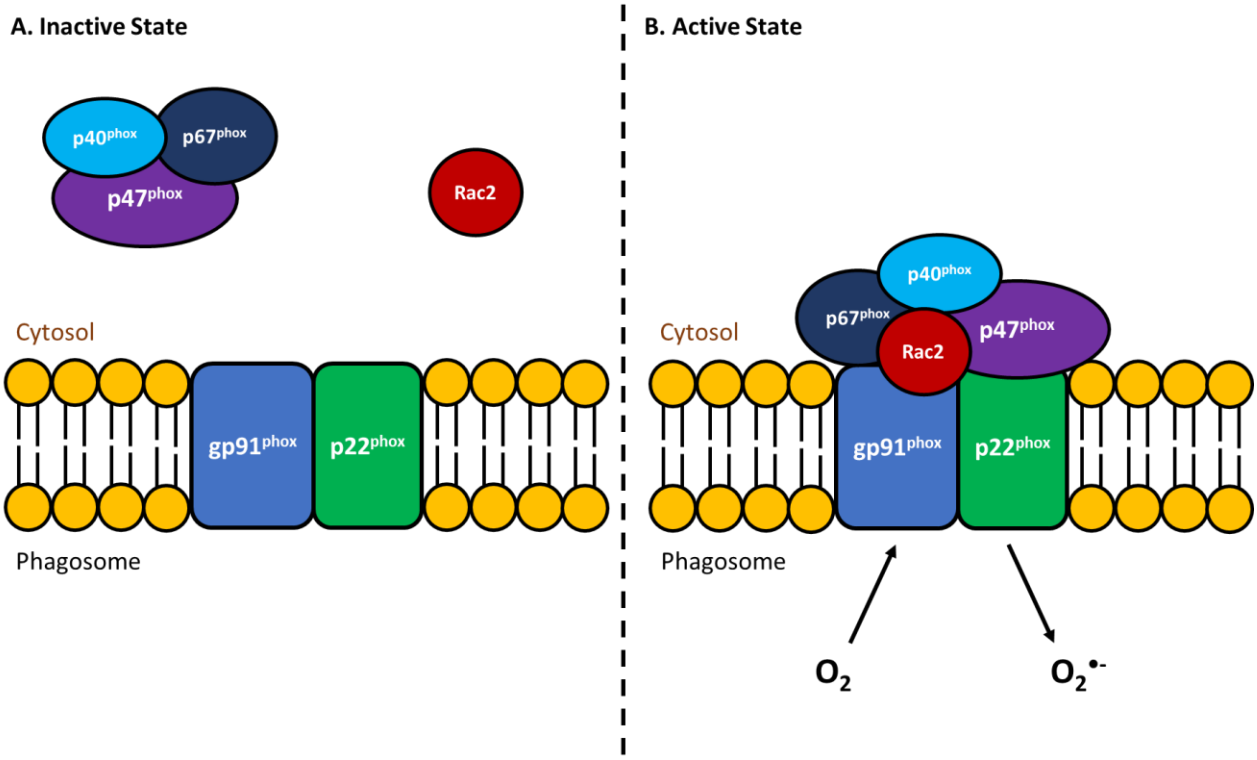

Figure 1.1. Schematic depiction of NADPH oxidase activation to enable the respiratory burst activity of phagocytes. The NADPH oxidase enzyme consists of six subunits. **(A)** When the phagocyte is in an inactive state, two of the subunits (gp91^{phox} and p22^{phox}) are transmembrane components while the remaining four components are cytosolic (p40^{phox}, p47^{phox}, p67^{phox} and Rac2). **(B)** Upon the phagocyte being activated by external stimuli, the four cytosolic components complex with gp91^{phox} and p22^{phox} to form the active NADPH oxidase. When in the active form, the now functional enzyme can convert molecular oxygen into superoxide anions to aid in degradation or killing of that which was phagocytosed.

1.7 Adaptive immunity of fish

All organisms have innate immune mechanisms, and while there are indications of adaptive immunity in invertebrates, this branch of the immune system appears to be an innovation specific to gnathostomes (jawed vertebrates). The adaptive immune system is remarkably flexible, capable of recognizing and initiating protective responses against specific foreign agents. Upon subsequent exposures, the adaptive immune system will remember antigens from a foreign invader, making it possible to mount a stronger and more efficient immune response (reviewed by Cooper & Alder, 2006; Flajnik & Kasahara, 2010). Though delayed when compared to mammalian counterparts, the specificity of the teleostean adaptive immune system is essential for long-lasting immunological memory. As such,

this branch of the immune system is critical for vaccine design, an enterprise in need of improvement for the aquaculture industry.

1.7.1 Cells of adaptive immunity

Much like in mammalian models, lymphocytes are considered the adaptive immune cells of fish. The two types of lymphocytes, T and B lymphocytes, represent the only cells capable of recognizing and responding specifically to an antigenic epitope. This antigen detection is based on compatibility with either the surface T cell receptor (TCR) or B cell receptor (BCR) depending on the lymphocyte. The genes that encode for these receptors undergo a series of DNA recombinations, providing them with immense phenotypic diversity to improve the likelihood of antigen recognition (reviewed by Nemazee, 2000). Depending on the type of activation, T lymphocytes can produce cytokines to direct immune responses ($CD4^+$ T cell, reviewed by Abbas et al., 1996) or induce programmed cell death in virally infected cells ($CD8^+$ T cell, reviewed by Barry & Bleackley, 2002). In comparison, B cells will transform into plasma cells following activation to produce antigen specific antibodies (reviewed by LeBien & Tedder, 2008). Though participating in a variety of different activities, the proficient function of both T and B lymphocytes are crucial for the success of the adaptive immune system.

In mammals, lymphocytes represent the largest blood cell in diameter (8-10 μm) and account for approximately 20-40% of WBCs (reviewed by Houwen, 2001; Mathur et al., 2013). This is quite different from teleost species where lymphocytes are smaller in size (5-8 μm) and represent the dominant circulating leukocyte at 83-90% of total WBCs (Pitombeira & Martins, 1970; Blaxhall & Daisley, 1973; Niimi & Lowe-Jinde, 1984). Regardless of the model system used, T and B lymphocytes appear identical when observed under a microscope, making it impossible to discern between the two without identifying cell surface markers. Though studied in detail for mammalian models, this was not possible in teleosts due to the absence of appropriate antibodies. Fortunately, with the recent development of antibodies specific for some of the cell surface markers on salmonid lymphocytes

(Zhang et al., 2010; Maisey et al., 2016; Takizawa et al., 2016), comparative immunologists are able to finally start understanding adaptive immune functions in teleosts.

1.7.2 Major histocompatibility (MH) genes

To effectively combat the inevitable interaction with foreign entities, vertebrates have evolved two distinct antigen presentation pathways that, when paired with effective innate immune system activation, can stimulate long-term immunological memory. The major histocompatibility complex (MHC) molecules are critical in this important immune process yet the MHC gene equivalents in teleosts are not clustered on a single chromosome as they are in mammals so they are not considered to be a "complex". Instead these genes can be found on more than one chromosome and as a result are simply referred to as Major Histocompatibility (MH) genes (reviewed by Dixon & Stet, 2001). In the mammalian model, the endogenous antigen presentation pathway includes MHC class I molecules which are found within all nucleated cells. This pathway involves the processing of antigens from intracellular pathogens and their presentation via MHC class I to $CD8^+$ cytotoxic T lymphocytes (reviewed by Neefjes et al., 2011). In comparison, the exogenous antigen presentation pathway uses MHC class II dimers which are generally only found on specific cell types, namely antigen presenting cells (APCs) that are capable of phagocytosis (reviewed by Landsverk et al., 2009). Once phagocytosed and processed, the antigens from extracellular sources are then loaded onto the MHC class II dimer and presented at the cell surface to $CD4^+$ T lymphocytes (reviewed by Blum et al., 2013). In fish, the MHC equivalents function in an identical matter to what has been observed in mammals. Because this thesis focuses on bacterial pathogens which are typically extracellular in nature, more emphasis will be placed on MH class II (**Figure 1.2**).

Per individual, the MH molecules play an important role by binding to and presenting well-matched peptides to appropriate T lymphocytes. The compatibility of these pathogen derived antigens to MH molecules is controlled by allelic variation at the peptide binding groove. There are many possible alleles for the peptide binding region and every individual within a species has a limited repertoire inherited from their parents in a Mendelian fashion (reviewed by Choo, 2007; Garcia et al.,

2012). The genetic polymorphism at the MH loci can provide more or less protection to pathogens and thus these genes are believed to be under strong selection pressure that is often governed by the surrounding habitat (reviewed by Yamaguchi & Dijkstra, 2019). Regardless of the species, individuals that are heterozygous at MH loci are believed to be better protected as the resulting molecules should be able to bind to and present a more extensive collection of antigens (Lens, 2011; Pierini & Lens, 2018). In fish, this is supported by heterozygous individuals presenting less infection and/or mortality when challenged with an infectious agent (Evans & Neff, 2009; Becker et al., 2014). Unlike what has been observed in some terrestrial species (Pazderka et al., 1975; Hill et al., 1992; Shen et al., 2014), specific MH alleles have not yet been shown to consistently predict resistance or susceptibility towards specific pathogens in fish, but perhaps more research is required

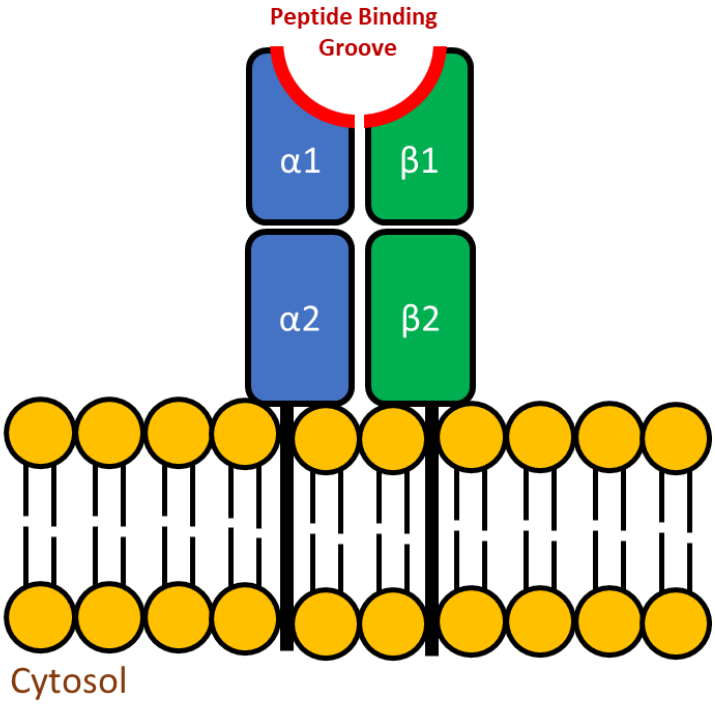

Figure 1.2. Structure of the MH class II molecule. The structure and function of the teleostean MH class II dimer is very similar to mammalian MHC class II. The molecule is a dimer, consisting of an alpha chain and beta chain, both of which have transmembrane regions. As outlined in red, both the α1 and β1 domains of this molecule have a hypervariable region that is part of the peptide binding groove. It is this region that is capable of binding to compatible antigens and presenting it to T cells. In this thesis, the hypervariable region of the β1 domain is used to genotype individuals and determine whether certain alleles at the peptide binding groove will infer resistance or susceptibility to bacterial pathogens.

1.7.3 Antibody development

Antibody development and production is of paramount importance in the humoral immune response of all jawed vertebrates, including bony fishes. This defense is particularly important when dealing with extracellular threats, as is the case with most bacterial pathogens. Antibodies prevent the growth and colonization of bacterial pathogens by neutralization, complement activation and/or opsonization to enhance phagocytosis (reviewed by Forthal, 2014). To date, there are three known antibody classes in teleosts based on differences in their constant region: IgM, IgD, and IgT (reviewed by Sunyer, 2013). IgM was the first isotype discovered in teleosts and can be found on B cells as well as secreted in the serum or mucus as a tetramer (reviewed by Mashoof & Criscitiello, 2016). The secreted form of teleost IgM is by far the most prevalent immunoglobulin in the serum and is responsible for systemic immunity of bony fishes (reviewed by Parra et al., 2015). At much lower concentrations, IgM is also present in the gut and skin mucosa. Similar to mammals, all mature IgM B cells in teleosts also express IgD, a class of antibody whose function is still not fully understood regardless of the model system used (reviewed by Sunyer, 2013). However, there have been slight improvements in elucidating mammalian IgD function recently (reviewed by Gutzeit et al., 2018). Lastly, IgT is a recently discovered antibody isotype exclusive to bony fishes (Hansen et al., 2005). IgT is present within the serum as monomers, while forming tetramers in the gut mucosa. With concentrations of IgT in the gut mucosa being double that observed in the serum (Zhang et al., 2010), it was believed that this Ig class likely had a vital role in mucosal immunity. This has been heavily supported since then with numerous studies demonstrating the role of IgT in teleostean mucosal immunity (Xu et al., 2013; Piazzon et al., 2016; Yu et al., 2018). When considering adaptive immune defenses that are important for combatting extracellular bacterial pathogens, effective antibody production and development is invaluable.

Although teleosts contain all of the components necessary for adaptive immune responses, opinion is divided as to whether they are capable of immunological memory. Rather than developing memory B cells, it has been proposed that the most mature stage of B lymphocytes in teleosts are long-

lived plasma cells (LLPCs). These cells reside within the head kidney, a primary immune organ of fish that is considered to be analogous to mammalian bone marrow. These LLPCs have been observed to secrete high amounts of antibody and appear to be the only detectable source of prolonged, high-titered antibody (Bromage et al., 2004). However, the idea of 'immunological memory' is called into question because the antibody response in fish shows poor affinity maturation and slow development of the secondary immune response, taking 3-4 weeks to initiate in fish (Ye et al., 2011a). This is a stark contrast to the rapid development anticipated based on the mammalian paradigm of immunological memory.

1.8 Cytokines

As described above, the immune system of vertebrates is a complex network connecting numerous cell types, barriers and specialized systems to prevent the entry and/or colonization of foreign entities within the host. The successful function of this multifaceted system depends on the ability of immune cells to migrate to and communicate with one another, a role fulfilled by extracellular mediators known as cytokines. Cytokines are a large family of small glycoproteins that are capable of acting in an autocrine, paracrine or endocrine fashion (reviewed by Zhang & An, 2009). These soluble proteins play crucial roles in regulating inflammation, haematopoiesis, cellular movements and immune cell activation (reviewed by Metcalf, 2008; Grayfer & Belosevic, 2012; Newton & Dixit, 2012; Hughes & Nibbs, 2018). As such, cytokines act as an essential link between the innate and adaptive arms of the immune system.

1.8.1 Pro-inflammatory

Inflammation is an essential response to combat tissue damage of any type whether it be from infection, trauma, foreign objects or toxins. Though there are many important components involved in an inflammatory response (reviewed by Martin & Leibovich, 2005, Newton & Dixit, 2012), the initiation and perpetuation of inflammation is governed primarily by pro-inflammatory cytokines produced by damaged cells and/or responding immune cells. In mammalian models, the three classical pro-inflammatory cytokines are interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)α. Upon

tissue damage, keratinocytes and fibroblasts release IL-1β inducing fever, T cell proliferation and increasing vascular permeability (Feghali & Wright, 1997). Meanwhile resident mast cells (MCs) degranulate in response to the mechanical trauma (reviewed by Watkins et al, 1995) releasing a wide variety of inflammatory mediators including, but not limited to, TNFα and IL-6 (reviewed by Theoharides et al, 2012, Wulff & Wilgus, 2013). The released cache of inflammatory mediators also aids in vascular permeability as well as the activation/recruitment of circulating immune cells which produce more pro-inflammatory cytokines. Leukocytes that are normally restricted to blood vessels will then gain access to the site of tissue injury and attempt to eliminate any invading targets (reviewed by Medzhitov, 2008). If the inflammatory response is successful in clearing the threat, it is followed by a resolution and repair phase mediated mainly by tissue-resident and recruited macrophages that shift the response from pro-inflammatory to anti-inflammatory (reviewed by Serhan & Savill, 2005). While fish immune systems are not as well characterized as mammalian equivalents, they possess all of the major pro-inflammatory cell types and cytokines (reviewed by Grayfer & Belosevic, 2012).

1.8.2 Anti-inflammatory

Though necessary for homeostatic maintenance, inflammatory responses can be quite damaging to surrounding tissues. This makes the control of such reactions essential for the day-to-day welfare of the host. Regulation and control of inflammatory responses requires a constant and ever-changing balance between pro-inflammatory and anti-inflammatory cytokines. Under normal circumstances, as the source of tissue damage is cleared, there is less stimuli available to induce a strong inflammatory response. This enables the constitutively produced anti-inflammatory cytokines to skew the reaction towards tissue repair mechanisms. Though there are several cytokines that are considered to have anti-inflammatory properties (reviewed by Zhang & An, 2009), IL-10 represents one that is believed to function solely as a potent anti-inflammatory cytokine. Produced by almost all leukocyte subsets including T cells, B cells, macrophages and mast cells (reviewed by Ouyang & O'Garra), IL-10 plays a vital role in ensuring that both innate and adaptive immune responses cannot have a strong reaction unless a true threat is present. The importance of controlling inflammation through endogenous

levels of IL-10 was made clear by Kuhn et al. (1993) when they generated IL-10 knockout mice. The IL-10 deficient mice spontaneously developed inflammatory enteritis, indicating that the mice were unable to prevent the inflammatory response towards their own commensal gut-associated bacteria (Kuhn et al., 1993). Presently, antibodies specific for teleostean cytokines are absent, thus current data pertaining to IL-10 and other cytokines are based solely on transcriptional analyses (Chettri et al., 2011). As with most components of the immune system, inflammatory responses must be tightly regulated.

1.9 Thesis Objectives

The overarching goal of my thesis was to enhance our understanding of salmonid immunity with regard to industrially relevant bacterial pathogens. More specifically, the outcomes of my research will help to understand salmonid immunity in response to *Flavobacterium psychrophilum* and *Vibrio anguillarum* with the potential to improve therapies for these bacterial infections. The three specific objectives of my PhD thesis were to:

- **i.** Determine whether selected salmonid immune markers could predict resistance and/or susceptibility to bacterial pathogens in whole animals (Chapters 2 and 5).
- **ii.** Understand the immunity to, and pathogenesis of, aquatic bacterial pathogens in relevant *in vitro* models (Chapters 3 and 6).
- **iii.** Assess the caliber of an alternative treatment option for a bacterial disease in aquaculture (Chapter 4).

To accomplish these objectives, five independent hypotheses were generated:

- **i.** Selectively breeding rainbow trout for resistance and susceptibility to BCWD will result in observable, family-specific differences in functional immune markers (MH class II β1 genotype, RBA or serum anti-*F. psychrophilum* IgM).
- **ii.** Exposing rainbow trout immune cells to the extracellular products of *F. psychrophilum* will stimulate and influence their function.

iii. Teleostean PACAP will be directly bactericidal to *F. psychrophilum* and will also display immunomodulatory effects on salmonid immune cells following exposure.

iv. Outbreeding of an inbred Chinook salmon population will confer hybrid vigor, as evidenced by survival to *V. anguillarum* infection and functional differences in immune performance.

v. Exposing a Chinook salmon spleen stromal cell line to heat-killed *V. anguillarum*, will stimulate a pro-inflammatory cytokine response.

Chapter 2: Serum IgM, MH class IIβ genotype and respiratory burst activity do not differ between rainbow trout families displaying resistance or susceptibility to the coldwater pathogen, *Flavobacterium psychrophilum*.

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2.1 Overview

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD) is a significant threat to global aquaculture. True to its name, BCWD tends to occur at temperatures between 8 and 12°C and presents as a systemic disease with characteristic skin ulcerations. Juvenile rainbow trout are particularly susceptible and in these fish the condition is referred to as rainbow trout fry syndrome (RTFS). Resistance to *F. psychrophilum* is heritable and is not adversely correlated with the growth of fish, thus selective breeding appears to be an achievable approach to its control. The current study explores the connection between resistance to BCWD and several immunological markers. After determining resistance/susceptibility to *F. psychrophilum* following experimental infection in 40 full-sibling families of rainbow trout, selected families were experimentally infected with *F. psychrophilum* and differences in antibody production, major histocompatibility (MH) class IIβ genotype and respiratory burst activity (RBA) throughout infection were compared. Serum IgM production increased over time but significant differences between resistant and susceptible families were not observed at either 28 days or 120 days. Of the six families that were genotyped for MH class IIβ, there did not appear to be specific genotypes that conferred resistance or susceptibility to *F. psychrophilum*. Further, the RBA of both head kidney leukocytes and whole blood was not significantly different between the resistant and susceptible rainbow trout families. Although the selected immune markers did not differ based on resistance status, the RBA of head kidney leukocytes in all families studied dramatically decreased seven days after infection while total blood RBA remained constant. Day seven was also when severe symptoms and/or mortality due to BCWD was first observed, thus these results may reveal information regarding the pathogenesis of the organism. A better understanding of appropriate immune defenses could provide the basis for breeding programs to effectively combat this costly pathogen, but further study of functional immune markers particularly during the fry stage of development is required.

2.2 Introduction

A large obstacle to overcome when dealing with aquaculture production of any type is the vulnerability to disease outbreaks. Like other forms of agriculture, aquaculture places animals in a setting that deviates significantly from that of their natural environment. The stress induced by conditions such as overcrowding, temperature fluctuations and handling can result in normally benign microorganisms becoming opportunistic pathogens (reviewed by Meyer, 1991). One such pathogen causing significant losses in the farming of rainbow trout (*Oncorhynchus mykiss*) is *Flavobacterium psychrophilum*, the etiological agent of bacterial cold-water disease (BCWD). Rainbow trout, a coldwater salmonid, is an important species of choice in global aquaculture, which has enabled BCWD to become a worldwide concern.

F. psychrophilum is widespread in freshwater aquatic environments but typically only causes infection in intensively reared salmonids at temperatures lower than 16°C (Holt, 1987). Although *F. psychrophilum* can infect many salmonid species and some non-salmonid species, juveniles of coho salmon (*Oncorhynchus kisutch*) and rainbow trout are particularly susceptible (reviewed by Nematollahi et al., 2003; Starliper, 2011). Clinical signs and manifestations vary depending on location as there appears to be geographic variability between isolates (Lumsden et al., 2004). In North America, the most common presentation of BCWD in fish larger than fingerling is an ulcerative dermatitis known as peduncle disease (Lumsden et al., 2004). The nature of this presentation means that the financial losses associated with BCWD are not due to mortalities alone as surviving fish often produce fillets that are less valuable or not marketable (Lumsden et al., 2004). In fry, the most important presentation is a systemic disease, often with exopthalmia and anemia, known as rainbow trout fry syndrome (RTFS) (Bebak et al., 2007). With no commercial vaccine available and mortality, although variable, has been reported to be as high as 50-85% (Brown et al., 1997; Cipriano & Holt, 2005), the financial losses due to *F. psychrophilum* infection can be devastating.

To combat the significant losses due to infectious disease, there has been increasing interest in selectively breeding aquaculture stocks for disease resistance. As has been demonstrated with many

other aquatic diseases (Gjedrem et al., 1991; Henryon et al., 2005; Kjoglum et al., 2006, 2008), resistance to BCWD does appear to be heritable (Silverstein et al., 2009), but there has been little research to identify the basis for this resistance. In addition, a method to consistently produce resistant crosses is yet to be developed for the vast majority of aquatic organisms. Creating and challenging families of rainbow trout takes a great deal of time and finances, thus identifying a marker for the observed resistance to BCWD would decrease costs and expedite this process immensely. With the immune system playing a vital role in the prevention and clearance of infection, it is reasonable to hypothesise that the heritable resistance to BCWD may be associated with teleostean immune function.

Like mammals, teleosts have both innate and adaptive immune systems. The innate immune response constitutes the first line of defense against infection and is activated through recognition of conserved molecular patterns common to pathogenic organisms (Uribe et al., 2011). Phagocytosis, or the engulfment of particles, is a major component of immunity in fish (Uribe et al., 2011). Circulating phagocytes will take up foreign bodies such as bacteria and eradicate them through the production of reactive oxygen intermediates (ROI) during a respiratory burst (Nikoskelainen et al., 2004). Phagocytosis, and the killing activity associated with it, is reported to be the mechanism of innate immunity that is the least influenced by low temperatures (Collazos et al., 1994a; Collazos et al., 1994b; Nikoskelainen et al., 2004), thus this process may be critical when dealing with a cold-water pathogen such as *F. psychrophilum*.

While the innate immune system is fast-acting, adaptive immunity requires more time to develop and has greater specificity. A key component of vertebrate adaptive immunity is the generation of antigen-specific antibodies from B cells. Antibodies promote the control and clearance of infection through such processes as opsonisation for phagocytes, complement activation and neutralization of microbes and toxins (Forthal, 2014). To date, three classes of immunoglobulins have been discovered in teleosts: IgM, IgT and IgD (reviewed by Sunyer, 2013). The most abundant immunoglobulin is a tetramer of the IgM class (Fillatreau et al., 2013). IgM is the first antibody class to appear after initial exposure to an antigen and may be part of an effective humoral response against invading pathogens

(Fillatreau et al., 2013; Reyes-Cerpa et al, 2012). With these protective properties in mind, there have been numerous attempts to identify antigens capable of inducing an effective antibody response to combat *F. psychrophilum* (Crump et al., 2005; Dumetz et al., 2006; LaFrentz et al., 2002; Sudheesh et al. 2007). Although antibody titres have been observed to increase after immunization with many of these antigens and whole-cell lysates, similar increases were often observed with adjuvant alone (LaFrentz et al., 2002). As there is limited information regarding consistent infection and vaccination models for *F. psychrophilum* (Gomez et al., 2014), there is a critical need to better understand the serum IgM response in fish presenting resistance/susceptibility to BCWD.

Although fish farmers can benefit from breeding programs based solely on survival phenotype, identifying genes (markers) correlated with enhanced immune performance could aid in the more rapid development of optimum stocks. One possible marker, and a focus of this study, is the major histocompatibility (MH) class II genes. As the class of molecules that recognize and present antigens from extracellular pathogens to tailor the resulting immune responses (Neefjes et al., 2011), there has been a great deal of interest in determining whether specific MH class II genotypes are linked to bacterial resistance/susceptibility in fish species (Dionne et al., 2009; Kjoglum et al., 2008; Rakus et al., 2009; Xu et al., 2010). Conversely, MH class I is associated with recognition and presentation of antigens from intracellular pathogens such as viruses (Neefjes et al., 2011). Because *F. psychrophilum* appears to have both intracellular and extracellular tendencies throughout its infection cycle (Decostere et al., 2001; Nematollahi et al., 2005; Nilsen et al., 2011b; Wood & Yasutake, 1956), it is not fully understood whether MH class I or MH class II genes would provide more protection towards BCWD. To date there has been some evidence of an association between MH class II variability and resistance to *F. psychrophilum* (Johnson et al., 2008), but there has been minimal research since then to relate specific alleles with BCWD resistance. As such, one component of this study aims to link MH class IIβ alleles with observed resistance to BCWD and determine whether this association goes further.

In the present study, full-sibling families of rainbow trout displaying resistance/susceptibility to BCWD were experimentally infected with *F. psychrophilum* so that differences in MH class IIβ

genotype, respiratory burst activity and antibody production could be observed and compared. As there is limited knowledge regarding both the pathogenesis of *F. psychrophilum* and what constitutes an effective immune response against the organism, this study aims to analyze functional immune differences in fish that are resistant/susceptible to provide information that could be used for the development of effective breeding programs.

2.3 Materials and Methods

2.3.1 *Fish*

Forty full-sibling families of rainbow trout were produced at Lyndon Fish Hatcheries (Dundee, Ont.). These families were transported to the Hagen Aqua Lab or the Alma Aquaculture Facility at the University of Guelph as eyed eggs. Susceptibility to *F. psychrophilum* experimental infection was compared among the 40 family groups. Six to ten families per experiment were divided into four 60 L tanks containing 40 fish (40 g each) per tank, which were supplied with single pass well-water at 11°C. Fish were fed a commercial pellet diet (Martin Mills Inc.). All maintenance and procedures were done in accordance with the University of Guelph Animal Care Guidelines.

2.3.2 *Culture and quantification of F. psychrophilum strain 101*

Frozen stocks of *F. psychrophilum* strain 101 (FPG 101) were sub-cultured onto cytophaga agar (CA) then following incubation at 15°C for 72 h, cells were harvested and suspended in cytophaga broth (CB) (Hesami et al., 2011). Cell suspensions were adjusted to an optical density of 0.6 at 600 nm using a Novaspec Plus spectrophotometer (GE Healthcare Life Sciences) and the suspension was then diluted to obtain a final concentration of ~1 x 10^9 colony forming units (CFU)/mL. The concentration was then adjusted so that the desired CFU/mL for each trial could be obtained. To confirm the cell numbers, suspensions were enumerated by plating 0.1 mL of ten-fold serial dilutions on CA plates in triplicate. After incubation at 15°C for 4 d, plates containing 25-250 colonies were counted and the number of CFU/mL calculated.

2.3.3 *Determining susceptibility/resistance to F. psychrophilum (Preliminary Trials)*

The Ontario *F. psychrophilum* isolate, FPG101, was used for all experimental infections as it had been shown to produce ~50% mortality in experimental trials of 30-40 g rainbow trout at doses similar to those used in the current study (JS Lumsden pers. comm.). After sedation with ~50 ppm benzocaine (Millipore Sigma) in well-aerated water, triplicate tanks (n = 40) of each family received intraperitoneal (i.p.) 0.1 mL injections ranging in concentration between 1.13 and 6.60 x 10^7 CFU/mL. With every family, a fourth tank of fish was sham-injected with 0.1 mL of sterile CB as a negative control. Following injection, all fish were observed three times a day and any mortalities were collected and saved at -20°C. In addition, fish with marked exopthalmia, hemorrhage or skin ulcerations were removed and euthanized using an overdose of benzocaine. These fish were counted as mortalities.

After the predetermined trial period (15 d), any surviving fish were euthanized with an overdose of benzocaine and were recorded as survivors. The caudal fins of mortalities and final survivors were collected, submerged in RNAlater and stored at -20°C for later use. Mortality was the measure used to rank the rainbow trout families for their resistance to *F. psychrophilum* infection. For subsequent experiments described below (sections 2.3.4 and 2.3.5), resistant and susceptible families were selected based on this resistance ranking.

2.3.4 *MH class II β1 genotyping*

2.3.4.1 DNA extraction

Based on the susceptibility results (section 2.3), caudal fins from survivors and mortalities of families 3, 4, 6, 7, 9, and 10 were collected for DNA extraction. DNA was extracted from all fin clips as described by Kellendonk, 2014. The final concentration of DNA was determined using the Take3 plate in a Synergy H1 plate reader (BioTek Instruments) before samples were moved to the -80°C for storage.

2.3.4.2 Amplification and sequencing of MH class II β1 genotypes

Primers were designed to amplify the MH class IIβ region for this study. The forward primer, OMYF (5'-TGCCAATTGCCTTCTACATTTGCCTG-3') was designed to a conserved region of MH class IIβ at the beginning of exon 1, while the reverse primer, OMYR (5'-TGGGGGCTCAACTGTCTTGTCCAGT-3') was designed to a conserved region at the end of exon 2 (Hodgins, 2011). These primers ensured the amplification of a portion of the peptide binding groove, the region that binds specifically to foreign antigens such as extracellular pathogens. For MH class IIβ amplification, the following PCR parameters were used: denaturation at 95°C for 2 min, followed by 24 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1.5 min. A final extension at 72°C was carried out for 8 min. Two PCR reactions were performed per individual. The amplified PCR products were separated on 1% agarose gels containing 3% Gel Red (Biotium Inc.). Bands of the desired size were cut out of the gel and extracted using the QIAquick Gel extraction kit (Qiagen). The resultant fragments were cloned into pGEM®-T Easy as per the manufacturer's instructions (Promega Corporation, Madison, WI). Competent *E. coli* XL1–blue cells were transformed using the Inoue procedure as described in Green and Sambrook, (2012) and grown on LB plates with 100 μg/mL of ampicillin, 240 ng/mL IPTG and 100 μg/mL X-gal. Ten colonies per PCR reaction were selected and grown in LB medium. Plasmids were extracted from the transformants using the GenElute Plasmid Miniprep Kit (Millipore Sigma) according to the manufacturer's instructions. Plasmids were sequenced using both T7 and SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, Ont.). The sequences were analyzed using both GENEious and the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A G-test was performed on the resulting contingency table using the SPSS statistical software. Significant differences were established at $p < 0.05$.

2.3.5 *Induction of F. psychrophilum antibody responses*

2.3.5.1 Infection

Two trials were performed to measure the levels of IgM antibody developed towards *F. psychrophilum* throughout infection. These trials were conducted in triplicate tanks (n = 40) when the fish were, on average, 108 g. The first trial (referred to as Trial 1) included two resistant families (F21 and F30), as well as two susceptible families (F11 and F17). After sedation with benzocaine, all fish received 100 µL injections i.p. of 1.10 x 10^7 CFU/mL of FPG101 suspended in CB. As a control, fish from the four families analyzed were mixed, then received 100 µL i.p. injections of sterile CB and separated into triplicate tanks. On days 0, 1, 7, 14, 21 and 28, blood was drawn from the caudal vein after anesthesia with ~50 ppm benzocaine. Fish were then euthanized using an overdose of benzocaine and whole-body weights and lengths were measured. Blood samples were left to clot overnight at 4°C before centrifugation at 1000 g for 10 min. The serum was then removed, aliquoted into 1.5 mL tubes and immediately stored at -20°C.

Because it has been reported that rainbow trout require up to 12 weeks to reach peak serum IgM levels when injected with killed *F. psychrophilum* (LaFrentz et al., 2002), a second trial (referred to as Trial 2) was conducted wherein fish were injected i.p. with 100 µL of a lower dose of live FPG101 (3.25 x 10^6 CFU/mL) and sampled over 140 days. In this trial, one resistant family (F32) and one susceptible family (F38) was used and all experiments were done in triplicate tanks (n = 40) where the fish were, on average, 85 g. For both families, triplicate control tanks were set up where all control fish received 100 µL i.p. injections of sterile CB broth. Serum samples were collected as described above. In Trial 2, the anterior head kidney was also removed from sampled individuals so that respiratory burst activity of head kidney leukocytes could be measured (described further in 2.3.6).

2.3.5.2 Isolating antigens from F. psychrophilum

Subcultures of glycerol stocks of FPG101 were grown on CA at 14°C and carefully checked for purity. An isolated colony was then used to inoculate 50 mL of CB, which was subsequently used to

inoculate 200 mL of CB after three days of incubation at 14°C. When the culture reached an optical density of approximately 0.8 at 525 nm, indicating a concentration of ~1 x 10^8 CFU/mL (Holt 1987), 0.5% formalin was added to the flask and cells were incubated at 14°C for 24 h with gentle stirring. The killed bacterial cells were then washed twice with 4 mL of phosphate buffered saline (PBS) then harvested by centrifugation (9,500 g at 4°C for 15 min) and the resultant pellets resuspended in 2 mL of PBS.

Six hundred microliters of 0.1 mm zirconia/silica beads (BioSpec Products) was added to a 1.5 mL screw cap tube followed by the addition of 600 µL of the inactivated *F. psychrophilum* culture. The tubes were placed in a BioSpec mini-beadbeater and agitated on full speed for 45 sec followed by 1 min of incubation on ice. This procedure was repeated five times for each tube before the samples were centrifuged at 9,500 g for 15 min. Following centrifugation, the supernatants were removed and combined in a sterile 15 mL Falcon tube. The protein concentration of the bacterial lysate was then quantified using a bicinchoninic acid assay (ThermoFisher Scientific) following the manufacturer's protocol. The bacterial lysate was then diluted to a final concentration of 0.5 µg/mL in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6).

2.3.5.3 Indirect ELISA assay to detect levels of F. psychrophilum antibodies in serum

One-hundred microliters of the whole-cell bacterial lysate preparation was used to coat the test wells of Nunc™ MicroWell™ 96-well microplates (ThermoFisher Scientific). Hyperimmune serum from rainbow trout injected with *F. psychrophilum* FPG25 (Hesami et al., 2008) was also used at a dilution of 1:50 as a positive control. To obtain hyperimmune serum, 200 g rainbow trout were injected in the dorsal sinus with 100 µl of formalin-killed FPG25. Three months later, fish received a 100 µl booster of formalin-killed *F. psychrophilum* FPG25. Serum samples were obtained at 5 months and 7 months following the initial exposure. Negative control wells were coated with the whole cell bacterial lysate but were incubated with 100 µl Tris-buffered saline supplemented with 0.5% Tween 80 (TBS-T) rather than rainbow trout serum to account for any non-specific binding of the detecting antibodies. The coated plates were incubated overnight at 4°C to enable adherence of the antigens.

The following day, all wells were washed three times with TBS-T. Three hundred microliters of blocking solution (2% laboratory grade gelatin (ThermoFisher Scientific) dissolved in TBS-T) was added to each well and incubated for 1 h at 37ºC. The plate was then washed three times with TBS-T and 100 µL of each trout serum (diluted 1:50) was added to triplicate test wells. After incubation for 1 h at room temperature (RT) the wells were washed thrice with TBS-T and 100 µL of mouse anti-rainbow trout IgM monoclonal antibody (Aquatic Diagnostics) at a dilution of 1:50 was added to each test well and hyperimmune serum control wells. The plate was incubated for 1 h at RT after which all wells were washed thrice with TBS-T. All test and the hyperimmune serum positive control wells received 100 µl of goat anti-mouse IgG conjugated to alkaline phosphatase (Millipore Sigma) at a dilution of 1:30 000. The plate was incubated for 1 h at RT and then washed three times with TBS-T. Fifty microliters of substrate, p-nitrophenyl phosphate (p-NPP), was added to each well and the plate was incubated in the dark for 30 min. The reaction was then stopped by the addition of 50 µl of 0.03M of NaOH to each well. The plate was read immediately at an absorbance of 405 nm in a Synergy H1 plate reader (BioTek). The negative control wells, which received TBS-T rather than trout serum, were averaged and subtracted from all other absorbance values. Plate variation was accounted for by normalizing each plate to the hyperimmune serum positive control. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA) where each of the triplicate tanks was considered an experimental unit (n = 3), and variation among tanks of each family receiving the same treatment was used as experimental error. This was then followed by Sidak's Multiple Comparisons Test with significant differences established at $p < 0.05$.

2.3.6 *Measuring Respiratory Burst Activity (RBA) of blood and head kidney leukocytes*

2.3.6.1 Infection

To determine whether RBA was associated with the observed resistance or susceptibility to *F. psychrophilum*, two infection trials were conducted. The families used for the first RBA trial are described above in section 2.3.5 (Trial 2). On days 0, 1, 7, 14 and 28, three fish per tank were euthanized with an overdose of benzocaine and the anterior head kidneys were collected and transferred to sterile

plasticware containing approximately 20 mL of L15 medium (Lonza) supplemented with 5% FBS, 100 U/mL of penicillin and 100 U/ml of streptomycin. These samples were stored on ice until the HKL could be isolated.

To validate the results observed for RBA in Trial 2, a repeat trial (Trial 3) was completed using the resistant family, F36 and the susceptible family, F38. Challenged fish received 100 µL i.p. injections of FPG101 (3.50×10^6 CFU/mL). This trial was conducted in triplicate tanks (n = 40) when the fish were, on average, 200 g. As a control, fish from the two families analyzed were mixed, received 100 µL i.p. injections of sterile CB, and were separated into triplicate tanks. On days 0, 3, 7, 14 and 20, three fish per tank were euthanized with an overdose of benzocaine so that the anterior head kidney of each individual could be removed and processed as described.

2.3.6.2 Isolation of head kidney leukocytes

The head kidney samples were vigorously vortexed 5 times at maximum speed for 30-60 sec to dissociate the cells from the underlying connective tissues. The dissociated cells were passed through a 40 µm sterile nylon mesh (ThermoFisher Scientific) then rinsed with 5 mL of sterile media. The resulting cell suspensions were brought up to a final volume of 28 mL and, after thorough mixing, divided into four 7 mL aliquots. The 7 mL cell suspensions were then layered on top of 3 mL of Histopaque 1077 (Millipore Sigma) and centrifuged for 500 x g for 30 min at 4°C. The leukocyte layer located at the medium:histopaque interface was collected and cells were pelleted by centrifugation at 500 x g for 5 min at 4°C. The supernatant was aspirated and the pellets were washed using 5 mL of PBS and again, harvested by centrifugation. The resultant cell pellets were resuspended in 1-3 mL of fresh 5% FBS/L15 so that cell concentration and viability could be determined using a trypan blue exclusion assay. After cell viability was determined to be above 95%, cell concentrations were adjusted to 7.0×10^6 cells/mL.

2.3.6.3 Real-time luminol-enhanced chemiluminescence assay for RBA

The RBA of both the whole blood samples and the head kidney leukocytes (HKL) was measured following the protocol of Jimenez-Vera et al. (2013) with minor variations. Briefly, Corning™ 96-well solid white polystyrene microplates were prepared with 6 wells containing either 100 µL of HKL (7.0 x 10^6 cells/mL) or 75 µL of blood sample from each individual. The first three wells per individual received 40 µL of luminol (10 mM, Millipore Sigma), 100 µL of zymosan A (20 mg/mL, Millipore Sigma), and were brought to a final volume of 300 µL using Hank's Balanced Salt Solution (HBSS, Millipore Sigma). To ensure that the increase in luminescence was due to stimulation, the remaining three wells per individual were used as unstimulated controls and received 100 µL of HBSS in place of zymosan A. The chemiluminescence of each well was measured with a Synergy H1 luminometer (Biotek) every 2 min for 90 min. The chemiluminescence emission of the HKL and blood samples was expressed as the integral of the relative light units (RLU) between 0 and 90 min. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA) where each of the triplicate tanks was considered an experimental unit (n = 3), and variation among tanks of each family receiving the same treatment was used as experimental error. This was followed by Sidak's Multiple Comparisons Test and significant differences were established at $p < 0.05$.

2.4 Results

2.4.1 Preliminary trials to determine rainbow trout families resistant/susceptible to F. psychrophilum

Fish from 40 families were infected with *F. psychrophilum* and survival was monitored. As **Figure 2.1A** displays, there was a broad range of survival between the families, with the most resistant family (F8) having 90.9% survival while the most susceptible family (F36) had only 2.5% survival. From the results presented in this study, families were chosen to conduct repeat infection trials so that both genetic (families presented in **Figure 2.1B**) and functional immune markers (families presented **Figure 2.1C**) could be assessed. To ensure that the observed resistance/susceptibility was consistent, the infection trial was repeated for a resistant family (F7) and a susceptible family (F10). In the initial trial F7 and F10 displayed 90.5% and 14.3% survival respectively. These results were confirmed to be robust as F7 and F10 displayed 86.0% and 19.9% survival respectively when the infection trial was repeated (data not shown). As the fish were approximately the same size (~30-50 g) during this repeat trial, these results provide evidence that susceptibility to FPG101 was consistent at this size/age.

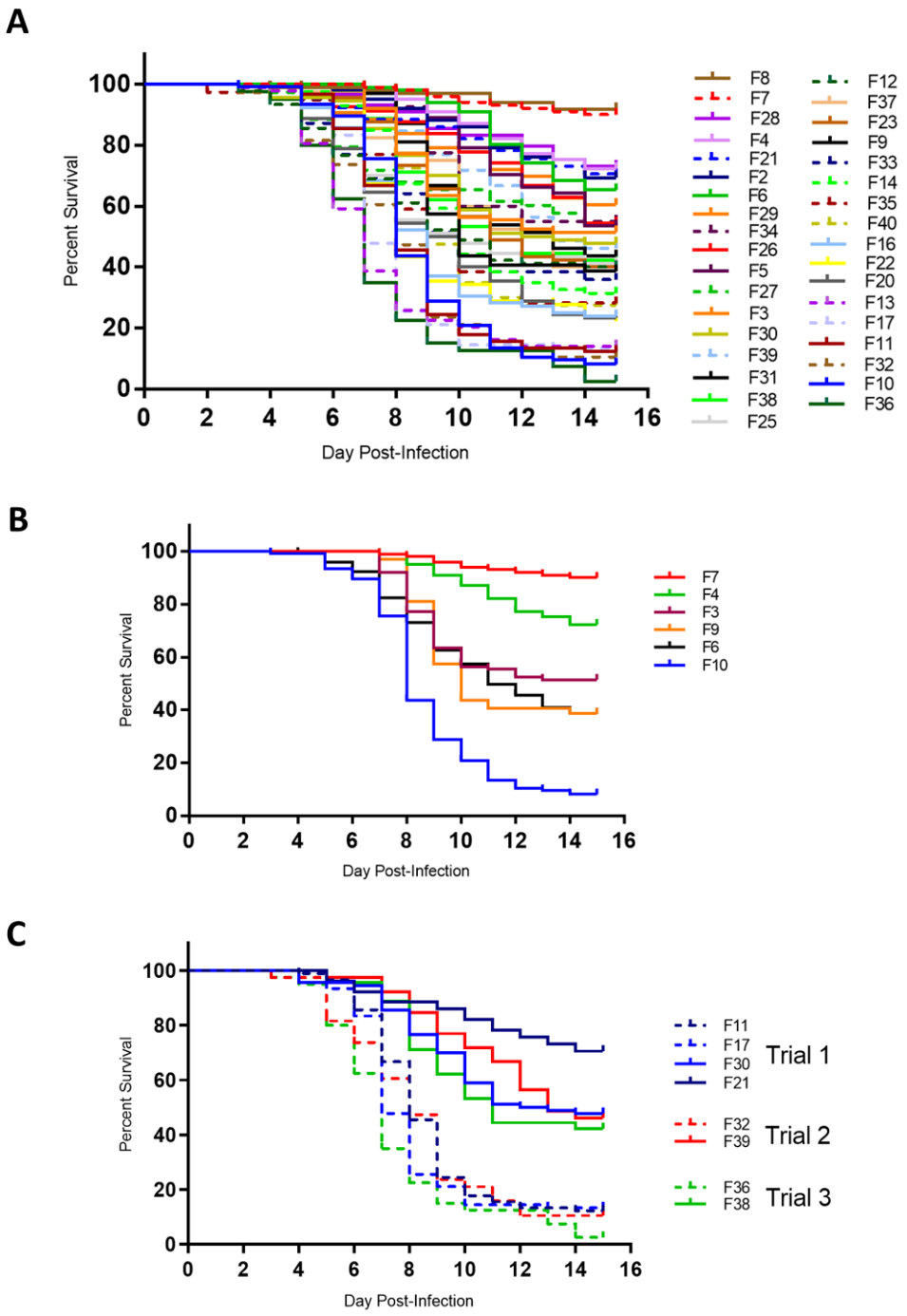

Figure 2.1. Survival curves throughout *F. psychrophilum* infection for the forty rainbow trout families analyzed for resistance/susceptibility to BCWD. Panel A: Survival curves for all rainbow trout families analyzed. Panel B: Survival curves for the six families used for MH class II β1 genotyping. Panel C: The survival curves for the eight families selected for the functional immune trials (Trials 1–3).

2.4.2 Relating MH class IIβ genotype to disease susceptibility

To explore whether there is a link between BCWD resistance and MH class IIβ alleles, a minimum of 18 individuals from six of the rainbow trout families studied were genotyped. The families selected (F3, F4, F6, F7, F9 and F10) had variable survival rates when challenged with *F. psychrophilum* (see **Figure 2.1B**) and thus were hypothesized to have variability in their MH genotypes. **Table 2.1** presents the MH class IIβ genotype frequencies observed between the six different families. Overall, there were five alleles identified which created a total of twelve MH class IIβ genotypes in the six families studied. When relating the allele prevalence to the observed number of survivors per family, as presented in **Table 2.2**, both DAB*1001/DAB*1001 and DAB*1001/DAB*0801 were observed at the highest frequency in both survivors and mortalities.

Table 2.1. MH Class IIβ genotype frequencies among the six families of *Oncorhynchus mykiss*. A total of 19 individuals were sequenced for families 4 and 9, and 18 individuals were sequenced for the other four families. Frequency was calculated using the formula f=x/n, where x is the number of times the genotype was observed and n is the number of individuals.

	Families					
	3	**4**	**6**	**7**	**9**	**10**
MH Class IIβ Genotype						
DAB*1001/DAB*1001	-	0.74	0.39	0.67	0.26	-
DAB*1001/DAB*1101	0.22	0.16	-	-	0.11	0.44
DAB*0801/DAB*0801	-	-	-	-	0.11	0.06
DAB*1001/DAB*0801	-	0.05	0.56	0.33	0.21	0.39
DAB*1101/DAB*0801	0.22	-	0.06	-	-	-
DAB*1201/DAB*1001	0.17	-	-	-	-	-
DAB*1201/DAB*0801	0.17	-	-	-	-	-
DAB*0401/DAB*1001	-	-	-	-	0.05	-
DAB*0801/1001/1101	0.11	0.05	-	-	0.05	0.11
DAB*0401/0801/1001	-	-	-	-	0.16	-
DAB*1001/1101/1201	0.06	-	-	-	-	-
DAB*0401/0801/1001/1101	-	-	-	-	0.05	-

Table 2.2. MH class IIβ genotype prevalence in mortalities and survivors to *F. psychrophilum* infection. A G-Test was performed on this contingency table using SPSS statistical software to identify any significant differences between genotypes.

Genotype	Percent of Mortalities	Percent of Survivors
DAB*1001/DAB*1001	32	40
DAB*1001/DAB*1101	18	13
DAB*0801/DAB*0801	3	2
DAB*1001/DAB*0801	19	31
DAB*1101/DAB*0801	6	2
DAB*1201/DAB*1001	3	2
DAB*1201/DAB*0801	5	2
DAB*0401/DAB*1001	2	0
DAB*0401/DAB*0801	2	0
DAB*0801/1001/1101	6	4
DAB*0404/0801/1001	3	0
DAB*1001/1101/1201	0	2
DAB*0401/0801/1001/1101	0	2

2.4.3 IgM antibody development in rainbow trout families resistant/susceptible to F. psychrophilum

The production of rainbow trout serum IgM against *F. psychrophilum* FPG101 appeared to increase over time regardless of the previously observed resistance in the six families studied. In Trial 1, anti-*F. psychrophilum* IgM levels increased over the 28-day infection but there was no significant difference in antibody production between the resistant (F21 and F30) and the susceptible (F11 and F17) families (**Figure 2.2A**). A similar trend was also seen in the 140-day infection trial, Trial 2 (**Figure 2.2B**), where serum IgM levels were also seen to increase over time with no significant difference between the resistant (F39) and susceptible (F32) families studied. As expected, the saline-injected controls in Trial 2 showed no increase in anti-*F. psychrophilum* serum IgM levels over time (**Figure 2.2B**).

The survival curves of families in both Trial 1 (**Figure 2.2C**) and Trial 2 (**Figure 2.2D**) did not reveal significant differences between resistant and susceptible families in contrast to the preliminary trials (**Figure 2.1C**). This can be seen in **Figure 2.2E** and **2.2F** where the percent mortality of families in the preliminary trial is compared to the percent mortality in Trial 1 (**Figure 2.2E**) and Trial 2 (**Figure 2.2F**). In the preliminary trials, the resistant families had low mortality rates (5 to 42%) while the rates in susceptible families were much higher (57 to 85%). However, upon completion of Trial 1 and Trial 2, the percent mortality was more similar regardless of whether the family was previously observed to be resistant or susceptible to *F. psychrophilum* (**Figure 2.2E** and **2.2F**). In Trial 1, the four families studied had observed mortalities between 70 and 80% while in Trial 2, the two families studied had mortalities of 25 and 30%.

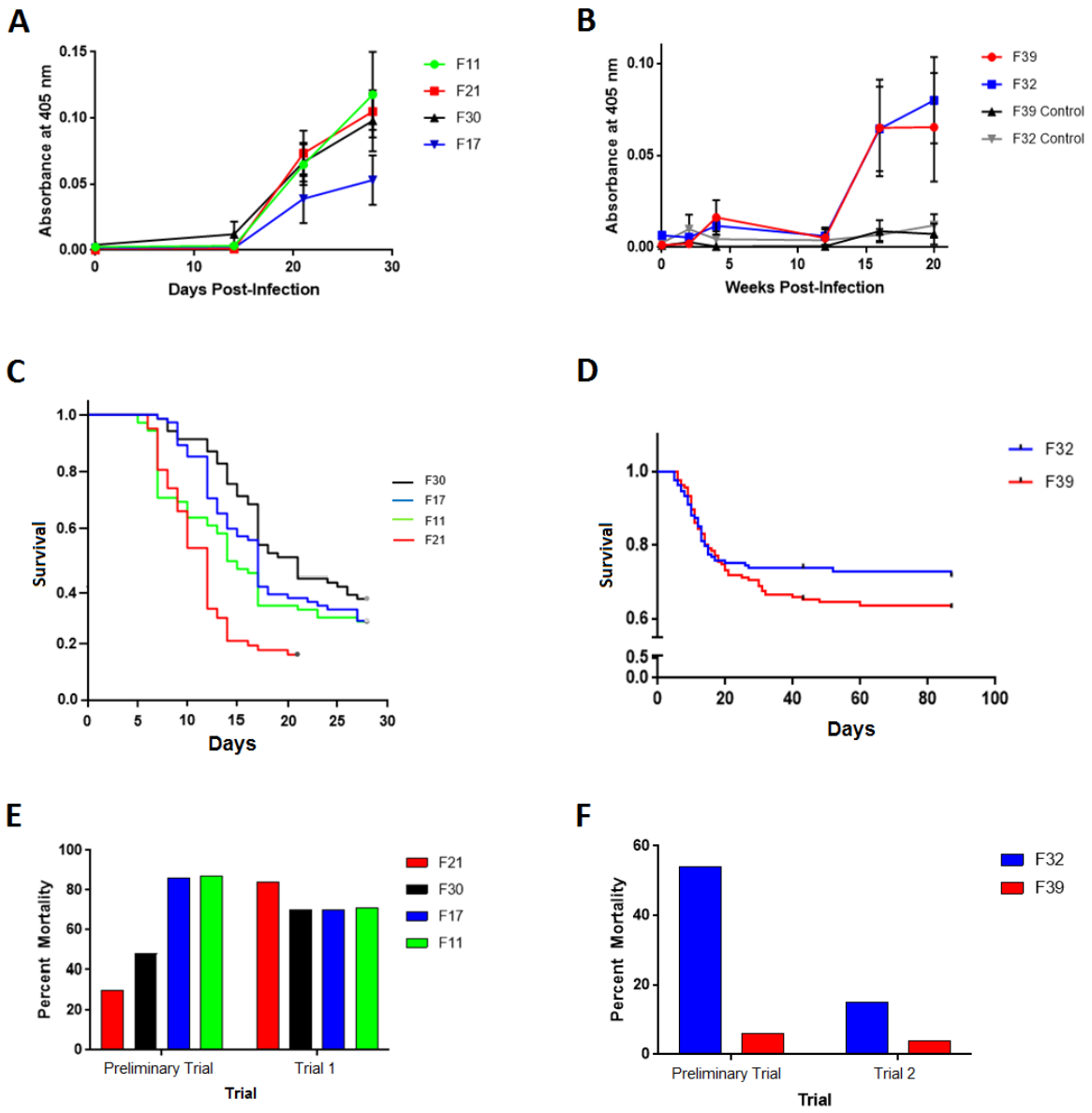

Figure 2.2. Indirect ELISA assay to compare serum IgM development against *F. psychrophilum* between resistant and susceptible families throughout infection. Indirect ELISA results for serum IgM levels developed against *F. psychrophilum* between two resistant (F21 and F30) and two susceptible (F11 and F17) rainbow trout families throughout a 28-day infection trial (A). Indirect ELISA results for serum IgM levels developed against *F. psychrophilum* between one resistant (F39) and one susceptible (F32) rainbow trout families throughout a 140-day infection trial. Serum from saline injected controls was also tested for antibody development to *F. psychrophilum* throughout the infection trial (B). Survival curve for the two resistant (F21 and F30) and two susceptible (F11 and F17) families during the 28-day infection trial (C). Survival curve for the resistant (39) and the susceptible (F32) families during the 140-day infection trial. Survival is only shown up to day 90 as there was no further mortality observed in this trial after this time (D). Comparison of the observed mortality during the preliminary trial to determine resistance/susceptibility and the trials used to measure serum IgM against *F. psychrophilum* (E-F). Error bars represent the standard error of the mean (SEM).

2.4.4 The effect of head kidney and total blood RBA on resistance to F. psychrophilum

The RBA of head kidney leukocytes from the infected resistant (F39) and susceptible (F32) families of Trial 2 were observed to differ significantly on day 14 (**Figure 2.3A**). Otherwise there was no significant difference in RBA between the groups. Throughout infection, the two families had very similar trends in RBA with high activity observed on days 0 and 1, followed by a significant decrease in activity on day 7 of the trial. For the final two weeks of the trial, RBA was observed to slowly recover until it was restored to pre-infection levels on day 28 (**Figure 2.3A**). The large drop in RBA on Day 7 was interesting as this was also the time when mortalities were first observed in Trial 2. On day 28 and beyond, when RBA activity appeared to have fully recovered, mortalities were rarely observed. The observed decrease in RBA of HKL in infected fish appears to be a consequence of *F. psychrophilum* infection as the sterile CB infected control fish had no significant difference in HKL RBA across all time points (**Figure 2.3A**). Much like what was observed in Trial 2, the RBA of the HKL did not differ significantly between the resistant (F36) and susceptible (F38) families studied. A significant decrease in RBA was observed at day 3 of Trial 3 (**Figure 2.3B**) and was maintained until trial completion on day 20. Further, there was no difference in total blood RBA between the two families studied throughout infection in Trial 3 (**Figure 2.3C**).

Although there was little to no difference in the RBA between the resistant and susceptible families studied, it is important to relate these results to the survival of each group throughout *F. psychrophilum* infection. In the preliminary trial, low mortality was observed in the resistant families, F32 and F36 (2-5%), while high mortality was noted in the susceptible families, F38 and F39 (42-55%) (**Figure 2.1C**). Despite these initial large differences in survival, upon completion of the RBA trials, all four of the families had consistent mortalities ranging between 1-10% (**Figure 2.3D**).

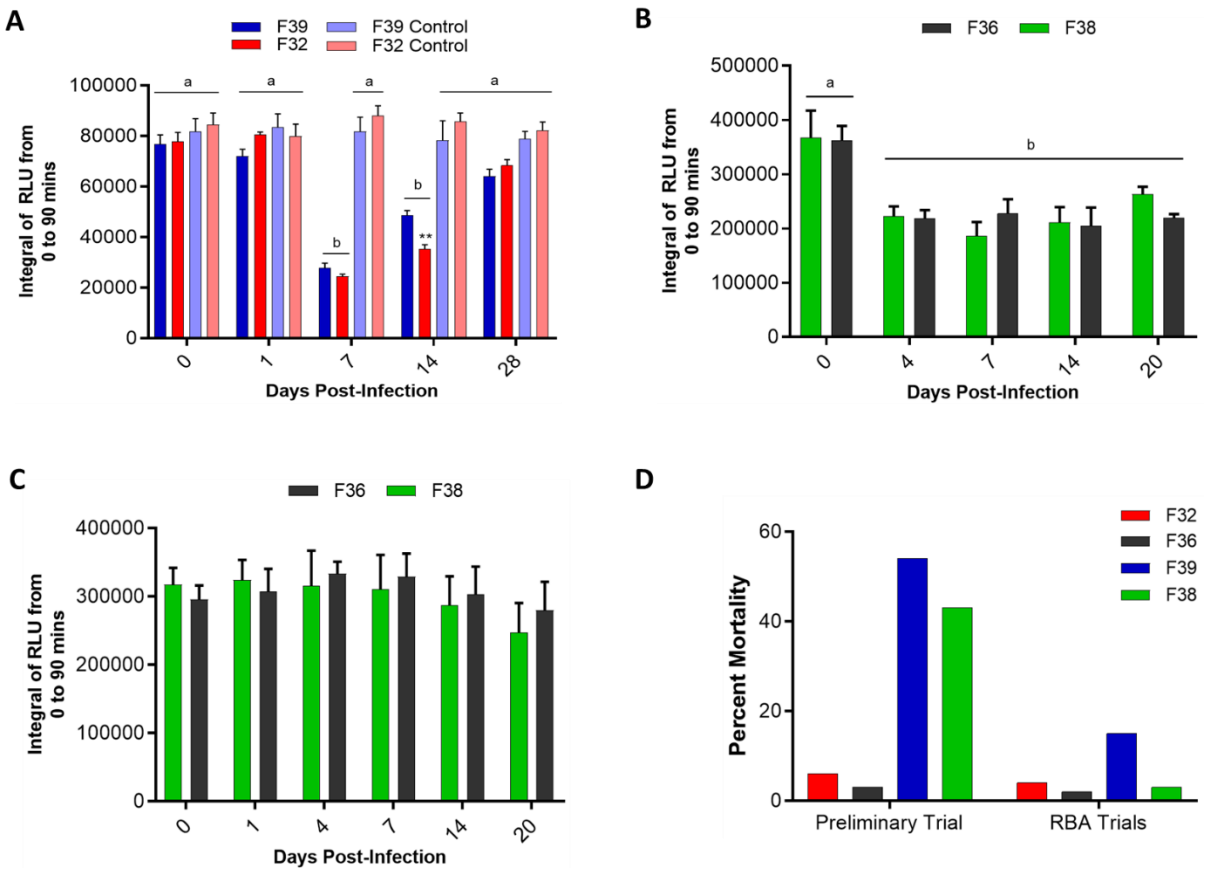

Figure 2.3. Respiratory burst activity of HKL and total blood in resistant and susceptible families of rainbow trout. RBA of HKL in one resistant (F39) and one susceptible (F32) rainbow trout family throughout *F. psychrophilum* infection. Control fish received i.p. injections of sterile CB (A). RBA of HKL in another resistant (F38) and susceptible (F36) rainbow trout family throughout *F. psychrophilum* infection (B). Impact of *F. psychrophilum* on the RBA of total blood in a resistant (F38) and a susceptible (F36) rainbow trout family throughout *F. psychrophilum* infection (C). Comparison of the observed mortality during the preliminary trial to determine resistance/susceptibility and the observed mortality in the trials used to measure RBA throughout *F. psychrophilum* infection (D). Error bars represent the SEM.

2.5 Discussion

The purpose of this study was to determine whether both functional and/or genetic immune markers could be used to predict resistance/susceptibility to the coldwater bacterial pathogen, *F. psychrophilum*. To assess this, forty full-sibling families of rainbow trout were created and when subsets of each were infected with *F. psychrophilum*, some families performed extremely well (90% survival), while others had very low survival (3% survival). This broad range in BCWD susceptibility has been observed in previous studies aimed at understanding and consistently standardizing the production of resistant rainbow trout (Hadidi et al., 2008; Wiens et al., 2013). This observed resistance/susceptibility to BCWD has been shown to be heritable through multiple generations of rainbow trout (Leeds et al., 2010; Silverstein et al., 2009; Wiens et al., 2013). Though promising, a great deal of time and resources is required to first identify resistant strains and then to subsequently propagate appropriate crosses until you have highly resistant stocks of fish. As a result, there is an undeniable benefit to identifying specific immune markers that are able to predict resistant or susceptible phenotypes towards BCWD.

Because of its role in the exogenous antigen presentation pathway, MH class II alleles are a genetic candidate of interest when attempting to select for resistance towards bacterial pathogens such as *F. psychrophilum*. In this study, when families shown to be resistant/susceptible to BCWD were genotyped for MH class II β1, only five alleles were observed resulting in a total of 12 different possible genotypes. This is a lower number than previously observed when wild fish populations were analyzed (Conejeros et al., 2008; Dorschner et al., 2000; Conejeros et al. 2012). However, because the rainbow trout families used in the current study were not from wild populations and thus had likely been selected for faster growth, better feed to weight conversion and better flesh quality, this greater degree of homogeneity was not unexpected. Increasing the variability of MH genotypes has not been a breeding focus for these families, therefore low diversity in MH genes may simply be a by-product of selection for other desirable traits. This has been reported in many other studies of production (Croisetiere et al.,

2008; Gomez et al., 2011; Grimholt et al., 2003; Kjoglum et al., 2008), thus the low allele frequencies presented in this study are likely representative of the commercial origin of the families that were used.

Remarkably, individuals with three or four allele genotypes for MH class IIβ were observed in this study but these generally occurred at very low frequencies in the families analyzed. The occurrence of two or more alleles per individual has been observed in previous MH studies (Conejeros et al., 2012; Conejeros et al., 2008; Dixon et al., 1996; Kruiswijk et al., 2004; Noakes et al., 2003) but this was usually attributed to high MH polymorphism. Although PCR artifacts and false alleles can arise due to the nature of MH sequences and the cloning process, precautions were taken in this study by modifying the PCR protocol to minimize their formation (Lenz & Becker, 2008). Accordingly, we have high confidence that the sequences presented in this study represent real genotypes, even those animals containing three to four alleles. Another explanation for these interesting genotypes is that the alleles come from two different loci. If this is the case, after phylogenetic tree analysis, researchers would anticipate seeing two major branches with clusters of alleles in each. Instead, researchers that have analyzed this phenomenon have observed a subdivided tree with many small clusters of alleles (Dorschner et al., 2000). As this has not been the central focus of MH analysis in teleost species, further research is required.

When relating the allele prevalence to the observed number of survivors per family, both DAB*1001/DAB*1001 and DAB*1001/DAB*0801 were observed at the highest frequency in both survivors and mortalities. These results are consistent with what has been previously observed in microsatellite and SNP analysis studies wherein classical MH class II genotypes were not found to be linked to BCWD resistance (Johnson et al., 2008) or only a weak correlation ($p < 0.15$) has been noted (Overturf et al., 2010). Despite these similar results, the MH genotypes presented in the current study represent full allele sequences rather than correlating a measure of MH genetic diversity with BCWD resistance. As a result, this is the first study in which specific amino acid sequences of the MH class IIβ peptide binding groove have been analyzed in rainbow trout to determine whether they are associated with resistance/susceptibility to *F. psychrophilum* infection. Based on the analysis of 110

rainbow trout from six different full-sibling families, the MH class II genotypes of these fish do not appear to be associated with resistance/susceptibility towards FPG101.

Although the pathogenesis of *F. psychrophilum* is not fully understood, the organism is known to have a systemic phase during infection (reviewed by Nilsen et al., 2011b; Starliper, 2011). Accordingly, it is reasonable to hypothesize that the humoral immune response, including antibody production, may be important for the control of BCWD. In a recent study by Marancik et al. (2014) comparing whole body transcriptomes between resistant and susceptible rainbow trout groups, higher *igm* gene transcript levels were observed in rainbow trout lines that were resistant when compared to their susceptible counterparts. Thus, serum IgM could play a role in the observed resistance to BCWD. Infection with *F. psychrophilum* was shown here to induce an antibody response, but the relative levels were not significantly different when comparing between resistant and susceptible fish. Although there has been an increasing interest in understanding the mucosal and systemic immunity in response to *F. psychrophilum* (Makesh et al., 2015), this is the first study in which serum IgM levels have been compared between families with established *F. psychrophilum* resistance and susceptibility phenotypes.

Depending on the organism in question, bacterial pathogens influence the respiratory burst activity of fish leukocytes in a variety of ways. The intracellular pathogen, *Aeromonas hydrophila* has been shown to increase the RBA of host leukocytes 7 days following infection (Biller-Takahashi et al., 2013). In contrast, both intracellular *Renibacterium salmoninarum* and extracellular *Vibrio anguillarum* have been shown to reduce the spleen leukocyte and HKL RBA of their hosts (Densmore et al., 1998; Sepulcre et al., 2007b). This is not surprising considering that *R. salmoninarum* and *V. anguillarum* both produce virulence factors that negatively impact fish leukocytes, p57 and RTX proteins respectively (Li et al., 2008; Wiens & Kaattari, 1991). Despite this, a comparable virulence factor of *F. psychrophilum* has not yet been discovered. To date there is little to no data regarding the effect of *F. psychrophilum* on total leukocyte populations but it has been previously shown that the organism can survive within, and be cytotoxic to, rainbow trout phagocytes (Decostere et al., 2001;

Lammens et al., 2000; Nematollahi et al., 2005; Wiklund & Dalsgaard, 2003). As HKL would contain phagocytes and the RBA of these cells was dramatically reduced in this study, perhaps this cytotoxic effect to phagocytes extends to other leukocytes. Further research is necessary to understand what aspect of *F. psychrophilum* is responsible for the decrease in HKL RBA that was observed in this study.

To ensure that the observed reduction in RBA was due to bacterial challenge, rather than leukocytes leaving the head kidney to monitor the periphery, a repeat trial (Trial 3) was conducted in which the respiratory burst activity of the HKL as well as in total blood from each fish was measured. If leukocytes had left the head kidney to monitor the periphery, an increase in the RBA of total blood would be anticipated. It is tempting to speculate that the HKL are not travelling to the bloodstream because the total blood RBA did not significantly change throughout infection. This is a possibility, but it is also feasible that immune cells were travelling to inflammatory sites such as the spleen or external lesions, and that their transit was not monitored at the time points selected for this trial. Although there is limited research on immune cell transit in salmonids, it has been shown in the embryonic zebrafish model that it can take as little as 4 hours for neutrophils to travel to the injection site of live *Pseudomonas aeruginosa* while no neutrophil transport is observed following sterile PBS injection (Deng et al., 2012). Similarly, phagocytes also migrate rapidly to sites of inflammation and bacterial infection (Davis et al., 2002; Herbomel et al., 1999; Zakrzewska et al., 2010) thus the time points selected for this study may not have been early enough to observe leukocyte migration. The results of the current study do suggest that *F. psychrophilum* may reduce the respiratory burst activity of head kidney leukocytes but this finding merits further research to validate whether the leukocytes are truly influenced by *F. psychrophilum*, or simply in transit to deal with the infection at other locations.

Though there were little to no differences in the immune parameters studied here between resistant and susceptible rainbow trout families, it is important to consider the massive variability in survival between each of the three trials. Furthermore, how these trials compare to the preliminary trial used to rank family resistance. The difference in mortality between Trial 1 and Trial 2 can be explained by the fact that the infectious dose used in Trial 2 was lower by a factor of ~10 which was used to

ensure enough fish survived for sampling throughout the longer (4 mo.) period of this trial. The large differences in mortality between Trials 1 and 2 also reflected differences in the size and age of the fish. In the preliminary trials, all fish were approximately 40 g in size, versus the 108 g or 85 g average size of the fish for Trials 1 and 2 respectively. This was also seen in the RBA trials where the susceptibility of F39 and F38 that was observed in the preliminary trial was almost nonexistent in Trial 3, when the fish had grown to an average size of 205 g. These results further support previous findings that the age of the host is very important in *F. psychrophilum* infection. For example, this age-related resistance has been observed in RTFS wherein macrophages obtained from susceptible 10-week-old rainbow trout fry were shown to contain viable, potentially replicating *F. psychrophilum*, while resilient fish aged 5 months and older did not (Decostere et al., 2001). Age-related disease susceptibility in salmonids has been observed for several other pathogens including the parasite *Hexamita salmonis* (Tojo & Santamarina, 1998), infectious pancreatic necrosis virus (IPNV, (Espinoza & Kuznar, 2002; Roberts & Pearson, 2005) and *Flavobacterium branchiophilum* (Good, Thorburn, & Stevenson, 2008). Thus, age/size differences have been shown to influence susceptibility to several aquatic diseases including *F. psychrophilum*, which may provide an explanation as to why the observed serum IgM for Trials 1 and 2 and the RBA for Trials 2 and 3 did not correlate with the resistance/susceptibility observed in the preliminary trial.

2.6 Conclusions

In this study, we showed that the resistance of rainbow trout fry to *F. psychrophilum* may be influenced by age/size as differences in susceptibility were not consistent at later life stages. Breeding for resistance in fry/fingerlings could still be beneficial for fish farmers as BCWD tends to be a disease of young salmonids. As such, losses of even young stock can be costly in terms of increased management costs, reduced feed conversion, etc. No clear genetic markers were identified that could be used for informed breeding strategies aimed at combatting BCWD. More specifically, MH class IIβ genotypes do not appear to correlate with the observed resistance/susceptibility to *F. psychrophilum*. In addition, neither anti-*F. psychrophilum* IgM levels nor the RBA of HKL and total blood were found

to differ between families previously observed to be resistant or susceptible to the organism. It is possible that the early observed differences in susceptibility would correlate with differences in these selected immune markers if tested during that earlier life stage. Accordingly, future research should emphasize the study of functional immune markers during the fry stage of development and additional immune/host markers should be evaluated. As RBA of HKL was significantly reduced in response to live infection with *F. psychrophilum*, future studies should also focus on understanding the pathogenesis of *Flavobacterium psychrophilum* FPG101 and its interaction with rainbow trout leukocytes.

Acknowledgements

Lyndon Hatcheries for creating the rainbow trout families and Alma Research Station at the University of Guelph for maintaining the crosses as they grew. Matt Cornish and Mike Davies of the University of Guelph's Hagen Aqualab for their helpful advice and technical insights when planning and executing the infection trials. And very importantly, thank-you to the many individuals who helped with infections and fish care throughout the numerous trials, including Paul Huber, Ehab Misk, Maureen Jarau, Juan-Ting Liu, Ryan Horricks and Ryan Eagleson. This work was supported by a grant from the Natural Sciences and Engineering Council entitled "Immunity, vaccination and rainbow trout family susceptibility to *Flavobacterium psychrophilum*" STPGP 413317-2011.

Chapter 3: Understanding the pathogenesis of *Flavobacterium psychrophilum* using the rainbow trout monocyte/macrophage-like cell line, RTS11, as an infection model.

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3.1 Overview

The life cycle of *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), appears to involve interactions with spleen and head kidney macrophages. To develop an in vitro model for studying this, *F. psychrophilum* was incubated with a rainbow trout monocyte/macrophage-like cell line (RTS11) in different ways and fundamental macrophage functions were evaluated. The animal cell basal medium, L15, supplemented with bovine serum (FBS) supports RTS11 maintenance, and surprisingly, L15 with 2% FBS (L15/FBS) also supported *F. psychrophilum* growth. L15/FBS in which the bacteria had been grown is referred to as *F. psychrophilum* conditioned medium (*Fp*CM). Adding *Fp*CM to RTS11 cultures caused a small percentage of cells to die, many cells to become more spread, and phagocytosis to be temporarily reduced. *Fp*CM also stimulated transcript expression for pro-inflammatory cytokines (IL-1β, TNFα and IL-6) and the anti-inflammatory cytokine (IL-10). Adding live *F. psychrophilum* to RTS11 cultures also altered cell shape, stimulated cytokine expression more profoundly than *Fp*CM, but phagocytosis was not impaired. Adding heat-killed bacteria to RTS11 cultures elicited few changes. These bacteria/RTS11 co-cultures should be useful for gaining a deeper understanding of the pathogenesis of *F. psychrophilum* which may aid in the development of effective measures to prevent infection and spread of this troublesome disease.

3.2 Introduction

The relatively recent development of salmonid aquaculture means that there are still many gaps in our knowledge regarding the challenges that this enterprise faces. Several of these obstacles can cause significant financial losses, but none are as severe as infectious disease. In 2014 the global loss due to infectious disease totalled approximately $6 billion dollars USD (World Bank, 2014), a significant deficit to the $70 billion dollars of product destined for human consumption (FAO, 2016). This has made both disease prevention and control a primary concern for aquaculture production. Yet when compared with our understanding of terrestrial pathogens, little is known about the life cycle and pathogenesis of the aquatic microorganisms that plague aquaculture. Furthermore, advances in salmonid immunology have called into question mammalian notions of what constitutes an effective immune response. This has left farmers with relatively few options outside of antibiotics to combat infectious agents, often exacerbating the problem by enhancing antibiotic resistance (reviewed by Santos & Ramos, 2018). Until we can advance our understanding of aquatic pathogens, both the development and improvement of disease control programs may be limited.

The bacterium, *Flavobacterium psychrophilum,* is one example of an aquatic pathogen causing financial losses for global rainbow trout aquaculture. But despite this, there is still very little known regarding its' infection process. Interestingly, differences in geographic location, bacterial isolate and host age can result in two separate disease presentations: bacterial coldwater disease (BCWD) or rainbow trout fry syndrome (RTFS, Lumsden et al., 2004). These conditions present as either an acute bacteremia primarily in small fish (RTFS) or as a more chronic disease characterized by an ulcerative dermatitis (BCWD) in larger fish (Lumsden et al., 2004; Starliper, 2011). Financial losses due to this organism can reach as high as 50-90% (Brown et al., 1997; Cipriano & Holt 2005; Nilsen et al., 2011b) resulting in several unsuccessful attempts to develop a vaccine (Hoare et al., 2017; Makesh et al., 2015). Because the infectious route of *F. psychrophilum* is unresolved, confirming the efficacy of vaccine candidates using methods that mimic natural infection has not been possible and has hindered vaccine design (reviewed by Gomez et al., 2014; Henriksen et al., 2013). As a result, antibiotic resistance has

increased in *F. psychrophilum* isolates and there are currently few other modes of combat or prevention (Hesami et al., 2010; Miranda et al., 2016; Ngo et al., 2017). When taking this all into consideration, it is clear that additional study of *F. psychrophilum*-host interactions within a relevant model system would prove invaluable.

In most animal models, it is well established that monocytes patrol the blood and blood associated tissues as immune effector cells. Upon damage or infiltration, monocytes are rapidly recruited to affected tissues where they can then differentiate into macrophages, the tissue resident phagocytic cells. Macrophages play many important roles in homeostatic maintenance but are also equipped with an arsenal of pattern recognition receptors (PRRs) to recognize any abnormalities or foreign entities and respond accordingly (reviewed by Geissman et al., 2010; Yang et al., 2014). Though its pathogenesis is not a common study focus, there is evidence to support that the infection cycle of *F. psychrophilum* is intricately involved with rainbow trout macrophages (Nematollahi et al., 2005; Semple et al., 2018a). As such, the rainbow trout splenic monocyte/macrophage-like cell line, RTS11 (Ganassin & Bols, 1998), would be an ideal model system to study this bacterial pathogen and confirm its interaction with these cells. To date, the contributions of RTS11 to teleostean immunology have been monumental in advancing our knowledge of salmonid gene expression (Alvarez et al., 2016; Semple et al., 2019), phagocytic activity (Montero et al., 2008; Leal et al., 2017), apoptosis (Rojas et al., 2010; Rojas et al., 2012), chemotaxis (Montero et al., 2008; Semple et al., 2018c) and more. Given all that has been accomplished with this cell line, RTS11 is likely to reveal more information regarding the pathologic process of *F. psychrophilum* as well as its impact on salmonid immune cells.

In an effort to combat *F. psychrophilum* infections, several studies have focused on breeding for resistance (Silverstein et al., 2009; Marancik et al., 2015), phage therapy (Castillo et al., 2012; Madsen et al., 2013) and/or vaccine development (Madetoja et al., 2005; LaFrentz et al., 2008; Hoare et al., 2017). Most of these methods have proven efficacious in examples of terrestrial disease (Bouters et al., 1973; Shurig et al., 1975; Briles et al., 1977) but as of yet, none have been successful in providing viable treatment options for BCWD/RTFS. The purpose of this study was to optimize and ultimately

use RTS11 to study the interaction of *F. psychrophilum* with rainbow trout immune cells. Additionally, because it is believed that extracellular protease production contributes significantly to its virulence (Duchaud et al., 2007; Hesami et al., 2011), this study also evaluated the impact of the bacterial culture supernatant on RTS11 survival and immune function. Revealing the direct impact of *F. psychrophilum* on rainbow trout immune cells could aid significantly in the development of effective vaccines and other prophylactic treatments for aquaculture.

3.3 Materials and Methods

3.3.1 Maintenance of RTS11

The rainbow trout monocyte/macrophage-like cell line, RTS11 (Ganassin & Bols, 1998), was maintained as described previously by (Sever et al, 2014).

3.3.2 F. psychrophilum growth in different media

F. psychrophilum strain 101 (FPG101) was grown as described previously by Semple et al (2018a) with minor adjustments. This Ontario isolate was originally cultured from the kidney of an infected rainbow trout presenting clinical signs of systemic BCWD/RTFS in 2008 (Hesami et al., 2011). Since then, FPG101 has been characterized as virulent in experimental trials by Jarau et al. (2018). Briefly, subcultures of FPG101 glycerol stocks were grown on cytophaga agar (CA) at 14°C and checked for purity. An isolated colony was then used to inoculate 3 mL of cytophaga broth (CB) and grown at 14°C for 72 h. Subsequently, 50 µL of the bacterial culture was used to inoculate either 40 mL of CB or 40 mL of L15 supplemented with 2% FBS. This was then separated into thirteen test tubes (150 mm tall x 16 mm) which each received 3 mL aliquots of the culture and were incubated at 14°C. Absorbance measurements were taken every 12 h for 6 d at an OD_{600}. This was experiment was repeated 3 times for both culture conditions.

3.3.3 Bacterial growth for RTS11 exposure conditions

3.3.3.1 Live and heat-killed F. psychrophilum

An isolated colony of *F. psychrophilum* strain 101 (FPG101) was used to inoculate 3 mL of cytophaga broth and was grown as described in section 3.3.2. After this time, the OD_{600} of the bacterial growth was consistently between 0.4-0.5, indicating a viable bacterial count of 2-5 x 10^8 CFU/mL. For every culture of FPG101, a standard plate count (SPC) was completed to confirm the anticipated bacterial concentration. Two 0.5 mL aliquots were removed from the 3 mL culture and centrifuged for 8 min at 8000 rpm. The resulting cell pellets were then washed twice with phosphate buffered saline (PBS). One cell pellet was resuspended in 0.5 mL of L15 supplemented with 2% fetal bovine serum (FBS) which was to be used to expose RTS11 to live bacteria.

The remaining cell pellet was resuspended in PBS and boiled for 20 minutes to kill any live bacteria. After boiling, 100 µL of the heat-killed FPG101 was spread onto a CA plate to confirm the absence of viable bacteria. Subsequently, the killed bacterial culture was centrifuged for 8 min at 8000 rpm and resuspended in 0.5 mL of L15 supplemented with 2% FBS which was to be used to expose RTS11 to heat-killed *F. psychrophilum.*

3.3.3.2 F. psychrophilum conditioned media (FpCM)

Following 72 h of growth at 14°C on CA, isolated colonies of FPG101 were used to inoculate multiple test tubes containing 3 mL of L15 media supplemented with 2% FBS. These cultures were grown at 14°C for 72 h after which a streak plate was performed for each culture to ensure purity. The cultures were then centrifuged for 8 min at 8000 rpm at 4°C so that supernatants could be removed and passed through a 0.22 µm filter to remove any residual bacteria. The isolated supernatants were combined and used to expose RTS11 to a final concentration of 50% supernatant (referred to as *Fp*CM).

3.3.4 Exposure of RTS11 to live, heat-killed and the supernatant of F. psychrophilum

In 6-well tissue culture plates (ThermoFisher), RTS11 was seeded at 1.5 x 10^6 cells/well in 2 mL of L15 media supplemented with 2% FBS and maintained overnight at 14ºC. Cells were exposed to a single dose of either live *F. psychrophilum* (MOI of 1), heat-killed *F. psychrophilum* (MOI of 1), *F. psychrophilum* supernatant (*Fp*CM) or a media alone control to a final volume of 4 mL per well. Following this single exposure to the appropriate condition, all experimental plates were returned to the 14ºC incubator. Unless otherwise described, cell pellets were collected for analyses. On Days 1, 2 and 3, the supernatant was collected from experimental wells and adherent cells were mechanically dislodged using a sterile 23 cm cell scraper (ThermoFisher) and added to the supernatant of respective wells. All wells were then washed with 1 mL of phosphate buffered saline (PBS, Gibco) which was also added to the appropriate supernatant/cell mixture. The cells were centrifuged (5 min, 500 x g, 4°C), washed three times with 5 mL of PBS, and the resulting cell pellets were stored at -80°C for future use.

3.3.5 Viability of RTS11 throughout exposure

To determine the impact that the organism has on RTS11 survival, the cells were exposed to a single dose of live *F. psychrophilum* (MOI of 1), killed *F. psychrophilum* (MOI of 1) or *Fp*CM (prepared as described in section 2.4). On Days 1, 2 and 3 following this exposure, the media was collected from experimental wells and any adherent cells were detached using 400 µL of 0.25% trypsin-EDTA (Gibco). All wells were then washed with 1 mL of PBS. The trypsinized cells were combined with the collected supernatant which was then centrifuged 500 x g for 5 min at 4ºC. The cell pellet was washed twice with 1 mL of PBS before resuspending in 200 µL of PBS. To determine RTS11 cell viability after the exposure conditions, a trypan blue (Sigma) exclusion test was performed using a haemocytometer under a phase contrast microscope (Leica). This experiment was repeated three times.

3.3.6 Measuring phagocytic function of RTS11 via flow cytometry

RTS11 was exposed to a single dose of live *F. psychrophilum* (MOI of 1), killed *F. psychrophilum* (MOI of 1), *Fp*CM or no treatment as described above in section 2.4. Immediately following exposure, each well received 4.6 x 10^7 FITC 2.0 µm latex beads (L4530, Sigma) after which, all plates were returned to a 14°C incubator. On 1, 2 and 3 d following exposure, the RTS11 cells were collected as described above in section 2.4. The final cell pellet was resuspended in 500 µL of PBS before transfer to a 5 mL polystyrene round-bottom tube (ThermoFisher).

The fluorescence of the beads within RTS11 cells was analyzed by flow cytometry using the FACSAria Fusion Cell Sorter with an integrated 3-laser and 9-color detection unit (BD Biosciences). The RTS11 cells that had phagocytosed the beads were gated to exclude the count of free FITC-conjugated beads from the measurement. Data is expressed as the percentage of RTS11 cells containing FITC-conjugated beads (10,000 events) after standardizing to the media only control. Data analysis was completed using the FlowJo® software (https://www.flowjo.com).

3.3.7 qRT-PCR

3.3.7.1 RNA extraction and cDNA synthesis

RNA was extracted from RTS11 cell pellets (1.5 x 10^6 cells) using an RNeasy RNA Extraction Kit (Qiagen) as described by the manufacturer. To remove any contaminating genomic DNA, all RNA samples were treated with DNase I (Thermo Scientific). RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at -80°C until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) in accordance to the manufacturer's instructions. For a no template control, 500 ng of RNA suspended in 20 uL of DEPC water was included in the cDNA synthesis reaction without reverse transcriptase.

3.3.7.2 qRT-PCR Reactions

To assess transcript levels of *IL-1β*, *TNFα*, *IL-6*, and *IL-10* in RTS11 cells, qRT-PCR analysis was completed. All PCR reactions were 10 µl and contained: 2.5 µl of cDNA (25 ng/µl diluted 1:10 in Rnase free water), 2x WISENT ADVANCED^{TM} qPCR mastermix (Wisent), and forward and reverse primers (Sigma Aldrich) to a final working concentration of 0.25 µM. All qPCR reactions were completed on the LightCycler® 480 II (Roche). The sequences for all primer sets are outlined in **Table 3.1**. Each experimental sample was run in triplicate. For each plate, triplicate wells of a calibrator, no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec and extension at 72°C for 8 sec. A melting curve was completed for every run from 65°C to 97°C with a read every 5 sec. Product specificity was determined through single PCR melting peaks. All qRT-PCR data was analyzed using the ΔΔCt method and is presented as the average of 3 experimental replicates with the standard deviation. Specifically, gene expression was normalized to the reference gene (EF1α) and expressed as fold change over the day 0 control group where control expression was set to 1.

Table 3.1. Primers used for qRT-PCR analysis of cytokine genes in RTS11 cells. Forward (F) and reverse (R) primer sequences for the genes analyzed are presented along with the base pair length for each amplicon and the original references or Genbank accession numbers for primer sets.

Primer Name	Sequence (5' – 3')	Length	Reference or Accession No.
IL-1β	**F:** CCACAAAGTGCATTTGAAC **R:** GCAACCTCCTCTAGGTGC	155	Semple et al, 2018b
TNFα	**F:** GTGCAAAAGATACCCACC **R:** CACTGCACGGTGTCAG	108	Semple et al, 2018b
IL-6	**F:** CTTCTACACGCTATCTCTCACTC **R:** CGTCTGTCCCGAGCT	128	Semple et al, 2018b
IL-10	**F:** GCCTTCTCCACCATCAGAGAC **R:** GATGCTGTCCATAGCGTGAC	120	NM_001245099.1
EF1α	**F:** CGCACAGTAACACCGAAACTAATTAAGC **R:** GCCTCCGCACTTGTAGATCAGATG	134	Semple et al, 2018b

3.3.8 Statistical analyses

After confirming a normal distribution and homogeneity of variance, statistical analyses were performed using a two-way ANOVA. This was then followed by a Tukey's posthoc test to determine significant differences between the exposure conditions at each individual timepoint. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were completed using the software Statistica version 7 (StatSoft, Tulsa, OK).

3.4 Results

3.4.1 Growth of F. psychrophilum in preferred and cell culture media

F. psychrophilum strain 101 was found to grow well in its preferred culture media, cytophaga broth with the logarithmic phase beginning at 24 h and ending at approximately 84 h (**Figure 3.1**). In comparison, although *F. psychrophilum* was capable of growth in L15 supplemented with 2% FBS, this growth was much slower and absorbance readings were significantly lower when compared to growth in cytophaga broth. Further, what would be considered the logarithmic phase during growth in 2% FBS was much more prolonged beginning at 36 h and reaching a plateau at 120 h (**Figure 1**).

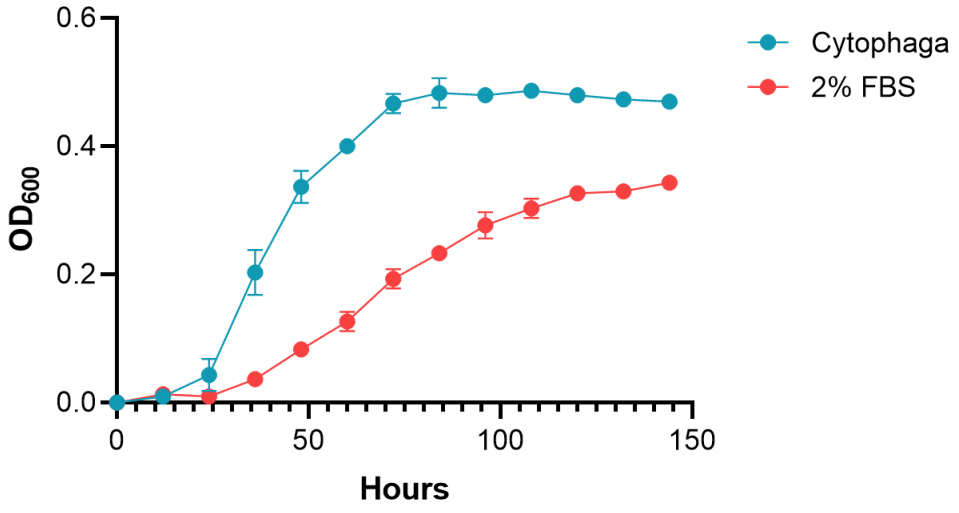

Figure 3.1. A comparison of *F. psychrophilum* growth in culture media for bacterial cells (cytophaga broth) and for animal cells (L15 with 2 % FBS). Growth was at 14°C and was monitored every 12 h for 144 h by measuring optical density at 600 nm. This figure represents three independent experiments for each culture condition. Data are presented as the mean with error bars representing ±SD.

3.4.2 Appearance of RTS11 when exposed to live, heat-killed and the supernatant of F. psychrophilum

To elucidate whether any stimulation of RTS11 by *F. psychrophilum* was due to products produced only by live growth, the exterior of the pathogen, or extracellular products produced by the organism, RTS11 was exposed to live, killed and the supernatant of the bacterium so that appearance of the cells could be assessed. When compared to the media only control (**Figure 3.2A-B**), RTS11 did appear to have morphological differences 2 d following exposure to these treatments. Specifically, when stimulated, the cells appeared to adhere and more cells transitioned to a "macrophage-like" morphology. When exposed to heat-killed *F. psychrophilum* there was a slight increase in adherent, macrophage-like cells (**Figure 3.2C-D**) but very few projections were observed. However, as time elapsed (Days 3 and 4), more macrophage-like cells were observed in this condition (data not shown). When RTS11 was exposed to live *F. psychrophilum* for 2 d, the majority of cells are adherent with elongated, complex projections (**Figure 3.2E-F**). Interestingly, when RTS11 was exposed to media consisting of 50% *F. psychrophilum* supernatant, the cells also became much more macrophage-like and presented the elongated projections (**Figure 3.2G-H**).

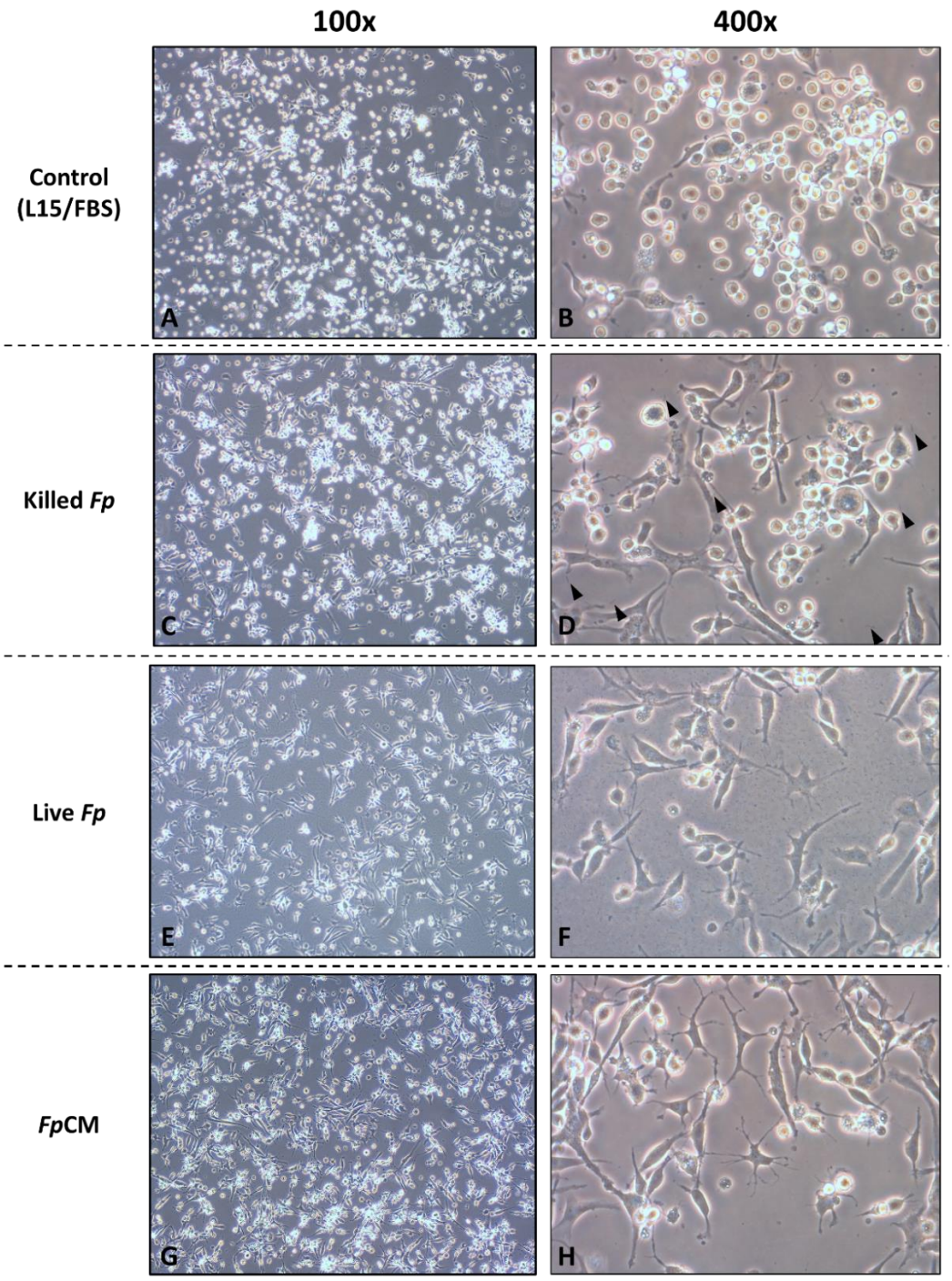

Figure 3.2. Influence of live, heat-killed and *F. psychrophilum* conditioned media (*Fp*CM) on the morphology of RTS11. RTS11 was exposed to either cell culture media alone (A, B), heat-killed *F. psychrophilum* at a MOI of 1 (C, D), live *F. psychrophilum* at an MOI of 1 (E, F), or *Fp*CM which refers to 50% media conditioned with 3 days of *F. psychrophilum* growth in L15/FBS (G, H). Black arrowheads indicate the *F. psychrophilum* bacteria. Representative pictures were collected to observe morphological differences in RTS11 at Day 2 following these exposure conditions.

3.4.3 Viability of RTS11 throughout bacterial exposure conditions

Survival of RTS11 was measured using a trypan blue exclusion assay following exposure to live or heat-killed bacteria, or *Fp*CM. On Day 1, percent mortality was significantly higher in RTS11 exposed to live bacteria and *Fp*CM, but these two conditions were not significantly different from one another. In comparison, viability was the least influenced by heat-killed *F. psychrophilum* on Day 1 (**Figure 3.3**). By Day 2, the live *F. psychrophilum* exposed cells presented significantly higher percent mortality when compared to the other two conditions. This trend continued to 3 d. Heat-killed and the supernatant of *F. psychrophilum* did not significantly differ from one another on both Days 2 and 3 (**Figure 3.3**).

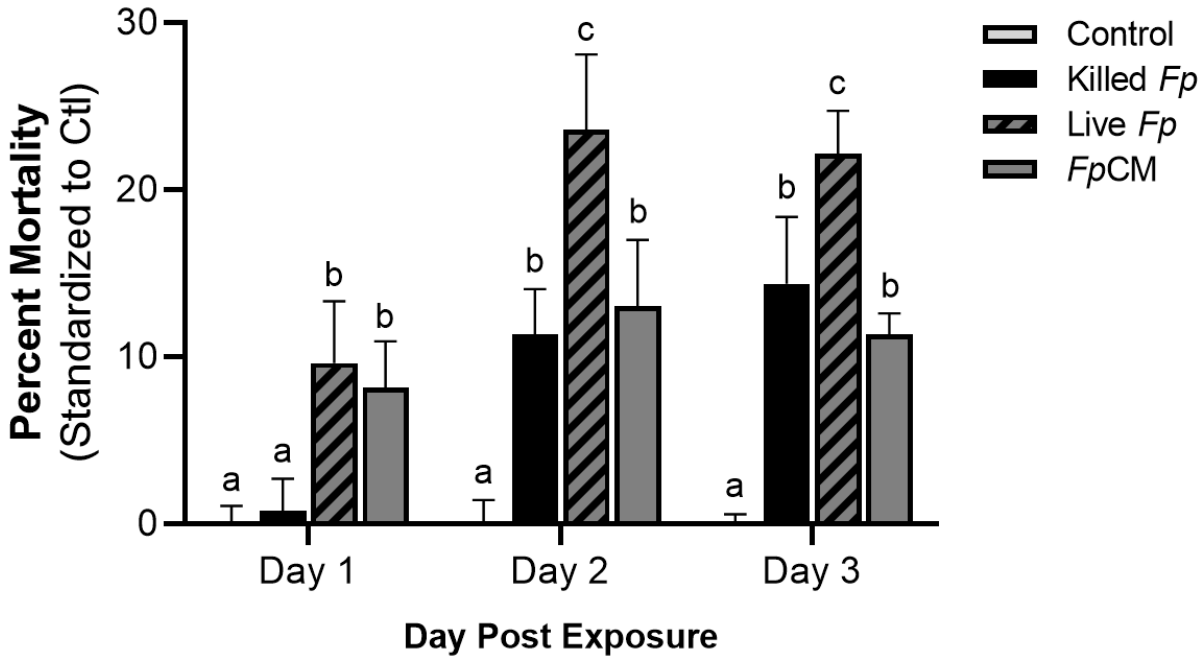

Figure 3.3. Relative survival of RTS11 when exposed to live, killed and the supernatant of *F. psychrophilum* (*Fp*CM). RTS11 was exposed to live, killed or *Fp*CM (following growth in L15/2% FBS) and the percent mortality of RTS11 was determined for each exposure condition using a trypan blue assay. This figure represents three independent experiments where a p-value of less than 0.05 was considered to be significantly different when compared between exposure conditions across all timepoints. Vertical error bars represent the standard deviation (SD).

3.4.4 Impact of live, killed and F. psychrophilum supernatant on phagocytic function

Following exposure to both FITC labelled beads and one of either live or killed bacteria or the supernatant of *F. psychrophilum*, flow cytometric analysis was conducted to determine the impact that these exposure conditions had on the phagocytic activity of RTS11. When treated with live and heat-killed *F. psychrophilum*, RTS11 did not differ from each other at all timepoints analyzed (**Figure 3.4A**). Interestingly, when RTS11 was exposed the *Fp*CM condition (50% of the *F. psychrophilum* conditioned media), phagocytic function was significantly suppressed at Day 1 (**Figure 3.4A**). Phagocytic function recovered in the *Fp*CM exposure condition by Day 2 and this condition, along with live *F. psychrophilum* exposure, actually increased phagocytic activity by Day 3. Representative images of RTS11 in the various exposure conditions reveal that some cells became engorged with the FITC labelled beads while others appeared to phagocytose very few (**Figure 3.4B**).

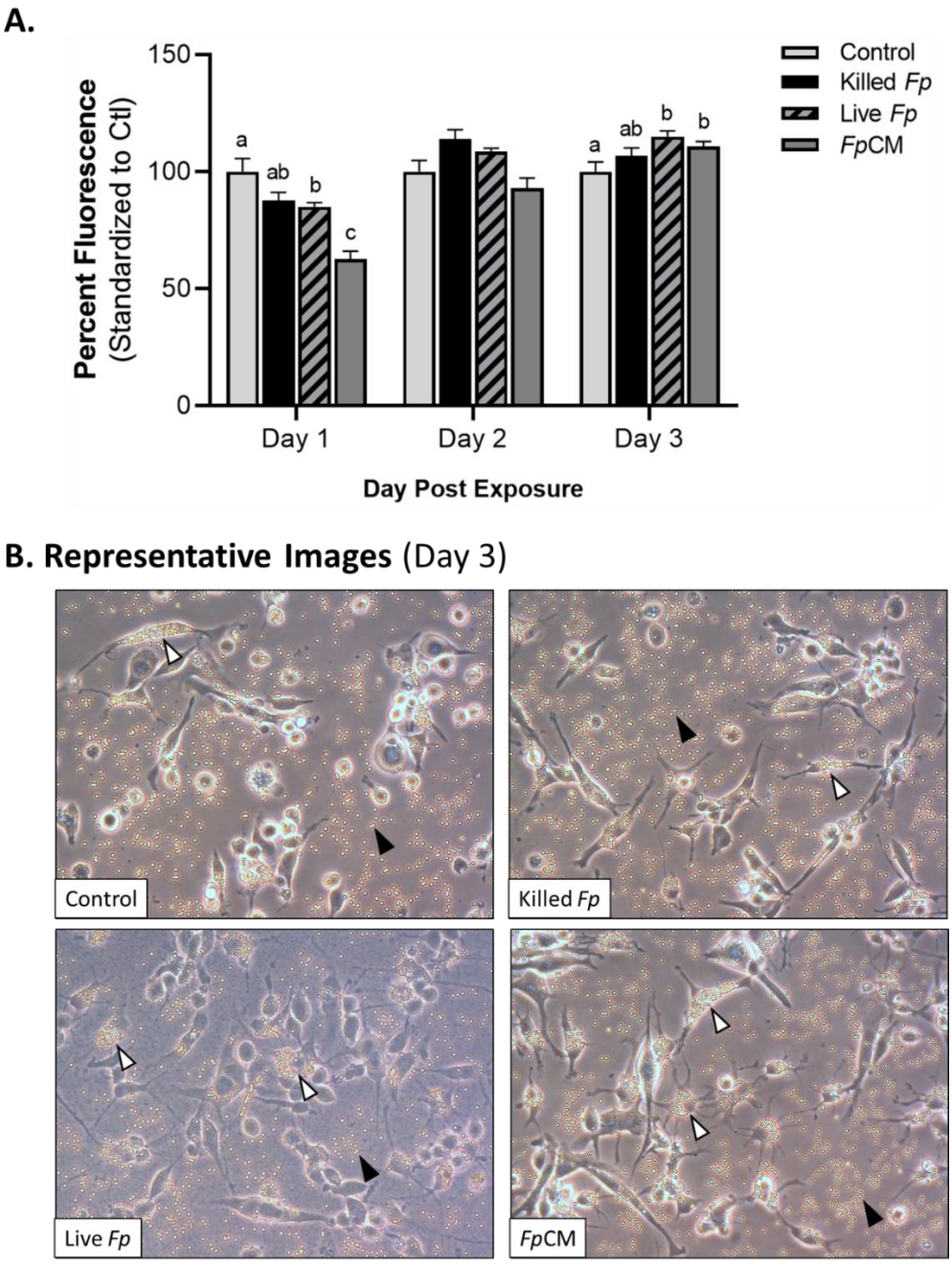

Figure 3.4. Assessment of RTS11 phagocytic function following exposure to live, heat-killed and the supernatant of *F. psychrophilum* (*Fp*CM). RTS11 was exposed to one of these three conditions in conjunction with FITC latex beads (beads marked by black arrows) and the ability of the cells to phagocytose these beads was assessed on Days 1, 2 and 3 via flow cytometry (A). Phase contrast photomicrographs (400x) of RTS11 cultures undergoing phagocytosis of FITC beads at Day 3 (B). Cells engorged with beads are marked by white arrows. This figure represents three independent experiments where a p-value of less than 0.05 was considered to be significant when compared to the control condition within each timepoint. All vertical error bars represent the standard deviation (SD).

3.4.5 Cytokine transcript expression throughout exposure conditions

When RTS11 was exposed to live, heat-killed or the supernatant of *F. psychrophilum* there were interesting differences in transcript expression between the bacterial exposure conditions. For *IL-1β*, cells exposed to live *F. psychrophilum* had significantly increased expression when compared to the other bacterial conditions at all three timepoints analyzed. RTS11 exposure to 50% supernatant resulted in significant upregulation of cellular *IL-1β* expression at Day 1 that decreased over time. Meanwhile, heat-killed *F. psychrophilum* did not stimulate upregulation of *IL-1β* at Day 1 post-exposure, but a slight increase was observed at 2 and 3 d when compared to the media only control (**Figure 3.5A**). RTS11 expression of *IL-6* showed a similar pattern with live bacteria inducing a significant upregulation at all three timepoints. Interestingly, RTS11 exposed to supernatant had as strong of an *IL-6* response as to live bacteria but this upregulation decreased over time. The heat-killed *F. psychrophilum* was not statistically different from the media only control at all timepoints analyzed (**Figure 3.5B**). For *TNFα* transcript expression, RTS11 exposed to live bacteria again induced a significantly higher increase in mRNA levels when compared to the other conditions studied. The supernatant condition showed only a significant upregulation on Day 1 but by Days 2 and 3 transcript levels of RTS11 were not different from cells exposed to the media control. Similar to what was observed with *IL-6*, cells exposed to the heat-killed *F. psychrophilum* did not differ from the media alone control at all timepoints assessed when observing *TNFa* expression (**Figure 3.5C**). When analyzing the impact of the exposure conditions on anti-inflammatory *IL-10* expression in RTS11, it was again observed that live bacteria followed by the bacterial supernatant resulted in the greatest upregulation at Day 1. As time progressed, live bacterial exposure resulted in a gradual increase in RTS11 *IL-10* expression, while exposure to the bacterial supernatant caused a steady decrease. In comparison, heat-killed *F. psychrophilum* did not cause RTS11 *IL-10* transcripts to significantly differ from the media alone control in all timepoints examined (**Figure 3.5D**).

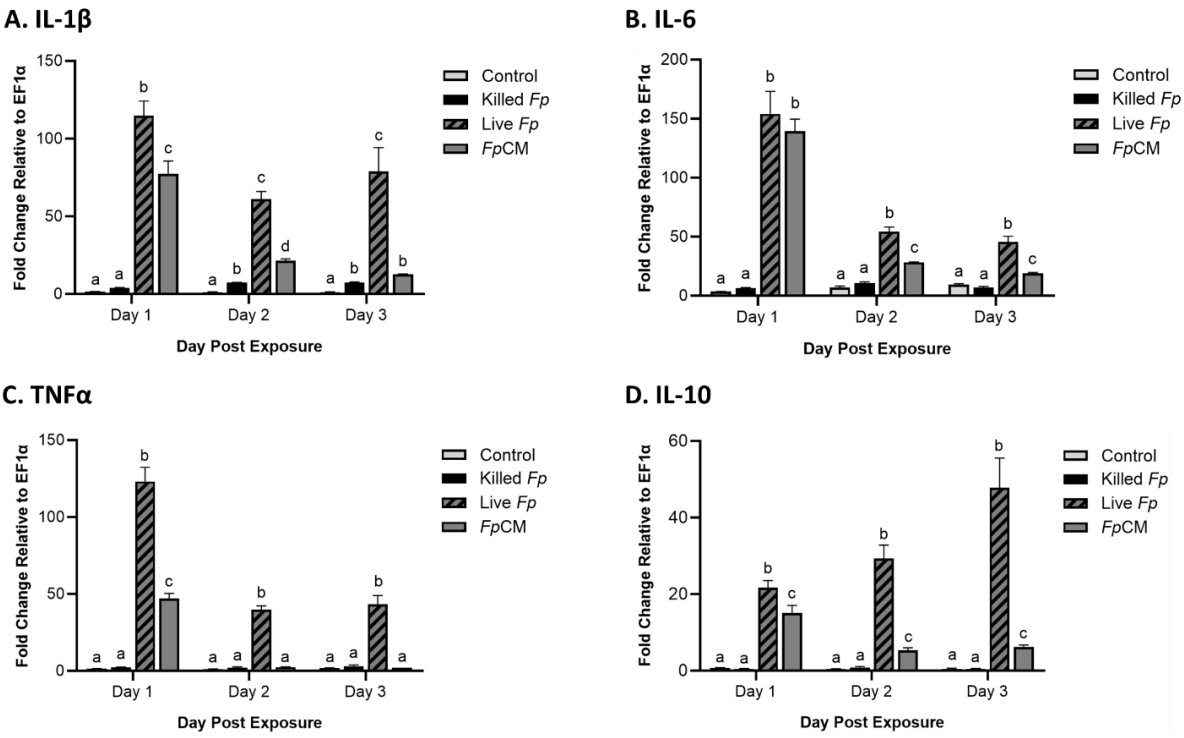

Figure 3.5. Influence of live, killed and the supernatant of *F. psychrophilum* (*Fp*CM) on RTS11 cytokine mRNA expression 1-3 Days following exposure. Transcript expression of pro-inflammatory cytokines *IL-1β* (A), *TNFα* (B), *IL-6* (C) as well as the anti-inflammatory cytokine *IL-10* (D) was assessed so that the effect of the different bacterial exposure conditions on immune function could be analyzed. All data was normalized to the reference gene (*EF1α*) and expressed as a fold change over the Day 0 control group where control expression was set to 1. All panels represent three independent experiments and are presented as means + SD. A p-value of less than 0.05 was considered to be statistically significant when compared to the media alone control for each timepoint.

3.5 Discussion

There has been a growing interest in the study of *F. psychrophilum* due to its harmful influence on global rainbow trout aquaculture. Initially, simply isolating and culturing the organism *in vitro* was problematic due to the absence of effective media (reviewed by Nematollahi et al., 2003; Starliper, 2011). Since then, there have been several media formulations that have dramatically improved experimental work with the bacterium including Shieh (Shieh, 1980), cytophaga (Wakabayashi & Egusa, 1974) and tryptone yeast extract salts broth (TYESB, Holt, 1987). But despite past complications surrounding the culture of this fastidious organism, the present study has revealed that *F. psychrophilum* is capable of considerable growth in cell culture media supplemented with 2% FBS.

This was unexpected as isolates of *F. psychrophilum* normally do not grow, or grow poorly, in high nutrient media (reviewed by Starliper, 2011). Though several types of animal sera have been used for cell culture work, fetal bovine serum is notoriously nutrient dense, contains many valuable growth factors and also presents low levels of antibodies (Bettger & McKeehan, 1986; Fang et al., 2017). These characteristics allow for considerable growth in essentially any cell culture system, which has made FBS the most commonly used media supplement (Fang et al., 2017). Because infection with *F. psychrophilum* can present as a septicaemia (Ekman & Norrgren, 2003), perhaps FBS provides a physiological environment that mimics blood and enables the organism to grow. Though only supplementation with 2% FBS was used here, further analyses should be completed to explore the maximum and minimum nutrient requirements for *F. psychrophilum* growth in this system. The ability of *F. psychrophilum* to grow in cell culture media means that live exposure of the pathogen is a possibility for *in vitro* cell culture systems. Additionally, further study of the bacterial supernatant (*Fp*CM) as well as its impact on eukaryotic cells can now be achieved.

The structure of any bacterium represents a complex mixture of pathogen associated molecular patterns (PAMPs) that can alert the host immune system of foreign invasion. To avoid this detection, many bacterial pathogens can generate exotoxins, cytolysins, proteases, and other extracellular substances that suppress and/or confuse immune responses (Esteve et al., 1995; Ruckdeschel et al., 1997; Hava et al., 2008). These factors prolong pathogen survival thus enhancing its virulence and fitness. When RTS11 was exposed to *F. psychrophilum* supernatant the cells became more macrophage-like, similar to when they were exposed to live pathogen alone. Therefore, it appears that both the bacterium, and something produced by the organism, are capable of stimulating RTS11. Under normal conditions, the composition of RTS11 is expected to be roughly 90% monocyte-like with the remaining 10% of cells presenting an adherent, macrophage-like morphology (Ganassin & Bols, 1998). But when RTS11 cells were stimulated with phorbol 12-myristate 13-acetate (PMA), these proportions changed so that a higher percentage of cells became macrophage-like (Ganassin & Bols, 1998), comparable to what was observed in the current study. Based on these collective results it seems that

the monocyte cells of RTS11 will differentiate in a manner analogous to what has been noted in whole animal models following appropriate stimulation (reviewed by Shi & Pamer, 2011). In the present study, it appears that live *F. psychrophilum*, the bacterial supernatant and, to a lesser extent, the heat-killed pathogen are able to provide this stimulatory effect. As a host model system for the study of *F. psychrophilum* infection, RTS11 provides the potential to learn more about this bacterial pathogen in relation to immune cells from a suitable host.

Several mammalian pathogens actively kill immune cells thereby preventing the host from mounting an effective response to control infection (reviewed by Menestrina et al., 1995; Narayanan et al., 2002; Alonzo & Torres, 2013). Quite often this strategy proves effective for the pathogen but there has been little observation of similar virulence factors in aquatic diseases. Using the *in vitro* model developed here, only live *F. psychrophilum* was found to consistently reduce the survival of RTS11 at all timepoints analyzed. Interestingly at Day 1, the supernatant alone was observed to induce RTS11 mortality akin to that of the live *F. psychrophilum* condition. Yet by Days 2 and 3, only live bacterial exposure significantly reduced RTS11 survival. Numerous bacterial extracellular products have been found to be cytotoxic to mammalian macrophages. The supernatant of *Pseudomonas aeruginosa* as well as an invasin produced by *Salmonella typhimurium* both induce apoptosis in murine macrophages (Hersh et al., 1999; Zhang et al., 2003). Because live *F. psychrophilum* would be expected to continually replenish the extracellular products when compared to supernatant exposure alone, it is possible that the aquatic pathogen secretes a product that reduces survival of rainbow trout immune cells. If this secreted substance was used up or degraded over time, it would explain why the supernatant alone condition only increased mortality at Day 1 while the live bacterial condition continued to have a greater impact on RTS11 survival throughout the trial. Further exploration regarding the type of cell death observed here would be required to fully understand the impact of *F. psychrophilum* on rainbow trout immune cells.

The defining feature of a macrophage is its ability to engulf and subsequently digest apoptotic cells as well as any abnormal or foreign entities (reviewed by Gordon & Pluddemann, 2017).

Depending on the constituents of that which was consumed, these highly versatile cells will then direct immune responses to ensure healing, homeostatic maintenance or pathogen clearance (reviewed by Flannagan et al., 2011). Given the importance of phagocytosis for averting microbial colonization within a host, it is not surprising that many bacterial pathogens develop virulence factors to inhibit this ability (reviewed by Ernst, 2001; Celli & Finlay, 2002; Sarantis & Grinstein, 2012). In the present study, it was observed that the supernatant of *F. psychrophilum* significantly suppressed the phagocytic activity of RTS11 at 1 d post-exposure when compared to the control condition. By Day 3, phagocytic activity of RTS11 recovered in the supernatant exposure, but perhaps it would have continued to impede had fresh supernatant been replaced over time. There are many ways that bacterial pathogens can suppress the phagocytic activity of macrophages. Virulent strains of the aquatic pathogen *Photobacterium damselae piscicida* produce a toxin called AIP56 (apoptosis inducing protein of 56 kDa) which causes apoptotic destruction of macrophages and neutrophils in infected sea bass (do Vale et al., 2003; do Vale et al., 2007; Costa-Ramos et al., 2011). This prevents phagocytosis by eliminating cell types capable of this function. Alternatively, some bacteria secrete products that inactivate the cellular machinery required to form phagosomes around extracellular pathogens. An emerging human pathogen, *Photorhabdus asymbiotica*, produces a toxin that causes actin disassembly so that phagocytes are unable to migrate and/or phagocytose effectively (Jank et al., 2013). Yet another strategy used to decrease the efficacy of phagocytosis is inhibition of opsonization. A secreted metalloprotease of *Staphylococcus aureus*, aureolysin, acts as an alternative C3 convertase. Aureolysin cleaves C3 into forms of C3a and C3b that degrade quickly without binding to bacteria, thus preventing the anticipated opsonization by these complement proteins (Laarman et al., 2011). *F. psychrophilum* is known to produce several extracellular enzymes (Duchaud et al., 2007; Hesami et al., 2011; Castillo et al., 2016) so it is possible that some may act as virulence factors to inhibit leukocyte function as noted here and in previous studies (Lammens et al., 2000; Semple et al., 2018a). Although reagents for teleost proteins and immune cells are not readily available for further validation, the results presented here connect the virulence of *F. psychrophilum* to impairment of phagocytosis in rainbow trout macrophages.

As a first line of defense in host tissues, macrophages have the responsibility of processing foreign substances and ensuring that the immune system responds accordingly. This task is mediated through cytokines produced by the macrophages to properly guide and activate immune cells (reviewed by Zhang & Wang, 2014). Quite often for bacterial pathogens, such as *F. psychrophilum*, it is anticipated that the initial reaction will be pro-inflammatory to combat the pathogen and inhibit its growth, followed by an anti-inflammatory response during the resolution of infection (reviewed by Wilson et al., 1998; Lauw et al., 2002). Because inflammatory responses can be quite damaging to the surrounding tissues, it is very important to have these reactions well controlled (reviewed by Lauw et al., 2002; Chen et al., 2018). When RTS11 was exposed to live *F. psychrophilum* the cytokine levels fit this described expression profile, and at Day 1 the bacterial supernatant was found to stimulate a similar, yet less intense, response. Unexpectedly, heat-killed *F. psychrophilum* resulted in very little to no significant differences in gene expression when compared to control RTS11 cells. Similar transcriptional trends have been observed in head kidney leukocytes isolated from rainbow trout susceptible to *F. psychrophilum* infection (Langevin et al., 2012). When measuring transcript expression 5 d following exposure to live *F. psychrophilum*, these primary leukocytes upregulated *IL-1β*, *TNFα* and *IL-10* (Langevin et al., 2012). Stimulation of pro-inflammatory cytokines was also observed in whole spleen tissue, where both *TNFα* and *IL-1β* transcript expression was induced upon live *F. psychrophilum* challenge in resistant and susceptible rainbow trout lines (Kutyrev et al., 2016). In fact, *IL-1β* gene expression has been observed to be overexpressed in multiple rainbow trout tissues (gills, liver, spleen and kidney) when naturally infected with *F. psychrophilum* (Orieux et al., 2013). These previous studies support the cytokine response that was observed here when co-culturing RTS11 with *F. psychrophilum*. Thus RTS11 is an affordable, reliable model for understanding both the immune response to, and the pathogenesis of, *F. psychrophilum*.

Many attempts to create an effective vaccine for BCWD/RTFS have focused on the use of killed whole-cell bacteria (Kirici et al., 2014; Hoare et al., 2017), likely because this vaccination method

has proven effective for other aquatic diseases such as vibriosis and its corresponding Vibrogen 2 vaccine (reviewed by Frans et al., 2011). Interestingly, though the killed *F. psychrophilum* used here could not stimulate a response in RTS11, our group has previously shown that the killed whole-cell bacteria of Vibrogen 2 can increase pro-inflammatory cytokine expression in the same cell line (Soulliere, 2017). In terrestrial pathogens, it has been shown that different species of heat-killed bacteria, even between strains of the same bacterial species, can result in very diverse gene expression profiles (Sela et al., 2018). This is likely due to the components of the bacterial membrane, thus *F. psychrophilum* cells alone may not be as immunogenic as those of *Vibrio anguillarum*. Perhaps future vaccine regimes for *F. psychrophilum* should incorporate the extracellular products of the organism to provide protection against its secreted virulence factors as well as the pathogen itself. This vaccination technique has proven protective for farmed sole when injected with sublethal doses of *Vibrio* extracellular products (Zorrilla et al., 2003). Based on the results presented here, it is evident that killed *F. psychrophilum* alone does not stimulate a strong response in rainbow trout immune cells, thus more research should be devoted to understanding the pathogen to streamline efforts for vaccine design.

3.6 Conclusions

Despite the damaging role that *F. psychrophilum* plays in the global rainbow trout aquaculture industry, there is very little known regarding the pathogenesis of this opportunistic pathogen. In the present study, a model system was developed using the rainbow trout monocyte/macrophage-like cell line, RTS11, to study the infection cycle of *F. psychrophilum*. The results presented show that the organism can grow in media used for successful culture of RTS11, making it possible to expose these cells to live, heat-killed and the supernatant of *F. psychrophilum*. When exposed to these different conditions, RTS11 responded by making a pronounced morphological change to become primarily macrophage-like. The live *F. psychrophilum* condition was shown to significantly decrease the survival of RTS11 with the bacterial supernatant having a negative impact only at earlier timepoints. Regarding cellular function, phagocytosis was inhibited primarily by the supernatant, indicating that the organism may produce virulence factors to evade the host immune cells. Finally, it was found that only live

bacteria and the supernatant of *F. psychrophilum* were able to stimulate a significant cytokine response in RTS11, which revealed that perhaps the use of killed whole-cell bacteria for vaccine development may not induce the protective response that is desired. Though further study is required, it is clear that gaining a deeper understanding of *F. psychrophilum* pathogenesis will aid massively in vaccine design and the development of other treatments options for BCWD/RTFS.

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Chapter 4: PACAP is lethal to *Flavobacterium psychrophilum* through direct membrane permeabilization and indirectly, by priming the immune response in RTS11.

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4.1 Overview

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional neuropeptide that is widely distributed in mammals and is capable of performing roles as a neurotransmitter, neuromodulator and vasodilator. This polypeptide belongs to the glucagon/secretin superfamily, of which some members have been shown to act as antimicrobial peptides in both mammalian and aquatic organisms. In teleosts, PACAP has been demonstrated to have direct antimicrobial activity against several aquatic pathogens, yet this phenomenon has never been studied throughout a live bacterial challenge. The present study focuses on the influence of synthetic *Clarias gariepinus* 38 amino acid PACAP on the rainbow trout monocyte/macrophage-like cell line, RTS11, when exposed to the coldwater bacterial pathogen *Flavobacterium psychrophilum*. PACAP was shown to have direct antimicrobial activity on *F. psychrophilum* when grown in both cytophaga broth and cell culture media (L15). Further, the ability of teleostean PACAP to permeabilize the membrane of an aquatic pathogen, *F. psychrophilum*, was demonstrated for the first time. The viability of RTS11 when exposed to PACAP was also observed using a trypan blue exclusion assay to determine optimal experimental doses of the antimicrobial peptide. This displayed that only concentrations lower than 0.1 μM did not have a negative impact on RTS11 survival. Interestingly, when RTS11 was pre-treated with PACAP for 24 h before experiencing infection with live *F. psychrophilum*, growth of the pathogen was severely inhibited in a dose-dependent manner when compared to cells receiving no pre-treatment with the polypeptide. Relative expression of pro-inflammatory cytokines (IL-1β, TNFα and IL-6) and PACAP receptors (VPAC1 and PAC1) was also analyzed in RTS11 following PACAP exposure alone and in conjunction with live *F. psychrophilum* challenge. These qRT-PCR findings revealed that PACAP may have a synergistic effect on RTS11 immune function. The results of this study provide evidence that PACAP has immunostimulatory activity on rainbow trout immune cells as well as antimicrobial activity against aquatic bacterial pathogens such as *F. psychrophilum*. As there are numerous pathogens that plague the aquaculture industry, PACAP may stimulate the teleost immune system while also providing an efficacious alternative to antibiotic use.

4.2 Introduction

Due to the rising demand for fish protein (FAO, 2018), aquaculture has become a necessary means to protect wild populations from irreversible overfishing. As such, it is imperative that these culture systems have a minimal impact on the environment while still being able to provide the high-quality product for market. To attain this goal, alternative methods must be developed to combat infectious disease as this is one of the greatest sources of instability and financial cost in aquaculture. Global losses due to aquatic infections total approximately $6 billion USD (World Bank, 2014) and currently, fish farmers have few methods outside of antibiotics to prevent/control outbreaks. With multi-drug resistance continually rising (reviewed by Santos & Ramos, 2018; Watts et al., 2017), antibiotic use in aquaculture is tightly regulated which often leaves farmers with few options when outbreaks do occur. This problem has led to an increased interest in the development of alternative approaches for disease prevention, including the use of naturally occurring antimicrobial peptides (AMPs). Though novel AMPs and their activities are continuously being discovered, one that has gained a lot of interest as a result of its vast pleiotropic effects is pituitary adenylate cyclase activating polypeptide (PACAP).

Initially, PACAP was discovered as a neuropeptide due to its ability to stimulate adenylate cyclase activity in ovine pituitary cell cultures (Miyata et al., 1989). Derived from a 175 amino acid precursor, functional PACAP has two molecular forms. The first has 38 amino acids (PACAP-38) while the other form is truncated containing only 27 residues (PACAP-27, Kimura et al., 1990; Ogi et al., 1990). Of the two, PACAP-38 is considered to be more bioactive as it has been shown to display 100-1000 times greater potency in stimulating cell proliferation, DNA synthesis and inositol phospholipid turnover in cells (Deutsch & Sun 1992; Matsumoto et al., 1993). Further analysis of PACAP-38 revealed that this peptide shared 68% sequence similarity with vasoactive intestinal polypeptide (VIP), thereby classifying PACAP as a member of the secretin/glucagon/growth hormone-releasing hormone/vasoactive intestinal peptide superfamily (Miyata et al., 1989). As this was the case, it is not surprising that PACAP-38 is able to bind with equal affinity to the same G-coupled protein receptors

(GCPRs) as VIP, vasoactive intestinal polypeptide receptor 1 (VPAC1) and vasoactive intestinal polypeptide receptor 2 (VPAC2), while also binding to its own receptor, pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1, Pisegna & Wank 1993; Shivers et al., 1991). All three of these receptors have a wide tissue distribution much like the neuropeptides themselves (Zhu et al., 2015). PACAP-38 in particular, displays a broad range of functions in multiple tissue types, including antimicrobial activity, growth, immunomodulation, neural development, anti-tumor activity and metabolism to name a few (Adams et al., 2008; Gray et al., 2001; Leyton et al,. 1999; Murakami et al., 1995; Starr et al. 2018). From an evolutionary perspective, the amino acid sequence of PACAP-38 is identical in all mammals with only a few amino acid substitutions when comparing to other species (e.g. frog, salmon, tunicate, etc.). PACAP-38 therefore must play a vital role in physiological function as it has remained essentially unchanged for ~700 million years (Arimura, 1998). This broad functional profile as well as its highly conserved nature has made PACAP-38 an attractive candidate for disease control and therapeutic use in aquaculture.

Though there are numerous pathogens that impact the aquaculture industry, *Flavobacterium psychrophilum* has proven to be a global threat in the culture of freshwater rainbow trout (*Oncorhynchus mykiss*). This gram-negative bacterial pathogen is the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), two separate conditions that can occur depending on the bacterial isolate, geographical location and age of the host (Lumsden et al., 2004). These conditions present as either an acute bacteremia primarily in small fish (RTSF) or as a more chronic disease most commonly characterized by an ulcerative dermatitis (BCWD) in larger fish (Lumsden et al., 2004; Starliper, 2011). Though variable, mortality resulting from these conditions without intervention generally ranges from 2-30% (Wiens et al., 2018) but in extreme cases can be as high as 50-90% (Brown et al., 1997; Cipriano & Holt 2005; Nilsen et al., 2011b). Despite concerted efforts to selectively breed for resistance to *F. psychrophilum* (Marancik et al., 2014; Silverstein et al., 2009; Vallejo et al., 2014; Wiens et al., 2018) and multiple attempts to develop an effective vaccine (reviewed by Gomez et al., 2014; Hoare et al., 2017; Makesh et al., 2015), there is little information

regarding the pathogenesis of the organism. Based on the data presented thus far, it appears that *F. psychrophilum* has an intricate relationship with the spleen and head kidney macrophages of rainbow trout (Nematollahi et al., 2005; Semple et al., 2018). As such, the spleen monocyte/macrophage-like cell line, RTS11 (Ganassin & Bols, 1998), is an ideal model system for studying *F. psychrophilum* infections. As a relevant immune cell line, RTS11 could provide further insight regarding the immunomodulatory effects of PACAP-38 as well as its antimicrobial function within an appropriate infection model.

Previous research involving teleostean PACAP-38 has focused on assessing its antimicrobial activity when directly dosing aquatic pathogens (Lugo et al., 2019), the growth/immunomodulatory effects of the peptide alone (Carpio et al., 2008; Lugo et al., 2013; Lugo et al., 2010a), or how viral/bacterial infection can influence gene expression of the peptide and its associated receptors (Gorgoglione et al., 2015). Though these results were promising, there is yet to be a study evaluating the activity of PACAP-38 in a live infection model. Furthermore, the effect of PACAP-38 has never been explored with respect to the industrially relevant pathogen, *F. psychrophilum*. The purpose of this study was to measure and understand the antimicrobial activity of PACAP-38 on *F. psychrophilum* as well as to determine whether PACAP could stimulate a protective immune response in RTS11 cells. Confirming the efficacy of PACAP in an *in vitro* infection model will provide further evidence to support its use in *in vivo* experiments. Additionally, the results of this work could provide valuable insights regarding the efficacy of PACAP-38 during live infections and thus aid in the development of a potential alternative for antibiotic use in aquaculture.

4.3 Materials and Methods

4.3.1 Maintenance of RTS11

The rainbow trout monocyte/macrophage-like cell line, RTS11 (Ganassin & Bols 1998), was maintained as described previously by (Sever et al., 2014).

4.3.2 Peptides

4.3.2.1 Synthetic PACAP from the teleost Clarias gariepinus

Clarias gariepinus synthetic PACAP-38 (amino acid sequence of HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK, MW of 4.7 kDa) was purchased from CS Bio (Shanghai) Ltd, China with 85% purity.

4.3.2.2 Synthetic HSP70 peptide fragment from rainbow trout

A synthetic peptide fragment of rainbow trout HSP70 (amino acid sequence of CGDQARTSSGASSQ, MW of 1.3 kDa) was purchased from Biomatik with 98% purity.

4.3.3 Growth of F. psychrophilum

F. psychrophilum strain 101 (FPG101) was grown as described previously by (Semple et al., 2018) with minor adjustments. This bacterial isolate has been characterized as virulent in experimental trials by Jarau and colleagues (Jarau et al., 2018). Briefly, subcultures of FPG101 glycerol stocks were grown on cytophaga agar (CA) at 14°C and checked for purity. An isolated colony was then used to inoculate 3 mL of cytophaga broth (CB) and grown at 14°C for 72 h. After this time, the OD_{600} of the bacterial growth was consistently between 0.4-0.5, indicating a viable bacterial count of 2 -5 x 10^8 CFU/mL. For every culture of FPG101, a standard plate count (SPC) was completed to confirm the anticipated bacterial concentration.

4.3.4 Minimum inhibitory concentration of C. gariepinus PACAP-38 on F. psychrophilum

The minimum inhibitory concentration (MIC) of *C. gariepinus* PACAP-38 on FPG101 was assessed by a broth microdilution peptide assay (BMPA) (Otvos & Cudic 2007). To prepare FPG101 for this assay, 3 mL of CB was inoculated with a single colony and allowed to grow overnight at 14°C.

After this time, 1 mL of the growth was centrifuged at 5,000 rpm for 5 min, the supernatant removed, and the pellet resuspended in 4 mL of fresh CB, to have an OD_{600} of 0.1-0.4. Finally, the bacterial suspension was diluted in CB to obtain a final OD_{600} of 0.001.

The BMPA was made using a flat-bottom 96-well plate (Fisher Scientific). The plate set up consisted of wells containing 90 µL of bacterial suspension and 10 µL of PACAP at 10 different final concentrations from 5 to 50 µM. In the positive control wells, PACAP was substituted with 10 µL of CB while the negative control wells contained 100 µL of CB only. All PACAP concentrations and controls were tested in triplicates. The bacterial growth was monitored after three days of incubation at 14°C, by measuring the change in the absorbance at 600 nm using a microplate reader (BioTek). The growth inhibition curves were generated by plotting the OD at 600 nm and the peptide concentration. The MIC was considered as the lowest concentration of PACAP at which no bacterial growth was detected (an OD_{600} of 0).

4.3.5 RTS11 Exposure Trials

4.3.5.1 Exposure to PACAP

In 6-well tissue culture plates (ThermoFisher), RTS11 was seeded at 1.5 x 10^6 cells/well in 1.5 mL of L15 media with no antibiotics and maintained overnight at 14°C. Cells were exposed to PACAP concentrations of either 0.0002 µM, 0.002 µM, 0.02 µM, 0.1 µM, 0.2 µM, 2 µM, 20 µM or a no PACAP control to a final volume of 4 mL per well. Following this single exposure to PACAP, all experimental plates were returned to the 14°C incubator. On days 1, 2 and 3, the supernatant was collected from experimental wells and adherent cells were mechanically dislodged using a sterile 23 cm cell scraper (ThermoFisher) and added to the supernatant of respective wells. All wells were then washed with 1 mL of phosphate buffered saline (PBS, Gibco) which was also added to the appropriate supernatant/cell mixture. The cells were centrifuged (5 min, 500 x g, 4°C), washed once with 5 mL of PBS, and the resulting cell pellets were stored at -80°C for future use.

4.3.5.2 Simultaneous exposure to both PACAP and live F. psychrophilum

In a second experiment, RTS11 was exposed to PACAP concentrations of 0.0002 µM, 0.002 µM, 0.02 µM and 0.1 µM in similar conditions as described above in the first PACAP trial (section 4.3.5.1). Prior to the single addition of PACAP, 0.5 mL of FPG101 was added to each well at bacterial concentrations ranging from 1.3-2.0 x 10^6 CFU/mL (multiplicity of infection [MOI] of 0.7-1.3). Sampling was completed as described above (section 4.3.5.1).

4.3.5.3 Pre-treatment with PACAP followed by infection with live F. psychrophilum

It was observed that *F. psychrophilum* grew rapidly in wells when exposed to RTS11 simultaneously with PACAP. Because it was possible that PACAP might not be able to influence either RTS11 alone, the *F. psychrophilum* alone, or both due to this rapid growth, PACAP was added to RTS11 wells 24 h before the addition of live *F. psychrophilum*. Otherwise all procedures for sample collection and exposure were identical to those described above in the first PACAP experiment (section 4.3.5.1).

4.3.6 Survival of RTS11 following PACAP exposure

To determine whether PACAP negatively influenced RTS11 viability, the cells were exposed to a single dose of 0.002 µM, 0.02 µM, 0.1 µM, 0.2 µM, 2 µM, 20 µM or a no PACAP control as described above. On days 1, 2 and 3 following this exposure, the supernatant was collected from experimental wells and any adherent cells were detached using 400 µL of 0.25% trypsin-EDTA (Gibco) and the wells washed with 1 mL of PBS. These trypsinized cells were combined with the collected supernatant which was then centrifuged 500 x g for 5 min at 4°C. The cell pellet was washed twice with 1 mL of PBS before resuspending in 200 µL of PBS. To determine RTS11 cell viability after exposure to PACAP, a trypan blue (Sigma) exclusion test was performed using a haemocytometer under a phase contrast microscope (Leica). This experiment was repeated three times.

4.3.7 Presence of live F. psychrophilum in RTS11 cell cultures following PACAP exposure

In six 6-well plates, quadruple wells of RTS11 cells were exposed to either PACAP and *F. psychrophilum* simultaneously or to 24 h pre-treatment of PACAP prior to the addition of *F.*

psychrophilum as described above. All experiments had a MOI of 1. On days 2 and 3 post-infection with live *F. psychrophilum*, 500 µL of the supernatant from each well was removed and serially diluted for an SPC assay to determine the number of viable bacterial cells in the supernatant. Otherwise the RTS11 cells for each day were collected as described above and pellets were frozen at -80°C for future RNA extraction.

4.3.8 Permeabilization assay

FPG101 was grown as described above for the MIC assay in section 2.4. One milliliter of the final bacterial culture was removed and boiled for 20 min to act as a heat-killed control. After boiling, 100 µL of the heat-killed FPG101 was spread onto a CA plate to confirm the absence of viable bacteria.

In a sterile, 96-well BioLite plate (ThermoFisher), 90 µL of live bacterial culture was added to all experimental wells. In triplicate, 10 µL of either PACAP or the synthetic HSP70 peptide fragment to reach final concentrations of 50 µM PACAP, 30 µM PACAP, 0.1 µM PACAP, and 50 µM HSP70. As a live bacteria control, 10 µL of cytophaga broth alone was added to triplicate wells of the live FPG101 culture. As a negative control, 90 µL of heat-killed FPG101 was added to triplicate wells and filled to 100 µL with CB. As a blank, triplicate wells received 100 µL of CB. The assay plate received gentle shaking to mix well contents and was incubated at 14°C for 72 h. Following incubation, each well received 100 µL of 2X BacLight solution (ThermoFisher, L13152) and was incubated in the dark for 15 min. Because the BacLight solution consists of both SYTO 9 (6 µM) and propidium iodide (30 µM), the plate was read at an excitation of 485 nm and an emission of 530 nm for SYTO 9 (green) as well as an excitation of 485 nm and an emission of 630 nm for propidium iodide (red). The reads were completed using a Synergy H1 plate reader (BioTek Instruments). The bacterial fluorescent intensities (F_{cell}) were calculated as a ratio of $F_{cell}530/F_{cell}630$ and presented as the green/red fluorescence ratio.

4.3.9 qRT-PCR

4.3.9.1 RNA extraction and cDNA synthesis

RNA was extracted from RTS11 cell pellets (1.5×10^6 cells) using an RNeasy RNA Extraction Kit (Qiagen) as described by the manufacturer. To remove any contaminating genomic DNA, all RNA

samples were treated with DNase I (Thermo Scientific). RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at -80ºC until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) in accordance to the manufacturer's instructions. For a no template control, 500 ng of RNA suspended in 20 uL of DEPC water was included in the cDNA synthesis reaction without reverse transcriptase.

4.3.9.2 qRT-PCR reactions

To assess transcript levels of *IL-1β*, *TNFα*, *IL-6*, *PAC1* and *VPAC1* in RTS11 cells, qRT-PCR analysis was completed. All PCR reactions were 10 µl and contained: 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water), 2x WISENT ADVANCED™ qPCR mastermix (Wisent), and forward and reverse primers (Sigma Aldrich) to a final working concentration of 0.25 µM. All qRT-PCR reactions were completed on the LightCycler® 480 II (Roche). The sequences for all primer sets are outlined in **Table 4.1**. Each experimental sample was run in triplicate. For each plate, triplicate wells of a calibrator, no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec and extension at 72°C for 8 sec. A melting curve was completed for every run from 65°C to 97°C with a read every 5 sec. Product specificity was determined through single PCR melting peaks. All qRT-PCR data was analyzed using the ΔΔCt method and is presented as the average of 3 experimental replicates with the standard deviation. Specifically, gene expression was normalized to the reference gene (EF1α) and expressed as fold change over the day 0 control group where control expression was set to 1.

Table 4.1. Primers used for qRT-PCR analysis of immune and receptor genes in RTS11. Forward (F) and reverse (R) primer sequences for the genes analyzed are presented along with the base pair length for each amplicon and the original references for primer sets.

Primer Name	Sequence (5' – 3')	Length	Reference
IL-1β	**F:** CCACAAAGTGCATTTGAAC **R:** GCAACCTCCTCTAGGTGC	155	(Semple et al., 2018b)
TNFα	**F:** GTGCAAAAGATACCCACC **R:** CACTGCACGGTGTCAG	108	(Semple et al., 2018b)
IL-6	**F:** CTTCTACACGCTATCTCTCACTC **R:** CGTCTGTCCCGAGCT	128	(Semple et al., 2018b)
VPAC1	**F:** CAGGTGAAAATTGGTTACACTGTTG **R:** TAGTTCCTAGTGCAGTGGAGTTTCC	139	(Lugo et al., 2011)
PAC1	**F:** TGAACCTGTTTGTGTCATTCATTCT **R:** ACACTCCACAGTGTGTAAGAAGCAG	110	(Lugo et al., 2011)
EF1α	**F:** CGCACAGTAACACCGAAACTAATTAAGC **R:** GCCTCCGCACTTGTAGATCAGATG	134	(Semple et al., 2018b)

4.3.10 Statistical analyses

All statistical analyses were completed using the statistical software Statistica version 7 (StatSoft, Tulsa, OK). Prior to the completion of the appropriate statistical test, a normal distribution and equal variance was confirmed. A one-way ANOVA was completed for the growth of *F. psychrophilum* in RTS11 cultures and the permeabilization assay. Alternatively, for all qRT-PCR results and analyzing the viability of RTS11 to various PACAP concentrations, a two-way ANOVA was completed. The appropriate ANOVA test was then followed by a Fisher's least significant difference (LSD) post-hoc test to determine significant differences.

4.4 Results

4.4.1 Minimum inhibitory concentration (MIC)

The MIC was analyzed using both the preferred growth medium of *F. psychrophilum*, cytophaga broth (CB), and the L15 cell culture media used to sustain the RTS11 cultures (**Figure 4.1**). For both CB (**Figure 4.1A**) and L15 (**Figure 4.1B**), the MIC was found to be 30 µM. It appears that PACAP can maintain its antimicrobial function in both media types, thus the function of this peptide could be assessed during *in vitro* live infection experiments with RTS11.

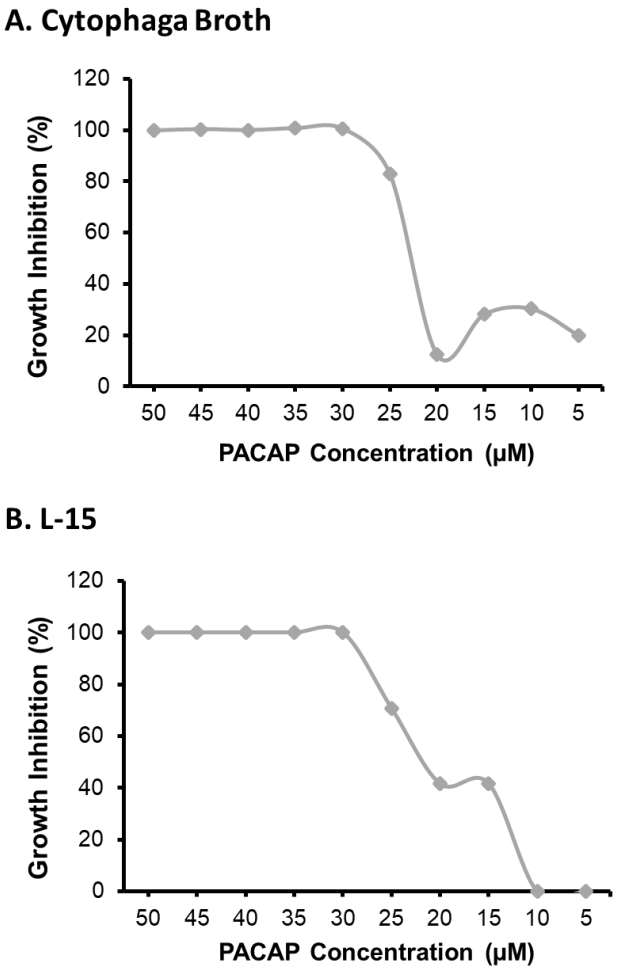

Figure 4.1. The minimum inhibitory concentration (MIC) of *C. gariepinus* PACAP-38 required to prevent the growth of *F. psychrophilum* alone. The MIC was determined following three days of growth in both cytophaga broth (A) and in Leibovitz-15 (L15) cell culture media (B). Each panel represents the results of three independent experiments.

4.4.2 Impact of PACAP concentrations on RTS11 survival

Even if PACAP is capable of killing aquatic bacterial pathogens, this ability has reduced value if the peptide negatively impacts the survival of rainbow trout immune cells as well. Based on the six concentrations of PACAP analyzed here (ranging from 0.002 µM – 20 µM), only PACAP concentrations of 0.2 µM and higher significantly decreased the viability of RTS11 (**Figure 4.2**). Furthermore, cell death was only observed on day 2 of exposure. Cell viability was not significantly different between PACAP concentrations on both days 1 and 3 of exposure.

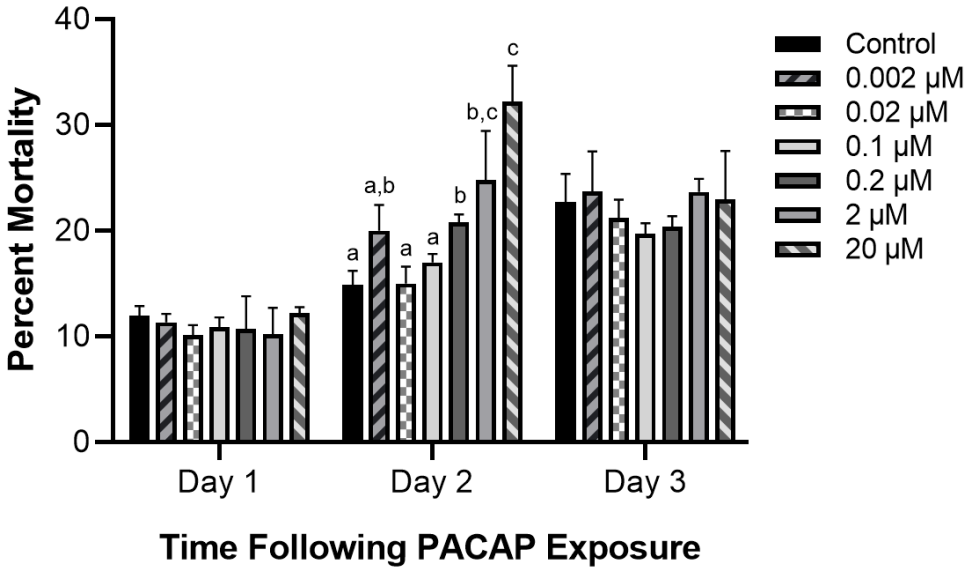

Figure 4.2. Impact of *C. gariepinus* PACAP-38 on RTS11 viability. RTS11 was exposed to varying concentrations of PACAP ranging from 0 µM – 20 µM and the percent mortality of RTS11 was determined for each concentration using a trypan blue assay. This figure represents three independent experiments where a p-value of less than 0.05 was considered to be significantly different when compared to the no PACAP control for individual timepoints. Significant differences at each timepoint are denoted by different letters (a, b, c). All vertical error bars represent the standard deviation (SD).

4.4.3 Effect of PACAP on F. psychrophilum growth throughout RTS11 infections

When RTS11 was exposed to live *F. psychrophilum* simultaneously with various PACAP concentrations, the number of viable bacteria present in the supernatant was not significantly different when compared to that of the no PACAP control on day 2 (**Figure 4.3A**). As it was possible that PACAP required time to stimulate a defensive immune state in RTS11, this experiment was repeated but this time the cells were exposed to PACAP concentrations 24 hours prior to receiving the infectious dose of *F. psychrophilum*. When using this experimental design, all three concentrations of PACAP (0.002 µM, 0.02 µM and 0.1 µM) were shown to significantly reduce the number of viable bacteria in the RTS11 on day 2 (**Figure 4.3B**). This reduction was still observed on day 3 but was only found to be significant in the two higher concentrations of PACAP at 0.02 µM and 0.1 µM (**Figure 4.3C**).

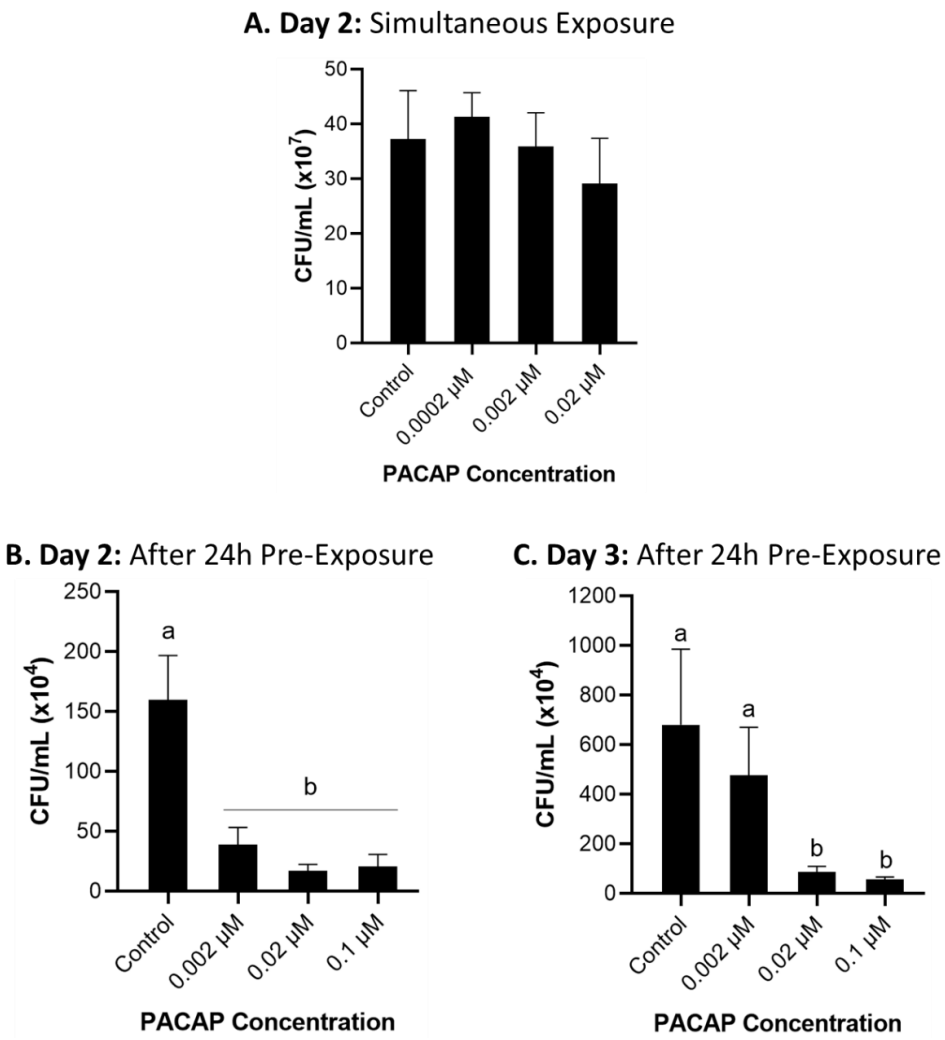

Figure 4.3. Quantification of *F. psychrophilum* by standard plate count (SPC) of cell culture media during live infection (MOI of 0.7-1.3) of PACAP-treated RTS11. RTS11 was exposed to live *F. psychrophilum* either alone or in combination with *C. gariepinus* PACAP-38 concentrations (0.0002 µM – 0.02 µM) and CFU/mL was calculated on day 3 (A). RTS11 pre-treated with PACAP-38 concentrations (0.002 µM – 0.1 µM) 24 h before exposure to live *F. psychrophilum* and the CFU/mL was calculated on day 2 post-infection (B) and day 3 post-infection (C). Each panel represents the results of three independent experiments where a p-value of less than 0.05 was considered to be significantly different when compared to the no PACAP control (i.e. RTS11 exposed to only live *F. psychrophilum*). All vertical error bars represent the SD.

4.4.4 Permeabilization of F. psychrophilum by PACAP

To establish whether the studied PACAP concentrations were either inducing direct lysis of *F. psychrophilum* or instead stimulating RTS11 to respond to and destroy the bacterial pathogen, a permeabilization assay was performed. At doses comparable to the MIC (50 µM and 30 µM), PACAP was shown to induce permeabilization of *F. psychrophilum* comparable to that observed when the bacterium was heat-killed (**Figure 4.4**). Interestingly, this ability was absent when using 0.1 µM of PACAP as, in this case, the bacteria presented reduced permeabilization similar to that of the live *F. psychrophilum* control (**Figure 4.4**). The permeabilization ability noted here was also specific to PACAP as 50 µM of a synthetic peptide fragment of comparable size (1.3 kDa), HSP70, was not able to permeabilize *F. psychrophilum*.

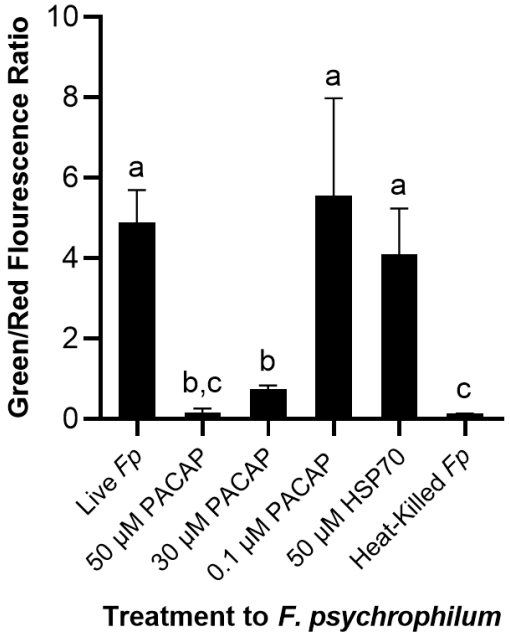

Figure 4.4. Disruption of *F. psychrophilum* membrane by PACAP-38 of *C. gariepinus*. Live *F. psychrophilum* was grown alone, in the presence of PACAP-38 (50 µM, 30 µM and 0.1 µM) and in the presence of a control synthetic peptide fragment of comparable size, 50 µM HSP70 (1.3 kDa). As a negative control, heat-killed *F. psychrophilum* was also included. Following a three day incubation at 14ºC, all experimental wells were exposed to BacLight which would cause live bacterial cells to fluoresce green (SYTO9) and permeabilized cells to fluoresce red (propidium iodide). The ratio of green/red fluorescence was calculated, and this value was compared between conditions. This experiment was replicated four times and the averages are presented as the means + SD. A p-value of less than 0.05 was considered to be statistically significant.

4.4.5 Influence of C. gariepinus PACAP-38 on RTS11 immune gene expression

4.4.5.1 Exposure to PACAP

To determine whether PACAP alone could stimulate a response in RTS11, the cells were exposed to various concentrations of the peptide (0.002 µM, 0.02 µM and 0.1 µM) over four days. Following this exposure, gene expression of pro-inflammatory cytokines (*IL-1β, TNFα* and *IL-6*) and PACAP receptors (*PAC1* and *VPAC1*) were measured using qRT-PCR. For all three of the pro-inflammatory cytokines measured, a significant difference was only seen on day 2 (**Figure 4.5A-C**). Furthermore, for *TNFα* and *IL-6*, this significant increase was only observed at the highest PACAP concentration of 0.1 µM. Meanwhile for *IL-1β*, a significant increase occurred at both 0.02 µM and 0.1 µM of PACAP. When regarding the PACAP receptors, there were no significant increases observed for *PAC1* but by day 2, *VPAC1* significantly increased at all of the concentrations studied (**Figure 4.5D-E**). To confirm that this response was specific to PACAP and not just a property of synthetic peptides in general, RTS11 was also exposed to 0.1 µM of a synthetic peptide fragment of rainbow trout HSP70, which was unable to induce significant expression differences in all of the genes selected for this study (**Figure 4.5F**).

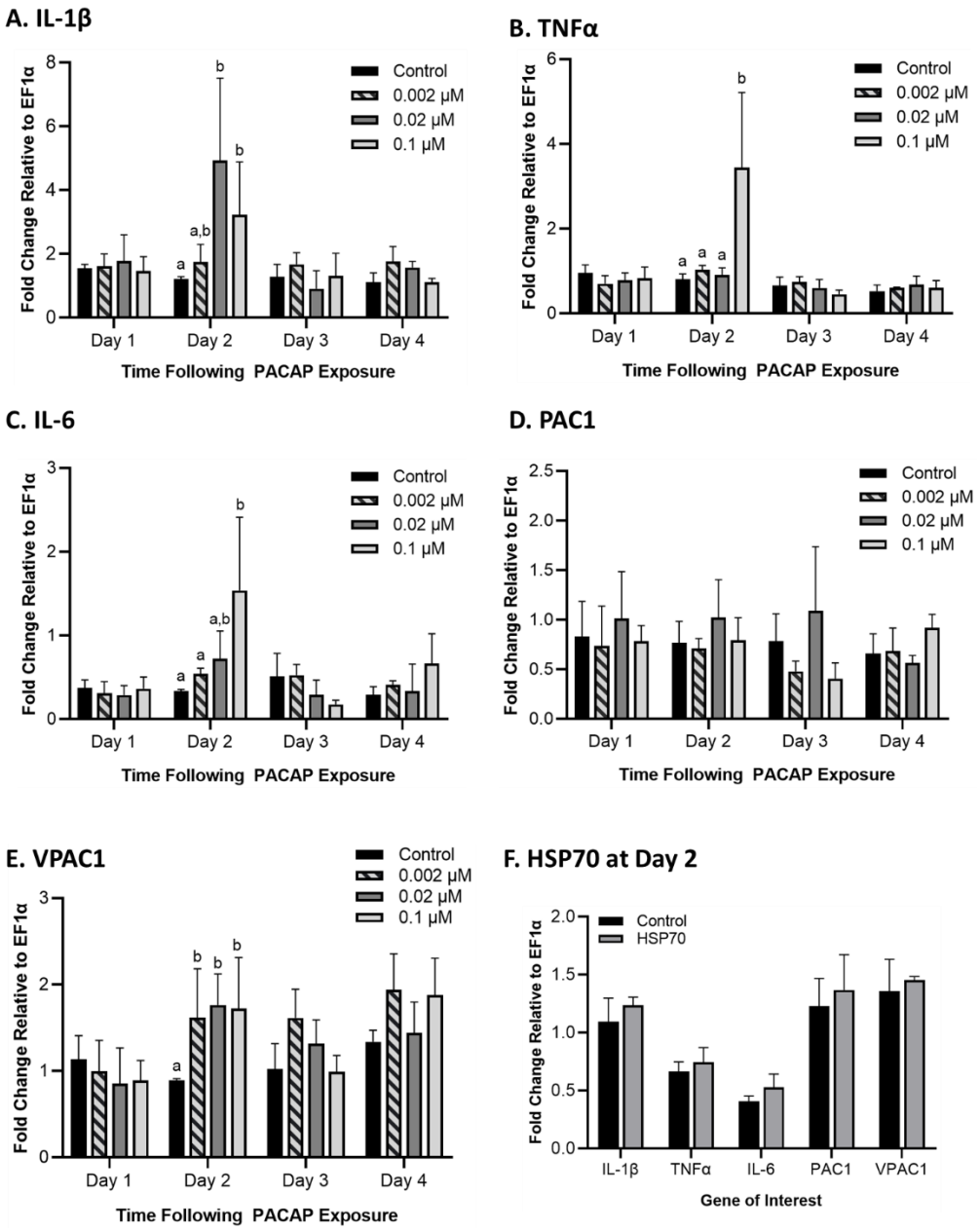

Figure 4.5. Impact of *C. gariepinus* PACAP-38 on RTS11 pro-inflammatory cytokines and PACAP receptor mRNA expression 1-4 days following peptide exposure. Transcript expression of pro-inflammatory cytokines *IL-1β* (A), *TNFα* (B) and *IL-6* (C) was measured so that the influence of PACAP alone on immune function could be assessed. The present PACAP receptors *PAC1* (D) and *VPAC1* (E) were also measured to determine whether PACAP could prime RTS11 cells to bind more PACAP. Because one day after the pre-exposure appeared to be the only timepoint with significant upregulation due to PACAP, RTS11 was also exposed to 0.1 μM of a synthetic HSP70 peptide fragment control of comparable size (1.3 kDa), for 48 hours to confirm that this stimulation was due to PACAP and not a property of synthetic peptides alone (F). All panels represent three independent experiments and are presented as means + SD. A p-value of less than 0.05 was considered to be statistically significant when compared to the no PACAP control for each timepoint.

4.4.5.2 Exposure to PACAP 24 hours before F. psychrophilum infection

When RTS11 was challenged with live *F. psychrophilum* infection 24 hours after exposure to PACAP, there were some interesting differences in transcript expression that were not observed during PACAP exposure alone (**Figure 4.5**). For *IL-1β* expression, there were no significant differences at 1 and 2 days post-infection when compared to the RTS11 cells exposed to live pathogen alone. However, by day 3 of infection, a significant increase in *IL-1β* transcripts was observed for all three concentrations of PACAP (**Figure 4.6A**). In comparison, *TNFα* expression was significantly upregulated at day 1, 2 and 3 post-infection but only at 0.1 µM of PACAP, the highest concentration of the AMP (**Figure 4.6B**). Interestingly, all three concentrations of PACAP showed a significant increase in *IL-6* expression on day 1 post-infection but by day 2 this upregulation was either lost at 0.002 µM or was significantly reduced in the two higher concentrations of PACAP (**Figure 4.6C**). When compared to the PACAP only expression (**Figure 4.5**), the PACAP receptors were also influenced differently during pathogen challenge. On day 1 post-infection, *PAC1* showed a significant increase only 0.1 µM (**Figure 4.6D**). Meanwhile, *VPAC1* showed a significant increase at day 2 in the two higher PACAP concentrations (0.02 µM and 0.1 µM) and on day 3, all three concentrations of PACAP presented a significant upregulation when compared to the control cells that were exposed to *F. psychrophilum* alone (**Figure 4.6E**).

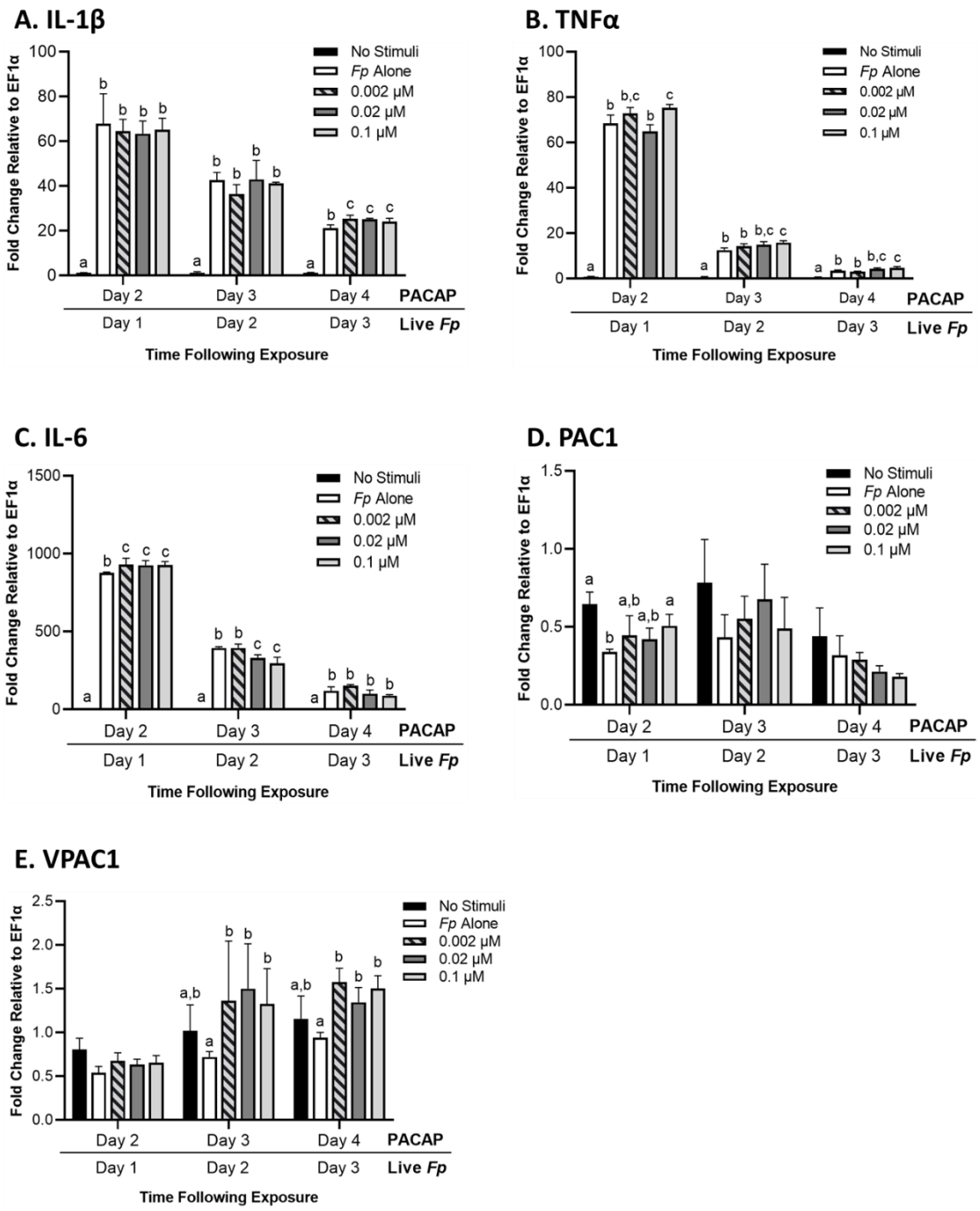

Figure 4.5. RTS11 transcript expression when challenged with live *F. psychrophilum* following 24 hr pre-treatment with PACAP concentrations (0.002 µM, 0.02 µM and 0.1 µM). RTS11 that was not exposed to PACAP or live *F. psychrophilum* was also included as a no stimuli control. To determine whether PACAP has immunomodulatory effects during live infection, transcripts of the pro-inflammatory cytokines *IL-1β* (A), *TNFα* (B) and *IL-6* (C) were measured in RTS11 on days 1, 2 and 3 of infection. The PACAP receptors that are found within RTS11, *PAC1* (D) and *VPAC1* (E) were also assessed throughout the live infection challenge. All panels represent three independent experiments and are presented as means + SD. A p-value of less than 0.05 was considered to be statistically significant when samples were compared within each timepoint.

4.5 Discussion

Currently aquaculture facilities have very few options outside of antibiotics to combat disease outbreaks. When this is combined with the rising incidence of multi-drug resistance, AMPs such as PACAP are promising alternatives for disease control/prevention. The purpose of this study was to evaluate the antimicrobial activity and immunomodulatory function of PACAP within a live infection model consisting of RTS11 and the coldwater pathogen, *F. psychrophilum*. To assess the efficacy of this proposed system, it was critical to determine the impact that PACAP alone had on both components of the infection model: the host and the bacterium. For several bacterial pathogens, PACAP has been shown to have a direct antimicrobial effect (Lugo et al., 2019; Starr et al., 2018) including those of aquatic origin (Lugo et al.. 2019). Thus it was not surprising that PACAP presented a similar result when *F. psychrophilum* was exposed to various concentrations in a preferred growth medium, cytophaga broth. Additionally, the MIC of PACAP was not influenced when *F. psychrophilum* was grown in cell culture media, a substance meant to mimic physiological conditions (reviewed by Yao & Asayama, 2017). This suggests that synthetic PACAP may maintain its antimicrobial effects in some physiological conditions, as may be the case when administered to live organisms. But despite this promising observation, it is important to remember that disease outbreaks in aquaculture settings would differ significantly from microbial culture settings in important ways. Namely the assumption of sterility and the resulting absence of competing microorganisms. As such, concentrations surrounding the observed MIC may not represent an effective therapeutic dose to control/prevent live infection and prove to be suboptimal for the host organism if not properly evaluated.

Aside from their potential as antimicrobials and immunomodulators, AMPs are also believed to have minimal negative effects on mammalian host cells (Deslouches et al., 2005) including those of immune origin (Zhu et al., 2015). Quite often this “cell selectivity” is based on the concentration required for the AMP to induce 50% hemolysis in host red blood cells (RBCs). If this concentration is much higher than what is required for the MIC, the peptide is considered to be essentially nontoxic to host cells (reviewed by Matsuzaki, 2009). This has been shown with *C. gariepinus* PACAP-38 when

both human and fish RBCs were exposed to the peptide, revealing only RBC lysis at extremely high concentrations (Lugo et al., 2019). Unfortunately, these methods of measurement are not always directly comparable as antimicrobial assays generally use a bacterial concentration of ~5 x 10^5 CFU/mL while hemolysis assays use what corresponds to be 6 x 10^8 cells/mL (reviewed by Matsuzaki 2009). In fact, when Imura and colleagues (2008) corrected for this concentration difference during their analyses of the antimicrobial peptide magainin, the MIC concentration of 10 µM was enough to completely lyse the host RBCs. In the present study, RTS11 was exposed to PACAP wherein higher concentrations (0.2 µM, 2 µM and 20 µM) had a significantly negative impact on RTS11 viability. Some studies have alluded to the idea that AMPs may be toxic to mammalian cells in the absence of microorganisms (Imura et al., 2008), thus it is possible that this may also be observed in fish cells. There are important differences between prokaryotic and eukaryotic membranes that may improve the chance that AMPs will preferentially bind to the membrane of microorganisms before host cells (reviewed by Huang et al., 2010). Prokaryotic membranes have a high negative charge due to being predominantly composed of phosphatidylglycerol, cardiolipin or phosphatidylserine, thus have a greater chance of attracting the cationic peptides that are AMPs. In comparison, mammalian cells may be less attractive for AMP penetration as they are enriched in zwitterionic phospholipids resulting in an overall neutral charge (reviewed by Huang et al., 2010; Matsuzaki, 1999). Furthermore, mammalian cell membranes contain cholesterol, something that is absent in prokaryotic membranes. Interestingly, it has been shown that cholesterol can dramatically reduce the activity of AMPs (Matsuzaki et al., 1995) providing another potential layer of protection for mammalian cells. But despite these important differences that may help with membrane selection of AMPs, complete protection of the host cells from AMP-induced cytotoxicity may not be possible at higher AMP concentrations. To even consider PACAP for use as a therapeutic agent in aquaculture settings, the therapeutic dose must not be cytotoxic to the host, whether infected or microbe-free.

In spite of the value that can be obtained from determining the MIC of PACAP in various culture media, as well as the ideal concentration for survival of host cells, the observed antimicrobial activity

is meaningless if it is lost or not effective during live pathogen challenge. The current study is the first of its kind that has demonstrated the antimicrobial activity of PACAP during an *in vitro* live infection model with an aquatic pathogen. But interestingly, the teleostean version of PACAP was only able to reduce the viable bacterial count of *F. psychrophilum* when RTS11 cells were pre-treated with PACAP for 24 hours. It appears that rainbow trout macrophages require time to respond and activate an effective immune response when exposed to *F. psychrophilum*. As obligate poikilotherms, metabolic rates in fish are heavily influenced by their environmental temperature (Gillooly et al., 2002; Patterson et al., 2013). Because cells derived from rainbow trout, a coldwater salmonid, are grown at much lower temperatures than their mammalian counterparts, it may take more time for these cells to respond to stimuli. Indeed this has been shown with both RTS11 and rainbow trout B cells where the chemoattractant ability of the chemokine CK9 strongly increased when the cells were pre-treated with T-independent antigen (Aquilino et al., 2016). Likewise, in rainbow trout primary head kidney culture, the cells sometimes required 48 hours before an increase in respiratory burst activity was observed (Novoa et al., 1996). In both of these examples, the immune cells were maintained at 18ºC, but in the current study, RTS11 was held at 14ºC as this is a relevant temperature at which BCWD occurs (Holt 1987). Thus, a longer pre-treatment time at this lower temperature may be required depending on the response that is being measured. Though a temperature between 8-14ºC would be optimal for testing the efficacy of PACAP in protecting rainbow trout from infection with *F. psychrophilum*, there are many infectious diseases that influence the culture of numerous aquatic organisms. As a result, experimental doses with therapeutic AMPs must be tested *in vivo* to ensure that they will provide protection and effective immune stimulation towards relevant pathogens within an applicable temperature range.

Cell culture systems provide a controlled, cost-effective method for exploring numerous biological phenomena, but it is important to recognize the limits of these models. In a cell culture setting, individual cells are directly exposed to the experimental stimulant, without physiological barriers or a complicated cellular milieu to overcome. As a result, *in vitro* systems often have much

lower doses than what is appropriate within the whole organism. This was displayed when Gotlieb et al (2015) used several methods to isolate and stimulate NK cells revealing that the cells were 10-30 times more susceptible to stress hormones *in vitro* than what was observed when stimulated in plasma, a much more biologically relevant medium. Though the effects of PACAP on fish infections *in vivo* have not yet been explored, there have been several studies to determine the impact of this AMP on growth, immunomodulation and physiology (Cardoso et al., 2015; Lugo et al., 2010a; Lugo et al., 2010b; Wang et al., 2013). One study by Lugo et al (2010a) exposed juvenile fish to an average of 4 µg of PACAP per fish, which significantly enhanced tilapia growth. In comparison, with the RTS11 infection model presented here, each well received 1.8 µg or less and antimicrobial activity was still observed. Though the PACAP doses optimized for the current *in vitro* study were very effective, an *in vivo* model would require further optimization to develop an efficacious exposure range.

When it comes to bacterial pathogens, the consensus regarding AMP function is that they are either membrane disrupting, or nonmembrane disrupting (reviewed by Bahar & Ren, 2013). Though PACAP was able to lower the number of viable bacteria when grown alone in media (for the MIC) and in RTS11 cultures throughout live infection, it was unclear whether this was due to the direct antimicrobial activity of PACAP acting on *F. psychrophilum*. Previous work with mammalian PACAP confirmed for the first time that the peptide was capable of disrupting membranes of relevant terrestrial bacterial pathogens (Starr et al., 2018). This permeabilization is a common mode of action for α-helical AMPs (Sato & Feix, 2006), including PACAP, but until now this ability has not been confirmed for the version of PACAP produced by fish. Previous bioinformatic analysis of PACAP-38 from *C. garipinus* provided evidence that this peptide was very likely to have cell penetrating properties (Lugo et al., 2019). The present study was able to functionally validate the ability of *C. gariepinus* PACAP-38 to permeabilize the membrane of *F. psychrophilum* at concentrations surrounding the MIC. Furthermore, this was an ability specific to PACAP as another synthetic peptide fragment of comparable size from a teleost, HSP70, did not induce permeabilization. Interestingly, at the highest concentration that reduced

the viable bacterial count during live infection, 0.1 µM, PACAP was unable to permeabilize the membrane of *F. psychrophilum*. This finding confirmed that at 0.1 µM, one of the many other effects that PACAP may have on teleostean immunity must have been responsible for stimulating RTS11 to destroy and/or slow the growth of the coldwater pathogen.

PACAP has been shown to have immunomodulatory effects on whole fish and in fish cells (Kasica-Jarosz et al., 2018; Lugo et al., 2010b; Lugo et al., 2019; Wang et al., 2013) but this has only been studied in the absence of live infection. Furthermore, there has been limited research regarding the activity of PACAP directly on teleost immune cells. Specifically with RTS11, the immunostimulatory effect of three other α-helical AMPs has been reported (Chiou et al., 2006) but not when dealing with a live bacterial challenge. The present study explores, for the first time, the impact of relevant doses of PACAP on the immune function of RTS11 in both the presence and absence of *F. psychrophilum.* In the absence of bacterial infection, PACAP was shown to stimulate pro-inflammatory cytokine expression 48 hours following PACAP treatment as well increasing the expression of one of the PACAP receptors, *VPAC1*. When analyzing PACAP receptor expression in RTS11, it is important to note that only *VPAC1* and *PAC1* receptor genes were measured as it has previously been shown by Lugo et al (Lugo et al., 2011) that these cells do not express the third receptor gene, *VPAC2*. Lugo and colleagues also showed that despite *PAC1* presenting the highest constitutive expression in all rainbow trout lymphoid tissues *in vivo*, this was not observed in RTS11 where *VPAC1* presents the greatest expression (Lugo et al., 2011). As *PAC1* has been found to be a fundamental type I receptor for PACAP this result was unexpected. Nonetheless, the current study validated this finding when *VPAC1* presented significant upregulation following RTS11 stimulation with PACAP while *PAC1* did not. As PACAP was shown to stimulate a slight increase in pro-inflammatory cytokines and upregulate the expression of *VPAC1*, pre-treatment with this AMP may stimulate a protective state within the rainbow trout immune cells.

In itself, the presence of live bacteria would be capable of inducing pro-inflammatory cytokine expression in RTS11. But when this was combined with 24-hour pre-treatments with PACAP, infection

with *F. psychrophilum* had an effect on transcript expression that was quite different from PACAP alone. Rather than all three of the studied pro-inflammatory cytokines increasing their expression at the same time, each one showed significant upregulation at different time points post-infection when compared to RTS11 exposed to the bacteria alone. The pattern of enhancing inflammatory cytokine production following pre-treatment is similar to that of trained immunity that has been observed in mammalian monocytes and macrophages (Quintin et al., 2012). Quintin and colleagues showed that when primed with β-glucan prior to exposure to LPS, monocytes and macrophages were able to induce a greater pro-inflammatory response than unprimed cells (Quintin et al., 2012). Perhaps PACAP has a similar function and is able to prime immune cells to produce a faster, more damaging response when they come into contact with a live pathogen. This would provide an explanation for the observed decrease in viable bacteria following PACAP exposure at doses that were not able to directly permeabilize the membrane of *F. psychrophilum.*

Despite the similarity to trained immunity, the actual function of PACAP in mammalian models appears to be contrary to what has been observed in bony fish. The vast majority of mammalian studies discuss the anti-inflammatory role of PACAP during experimental bacterial infection. These experiments often involve exposure to bacterial products (such as LPS) simultaneously with PACAP, after which various immune parameters are observed (Delgado et al., 1999a; Delgado & Ganea, 2001; Martinez et al., 2002). This has led to the belief that many AMPs, including PACAP, play important anti-inflammatory roles to protect the host from dangerous, over-reactive inflammatory responses (Delgado et al., 1999b). Though very valuable, these mammalian studies are not directly comparable to a live, growing infection within an organism or cell culture. Additionally, aside from zebrafish, teleosts appear to be lacking TLR4, which binds and responds to LPS (Ali et al., 2014; Sepulcre et al., 2009). As a result, observations in mammalian study systems may not be directly transferrable to those of fish. The immunostimulatory effect of PACAP on rainbow trout immune cells as observed in this study has been previously reported in head kidney leukocytes derived from another bony fish, the grass carp (Wang et al., 2013). Wang and colleagues found that when these immune cells were exposed to

bacterial products, PACAP induced inflammatory cytokine expression while having no impact on the expression of the anti-inflammatory cytokine, *IL-10* (Wang et al., 2013). When all of this information is taken together, it appears that PACAP may play a different, yet valuable role in the immunomodulation of teleosts when compared to what has been observed in mammals.

4.6 Conclusions

Antimicrobial peptides (AMPs) are promising alternatives to antibiotics in the ongoing battle between aquaculture facilities and infectious agents. One AMP that has received a lot of attention due to its pleiotropic effects in aquatic species is PACAP. The results of the present study revealed that PACAP derived from the teleost *Clarias gariepinus* acts as a potent antimicrobial peptide against the causative agent of BCWD, *F. psychrophilum*. Furthermore, its mode of action was confirmed to be permeabilization of the bacterial membrane. When a live infection model was developed with this pathogen and the monocyte/macrophage-like cell line, RTS11, 24 h pre-exposure of PACAP appeared to protect RTS11 by significantly reducing the number of viable bacteria in the culture system. Based on transcript levels of pro-inflammatory cytokines and receptors for the AMP, PACAP was also shown to have an immunostimulatory effect on RTS11 whether exposed to the AMP alone or exposed to both PACAP and live *F. psychrophilum* challenge. Overall, this study was able to provide further validation regarding the antimicrobial effect of PACAP on aquatic pathogens as well as its immunomodulatory activity on teleost immune cells. As a promising candidate for use in aquatic models, future studies should focus on confirming these valuable functions of PACAP throughout live infection models *in vivo*.

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Dr. Niels Bols for kindly allowing the use of his cell culture facility.

Chapter 5: Revealing immune differences between eight outbred Chinook salmon populations when challenged with live *Vibrio anguillarum*.

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5.1 Overview

Aquaculture in North America is currently dominated by Atlantic salmon, but there has been an increasing interest in the production of species native to the Pacific coast such as Chinook salmon (*Oncorhynchus tshawytcha*). Chinook salmon is relatively new to culture production; therefore, the selection of appropriate stocks is critical for the success of this developing species. In many cases genes from wild populations are incorporated into farmed stocks to avoid the decrease in performance associated with inbreeding. The current study focuses on assessing the immunological performance of eight outbred Chinook salmon stocks after challenge with the marine pathogen, *Vibrio anguillarum*. Throughout exposure challenge to both the pathogen and sterile PBS, significant differences in mortality were observed. Fish were also assessed for MH class II β1 genotype to determine whether there were specific alleles that could predict resistance/susceptibility. Although unique MH alleles did not appear to confer resistance, the crosses with better survival presented a higher percentage of individuals that possessed a heterozygous MH genotype. The stress induced during infection resulted in several individuals presenting signs of Bacterial Kidney Disease (*Renibacterium salmoninarum*) indicating that chronic co-infection may have played a role in susceptibility. This was supported by the highest and lowest performing populations presenting the lowest and highest prevalence of BKD respectively as validated through an ELISA-based assay of head kidney samples. When spleen samples from the highest and lowest performing populations were assessed for cytokine and respiratory burst gene expression throughout bacterial challenge, the high surviving stocks presented lower expression of inflammatory cytokine transcripts (*IL-1β*, *IL-6* and *TNFα*) when compared to the low performing stocks. Interestingly, microsatellite analysis revealed that there were little to no differences in heterozygosity among these eight populations despite the variations observed regarding immune performance. Understanding the impact of outbreeding on the immune function of farmed, and often inbred, Chinook salmon could aid in the future development of high-quality aquaculture stocks for this species.

5.2 Introduction

The high demand for alternative and healthy protein options has resulted in aquaculture becoming the fastest growing animal food sector for the past 40 years (FAO, 2018). North American aquaculture is currently dominated by Atlantic salmon, resulting in production of this species on both the East and West coasts of Canada. However, because Atlantic salmon are not indigenous to the West coast, there is public concern regarding the introduction of foreign diseases to wild populations as well as the exposure of native salmon species to ecological niche competition through farmed escapees (reviewed by Noakes et al., 2000). Despite the negative publicity associated with farming Atlantic salmon on the West coast, a staggering 71% of Canadian salmon production occurs in British Columbia (Statistics Canada, 2017). Thus, the majority of Atlantic salmon are actually grown on the Pacific coast rather than their native coast in Canada. To alleviate some of the public apprehension, there have been numerous attempts to domesticate and farm Pacific salmon species for aquaculture production.

While there are five major Pacific salmon species, Chinook salmon (*Oncorhynchus tshawytscha*) is the largest and most highly valued in North America (Christensen et al., 2018; Ohlberger et al., 2018), making it a leading candidate for use in aquaculture. The farming of Chinook salmon on the West coast offers the benefit of potentially higher disease resistance resulting from accumulated adaptations to resident pathogens (Evans & Neff, 2009). Unfortunately, when compared to the highly domesticated Atlantic salmon species, Chinook salmon are currently unable to tolerate the high densities used for commercial salmon production (DFO, 1991). So, from an economic perspective, the culture of Chinook salmon would not appear profitable enough to convince farmers to convert to such low-density farming despite a more environmentally friendly product. Given that Atlantic salmon underwent over 50 years of domestication in aquaculture (Bicskei et al., 2014), it is reasonable that both time and concerted efforts would be required to prepare Chinook salmon for comparable large-scale production.

A common method used to generate domesticated stocks for any species of interest is selective breeding. This involves identifying individuals whose phenotype is optimal (high growth, disease

resistance, feed conversion, etc.) and using these animals to breed the next generation. Over successive iterations of this process, traits of interest can be enhanced however this strategy can also result in a phenomenon known as inbreeding depression. Inbreeding depression refers to the reduced fitness observed in the progeny of individuals that are closely related (Conner & Hartl, 2004). For agricultural species this can present itself as a reduction in overall yields, reproduction and survival (reviewed by Charlesworth & Willis, 2009). These negative effects occur because inbreeding increases homozygosity at all loci, thus enhancing the probability of unmasking deleterious alleles that would not present themselves within heterozygous individuals (reviewed by Paige, 2010). Fortunately, inbreeding depression can be countered by the introduction of outside genetic material through a practise known as outbreeding.

When it comes to farming native species on the Pacific coast, attempts to domesticate Chinook salmon have resulted in farmed stocks becoming increasingly inbred. It is possible that these stocks are suffering inbreeding depression, thus impeding their survival at higher densities. Though there are many factors that can contribute to this reduction in fitness, susceptibility to disease is a significant challenge continuously faced by fish farmers (reviewed by Assefa & Abunna, 2018). The negative impact associated with infectious disease is high and is often considered to be the leading cause of financial loss for several aquaculture sectors (Leung & Bates, 2012; reviewed by Stentiford et al., 2012; Stentiford et al., 2017). Moreover, there is limited understanding of aquatic pathogens and minimal success in selecting for disease resistance when compared to terrestrial counterparts (Pazderka et al., 1975; Snowder et al., 2005; Odegard et al., 2011). This may reflect a general lack of insight regarding aquatic environments including the many microbial pathogens that influence the aquaculture industry.

One particularly costly infectious agent is the gram-negative bacterium, *Vibrio anguillarum*. This saltwater pathogen is the causative agent of a highly fatal hemorrhagic septicaemia known as vibriosis (reviewed by Frans et al., 2011). As an opportunistic pathogen, outbreaks tend to coincide with environmental and/or other stressors (Austin & Austin, 2007). Though symptoms of vibriosis are typical of a hemorrhagic septicemia (bleeding vent, swollen ulcerated lesions, bleeding eyes,

exopthalmia, etc.), acute outbreaks can result in extremely high mortality without the presentation of any clinical signs (reviewed by Frans et al., 2011; Austin & Austin, 2007). Given that the organism can infect over 90 different aquatic species (reviewed by Hickey & Lee, 2018), *V. anguillarum* has historically been, and continues to be, responsible for significant financial losses to the aquaculture industry. In Canada, the first reported case of vibriosis in a Pacific salmon species was in 1971 (Evelyn, 1971). Since then, the pathogen has plagued Canadian salmon culture making vibriosis a highly relevant disease model for evaluating Chinook salmon stock quality.

The present study describes the crossing of an inbred Chinook salmon stock with seven wild populations and the subsequent evaluation of offspring immune performance when challenged with live *V. anguillarum*. The seven outbred populations and the control inbred stock had variable survival to *V. anguillarum*, indicating that there may be identifiable immune components used to cope with infection. Aspects of both innate and adaptive immunity were explored at functional and genetic levels. More specifically, differences in major histocompatibility (MH) class II β1 genotypes, serum IgM, co-infection with *Renibacterium salmoninarum* and immune gene expression were compared between crosses. Additionally, microsatellite analysis was completed to determine the level of heterozygosity between the seven outbred crosses and the control inbred stock. It is possible that the differences observed here indicate hybrid vigor and could potentially be targets of selection to improve the performance of Chinook salmon stocks for future production measures.

5.3 Materials and Methods

5.3.1 Breeding design

Breeding and rearing occurred at Yellow Island Aquaculture Ltd. (YIAL), an organic Chinook salmon farm located on Quadra Island, British Columbia, Canada. The YIAL Chinook salmon stock has been in production since 1985 and originated from the Robertson Creek and Big Qualicum River (Vancouver Island, British Columbia, Canada) Chinook salmon stocks. To generate outbred crosses with the YIAL production stock, milt from males taken from seven wild populations was collected from across Vancouver Island and lower mainland British Columbia (**Figure 5.1**). The target populations used to provide the milt were: Big Qualicum River (BQ), Capilano River (Cap), Chilliwack River (Chil), Nitinat River (Nit), Puntledge River (Punt), Robertson Creek (RC) and Quinsam River (Quin). The domestic production cross (YIAL x YIAL) would serve as an internal control for comparative assessment between the stocks. Milt collection and all associated procedures are as described in Semeniuk et al. (2019).

To minimize maternal effects (Wellband et al., 2017), eggs from 17 highly inbred female offspring of one self-fertilizing hermaphrodite Chinook salmon from YIAL were used (see Komsa, 2012). Briefly, hermaphrodite fish (genetically female, but phenotypically both male and female) were generated from incomplete sex reversal by exposing female larvae to 17-alpha-methyltestosterone (17aMT) treatments. Female offspring from the hermaphrodite salmon had an average inbreeding coefficient of 0.50. Fertilization of all crosses took place on November 1, 2013 and was performed as described by Semeniuk et al. (2019).

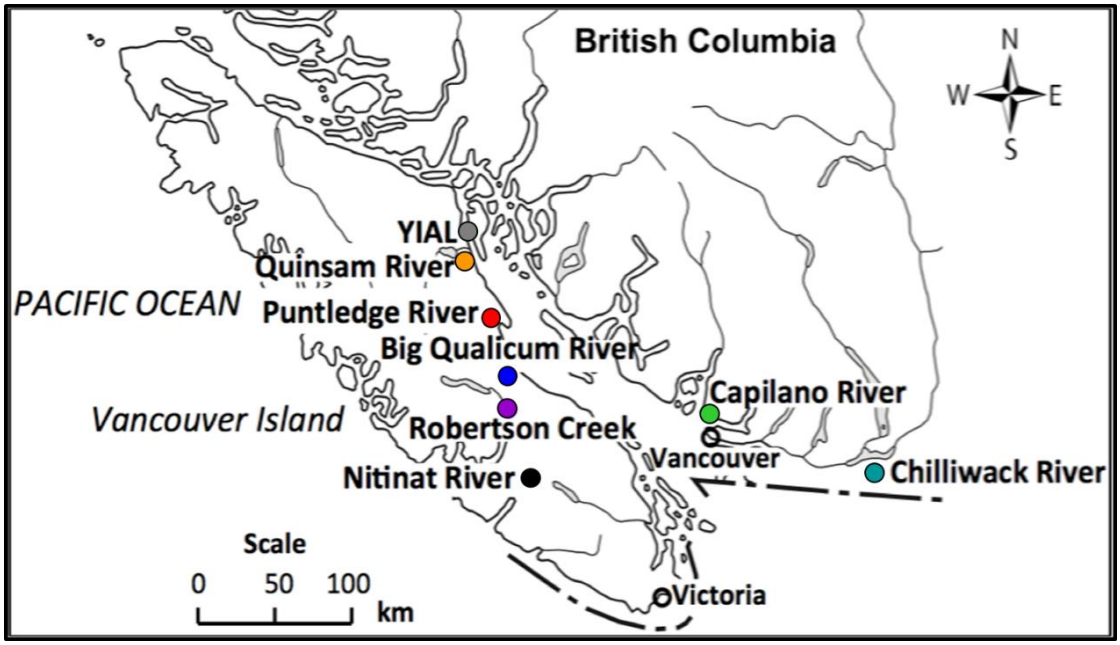

Figure 5.1. Geographical location of the British Columbia rivers from which milt was collected to produce the seven outbred Chinook salmon populations. The location denoted as YIAL (Yellow Island Aquaculture Ltd.) indicates the source of the farmed salmon milt used as the inbred population control. This Chinook salmon farm was also the location for the live *V. anguillarum* infection trial used to produce samples for this study.

5.3.2 Rearing

Rearing of the eight different crosses during both fresh- and saltwater phases of growth were as described by Semeniuk et al. (2019). Prior to sea transfer, a subset of each cross received passive integrated transponders (PIT) identification tags.

5.3.3 Culture and quantification of V. anguillarum

V. anguillarum serotype O1 was kindly provided by Simon Jones of Pacific Biological Station (Nanaimo, BC). This particular strain of *V. anguillarum* was isolated from a diseased winter steelhead trout obtained from Little Campbell River, BC. Frozen stocks of the *V. anguillarum* were subcultured onto tryptic soy agar (TSA) supplemented with 2% NaCl. Following incubation at room temperature (RT) for 48 h, a single colony was used to inoculate 2.5 mL of tryptic soy broth (TSB) supplemented with 2% NaCl and incubated for 17 h at RT and 200 rpm. Following this incubation, 100 µL of this culture was used to inoculate 100 mL of TSB with 2% NaCl and incubated at RT for 24 h while shaking at 200 rpm. The resulting culture had an optical density of 0.25 which was confirmed to be 2.0×10^9

CFU/mL by a standard plate count (SPC). Specifically, bacterial numbers were enumerated by plating 0.05 mL of ten-fold serial dilutions on TSA with 2% NaCl plates in triplicate. After incubation at RT for 30 hours, plates containing 25-250 colonies were counted and the number of CFU/mL calculated.

5.3.4 Infection with live V. anguillarum and sample collection

On July 16, 2015, two weeks before the start date of the infection trial, 160 fish from each of the 8 different populations were transferred from net pens to 2000 L troughs. Because each fish was PIT tagged, 40 fish from four of the populations were in each of the eight troughs (n = 160). Each trough was replicated four times, with two of the trough replicates being used for live *V. anguillarum* infection, and the remaining two being used for sham injection with sterile phosphate buffered saline (PBS, Lonza). Two days prior to challenge (day 0), blood, gill, spleen, head kidney, tail fin, and muscle samples were collected from two individuals per family per trough to act as Day 0 unstimulated controls.

Following sedation with clove oil, all fish received 100 µL intraperitoneal (i.p.) injections of 8.0 x 10^4 CFU/mL of *V. anguillarum* suspended in sterile PBS. In comparison, all fish in the control troughs received i.p. injections of 100 µL sterile PBS. The water outflow from all challenged troughs underwent UV sterilization (493.5 mJ/cm^2) which ensured the elimination of the infectious agent. On days -2, 1, 7, 12, and 28, three fish from every population within each trough were sampled and blood was drawn from the caudal vein after anesthesia with clove oil. Fish were then euthanized with an overdose of clove oil and whole-body weights and lengths were measured. Gill, spleen, head kidney (HK), tail fin, and muscle samples were collected for each individual and stored in RNA Later at -20°C for future use. Blood samples were left to clot overnight at 4°C before centrifugation at 1000 g for 10 min. The serum was then removed, aliquoted into 1.5 mL low protein binding tubes (Costar) and immediately stored at -20°C.

5.3.5 Microsatellite analysis

To further explore the differences between the single hatchery (YIAL) and the seven outcross populations, estimates of percent heterozygosity were obtained using DNA extracted from fin clips.

Fin clips were collected and preserved from the seven outbred and the YIAL populations. DNA was extracted from fin clips using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at -80°C until further use. Individual genotypes were determined through polymerase chain reactions (PCR) at 9 previously described microsatellite loci, specifically Oneu3, Oneu8 (Scribner et al., 1996), Omm1135 (Rexroad et al., 2001), Omy325 (O'Connell et al., 1997), OtsG432, OtsG474 (Williamson et al., 2002), Ots1, Ots4 (Banks et al. 1999) and Ogo4 (Olsen et al., 1998). PCR conditions included: a 5-min denaturation step (94°C), followed by 30 cycles of a 20-s denaturation step (94°C), a 20-s annealing step (64.6°C – Omy325, Ots4; 63.5°C – Oneu8, Ogo4; 54.3°C – Oneu3; 58.3°C – Omm1135, Ots1; 60.2°C – OtsG474, OtsG432) and a 30-s extension step (72 °C), followed by a final extension step of 3 min. PCR products at all 9 loci were pooled by individual, cleaned by precipitation with isopropanol, resuspended in milliQ water and then individually barcoded. The barcoding PCR included: a 2-min denaturation step (94 °C), followed by 8 cycles of a 30-s denaturation step (94 °C), a 30-s annealing step (60 °C) and a 1-min extension step (72 °C), followed by a final extension of 5 min. The barcoded amplicons were then pooled and gel-extracted using a Qiaquick Gel Purification Kit (Qiagen).

The library was assessed and quantified using the High Sensitivity DNA Reagents Kit (Agilent Technologies) on a Bioanalyser 2100 (Agilent Technologies) and was then diluted to 60 pM for template preparation using the 400 bp Hi-Q View Kit (Life Technologies). Sequencing of the library was performed on the Ion Torrent Personal Genome Machine (Life Technologies) using a 318 v2™ chip (Life Technologies). Truncated and low-quality sequences were removed from the data and the remaining sequences were separated based on individual and microsatellite loci using Mothur (Schloss et al., 2009). Fragment sizes (alleles) were then identified using an R based bioinformatics framework developed to score microsatellites generated from next generation sequencing platforms (R Core Team, 2014; Roy et al., 2017).

5.3.6 MH class II β1 genotyping

DNA was extracted from fin clip samples as described above in section 5.3.5. Primers that were previously validated by Becker et al (Becker et al., 2014) to amplify the hypervariable region of MHC class II β1 (exon 2) of Chinook salmon were used for this study. Specifically, the forward primer used was 5'-CTTGGTCTTGACTTGMTCAGTCA-3' and the reverse primer was 5'CCCGAGAAGCTTCCGATACTCCTCAAAGGACCTGCA-3'. For amplification, the following PCR parameters were used: denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 57°C for 30 s and extension at 72°C for 45 s. A final extension at 72°C was carried out for 5 min. Two PCR reactions were performed per individual. The amplified PCR products were separated on 1% agarose gels containing 1% GelGreen (Biotium Inc.) and bands of the desired size (310 bp) were cut out of the gel and extracted using the QIAquick Gel extraction kit (Qiagen). The resultant fragments were cloned into pGEM®-T Easy and transformed into competent *Escherichia coli* XL1-blue cells as described previously by Semple and colleagues (Semple et al., 2018a). Following extraction from a minimum of 10 different clones per PCR reaction, plasmids were sequenced using SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, Ont.). The sequences were analyzed using both Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) and the Basic Local Alignment Search Tool (BLAST) software (blast.ncbi.nlm.nih. gov/Blast.cgi). All DNA sequences were then converted to amino acid sequences using the ExPASy Bioinformatics Resource Portal (web.expasy.org/translate/). Only functional amino acid sequences that were observed in both PCR reactions for each individual were considered to be representative of true MHC class II β1 alleles.

5.3.7 Measuring the serum antibody response to V. anguillarum

5.3.7.1 Isolating antigens from V. anguillarum

Subcultures from glycerol stocks of *V. anguillarum* were grown on TSA supplemented with 2% NaCl. An isolated colony was used to inoculate 50 mL of TSB with 2% NaCl and grown for 24 hr at RT while shaking at 200 rpm. When the culture reached an optical density of approximately 0.24 at 600 nm, indicating a concentration of approximately 3.6 x 10^9 CFU/mL, 0.5% formalin was added to the flask and the cells were incubated at RT for 24 hr while shaking. The killed bacterial cells were then washed three times with PBS, harvested by centrifugation (10,000 rpm at 4°C for 10 min) and the subsequent pellets were resuspended in 2 mL of PBS. The resulting formalin-killed *V. anguillarum* culture was then bead-beaten to liberate whole cell antigens as described previously by Semple et al., 2018a. The bacterial lysate was then diluted to a final concentration of 5 µg/mL in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6).

5.3.7.2 Indirect subtractive ELISA to measure serum IgM between the crosses

For each sample being tested, six wells of an Immulon HBX 96-well polystyrene plate (ThermoFisher) were prepared wherein three wells were coated with 100 µL of whole cell bacterial lysate (5 µg/mL) and the remaining triplicate wells received 100 µL of coating buffer alone. The plates were then incubated overnight at 4°C. Following coating and in between all subsequent steps, solutions were removed and all wells on the plate were washed three times with 300 µL of tris-buffered saline with 1% Tween 80 (TBS-T). Prior to samples being added, all wells on the plate were blocked with 300 µL of blocking solution (5% skim milk dissolved in TBS) for 1 hr at RT. After blocking, Chinook serum samples were prepared as a 1:50 dilution in sterile-filtered phosphate buffered saline (PBS). Serum samples were added to the plate in triplicates of 100 µL for both the bacterial antigen coated and uncoated wells. Plates were then incubated at RT for 1 hr. Following removal of samples and washing, 100 µL of rabbit anti-salmonid immunoglobulin primary detection antibody (1:2500 dilution in blocking solution; Cedarlane) was added to all wells and incubated at RT for 1 hr. The primary antibody

was removed and the plate was washed followed by a 1 hr incubation at RT with 100 µL of goat anti-rabbit secondary detection antibody conjugated to biotin (1:2000 dilution in blocking buffer; Cedarlane). After removal of the secondary antibody and washing, 100 µL of streptavidin conjugated to horse-radish peroxidase (HRP, Biolegend) at a 1:1000 dilution in blocking buffer was added and incubated at RT in the dark for 1 hr. Following washing, 100 µL of TMB SENS™ (Cedarlane) was added to all wells and the plate was incubated at RT in the dark for 1 hr. At the end of this incubation, 100 µl of stop solution (0.3M H_2SO_4) was added to each well to prevent further development of colored product. The resulting color development was measured at 450 nm using a BioTek Synergy H1 Hybrid Plate reader (BioTek). For each sample, raw absorbance values from wells with no antigen were subtracted from raw absorbance values of wells coated with bacterial antigen. This value was taken as a relative measure of individual serum IgM antibody developed against *V. anguillarum*.

5.3.8 qRT-PCR of spleen transcripts throughout V. anguillarum infection

5.3.8.1 RNA extraction and cDNA synthesis

RNA was extracted from 20-40 mg of spleen tissue using 1 mL of Trizol (Life Technologies) as described by the manufacturer. To remove any contaminating genomic DNA, RNA samples were treated with DNase I (Thermo Scientific). RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at -80°C until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) in accordance to the manufacturer's instructions. For a no template control, 500 ng of RNA suspended in 20 uL of diethyl pyrocarbonate (DEPC) water was included in the cDNA synthesis reaction without reverse transcriptase.

5.3.8.2 Validatoin of qRT-PCR primers for Chinook salmon

Many of the qRT-PCR primers used for this study had only been validated in rainbow trout (Semple et al., 2018b) making it necessary to confirm their specificity when using a cDNA template obtained from Chinook salmon. Specificity of each primer set was confirmed using cDNA obtained

from the spleen of Chinook salmon challenged with *V. anguillarum*. Sequence validation was confirmed as described above in section 5.3.6.

5.3.8.3 qRT-PCR reactions

To assess transcript levels of *IL-1β, TNFα, IL-6, IL-10, NADPH p22^{phox}* and *NADPH p40^{phox}* in Chinook salmon spleen samples, qRT-PCR analysis was completed. All PCR reactions were 10 µl and contained: 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water), 2x WISENT ADVANCED™ qPCR mastermix (Wisent), and forward and reverse primers (Sigma Aldrich) to a final working concentration of 0.25 µM. All qRT-PCR reactions were completed on the LightCycler® 480 II (Roche). The sequences for all primer sets are outlined in Table 5.1. Each experimental sample was run in triplicate. For each plate, triplicate wells of a calibrator, no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec and extension at 72°C for 8 sec. A melting curve was completed for every run from 65°C to 97°C with a read every 5 sec. Product specificity was determined through single PCR melting peaks. All qRT-PCR data was analyzed using the ΔΔCt method while incorporating individual primer efficiencies as described by Pfaffl (2001) and is presented as the average of 3 experimental replicates for each individual (n = 6) with the standard error of the mean (SEM). All primer efficiencies were above 1.87 and below 2.12. Specifically, gene expression was normalized to the reference gene (EF1α) and expressed as fold change over the day -2 control group where control expression was set to 1. Multiple reference gene primer sets were developed but EF1α was selected as it was observed to have consistent Cp values across a subset of samples (data not shown).

Table 5.1. Primer sequences used for qRT-PCR analysis of immune genes in Chinook salmon. Forward (F) and reverse (R) primer sequences for the genes analyzed are presented along with the original references/accession numbers for the primer sets.

Primer Name	Sequence (5' – 3')	Accession No.
NADPH $p22^{phox}$	**F:** TGGGAGTTTGCTGCTTATGCT **R:** TGGGCCGAATGACTTCACAC	XM_024440320.1
NADPH $p40^{phox}$	**F:** GTCTTACAGCTGCCTGCGC **R:** CACATCTCTCATGCGTGCCAAT	XM_024409180.1
IL-1β	**F:** CCACAAAGTGCATTTGAAC **R:** GCAACCTCCTCTAGGTGC	XM_024418276.1
TNFα	**F:** GTGCAAAAGATACCCACC **R:** CACTGCACGGTGTCAG	XM_024396390.1
IL-6	**F:** CTTCTACACGCTATCTCTCACTC **R:** CGTCTGTCCCGAGCT	XM_024404411.1
IL-10	**F:** GCCTTCTCCACCATCAGAGAC **R:** GATGCTGTCCATAGCGTGAC	XM_024405117.1
EF1α	**F:** CGCACAGTAACACCGAAACTAATTAAGC **R:** GCCTCCGCACTTGTAGATCAGATG	XM_024396038.1

5.3.9 Measuring co-infection with bacterial kidney disease (R. salmoninarum)

Infection status with *R. salmoninarum*, the causative agent of bacterial kidney disease (BKD), was determined for the highest performing (YIAL) and the lowest performing (Punt) Chinook salmon crosses. Head kidney samples from these populations were processed for use in a commercial *R. salmoninarum* ELISA assay (Ango) as described by the manufacturer. Head kidney samples that had an absorbance equal to or higher than that of the provided positive control were considered to represent individuals infected with *R. salmoninarum*.

5.3.10 Statistical analyses

For exposure trials to live *V. anguillarum* and sterile PBS, statistical analyses of survival curves was conducted. To determine significant differences in survival between the eight different crosses for each exposure condition, a Mantel-Cox test was completed with an adjusted alpha level of 0.0018 ($p = 0.05/28$) given the multiple pairwise comparisons among the eight populations. These statistical analyses were completed using GraphPad Prism 8.1.0.

For the microsatellite analysis, individuals that were genotyped at fewer than 6 loci were removed from subsequent analyses. As a result, all microsatellite genetic analyses included 19 to 28

individuals for each of the eight populations. At all loci, significant deviations from Hardy-Weinberg Equilibrium (HWE) were tested using Genepop version 4.2 (Raymond & Rousset, 1995; Rousset, 2008). Significant linkage disequilibrium was also analyzed using Genepop version 4.2 (Raymond & Rousset, 1995; Rousset, 2008) with an adjusted alpha level of 0.006 ($p = 0.05/9$) given multiple pairwise comparisons among the 9 loci. The mean observed (H_o) and mean expected (H_e) heterozygosity across all loci were calculated using GenAlEx version 6.5 (Peakall & Smouse 2012). After confirming normal distributions and homogeneity of variance in each of the populations, heterozygosity estimates were then compared among the eight groups using a one-way ANOVA in the statistical software, Statistica version 7 (StatSoft, Tulsa, OK).

When analyzing the data for serum IgM and immune transcript expression, after confirming a normal distribution and homogeneity of variance, statistical analyses were performed using a two-way ANOVA. This was then followed by a Tukey's posthoc test to determine significant differences between the exposure conditions at each individual timepoint. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were completed using the software Statistica version 7 (StatSoft, Tulsa, OK).

5.4 Results

5.4.1 Survival of Chinook salmon crosses when challenged with live V. anguillarum or sham injected

When experiencing live *V. anguillarum* challenge, the eight populations analyzed showed high variability in survival (**Figure 5.2A**). The two highest performing crosses were YIAL (63% survival) and Nitinat (60% survival), while the two lowest performing crosses consisted of Big Qualicum (22% survival) and Puntledge (18% survival). The two highest and lowest performing crosses were significantly different from one another (see Appendix A1). From days 0 to 2, a reduction in survival was observed in several of the populations likely due to the handling stress when receiving the i.p. injections. A much more dramatic decrease in survival was observed between day 4 and day 7, this was also the time when symptoms of vibriosis were first observed. Similar to the live infection, the PBS sham injected fish also presented variability in survival (**Figure 5.2B**). The highest performing crosses in the sham exposed individuals were Nitinat (69% survival) and Chilliwack (67% survival) while the lowest performing were Capilano (26% survival) and Quinsam (10% survival). Mortality due to the initial handling stress was also observed in the sham injected controls from days 0 to 2. For significance between the crosses of the shame injected exposure treatment, see Appendix A2.

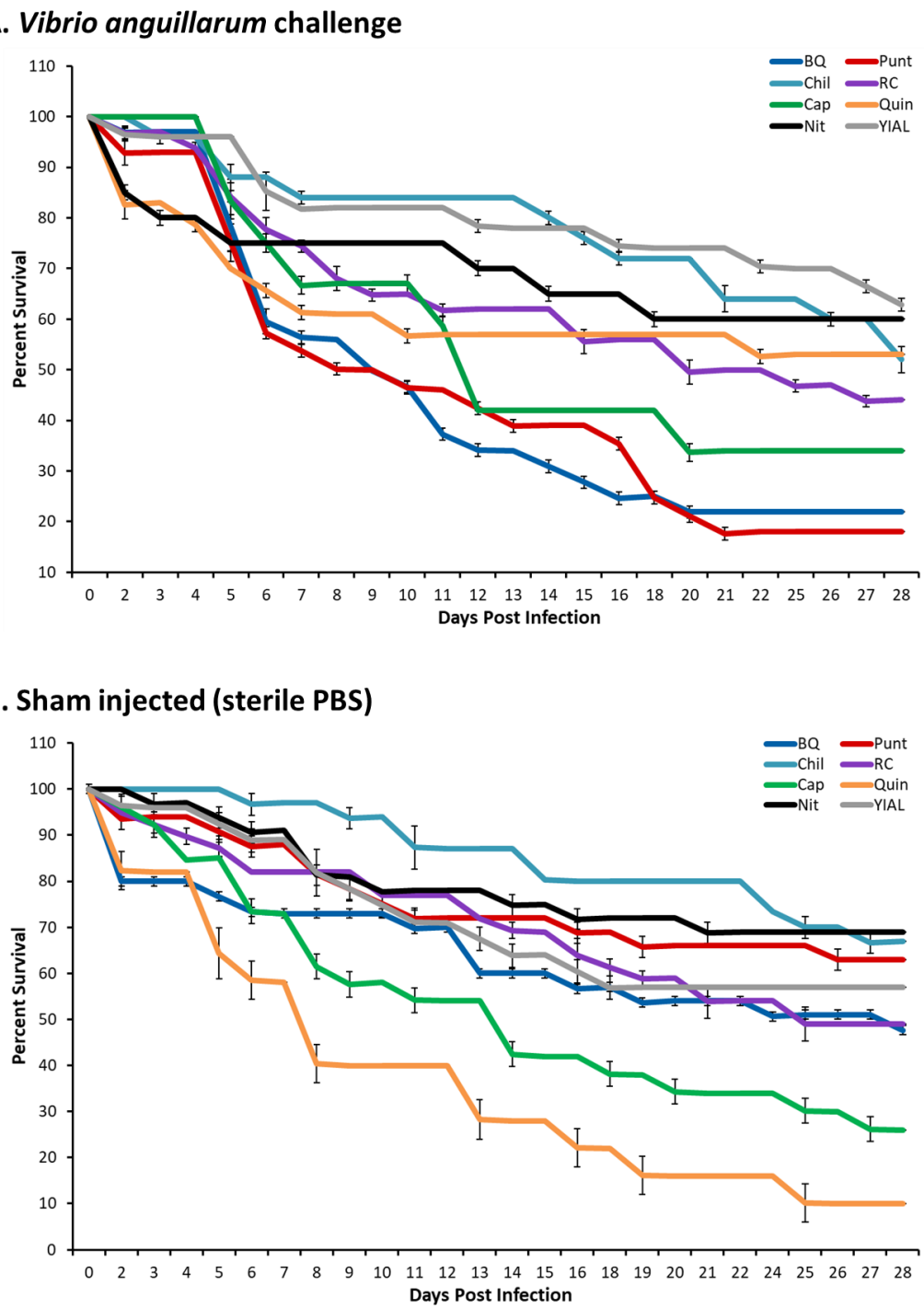

Figure 5.2. Survival curves for the eight outbred Chinook salmon populations during exposure challenges. Individuals within the eight Chinook salmon crosses received either 100 µL of *Vibrio anguillarum* at a concentration of 8.0 x 10^4 CFU/mL (A) or 100 µL of sterile PBS to act as a sham injected control (B). Both exposure conditions were completed in duplicate troughs (2000 L) that housed a mixture of four of the populations in each trough (n = 40 per population). Error bars represent ± SD.

5.4.2 Comparing the heterozygosity between the eight populations

None of the loci had significant deviations from HWE in all eight groups and no pairs of loci had significant linkage disequilibrium. Observed heterozygosity ranged from 53.9% to 63.6% but there were no significant differences between any of the eight populations (Table 5.2; p = 0.8137). Expected heterozygosity was also not significantly different between the groups (Table 5.2, p = 0.9883).

Table 5.2. Level of heterozygosity calculated between the eight Chinook salmon populations based on microsatellite analysis. The values for observed (H_o) and expected (H_e) heterozygosity as well as the number of individuals genotyped (N) are presented.

Group	N	H_o	H_e
Big Qualicum	27	0.636	0.624
Puntledge	28	0.631	0.613
Robertson Creek	24	0.634	0.609
Nitinat	26	0.580	0.561
Quinsam	27	0.602	0.591
Chilliwack	27	0.614	0.600
Capilano	19	0.586	0.584
YIAL	22	0.539	0.566

5.4.3 MH class II β1 genotypes of the outbred Chinook salmon populations

Following MH class II β1 genotyping of 30 individuals per cross, the eight populations did not present large differences in the number of MH class II β1 alleles. All crosses had 2-3 alleles with the exception of the lowest performing cross, Punt, which presented 5 individual alleles (**Figure 5.3A**). When comparing the percentage of fish that were heterozygous for multiple MHC class II β1 alleles, it was observed that the two highest performing populations, YIAL and Nit, also had the highest percentage of heterozygous fish. Meanwhile, the two lowest performing populations, BQ and Punt, had very few fish that were heterozygous for this gene (**Figure 5.3B**). Following the conversion of nucleotide sequence to amino acid sequence, a total of nine individual alleles were identified in the 240 fish that were assessed. When comparing the number of unique alleles observed between the eight different crosses, most of the nine alleles were shared with only Punt, Chil and YIAL presenting unique alleles (**Table 5.3**).

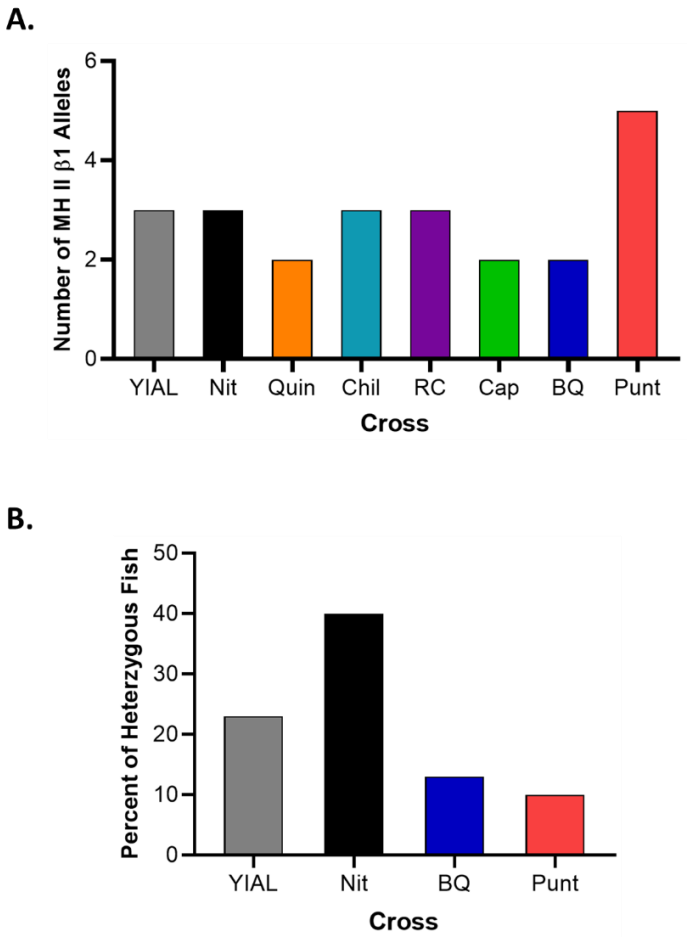

Figure 5.3. Differences in MH class II β1 genotypes between the Chinook salmon crosses. Following the genotyping of 30 individuals from each of the 8 crosses studied, nucleotide sequences were converted to amino acids and the number of alleles observed in each population was reported (A). In the two highest and two lowest performing populations when subjected to live *V. anguillarum* challenge, the percent of individuals that were heterozygous for MH class II β1 genotype is presented (B).

Table 5.3. Record of shared and unique MH class II β1 alleles between the eight Chinook salmon crosses. Following conversion to amino acid sequences, a total of 9 alleles were observed in the 240 fish assessed (n=30 per population).

Population	Onts1	Onts2	Onts7	Onts9	Unique
Big Qualicum	✓	✓			0
Puntledge	✓			✓	3
Capilano	✓	✓			0
Robertson Creek	✓	✓	✓		0
Quinsam	✓			✓	0
Chillawack	✓	✓			1
Nitinat	✓	✓	✓		0
YIAL	✓	✓			1

5.4.4 IgM antibody development to V. anguillarum throughout infection

When observing specific anti-*Vibrio* antibody titres throughout the live exposure challenge, most of the populations had an increase in serum IgM towards *V. anguillarum* over time (**Figure 5.4A**). At the final timepoint of 28 d, only Nit was observed to be significantly different from the outbred Quin and BQ crosses (**Figure 5.4A**). In comparison, when each stock received i.p. injections of sterile PBS, there were no significant differences in serum anti-*V. anguillarum* IgM between any populations at the timepoints analyzed (**Figure 5.4B**).

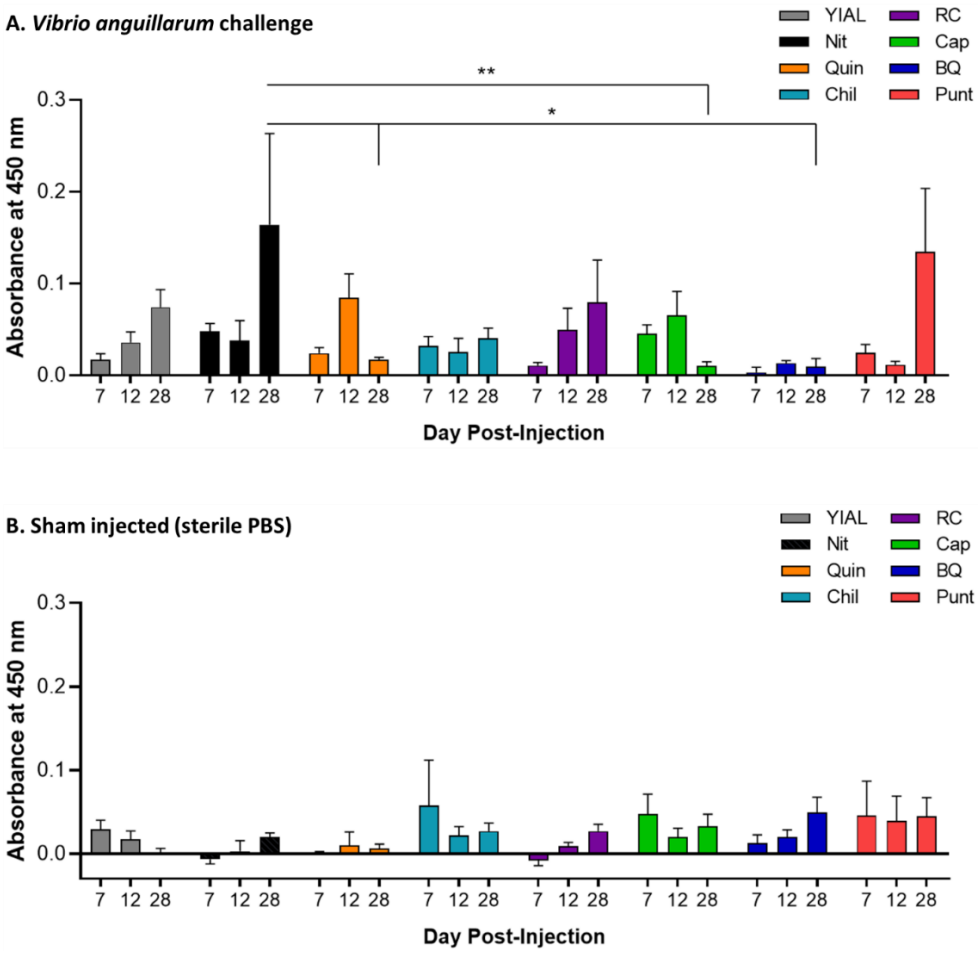

Figure 5.4. Serum IgM developed towards *V. anguillarum* in Chinook salmon crosses throughout the exposure trial. Levels of serum IgM were determined using a subtractive indirect ELISA assay for the Chinook salmon populations when receiving i.p. injections of either live V. anguillarum (A) or sterile PBS which acted as sham injected controls (B). Error bars represent +SEM and each data point is the average of 6 individuals. Significant differences were only reported if they were observed between populations at the same timepoint. A p-value of less than 0.06 is represented by * while a p-value of less than 0.01 is represented by a ** symbol.

5.4.5 Induction of immune transcripts within spleen during V. anguillarum infection

When infected with live *V. anguillarum*, several differences in immune transcript expression of the spleen between the highest and lowest performing Chinook salmon crosses were observed. One of the lowest performing populations, BQ, had an upregulation of the proinflammatory cytokines *IL-1β* and *TNFα* at Day 7 when compared to the three other populations, otherwise the four populations had similar expression patterns in these two genes (**Figure 5.5A-B**). Though there appeared to be a slight increase in *IL-6* expression at Day 7 for all four populations, there were no significant differences observed across any of the timepoints or between crosses (**Figure 5.5C**). Meanwhile the anti-inflammatory cytokine, *IL-10*, did not present significant differences in expression between the populations until 12 d where the lowest performing population, Punt, had significantly higher expression when compared to Nit, one of the populations that presented the highest survival throughout *V. anguillarum* infection (**Figure 5.5C**). When assessing the transcript expression of the NADPH oxidase subunit $p22^{phox}$, only the highest performing population, YIAL, presented an increase in gene expression on Days 1 and 7 when compared to both Nit and Punt (**Figure 5.5D**). YIAL was also the only population to show a significant increase at Day 1 in the other NADPH oxidase subunit, $p40^{phox}$, but this was only when compared to the lowest performing population, Punt. However, by Day 12, Nit showed a significant decrease in $p40^{phox}$ when compared to Punt (**Figure 5.5E**).

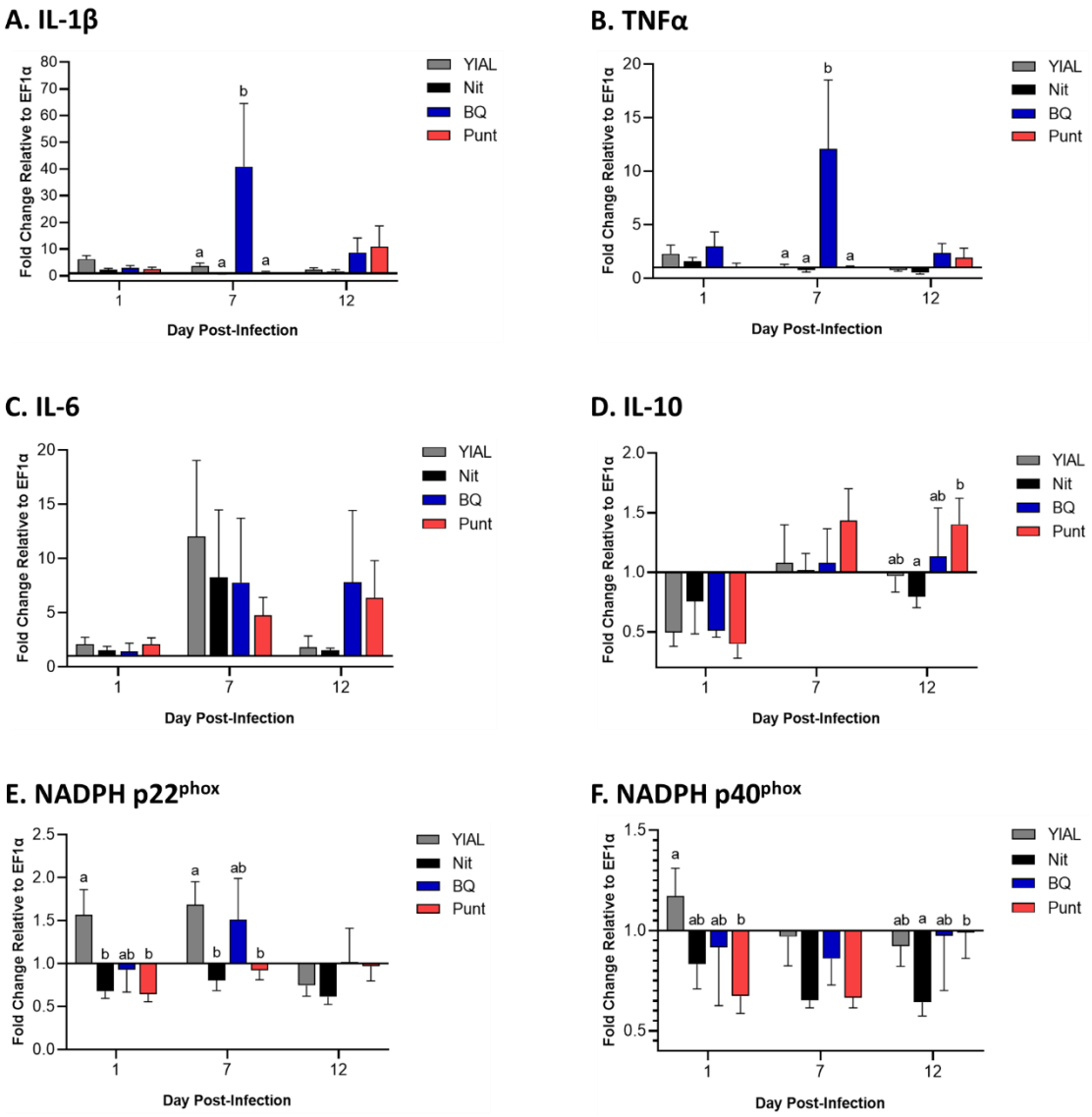

Figure 5.5. Spleen immune transcript expression within the two highest and two lowest performing populations throughout live *V. anguillarum* challenge. Transcript expression of cytokines *IL-1β* (A), *TNFα* (B), *IL-6* (C) and *IL-10* (D) as well as the NADPH oxidase subunits *p22*^{phox} (E) and *p40*^{phox} (F) was assessed via qRT-PCR analysis on Days 1, 7 and 12 following injection with live *V. anguillarum*. Only individuals from the two highest performing crosses (YIAL and Nit) and the two lowest performing crosses (BQ and Punt) was evaluated. All data was normalized to the reference gene (*EF1α*) and expressed as a fold change over the Day 0 control group where control expression was set to 1. Error bars represent +SEM and each data point is the average of 6 individuals. A p-value of less than 0.05 was considered to be statistically significant.

5.4.6 Co-infection with R. salmoninarum in highest and lowest performing crosses

Because *R. salmoninarum* is another common pathogen in Chinook salmon aquaculture, an ELISA-based assay was used to determine whether the highest performing (YIAL) and lowest performing (Punt) crosses had differences in the number of fish that were positive for BKD infection. Punt had the lowest survival following challenge with live *V. anguillarum* and this 85% of the fish tested (n = 20) in this cross were also positive for BKD (**Figure 5.6**). In comparison, YIAL had the highest survival throughout *V. anguillarum* infection and only 55% (n = 20) of the fish from this population were positive for BKD (**Figure 5.6**).

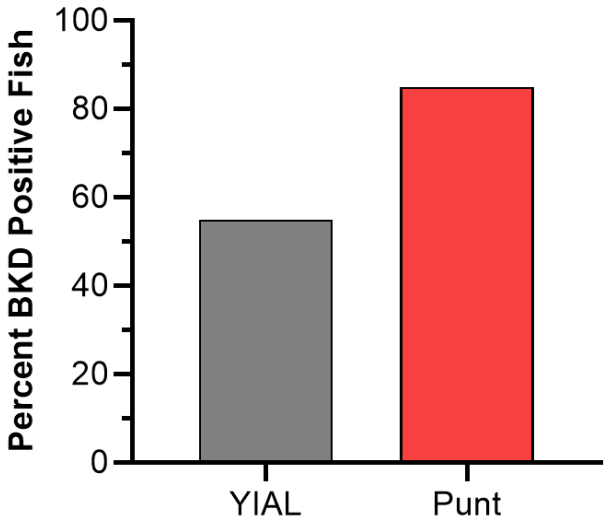

Figure 5.6. Determining co-infection with *R. salmoninarum* in the highest and lowest performing Chinook salmon crosses. Head kidney samples of crosses that presented the highest and lowest survival to *V. anguillarum* infection (YIAL and Punt respectively) were tested for co-infection of *R. salmoninarum*, the causative agent of BKD.

5.5 Discussion

Reintroducing genetic diversity into domesticated or captive populations has been shown to enhance immune performance in several species (Pinard-Van der Laan et al., 1997; Spielman et al., 2004; Smallbone et al., 2016), yet precise improvements for desired immune traits are not guaranteed by this strategy. Thus, even though outbreeding is a common technique for the improvement of inbred animals, there has been limited application of this practise in aquaculture. The primary goal of the current study was to improve immune performance of the domesticated YIAL Chinook salmon stock by outbreeding and identify potential genetic or functional immune markers that could predict any resulting immune differences. Given that the YIAL production stock has been bred for over 30 years without outside genetic input, it was our prediction that some outbred crosses would maintain the desired phenotypes of domestication while also exhibiting improved responses to disease challenge indicative of hybrid vigor.

When challenged with live *V. anguillarum*, there was variable survival across all eight groups studied with the pure domesticated cross (YIAL) presenting the highest survival. Chinook salmon production at YIAL follows organic standards and consequently, through the avoidance of antibiotic use, has selected for disease resistance over multiple generations. It was anticipated that the YIAL stock would contribute valuable traits for disease resistance, however, it was unknown whether the introduction of wild genes would further enhance or inhibit this. The results presented here reveal that only the Punt and BQ outbred crosses were significantly more susceptible to *V. anguillarum* infection when compared to YIAL or Nit. Differences in susceptibility to vibriosis have been observed in BC Pacific salmon populations previously (Beacham & Evelyn, 1991; Beacham & Evelyn, 1992a; Balfry et al., 2001; Balfry et al., 2008). One study by Beacham & Evelyn (1991) showed significant variation in mortality between three populations of Chinook salmon originating from the Kitimat, Nitinat and Quinsam rivers of BC after receiving i.p. injection of live *V. anguillarum*. Surprisingly, this study reported that Nitinat mortality was more than double that of Quinsam and Kitimat (Beacham & Evelyn, 1991). This is contrary to the results presented here wherein the outbred Nitinat was one of the highest

surviving crosses when challenged with *V. anguillarum*. This contradictory result for Nitinat could reflect that a high proportion of this population died when transferred to saltwater (Semeniuk et al., 2019). As a result, the outbred Nitinat fish used in this study may have actually represented the most resilient of that cross thus providing an explanation for the unexpected high performance. Overall, the variability in survival to *V. anguillarum* challenge that was observed between the eight crosses could reflect interesting immune differences for discovery.

Though the focus of this study was to compare differences in immune performance throughout infection challenge, it was observed that even when injected with sterile PBS there was disparity in survival among the eight crosses. When comparing the effect of handling stress between wild and hatchery-reared Chinook salmon, Mazur & Iwama (1993) found that hatchery-reared salmon had lower plasma cortisol levels and lower hematocrits when compared to their wild counterparts. Similar trends were shown in other salmonids such as sea trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorynchus mykiss*, Woodward & Strange, 1987; Johnsson et al., 2001; Lepage et al., 2001) indicating that domesticated stocks may be better able to cope with and overcome stressful situations. Despite these observations, the highly domesticated YIAL stock presented lower survival in the sham injected controls than three of the outbred populations. Though YIAL was only significantly different from one outbred cross, it was anticipated that the more domesticated group would outperform the other crosses as these fish spent the longest duration in captivity. Because the outbred populations would maintain some traits from the captive YIAL population parent, it is possible that this unexpected result was an example of heterosis.

When the host encounters bacterial pathogens, like *V. anguillarum*, the major histocompatibility (MH) class II molecules are responsible for the recognition and presentation of antigens to helper T lymphocytes. In doing so, this class of MH directs the immune response to effectively combat particular extracellular threats (reviewed by Grimholt, 2016). The binding of pathogen derived antigens to MH molecules is governed by allelic variation at the peptide binding groove. There are many such alleles and every individual within a species has a limited repertoire. The genetic polymorphism at the MH

loci can provide more or less protection towards relevant pathogens and thus these genes are believed to be under strong selection pressure (reviewed by Yamaguchi & Dijkstra, 2019). Despite this, the results of the current study revealed that outbreeding a domesticated stock with seven wild populations did not increase the allelic diversity of MH class II β1 genotypes. Across all eight groups, only 9 alleles were detected in the 240 individuals assessed. This level of allelic variation is similar to what has been reported previously for wild Chinook salmon populations (Kim et al., 1999; Evans et al., 2010; Lehnert et al., 2016). Interestingly, the lowest performing cross, Punt, presented the greatest number of unique alleles. This is contradictory to the belief that more allelic diversity in MH genotypes will better protect a population as a whole (reviewed by Bernatchez & Landry, 2003; Spurgin & Richardson, 2010), but this could be pathogen dependent. It was also observed that a greater percentage of individuals were heterozygous for MH class II β1 genotype in the two highest performing crosses when compared to the two lowest performing crosses. This may support the notion that heterozygotes are able to detect a broader range of pathogen-driven antigens because they have greater variability in their MH molecules, a form of balancing selection referred to as heterozygote advantage (reviewed by Sommer, 2005; Spurgin & Richardson, 2010). Indeed, heterozygosity in MH genotype has been associated with better survival in Chinook salmon when challenged with infectious hematopoietic necrosis virus (IHNV, Arkush et al., 2001) and *V. anguillarum* (Becker et al., 2014) as well as heterozygous fry exhibiting fewer bacterial infections overall when compared to their homozygous counterparts (Evans & Neff, 2009). As of yet, specific alleles at the MH class II loci have not been associated with resistance or susceptibility to individual pathogens in salmonids. This was also observed here wherein individual MHC class II alleles could not be attributed to the variable survival to *V. anguillarum* infection, but further support for heterozygote advantage hypothesis in Chinook salmon was observed.

Because vibriosis is a bacterial septicaemia, host development of pathogen-specific antibodies should be crucial for limiting the spread of infection. To date, teleosts are known to have three classes of antibody, with IgM being the dominant class within the blood (reviewed by Fillatreau et al., 2013; Sunyer, 2013). In the current study, a subtractive indirect ELISA assay was developed so that serum

IgM levels could be compared between the seven outbred crosses and the domesticated control group. When serum IgM was measured throughout *V. anguillarum* challenge, only the outbred Nit group was observed to have significantly higher levels of serum IgM when compared to outbred Quin, Cap and BQ at Day 28. Given that survival between these four outbred crosses was variable, it does not seem that higher levels of serum IgM conferred protection from this isolate of *V. anguillarum.* Serum IgM levels in salmonids have been studied previously in response to bacterial pathogens using ELISA-based assays (Boesen et al., 1997; Johnson et al., 2004; Becker et al., 2014; Semple et al., 2018a). Though significant differences have been observed previously in Chinook salmon serum IgM (Becker et al., 2014), but quite often the large variability between individuals masks any obvious trends between groups. With regard to the results presented here, it does not appear that serum IgM levels corresponded to or could predict survival with regards to *V. anguillarum*, despite some significant differences being observed.

The teleost spleen is responsible for filtering blood and initiating immune reactions to blood-borne antigens/threats (reviewed by Secombes & Wang, 2012). Consequently, this organ can be a major reservoir for extracellular pathogens that replicate in the blood, such as *V. anguillarum*. As a result, transcript expression of the spleen may reflect the immune status of infected fish. When spleen tissues from the two highest and two lowest performing crosses were assessed for gene expression of pro-inflammatory cytokines, the only significant difference was observed in the low performing BQ population which had a significant upregulation in *IL-1β* and *TNFα* at 7 d. Additionally, though this was not significant, it does appear that the lower surviving crosses had an enhanced pro-inflammatory response in all three genes at Day 12. Given this trend, it is possible that the higher inflammatory response observed in the lower performing crosses depleted energy resources, making these fish more susceptible to vibriosis. As has been shown with septic shock in mammals, a greater cytokine response is not always beneficial for the host (Waage et al., 1989; Pinsky et al., 1993; Li et al., 1995). Findings like these are absent from teleost literature but there has been a large body of research devoted to understanding the antagonistic mechanisms controlling inflammation in fish (reviewed by Rebl &

Goldammer, 2018). These studies provide evidence that inflammatory responses in teleosts also must be tightly regulated. Thus, it is reasonable to suggest that the cytokine dysregulation observed in the present trial could be related to poor survival during bacterial infection. Additionally, IL-10 has been shown to protect both mice and humans from lethal sepsis (Howard et al., 1993; Li et al., 2017). Yet the results presented here show that higher *IL-10* expression in Punt did not protect this cross as it was the lowest performing in the *V. anguillarum* challenge condition. It seems that when it comes to cytokine levels for Chinook salmon infected with *V. anguillarum*, a lower response may provide greater protection.

The respiratory burst activity of phagocytes is an essential immunological response to eliminate bacterial pathogens in all vertebrate species. Following ingestion, phagocytic leukocytes can kill most bacteria by producing reactive oxygen intermediates (ROI). This process requires NADPH oxidase (NOX), which catalyzes the conversion of molecular oxygen into superoxide anions (reviewed by Panday et al., 2015; Nguyen et al., 2017). Previous work with sea bass (*Dicentrarchus labrax* L.) has shown that *V. anguillarum* inhibits the respiratory burst of head kidney leukocytes when measured by a functional chemiluminescent assay (Sepulcre et al., 2007b). Sepulcre and colleagues (2007b) were then able to link this functional decrease in RBA to downregulation in gene expression of NOX subunits, $p22^{phox}$ and $p40^{phox}$, during live *V. anguillarum* infection. In our study, transcript expression of $p22^{phox}$ and $p40^{phox}$ was analyzed and compared between the two highest and two lowest performing crosses. YIAL was observed to have significantly higher expression of these genes at earlier timepoints when compared to the outbred groups. Decreases in circulating leukocytes has been reported previously in rainbow trout with vibriosis (Lamas et al., 1994). Thus, reduced expression of NOX genes could be a sign of immune cells succumbing to the associated virulence factors. Because Chinook salmon from the YIAL cross presented the greatest survival to *V. anguillarum* challenge and this group has undergone over 30 years of selection without antibiotics, it is possible that these animals are able to mount a more effective and rapid ROI response. If this was the case, it may help explain the greater survival of YIAL when challenged with such a fast-acting pathogen.

The results of this study thus far have focused on linking the immune response of Chinook salmon crosses to their survival throughout *V. anguillarum* infection. However, when the animals are grown in sea-pens, *V. anguillarum* is not the only microorganism that they will come into contact with. Another common pathogen in salmon culture is *R. salmoninarum*, the causative agent of bacterial kidney disease (BKD). Because *R. salmoninarum* is an intracellular pathogen (Gutenberger et al., 1997), the requirements of the immune system to clear and/or inhibit disease presentation are different than for that of the extracellular *V. anguillarum*. This means that if the animals were already dealing with *R. salmoninarum* infection, they may have had less energetic resources remaining to combat the live *V. anguillarum* administered in this study. Indeed, it was observed in the present study that the highest performing group (YIAL) had a much lower percentage of fish positive for BKD when compared to the lowest surviving cross (Punt). This would not be the first time that co-infection has been seen to inhibit the survival of salmonid species to infection (Johansen et al., 2009; Roon et al., 2015; Figueroa et al., 2017) and has been studied extensively in fish (reviewed by Kotob et al., 2016). When Chinook salmon are infected with the parasite, *Nanophyetus salmincola*, significantly greater susceptibility to *V. anguillarum* has been observed (Jacobson et al., 2003). Given the consistently negative impact of co-infection on survival in fish, it was not surprising that high co-infection coincided with reduced performance in the current study.

5.6 Conclusions

In this study, a domesticated stock of Chinook salmon was crossed with seven wild population so that immunological performance could be assessed when challenged with the marine pathogen *V. anguillarum*. Significant differences in survival were observed with the domesticated YIAL population and the outbred Nitinat group having the highest survival while the lowest performance was observed in the outbred Puntledge and Big Qualicam crosses. Specific MH class II β1 alleles were not found to predict survival to *V. anguillarum* infection but crosses that had a higher percentage of fish presenting a heterozygous genotype had greater survival. When assessing serum IgM levels between the crosses, only Nitinat showed a significant increase when compared to Quinsam, Big Qualicum and Capilano,

thus this did not seem to coincide with the observed survival. Notable differences were observed in spleen transcript levels between the two highest and two lowest performing where a stronger cytokine response predicted lower survival to *V. anguillarum* infection, possibly due to depletion of energetic resources. Meanwhile, upregulation of RBA genes at earlier timepoints was observed in the highest performing cross, YIAL. Lastly, it was observed that the lowest performing cross had a higher percentage of fish infected with *R. salmoninarum* when compared to the highest surviving cross. Thus, co-infection may have impacted the observed survival to *V. anguillarum* exposure. This study represents the first of its kind to outbreed a domesticated stock with wild populations of Chinook salmon and compare the immune function of resulting offspring. Though clear immune markers for resistance/susceptibility to vibriosis were not determined, there were obvious differences between the crosses regarding their immune function. However, the inbred, domesticated stock had the highest performance overall. As a result, it appears possible that Chinook salmon stocks could be enhanced for commercial culture, but outbreeding may not be the method of choice.

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Chapter 6: Development and characterization of a Chinook salmon spleen stromal cell line to study the cellular immune response to *Vibrio anguillarum*.

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6.1 Overview

Despite increased interest in Chinook salmon aquaculture, there is inadequate understanding regarding the bacterial immune defenses of this species. This study describes the establishment and characterization of a continuous stromal cell line derived from Chinook salmon spleen, CHST, and its response to heat-killed bacterial exposure challenge. Optimal growth of CHST was seen at 18°C when grown in Leibovitz's L15 media supplemented with 20% fetal bovine serum. DNA analyses confirmed that CHST did originate from Chinook salmon tissue. The bacterial species used for exposure studies was *Vibrio anguillarum*, a common pathogen of marine aquaculture and the causative agent of an acute hemorrhagic septicaemia known as vibriosis. *V. anguillarum* was observed to grow rapidly in cell culture media and is known to produce a plethora of extracellular toxins, thus live bacterial challenge was not used for this study. Instead, *V. anguillarum* was heat-killed and CHST was challenged so that pro-inflammatory (IL-1β, IL-6 and TNFα) as well as anti-inflammatory (IL-10) cytokine transcript levels could be measured at 1, 4, 12, 24 and 96 hr post-exposure. The heat-killed bacteria was observed to significantly stimulate the expression of all three pro-inflammatory cytokines at 4, 12, 24 and 96 hr post-exposure with the peak in expression occurring at 12 and 24 hr. Meanwhile, *IL-10* was not observed to significantly increase until 96 hr post-stimulation, which was also the time when the inflammatory cytokine expression was decreasing. The establishment and characterization of CHST provides a valuable model for studying the immune response of Chinook salmon stromal cells in response to stimuli, including those of bacterial origin.

6.2 Introduction

Due to their large size and high market value, Chinook salmon (*Oncorhynchus tshawytcha*) have historically been a focus for capture fishery production on the West coast of Canada (Christensen et al., 2018, Ohlberger et al., 2018). This is rapidly changing though, because declining numbers in native populations have resulted in the inability of fisheries to meet the rising demands for Chinook salmon consumption (Johnson et al., 2017). Additionally, since the majority of salmon farmed in British Columbia are Atlantic (Statistics Canada, 2017), there is growing apprehension that escapees and the introduction of novel diseases could be implicated in the diminishing numbers of wild Chinook salmon (reviewed by Noakes et al., 2000; Naylor et al., 2003; Ayer & Tyedmers, 2009). All of these concerns have resulted in increased public awareness promoting the culture of Chinook salmon on the West coast to hopefully ease some of the pressure experienced by wild populations. Unfortunately, because this species is not as well domesticated as their Atlantic counterparts, there are many gaps in knowledge regarding their efficient culture which makes this endeavor difficult. Obtaining and applying a deeper understanding Chinook salmon immunity is one way to help make this species more robust in intensive culture situations.

Mortality due to infectious disease translates into one of the greatest sources of financial loss for global aquaculture (World Bank, 2014; reviewed by Stentiford et al., 2017). With countless bacterial species inhabiting aquatic environments, some are able to take advantage of host stress and/or immune suppression to become opportunistic pathogens (Sundberg et al., 2016). Though there are numerous bacterial infectious agents in marine environments (reviewed by Toranzo et al., 2005), *Vibrio anguillarum* is one responsible for significant financial losses to salmon aquaculture (reviewed by Frans et al., 2011; Hickey & Lee, 2018). This gram negative bacterium is the causative agent of vibriosis, a fatal hemorrhagic septicaemia capable of infecting over 90 different aquatic species (reviewed by Hickey & Lee, 2018). Acute outbreaks of the disease can be particularly problematic as these can result in high mortalities without the presentation of any clinical signs (reviewed by Frans et al., 2011). Despite the presence of several commercial vaccines (reviewed by Hickey & Lee, 2018), vibriosis is

still a prevalent disease in salmon aquaculture, outlining that there is more to learn of this pathogen and what constitutes an effective immune response towards it.

As a bacterial septicaemia, *V. anguillarum* can be found throughout the blood of infected individuals, making the spleen an important site for immune defenses. Like mammals, the spleen of teleosts is an important haematopoietic organ that can be separated into ellipsoids, red pulp and white pulp (reviewed by Fange & Nilsson, 1985; Espenes et al., 1995; Genten et al., 2009). However, fish also have the additional element of melanomacrophage centres (MMCs) which are believed to be primitive analogues of mammalian germinal centres (GCs, reviewed by Stosik et al., 2019). The red pulp is a macrophage and erythrocyte dense region that plays an important role in iron recycling and surveillance of blood-borne pathogens. In comparison the white pulp is a lymphocyte-rich area that supports antigen specific immune responses (reviewed by Klei et al., 2017; Golub et al., 2018). As observed in all tissues, the functional cells of the spleen are supported by an intricate network of stromal cells. More specifically, the immune cells within the spleen require the signals that are produced by stromal cells in response to their surroundings. As a result, splenic stromal cells provide a crucial microenvironment to support haematopoiesis along with the proliferation and development of immune cells (Wilson et al., 2000; O'Neill et al., 2011). Thus, splenic stromal cells enable the establishment and continuation of effective immune responses, particularly when dealing with a bacterial septicaemia like *V. anguillarum*.

The present study describes the establishment and characterization of CHST, a stromal cell line derived from the spleen of an adult Chinook salmon. The species of origin for CHST was confirmed to be Chinook salmon and optimal growth conditions were determined. Additionally, various animal cell culture media formulations were assessed to determine if they could support the growth of *V. anguillarum* for potential live challenges *in vitro*. Following a 96 hr exposure challenge to heat-killed *V. anguillarum*, significant induction of CHST pro-inflammatory (*IL-1β*, *IL-6*, *TNFα*) and anti-inflammatory (*IL-10*) cytokine expression was observed. As CHST is the only known stromal cell line

created from Chinook salmon, these cells are very relevant for understanding species specific aspects of immunity and cellular function.

6.3 Materials and Methods

6.3.1 Primary cultures

In September of 2016, a 200 g Chinook salmon was retrieved from a net-pen that was produced and maintained at Yellow Island Aquaculture Ltd (YIAL, Quadra Island, BC, Canada). The fish was euthanized with an overdose of clove oil prior to tissue collection. All procedures were performed following the guidelines of the Animal Care Committee at the University of Waterloo. The spleen, caudal fin and gills were collected and stored in L15 media supplemented with 20% fetal bovine serum (FBS, Gibco), 200 U/mL of penicillin and 200 U/mL streptomycin (Thermo Scientific). Upon arrival to the University of Waterloo, each tissue type was diced into small pieces in a laminar flow hood and rinsed three times with buffered saline solution (PBS, Lonza) containing the same antibiotics as noted above. Tissue pieces were then transferred to 25 cm^2 flasks (BD Falcon) where approximately 1–2 mL of L15 supplemented with 20% FBS and the previously described antibiotic concentrations. Primary cultures were established by the explant outgrowth method as previously demonstrated with walleye spleens and fins (Vo et al., 2015, Vo et al., 2016). Flasks were then incubated at 18°C with media changes occurring every 2–3 days. It was eventually observed that some flasks were developing cell populations that were hematopoietic in nature. The media supplementation to these flasks was changed to L15 with 30% FBS to promote these cell populations. In an effort to isolate the immune cells, only the floating cells were transferred to new cell culture flasks. Eventually the floating cells adhered to the flask and took on a fibroblastic-like morphology. These fibroblastic-like cells would eventually develop into the CHST cell line.

6.3.2 Maintenance of CHST

Initially, CHST was routinely grown in L15 supplemented with 30% FBS at 18°C to maintain its hematopoietic cell populations. Following the first passage, the adherent CHST cells were subcultured at a 1:2 ratio on a weekly or bi-weekly basis using trypsin (Lonza) and maintained in

L15/20% FBS. CHST has been maintained for over three years and has undergone more than 30 passages.

6.3.3 Cryopreservation of CHST

Approximately 4 x 10^6 CHST cells at multiple passages were cryogenically frozen in L15 containing 20% FBS and 10% dimethyl sulphoxide (DMSO, Sigma). The cells were frozen at -60°C overnight and subsequently immersed in liquid nitrogen (-196°C) for long-term storage. To determine cell viability upon thawing, a trypan blue (Sigma) exclusion assay was performed using a haemocytometer under a phase contrast microscope (Leica).

6.3.4 Optimal growth conditions of CHST

Optimal temperature of growth for CHST was analyzed between 4 and 26°C. CHST cells were seeded into five 6-well plates (Fisher Scientific) at a concentration of 2×10^5 cells/well and incubated overnight at 18°C. Three wells of one plate were used to provide the day 0 cell counts. Two 6-well plates were incubated at each of the four temperatures studied (4, 14, 18 and 26°C) for 6 days. On days 0, 3 and 6, triplicate wells from each temperature were washed with 1 mL of PBS and cells were dissociated using 300 μL of trypsin (Gibco). Cell counts for each well were determined using a hemocytometer under a phase contrast microscope (Leica). Cell counts for each time interval were averaged and calculated as a percentage of the day 0 growth to determine the percent growth.

To determine the optimal FBS concentration for maintenance of CHST, five 6-well plates were seeded with cells as described above. Following overnight adherence at 18°C, media was removed from all plates and one 6-well plate received 2 mL of either 5% FBS, 10% FBS, 15% FBS or 20% FBS media. All plates were then returned to the 18°C incubator. On days 0, 3 and 6, triplicate wells from each FBS concentration were washed with 1 mL of PBS so that cells could be dissociated and counted as described above.

6.3.5 Confirming species of origin

Genomic DNA was extracted from CHST cell pellets using the DNeasy Blood and Tissue Kit (Qiagen) as described by the manufacturer. To validate the origin of the CHST cells, primers previously shown to amplify a segment of the Chinook salmon mitochondrial cytochrome c oxidase subunit (cox1, **Table 6.1**) were used as described previously by Semple et al (2018d). The PCR product was then run on a 1.5% agarose gel with 1X GelGreen (Biotium). Bands of the desired size were cut out of the gel and extracted using the QIAquick Gel extraction kit (Qiagen). The resultant fragments were cloned into pGEM®-T Easy plasmids (Promega Corporation) and transformed as previously described by Semple et al. (2018a). Five colonies per PCR reaction were selected and grown in LB medium. Plasmids were extracted from the transformants using the GenElute Plasmid Miniprep Kit (Millipore Sigma) according to the manufacturer's instructions. Plasmids were sequenced using SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, Ont.). The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.3.6 V. anguillarum growth in bacterial and animal cell media

Frozen stocks of the *V. anguillarum* were subcultured onto tryptic soy agar (TSA) with 2% NaCl. Following incubation at room temperature (RT) for 48 h, a single colony was used to inoculate 3 mL of tryptic soy broth (TSB) supplemented with 2% NaCl and incubated for 10 h at RT and 200 rpm. After this incubation, 1 mL of the bacterial culture was centrifuged (8 min at 8000 rpm) and was washed twice with 1 mL of PBS. The bacterial pellet was then resuspended in 1 mL of PBS.

To determine the growth of *V. anguillarum* in its preferred culture media (TSB with 2% NaCl) as well as in animal culture media formulations (L15 with no FBS, 2% FBS, 10% FBS or 20% FBS), 1 µL of the bacterial growth was added to 1 mL of the different media types. In a sterile, 96-well BioLite plate (ThermoFisher), 100 µL of each inoculum was added to 6 wells of the plate. For each media type, four wells received 100 µL of the media alone to act as blanks. All perimeter wells received 300 µL of sterile water to prevent evaporation of experimental wells. Once all wells were prepared, the absorbance

at 600 nm was read for each well every hour for 25 hr using a Synergy H1 plate reader (BioTek instruments). For every absorbance reading, the blank culture absorbance value was subtracted from the six bacterial growth wells for that respective media type.

6.3.7 Preparation of heat-killed V. anguillarum

Three milliliters of *V. anguillarum* bacterial culture was prepared as described above in section 6.3.6. The resulting culture had an optical density of 0.9 which was confirmed to be 1.08×10^{10} CFU/mL by a standard plate count (SPC). Specifically, bacterial numbers were enumerated by plating 0.1 mL of ten-fold serial dilutions on TSA with 2% NaCl plates in triplicate. After incubation at RT for 30 hours, plates containing 25-250 colonies were counted and the number of CFU/mL calculated. One 1 mL aliquot was removed from the *V. anguillarum* growth and centrifuged for 8 min at 8000 rpm. The resulting cell pellet was then washed twice with 1 mL of PBS before being resuspended in 1 mL of PBS. The *V. anguillarum* culture was then boiled for 25 min to kill any live bacteria. After boiling, 100 µL of the heat-killed *V. anguillarum* was spread onto a TSA with 2% NaCl plate to confirm the absence of viable bacteria. Subsequently, the killed bacterial culture was centrifuged for 8 min at 8000 rpm and resuspended in 900 µL of L15 supplemented with 20% FBS which was to be used to expose CHST to the heat-killed bacterium.

6.3.8 Exposure of CHST to heat-killed V. anguillarum

In 6-well tissue culture plates (ThermoFisher), CHST was seeded at 4×10^5 cells/well and maintained overnight at 18°C. The cells were then exposed to either heat-killed *V. anguillarum* at an MOI of 50, or media alone to act as unstimulated controls. Following this single exposure, all experimental plates were returned to the 18°C incubator. At 1 hr, 4 hr, 12 hr, 24 hr and 96 hr following stimulation, CHST cells were mechanically dislodged using sterile 23 cm cell scrapers (ThermoFisher) and transferred to a sterile, 15 mL conical tube (Falcon). The cells were centrifuged (5 min, 500 x g, 4°C), washed once with 5 mL of PBS, and resulting cell pellets were stored at -80°C.

6.3.9 qRT-PCR

6.3.9.1 RNA extraction and cDNA synthesis

RNA was extracted from CHST cell pellets (4 x 10^5 cells) using an RNeasy RNA Extraction Kit (Qiagen) as described by the manufacturer. To remove any contaminating genomic DNA, all RNA samples were treated with DNase I (Thermo Scientific). RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at -80ºC until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) in accordance to the manufacturer's instructions. For a no template control, 500 ng of RNA suspended in 20 uL of DEPC water was included in the cDNA synthesis reaction without reverse transcriptase

6.3.9.2 qRT-PCR reactions

To assess transcript levels of *IL-1β*, *TNFα*, *IL-6*, and *IL-10* in CHST cells, qRT-PCR analysis was completed. All PCR reactions were 10 µl and contained: 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water), 2x WISENT ADVANCED™ qRT-PCR mastermix (Wisent), and forward and reverse primers (Sigma Aldrich) to a final working concentration of 0.25 µM. All qPCR reactions were completed on the LightCycler® 480 II (Roche). The sequences for all primer sets are outlined in **Table 6.1**. Each experimental sample was run in triplicate. For each plate, triplicate wells of a calibrator, no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec and extension at 72°C for 8 sec. A melting curve was completed for every run from 65°C to 97°C with a read every 5 sec. Product specificity was determined through single PCR melting peaks. All qRT-PCR data was analyzed using the ΔΔCt method while incorporating individual primer efficiencies into the equation as described by Pfaffl (2001) and is presented as the average of 3 experimental replicates with the standard deviation. Specifically, gene expression was

normalized to the reference gene (EF1α) and expressed as fold change over the day 0 control group where control expression was set to 1.

Table 6.1. Primer sequences used to amplify Chinook salmon genes. Forward (F) and reverse (R) primer sequences for the genes analyzed are presented along with the base pair length for each amplicon and the original references or Genbank accession numbers for primer sets.

Primer Name	Sequence (5' – 3')	Length	Reference or Accession No.
Cox1	**F:** CCTCAGTTGATCTGACGA **R:** CACGAGTGTCAACGTCTA	481	Semple et al, 2018d
IL-1β	**F:** CCACAAAGTGCATTTGAAC **R:** GCAACCTCCTCTAGGTGC	155	XM_024418276.1
TNFα	**F:** GTGCAAAAGATACCCACC **R:** CACTGCACGGTGTCAG	108	XM_024396390.1
IL-6	**F:** CTTCTACACGCTATCTCTCACTC **R:** CGTCTGTCCCGAGCT	128	XM_024404411.1
IL-10	**F:** GCCTTCTCCACCATCAGAGAC **R:** GATGCTGTCCATAGCGTGAC	120	XM_024405117.1
EF1α	**F:** CGCACAGTAACACCGAAACTAATTAAGC **R:** GCCTCCGCACTTGTAGATCAGATG	134	XM_024396038.1

6.3.10 Statistical analyses

After confirming a normal distribution and homogeneity of variance, statistical analyses were performed using a two-way ANOVA. This was then followed by a Tukey's posthoc test to determine significant differences between the exposure conditions at each individual timepoint. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were completed using the software Statistica version 7 (StatSoft, Tulsa, OK).

6.4 Results

6.4.1 Development of the CHST cell line

Primary cultures of Chinook salmon spleen were initiated by the explant outgrowth method. Initially, the primary cells migrated out and away from the spleen tissue explants to form islands of cells surrounding the tissue. Over time, these cells became hematopoietic in morphology and were able to produce a large number of floating immune cells (**Figure 1A**). In an attempt to propagate the immune cell population, only the non-adherent cells were transferred to new flasks. Surprisingly, some of these floating cells became adherent and presented a more fibroblastic-like morphology as they grew (**Figure**

1B). Earlier passages of these adherent cells were grown using L15/20% FBS and were a mixed cell population consisting of both epithelial-like and fibroblastic-like cells (**Figure 1C**). However, subsequent passages of the cells resulted in the loss of the fibroblast-like morphology so that the resulting CHST cell cultures became predominantly epithelial-like (**Figure 1D**). CHST was also able to withstand cryogenic storage presenting on average 83% viability upon thawing (data not shown).

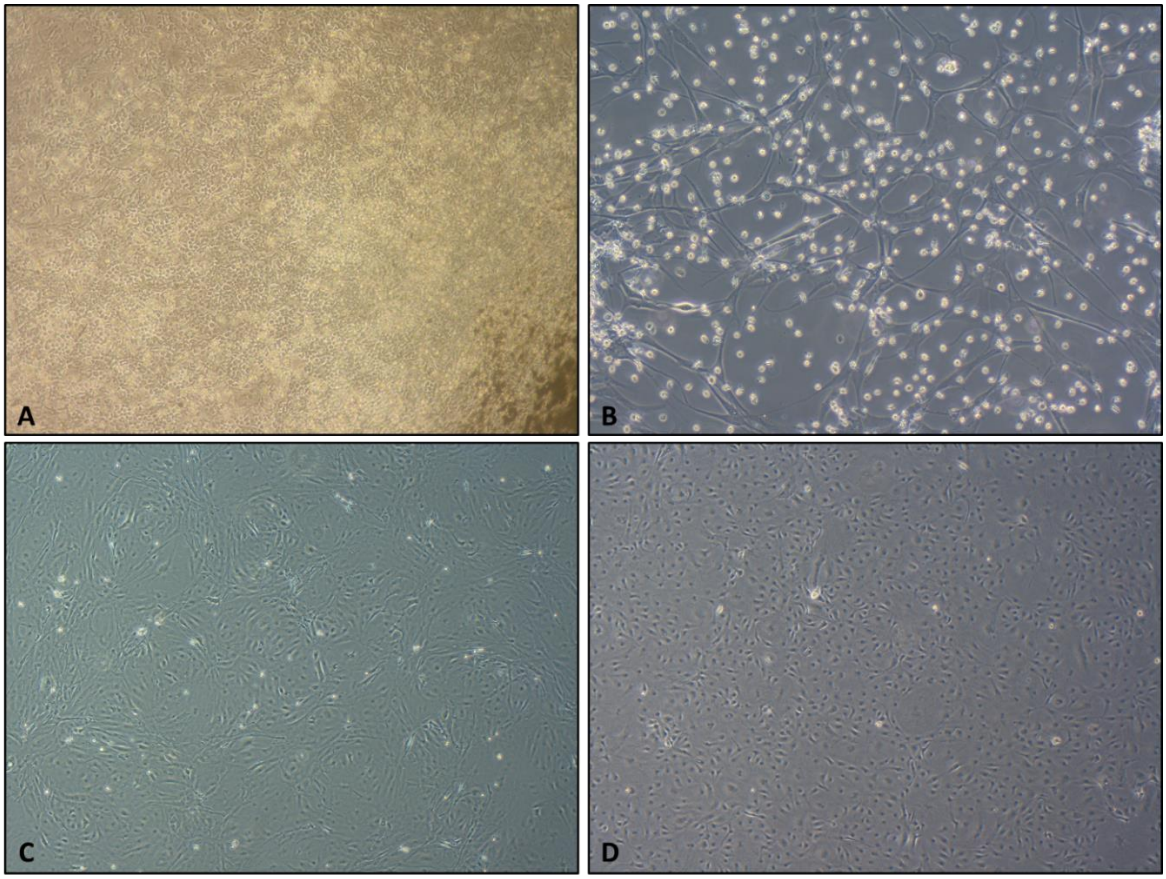

Figure 6.1. Development of the Chinook salmon spleen stromal cell line, CHST. (A-100x) Initial 3 month tissue explant outgrowth from the Chinook salmon spleen when supplemented with L15/30% FBS. The preliminary cell populations were hematopoietic in nature with many non-adherent immune cells. (B-400x) When attempting to passage only the non-adherent cells, some became adherent and continued to proliferate presenting a fibroblastic-like morphology. Meanwhile, the floating cells were not able to propagate when removed from the initial hematopoietic cell populations. (C-100x) In earlier passages (passage 3), both fibroblastic-like and epithelial-like cell morphologies could be observed in the cultures that would eventually become CHST. (D-100x) Upon subsequent passages (passage 16), the epithelial-like morphology became predominant.

6.4.2 Confirming the species of origin of CHST

Confirming the species identity of CHST was accomplished through the amplification of the cox1 gene. Using BLAST, the sequence obtained from CHST were searched and found to have the highest identity and query cover with Chinook salmon sequences (**Table 6.2**).

Table 6.2. Validating the species identity of CHST through cox1 barcoding. The cox1 gene was amplified and sequenced to confirm the species of origin for CHST. The four known sequences that had the highest similarity to the CHST cox1 sequence are presented.

Gene	Length (bp)	% Identity to Chinook salmon Sequences
Cytochrome C Oxidase Subunit 1 (Cox1)	478	100%, JX960927.1 99.79%, HQ167683.1 99.79%, AF392054.1 99.58%, KX958414.1

6.4.3 Determining optimal growth conditions for CHST

To assess optimal growth conditions of the newly established CHST, effect of temperature and FBS supplementation on growth was explored. Over a six day period, CHST displayed the highest growth rate at 18°C with significantly less growth being observed at 4°C, 14°C and 18°C (**Figure 6.2A**). When characterizing media supplementation, CHST displayed the greatest proliferation in L15 media supplemented with 20% FBS at 18°C (**Figure 6.2B**). As there was no significant difference in cellular growth between 20% and 15% FBS, growing CHST at the lower FBS concentration would be a cost-effective alternative pending consistent passaging of the culture.

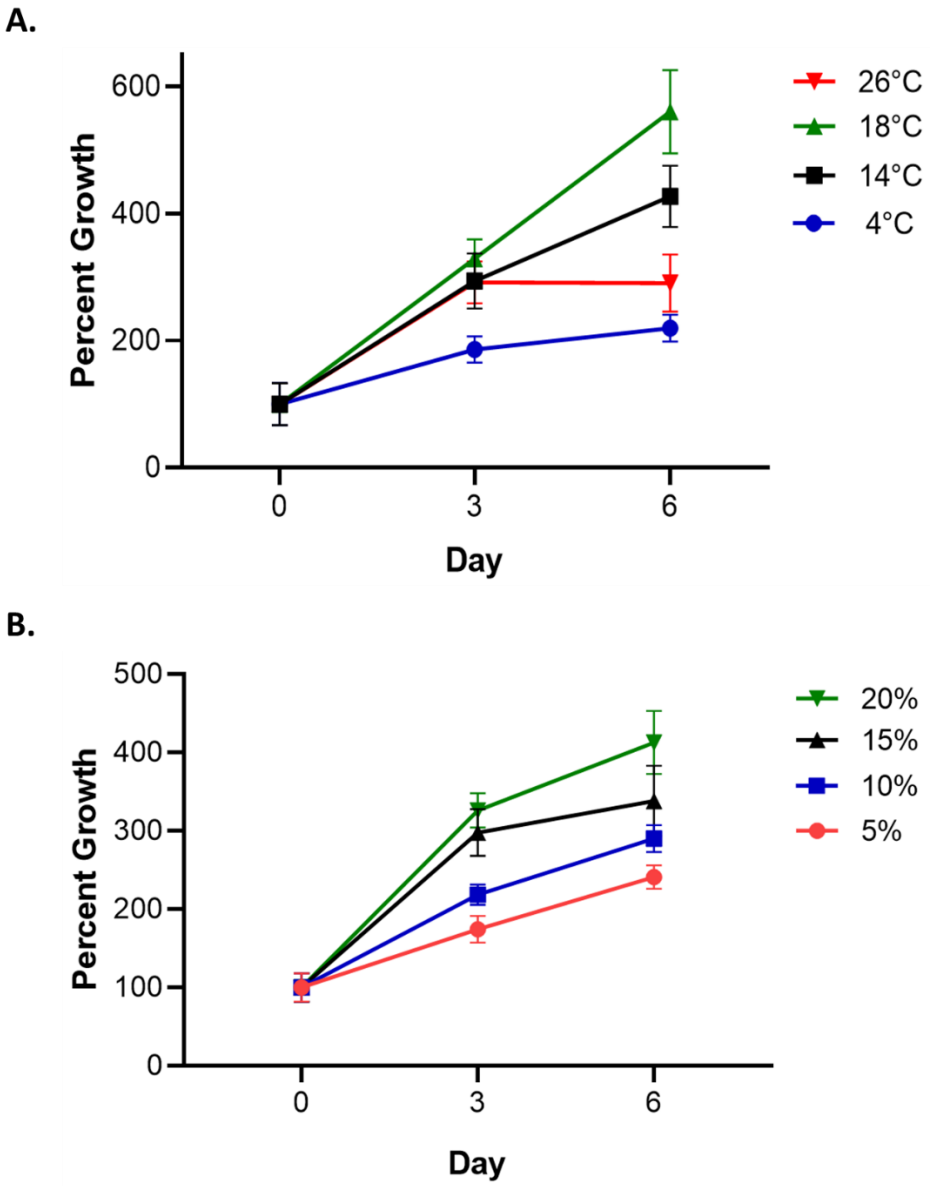

Figure 6.2. Optimal culture conditions for growth of CHST. (A) Impact of temperature on cell growth. CHST cells were plated at 200,000 cells/well in 20% FBS supplemented media, incubated at 4, 14, 18 or 26°C and counted on days 0, 3 and 6. (B) Influence of FBS concentration on CHST proliferation. Cells were plated at 200,000 cells/well. Following overnight adherence, triplicate wells received media supplemented with either 5%, 10%, 15% or 20% FBS and were then counted on days 0, 3 and 6. For both experiments, percent growth was calculated with respect to the average day 0 cell count. All cell counts were done in triplicate and are represented as means ± SD.

6.4.4 Growth of V. anguillarum in animal cell culture media

V. anguillarum was found to grow well in its preferred culture media, 2% NaCl supplemented TSB, with the logarithmic phase beginning at 4 h and ending at approximately 13 h (**Figure 6.3**). In comparison, although *V. anguillarum* was capable of growth in L15 alone and when supplemented with 2% FBS, this growth was much slower and absorbance readings were lower when compared to growth in its preferred culture medium. Further, what would be considered the logarithmic phase during growth in L15 alone and when supplemented with 2% FBS was prolonged with both beginning at 5 h and reaching a plateau at 18 h (**Figure 6.3**). The plateau phase of bacterial growth in L15 alone was very difficult to distinguish as the *V. anguillarum* appeared to be slowly growing over time. When *V. anguillarum* was grown in L15 supplemented with 10% and 20% FBS, no bacterial growth was observed. (**Figure 6.3**). Though *V. anguillarum* was capable of growth in cell culture media, CHST was not exposed to the live pathogen as the organism grew rapidly in media and proving to be lethal to the cells (data not shown).

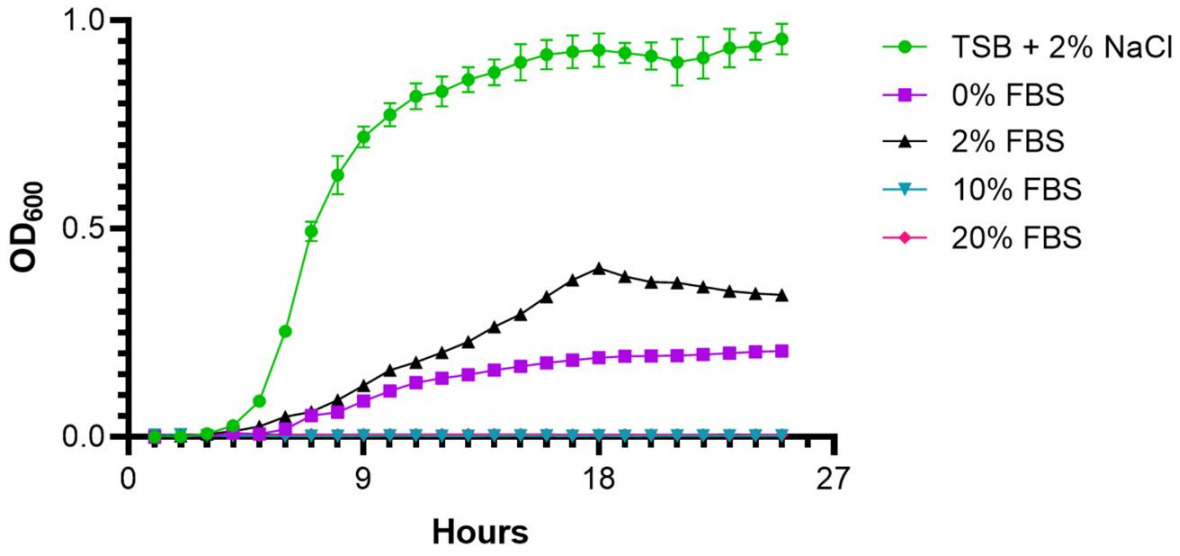

Figure 6.3. A comparison of *V. anguillarum* growth in culture media for bacterial cells and for animal cells (L15 supplemented with 0%, 2%, 10% or 20% FBS). Growth was at RT and monitored every hour for 25 h by measuring optical density at 600 nm. This figure represents three independent experiments for each culture condition. Data are presented as the mean with error bars representing ±SD.

6.4.5 Cytokine expression throughout exposure to heat-killed V. anguillarum

When CHST was exposed to heat-killed *V. anguillarum*, stimulation of pro-inflammatory and anti-inflammatory cytokines was observed over the 96 hr experiment. Transcript expression of *IL-1β, IL-6* and *TNFα* was observed to significantly increase at all timepoints other than 1 hr when compared to the control (**Figure 6.4A-B**). More specifically, *IL-1β* transcript expression was observed to increase over time until it reached a peak at 24 hr before dramatically decreasing in expression at 96 hr (**Figure 6.4A**). Meanwhile, *IL-6* expression increased with a peak observed at 12 hr and then decreasing over time until 96 hr (**Figure 6.4B**). The final pro-inflammatory cytokine assessed, *TNFα*, followed a similar trend to *IL-6* but the peak was observed at both 12 and 24 hr before a reduction in expression was noted at 96 hr (**Figure 6.4C**). The anti-inflammatory cytokine, *IL-10*, was not observed to increase in gene expression significantly when compared to the control until 96 hr (**Figure 6.4D**).

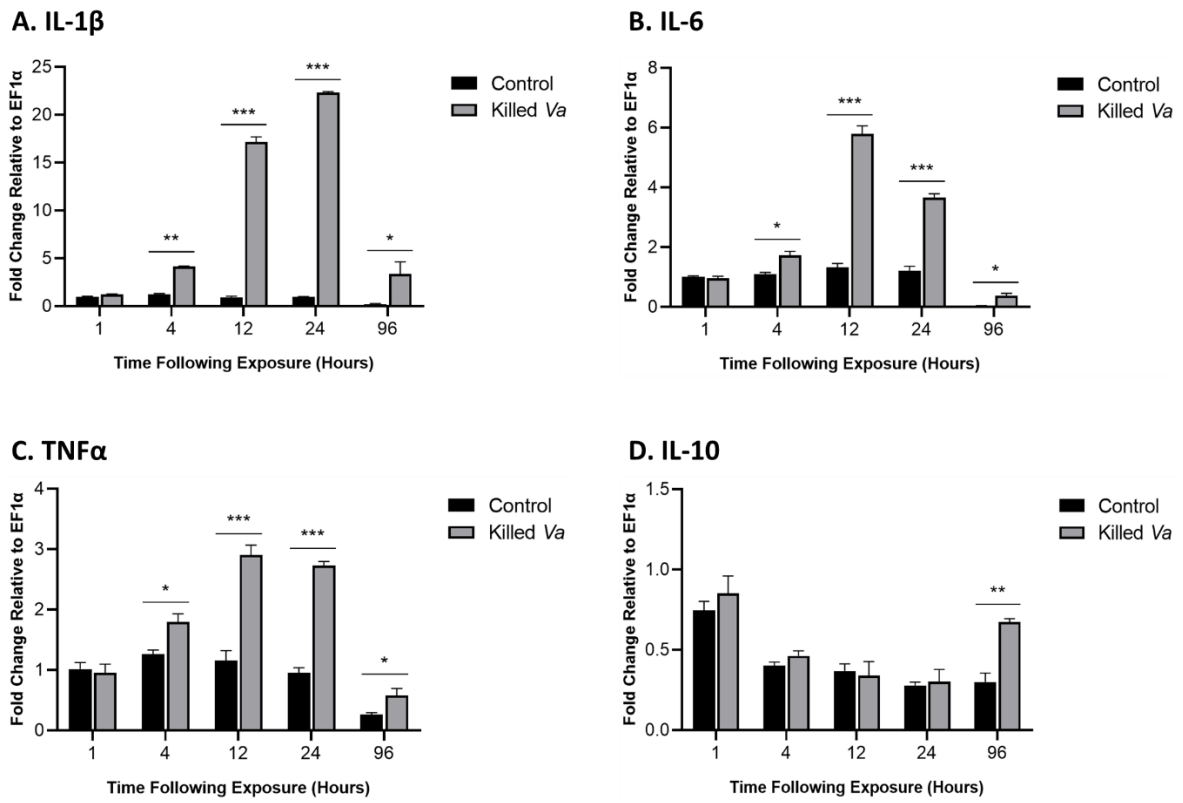

Figure 6.4. Influence of heat-killed *V. anguillarum* on pro- and anti-inflammatory cytokine expression in CHST throughout 96 h of exposure. Transcript expression of pro-inflammatory cytokines *IL-1β* (A), *TNFα* (B), *IL-6* (C) as well as the anti-inflammatory cytokine *IL-10* (D) was assessed so that the effect of killed *V. anguillarum* on immune function could be analyzed. All data was normalized to the reference gene (*EF1α*) and expressed as a fold change over the day 0 control group where control expression was set to 1. All panels represent three independent experiments and are presented as means + SD. A p-value of less than 0.05 was considered to be statistically significant when compared to the media alone control for each timepoint.

6.5 Discussion

In the present study, a Chinook salmon-derived spleen stromal cell line, CHST, was characterized and its transcriptional response to heat-killed *V. anguillarum* was assessed. Currently there are only two Chinook salmon cell lines available, the embryonic epithelial cell line CHSE-214 (McCain, 1970) and an epithelial spleen cell line derived from a triploid Chinook salmon, CHSS (Semple et al., 2018d). Though both of these are excellent tools for understanding Pacific salmonid immunity, CHST originated from non-adherent immune cells of a haematopoietic culture. These cells were surrounded with a microenvironment meant to support haematopoiesis, thereby increasing the likelihood that when these cells differentiated, they would become stromal in nature. As such, CHST

can further the understanding of Chinook salmon immunity in regards to the cells that are meant to support immune function and differentiation, as these have been shown to play meaningful roles in the immunity of other species (reviewed by Roozendaal & Mebius, 2011; Crowley et al., 2018).

Following the establishment of novel cell lines, it is important to report the optimal growth conditions so that future experiments using these cell cultures can be repeated reliably. Ideally, cell lines will have thermal temperature ranges that reflect their species of origin but because both the culture system and external growth factors differ significantly from that of the whole animal, this is not always the case. Adult Chinook salmon are reported to have a preferred temperature range between 12 and 15°C (Richter and Kolmes, 2005) but optimal growth of this species is seen at 15-19°C depending on feeding rates (Brett et al., 1982; Marine, 1997). As CHST was derived from an adult Chinook salmon, an optimal growth temperature of 18°C fits within the anticipated thermal optimum for this species and is comparable to other salmonid *in vitro* models (Semple et al., 2018d).

The strain of *V. anguillarum* used for this study was observed to grow in both its preferred culture media, and in formulations of animal cell basal media that had less supplementation with FBS. As a bacterial septicaemia that flourishes in the blood of teleosts, greater growth in low serum solutions was unexpected. However, given that mammals and fish and the vast differences in their habitats, the components of serum between these two taxa is likely to differ significantly. It has been shown that total plasma protein and albumin content of mammalian serum is twice and four times that of fish respectively (Zhang et al., 2013). Though variable, lower values of albumin and plasma protein when compared to mammals have been consistently shown in sera from fish species (Metcalf et al., 1998; Manera & Britti, 2008; Silva et al., 2015). Thus, *V. anguillarum* may be better able to grow at lower concentrations of FBS as this would be comparable to both its native marine environment and the blood of potential teleostean host species. Additionally, *V. anguillarum* is considered an opportunistic pathogen capable of long-term growth in saltwater (Enger et al., 1990; Eguchi et al., 2003), so the organism does not have a strict requirement for the added nutritional composition of serum to guarantee its survival. This means that the salt and nutrient content of L15 media alone (reviewed by Bols & Lee,

1991) is enough to support of *V. anguillarum*, though not to a degree that is comparable to its preferred culture media.

When an individual is infected with a bacterial pathogen the stromal cells of the spleen interact with blood borne lymphocytes through cytokine signalling. In our study, when challenged with heat-killed *V. anguillarum*, CHST was observed to increase expression of pro-inflammatory cytokines (*IL-1β*, *IL-6* and *TNFα*) significantly when compared to unstimulated controls. Over time, this induction was countered by an increase in *IL-10* cytokine expression. The cytokine response of rainbow trout stromal cells has also been explored using the stromal cell line, TSS, when exposed to the stimulants LPS, polyinosinic polycytidylic acid (poly I:C) or levamisole (Fierro-Castro et al., 2012; Fierro-Castro et al., 2013). Fierro-Castro and colleagues (2013) found that the stromal cells responded to poly I:C and levamisole in a manner that was comparable to rainbow trout macrophage cells for TN*Fα, IL-10 and IL-1β*. However, when exposed to LPS the TSS cells showed little to no induction of transcripts. This may reflect the fact that LPS is often considered an imperfect stimuli for some fish species as the toll-like receptor that binds and responds to LPS, TLR4, has only been found in zebrafish and no other teleostean species (Sepulcre et al., 2009; Ali et al., 2014). This outlines the importance of using whole-killed or live bacteria stimuli when possible. IL-6 is often not the focus of studies using fish stromal cells but this cytokine has been shown to be produced in splenic stromal cells isolated from mice with expression increasing as the animals age (Park et al., 2014). When cells were isolated from the peritoneal membrane of gilthead seabream they were shown to upregulate gene expression of *IL-6* when stimulated with genomic DNA isolated from *V. anguillarum* (Roca et al., 2008). When all of this is taken together, it appears that the structural stromal cells necessary to support immune cell function can sometimes play vital roles in the continuation and regulation of immune function.

6.6 Conclusions

CHST is a valuable tool for understanding the cellular physiology and, as described in this study, the immune function of Chinook salmon. The results presented here describe the characterization of a stromal cell line derived from the spleen of an adult Chinook salmon. Though CHST was not able to be exposed to live pathogen, heat-killed *V. anguillarum* was able to be used as a stimuli and was much more effective than previous studies using LPS for stromal cell stimulation. Further, CHST was found to respond significantly to heat-killed *V. anguillarum* when analyzing transcript expression of both pro- and anti-inflammatory cytokines over time. When it comes to understanding the stromal cells necessary to induce the development and differentiation of immune cells in response to stimuli, CHST provides an excellent model system.

Acknowledgements

Dr. Niels Bols for kindly allowing the use of his cell culture facility and Dr. Nguyen (Nathan) Vo for his technical insights regarding the initiation of salmonid cell lines.

Chapter 7: General Discussion and Future Directions

7.1 The importance of understanding bony fish immunology

Human beings have relied on fish populations for sustenance throughout history. In regard to the environment, fish play a vital role in natural food webs supporting the growth and survival of numerous species (Pauly et al., 1998; Wipfli & Baxter, 2011). From an economic perspective, fish represent a valuable commercial product providing many employment options while also ensuring that people have access to healthy protein sources worldwide. Aquaculture production also has the added benefit of ensuring that wild populations will not be dangerously overfished in order to meet the rising demand for this food source. As the intensive culture of fish is still in its infancy when compared to terrestrial agriculture, there are still many developments to be made. One way to help enhance the productivity of aquaculture efforts is to obtain a deeper understanding of teleostean immunity. Advances here will lead to improved vaccine design and therapeutic options, thereby augmenting yields and strengthening fish health in these facilities.

Though there are many similarities between the immune system of fish and mammals, there are also notable differences. Despite the highly studied mammalian model providing a solid baseline, comparative immunologists must experimentally confirm whether mammalian immune responses and tissues are analogous in fish. As an example, the bone marrow of mammals is the site of haematopoiesis and where B cells develop (reviewed by Mercier et al, 2012). However, fish do not have bone marrow, so instead this essential process occurs in the anterior portion of their kidney (Secombes & Wang, 2012). When it comes to specific immune receptors, fish have many similarities to those found in mammals (reviewed by Uribe et al., 2011). Nevertheless, teleostean equivalents quite often vary in number and ligand specificity. This can be seen when comparing toll-like receptors (TLRs) between these two taxa. One mammalian example, humans, are known to have 10 different TLRs while the majority of fish have at least 16 (reviewed by Palti, 2011; Nie et al., 2018). Some of these human TLRs can be found in fish, while others have been lost in many bony fish species, such as TLR6 and TLR10 (reviewed by Palti, 2011). Yet despite these interesting observations between fish and mammalian

TLRs, direct evidence of ligand specificity has only been shown in five of the sixteen TLRs found in fish (Tsujita et al., 2004; Phelan et al., 2005; Matsuo et al., 2008; Ribeiro et al., 2010). Given the large differences in environment, combined with the fact that fish have undergone at least one, if not two, whole genome duplications (WGD) not experienced by mammalian species (reviewed by Glasauer & Neuhauss, 2014), it is not surprising that there are significant differences both functionally and genetically between fish and mammals. This emphasizes the importance of validating immune paradigms in teleosts before basing therapies on concepts that have only been confirmed in mammalian models.

Teleosts represent approximately half of all surviving vertebrate species, making them the largest and most diverse group of this subphylum (Volff, 2005). Just as there are differences between species within the taxa Mammalia (reviewed by Zschaler et al., 2014; Tao & Reese, 2017), there are also distinct differences between species of fish, indeed these differences are bigger given fish emerged and diversified close to 400 million years ago (reviewed by Brazeau & Friedman, 2015). As an example, the majority of fish studied have genes for both MH class I and MH class II molecules but the Atlantic cod (*Gadus morhua*) has lost the genes for MH class II and its accessory molecules (Star et al., 2011). Furthermore, the cod-like fish, broadnosed pipefish (*Syngnathus typhle*), has lost MH class II function while still maintaining some of the genetic information (Haase et al., 2013). When the whole genome of cod was compared to other sequenced fish species, it was observed that cod has large gene expansions and several gene losses in the TLR repertoire (Solbakken et al., 2016). It is possible that the loss of such a large component of adaptive immunity resulted in a greater dependence on the innate immune system in this particular species. Understanding the interspecies differences in immunity such as these will help increase the efficacy of future therapies and vaccine initiatives. As this thesis focused strictly on salmonid immune function in response to bacterial pathogens, reactions of rainbow trout and Chinook salmon were explored at both the innate and adaptive levels of immunity. This has provided valuable information regarding these industrially relevant species and their response to bacterial pathogens.

7.2 Heritable differences in selectively bred fish

There are a number of ways in which the breeding of fish differs from other livestock, and this is often due to the high fecundity of aquatic species. This allows for strong selection intensity and results in large families, which can facilitate the extensive collection of phenotypic records of close relatives for selection candidates within breeding programs (reviewed by Gjedrem & Robinson, 2014). As it relates directly to the economic potential of an aquaculture facility, growth is often the initial focus of selective breeding endeavors. Heritability of growth has been observed in several fish species including tilapia (*Oreochromis niloticus*), rainbow trout, Atlantic salmon, Asian seabass (*Lates calcarifer*), as well as many others (Thodensen et al., 1999; Perry et al., 2005; Bentsen et al., 2017; Ye et al., 2017). Though now well established, the first breeding programs for aquatic species used mass selection and were generally unsuccessful (Moav & Wohlfarth, 1976; Hulata et al., 1986). It is now understood that this was likely due to the accumulated effects of inbreeding as they are known to influence several economic traits such as growth (Bentsen et al., 1998; Bentsen & Oleson, 2002; Rodgveller et al., 2005; Bentsen et al., 2017). Fortunately, with the development of creative breeding programs, a reduction in inbreeding can be consistently predicted for some aquatic species (D'Ambrosio et al., 2019). Yet, because the effects of heterosis tends to vary between species and individual stocks, confirmation of genetic improvements should be analyzed on a case-by-case basis.

Despite being extremely valuable, growth certainly is not the only factor selected for in aquaculture. Given the large losses due to infectious disease (World Bank, 2014), many breeding programs have been adopted to maintain improved growth while also selecting for resistance to problematic pathogens (reviewed by Yanez et al., 2014). In some cases, heritability of disease resistance is observed without negatively influencing animal growth (Imsland et al., 2002), such as has been observed in rainbow trout with resistance towards *F. psychrophilum* (Silverstein et al., 2009) or *F. columnare* (Evenhuis et al., 2015). However, it has also been shown that selecting for resistance to a single pathogen can sometimes result in increased susceptibility to another or decreased growth (Fevolden et al., 1992; Gjoen et al., 1997; Grimholt et al., 2003; Yanez et al., 2016; Trang et al., 2019).

Additionally, the gold standard for determining disease resistance is survival comparisons throughout live infection challenges (reviewed in Odegard et al., 2011). However, the subsequent results may not be representative as experimental infection models are often not comparable to what would be observed in a natural infection. For some aquatic pathogens, experimental models of infection that appropriately mimic live infection have still not been established. This has led to several alternative approaches for mimicking the outbreaks that are observed in aquaculture facilities such as co-habitation (Murray et al., 1992; Munang'andu et al., 2016), waterborne (Long et al., 2014; Zhang et al., 2016) and stress induction challenges (Taksdal et al., 1998; Henriksen et al., 2013). The major issue with these approaches is that they often are not repeatable if they are successful in producing disease symptoms. Intraperitoneal injection, such as that used in this thesis, will result in a much more consistent infection status for disease challenge trials. However, this method is not representative of a natural outbreak as the integument and mucous barriers are bypassed. Lack of appropriate challenge models has been a confounding factor for determining whether disease resistance observed in a lab setting will translate to disease resistance in an intensive culture situation. Understandably, this has also made it difficult to determine the true efficacy of vaccines and other therapeutics.

In an attempt to make salmonid stocks that were more robust and/or resilient in the face of bacterial disease, two different breeding strategies were used within this thesis. Initially, selective breeding to determine functional immune parameters that could predict resistance to BCWD was completed for rainbow trout. Though there was large variability in survival observed, this was not the case as the fish grew larger in size. This phenomenon of age-related disease susceptibility has been reported with other aquatic diseases including *F. branchiophilum* (Good et al., 2008) and infectious pancreatic necrosis virus (IPNV, Roberts & Pearson, 2005). Several long-term breeding studies have shown that resistance to BCWD is heritable and also that breeding over multiple generations can achieve consistently high resistance (Weins et al., 2013; Marancik et al., 2014; Wiens et al., 2018). The specific immune function components responsible for this resistance have not yet been identified, though there is some evidence to support the hypotheses that MH class IB (Johnson et al., 2008), spleen

size (Hadidi et al., 2008), IgT^+ B cells (Zwollo et al., 2017) and the microbiome of mucosal tissues (Brown et al., 2019) could be implicated. But because correlation is not always causation, some of these examples have already been debunked (Wiens et al., 2015), emphasizing the importance of trial repetition and design in future experiments. The data presented in this thesis does not support the idea that resistance to BCWD is related to MH class II β1 genotype, serum IgM production or respiratory burst activity. However, this may be due to the age of the fish or the fact that the families used here were only of the first generation. Thus, to confidently conclude that these immune components have no influence on the heritable resistance to BCWD, repeated experiments would need to be conducted wherein the immune parameters are measured immediately following assessment of resistance. The results presented here provide data important to the understanding of host response to *F. psychrophilum* throughout live infection. Ideally, once immune factors are identified that can predict resistance to BCWD, selective breeding programs will then be simplified, and the rainbow trout industry will have one less pathogen to be concerned about.

Although selective breeding for resistance has shown promise, this technique can be difficult to accomplish when aquaculture practises have propagated inbred stocks over time. Another breeding strategy used in this thesis was outbreeding to improve the biological fitness of a highly inbred Chinook salmon stock. There are multiple ways to evaluate the fitness of a group, but because this thesis focuses on bacterial pathogens, survival and immune performance following *V. anguillarum* challenge was assessed between seven outbred crosses and the control inbred stock. Significant differences between the eight populations in survival, immune transcript expression and serum IgM antibody to *V. anguillarum* were observed. Interestingly, it was the control inbred and one outbred population that presented the highest performance but overall there was a large degree of variability in survival across the groups. For aquaculture purposes it would be important to determine if these immunological differences are heritable in subsequent generations. Historically, heritability estimates for vibriosis have been low in Atlantic salmon but significant differences have been reported specifically for *V. anguillarum* (Gjedrem & Aulstad, 1973; Gjoen et al., 1997) and other hemorrhagic bacterial diseases

(Standal & Gjerde, 1987). This indicates that selection against such conditions could result in genetic changes in salmonid species. Yet despite this, a comparable study was completed using several Pacific salmon species wherein these claims of heritability to vibriosis were contradicted (Beacham & Evelyn, 1992b). Following analyses of specific immune parameters, it was observed that specific MH class II β1 alleles could not predict resistance to *V. anguillarum*, similarly to the genotyping results obtained from resistant/susceptible rainbow trout infected with BCWD. This is contrary to what has been observed in another teleost species, such as Japanese flounder (*Paralichthys olivaceus*), wherein certain MH class IIβ alleles displayed higher resistance to *V. anguillarum* infection and were also heritable (Xu et al., 2008). Though serum IgM was not observed to predict resistance to *F. psychrophilum* in rainbow trout, some significant differences between the outbred crosses were observed in Chinook salmon challenged with *V. anguillarum*. Unfortunately, serum IgM response to *V. anguillarum* does not appear to be very heritable based on previous studies in Atlantic salmon (Stromsheim et al., 1994; Fjalestad et al., 1996). These studies only analyzed antibody development to one antigen, the O-antigen, thus it is possible that serum IgM developed to the entire pathogen could be heritable in Pacific salmon species such as Chinook salmon. Although significant immune differences were observed here between the eight outbred crosses, much like what was observed with selectively breeding rainbow trout, future studies and more generations of offspring would be required to determine the heritability of these traits in Chinook salmon.

7.3 Vaccines for bacterial pathogens

Though there are several different types of vaccines produced for aquaculture (Assefa & Abunna, 2018), the majority used for bacterial pathogens are killed whole-cell preparations. These do provide some protection but when compared to the successes of terrestrial vaccine formulations, are limited at best. This lower efficacy is likely due to fundamental differences between the teleostean and mammalian adaptive immune response and how it translates into immunological memory. Because fish do not have IgG as an antibody isotype (reviewed by Sunyer, 2013), any secondary antibody response observed is slight and uses a different approach than the canonical mammalian definition of

immunological memory. This means that fish depend only on the low affinity but high avidity of IgM for repeated exposure to antigen, and thus a less intense secondary serum antibody response is observed in these animals (Kaattari et al., 2002; reviewed by Solem & Stenvik, 2006). Additionally, fish do not appear to go through class switch recombination (CSR), despite having all of the necessary components and enzymes (i.e. AID, RAG1/2, Ikaros, TdT, etc.) required to complete this process (reviewed by Dickerson & Findly, 2017), due to the structure of their heavy chain genes. Interestingly, even though the catalytic domain of activation-induced (Cytidine) deaminase (AID) differs from tetrapods, when this enzyme is transfected into murine B cells it is still able to catalyze CSR (Barreto et al., 2005). Since fish do not appear capable of CSR, this finding revealed that the actual process of CSR must have evolved separately from the AID enzyme itself (Barreto et al., 2005; Wake et al., 2006). Though protective responses have been observed in aquatic species years following initial exposure to antigen (Findly et al., 2013), the requirements to consistently stimulate immunological memory/protection to specific pathogens must still be scientifically confirmed.

Rather than the rapid isotype switching following initial exposure to a foreign entity, fish rely heavily on both IgM and IgT antibody responses (reviewed by Sunyer, 2013). This reveals the importance of stimulating mucosal immunity for future vaccine design regimes, something that was not a central focus historically (reviewed by Munang'andu et al., 2015). For certain bacterial pathogens, immersion vaccination of fish has been found to be effective in inducing a mucosal response (Liu et al., 2014; Hoare et al., 2017). However, in many of these cases boosters are required and such application routines for cultured fish have not been developed (reviewed by Sudheesh & Cain, 2017). In mammals, high antibody levels can be observed as early as three weeks following antigen exposure in what is known as the primary antibody response (Punt et al., 2019). Yet based on the data presented in this thesis, even after four weeks and/or four months following live infection there was large variability in response wherein many individuals present low antibody production. Additionally, in the Chinook salmon model, the fish were vaccinated against *V. anguillarum* and were then maintained in net pens for multiple years where they would have likely been exposed to environmental *V.*

anguillarum. Despite this, they still did not present a strong memory response. This low level of antibody production is consistent with what has been observed in other salmonid models where antibody titers can require 12 weeks or longer to reach peak levels in rainbow trout (LaFrentz et al., 2002, Ye et al., 2011b). Given that delayed antibody production is a consistent finding in salmonid species, perhaps alternative approaches must be made to successfully stimulate protective responses towards bacterial pathogens.

Surprisingly, despite the negative impact that bacterial pathogens have on aquaculture facilities, there is limited research devoted to understanding the pathogenesis of these organisms. Elucidating the pathologic cycle has been instrumental in developing effective vaccines for several terrestrial pathogens. The vaccines for both tetanus (*Clostridium tetani*) and diphtheria (*Corynebacterium diphtheriae*) are toxoids as it is the toxin produced by the bacterium that causes fatalities, not the organism itself (Kretsinger et al., 2006). The influence of extracellular products on disease state has been observed with aquatic pathogens both as described here for *F. psychrophilum*, and as observed previously when conducting *in vivo* studies with *Moritella viscosa* in Atlantic salmon (MacKinnon et al., 2019). Here, the conditioned media of *F. psychrophilum* alone was shown to stimulate cytokine pro-inflammatory transcript levels in immune cells and significantly inhibit their phagocytic activity. These extracellular products of *F. psychrophilum* have historically been a focus regarding the pathogenesis of the organism. Ostland and colleagues (2000) previously demonstrated that when a crude extracellular preparation of *F. psychrophilum* was injected directly into the muscle of rainbow trout, muscle necrosis was observed. This led to further studies attempting to determine virulence factors of *F. psychrophilum,* including the identification of extracellular proteases and mutation analyses using the identified secreted proteases (Secades et al., 2001; Secades et al., 2003; Perez-Pascual et al., 2011; Perez-Pascual et al., 2017). Unfortunately identification of individual virulence factors has had limited success, but loss of virulence has been observed in some cases (Perez-Pascual et al., 2015; Perez-Pascual et al., 2017). Most of these experiments looked directly at the resulting virulence of the pathogen. Some analyses have observed the immune function of the host, but

these represent very few in the field. Therefore, studies such as those presented in this thesis will eventually explain the pathogenesis of *F. psychrophilum*, and better treatment options will be developed.

Like many pathogens, it appears that *F. psychrophilum* has both intracellular and extracellular components of its pathologic cycle (Ekman & Norrgren, 2003; Nematollahi et al., 2005; Nilsen et al., 2011b). This may further impede design of BCWD therapies as vaccines for intracellular bacterial pathogens, such as *Mycobacterium tuberculosis*, have shown variable efficacy (reviewed by Henao-Tamayo et al., 2014; de la Maza et al., 2017). In these situations, a typical humoral response will not successfully eliminate the pathogen of interest so alternative methods of immune stimulation must be made (reviewed by Titball, 2008; Griffiths & Khader, 2014). This mirrors the difficulties and variable success observed when attempting to develop a fish vaccine for *R. salmoninarum*, an intracellular bacterial pathogen (Burnley et al., 2010). Despite all that is unknown regarding *F. psychrophilum*, there have been some promising vaccine candidates developed recently for BCWD (Fredriksen et al., 2013; Ma et al., 2019; Hoare et al., 2019) but these studies must be repeated and tested during natural outbreak/exposure conditions. For many bacterial pathogens, developing an effective vaccine or improving current formulations has required an understanding of the bacterial pathogenesis. This is precisely the information that is lacking with regard to many salmonid pathogens afflicting aquaculture and also why one aim of this thesis was to understand the pathologic cycle of *F. psychrophilum*.

7.4 AMPs as adjuvants/immunostimulants for aquaculture

As seen in successful mammalian vaccine preparations, the presence of the antigen itself is important driving the specificity of immunological memory, but appropriate adjuvants are key to ensure that the resulting immune response is protective. If a vaccine contained purified protein antigens alone, this would result in only a slight antibody response with little to no T cell activation. Thus, multiple immunizations would likely be required to stimulate a sufficient antibody memory response (reviewed by Reed et al., 2013). For terrestrial animals, there are a variety of different and effective adjuvants used for vaccination programs (reviewed by Di Pasquale et al., 2015). Presently for fish, commercial

vaccines consist mainly of purified antigens for the pathogen of interest as well as an emulsifying agent (reviewed by Tafalla et al., 2013). However, there has been significant research regarding the use of PAMPs, cytokines and other immunostimulants to enhance vaccine efficacy. For many of these studies, the protective response has been improved during experimental challenge with the pathogen of interest. The addition of flagellin along with Hsp60 and Hsp70 chaperonins to a subunit vaccine for *P. salmonis* resulted in a high protective response in Atlantic salmon (Wilhelm et al., 2006). One study used aluminum sulphate (alum), a common adjuvant of mammalian vaccines, in conjunction with an *Escherichia coli* mutant to be used to vaccinate for *Edwardsiella ictaluri* in catfish (*Ictalurus punctatus*). Following disease challenge, the alum adjuvant resulted in 92% survival when compared to the 54% survival observed no adjuvant control (Tyler & Klesius, 1994). Recombinant cytokines such as IL-8 and IL-1β have also shown promise as adjuvants for fish vaccines (Wang et al., 2016; Cao et al., 2017). Adjuvants have been an essential component for the successes of mammalian vaccines. Their addition to vaccine candidates for fish have had promising results but further study is required to determine whether these results are reliable or repeatable.

The production of AMPs by fish species has been extensively studied as an alternative treatment for antibiotics (reviewed by Chaturvedi et al., 2018). Aside from their direct impact on pathogens, AMPs have been shown to significantly influence the immune system of fish, making them prime candidates as indirect therapeutic agents and/or adjuvants. In fact, co-administration of AMPs with antigens from pathogens has been shown to boost immunogenicity in tilapia (Acosta et al., 2014). In salmonids, there have been few studies involving co-administration with vaccine candidates, but exposure to AMPs alone has been consistently shown to stimulate pro-inflammatory responses *in vitro* and *in vivo* (Chiou et al., 2006; Bridle et al., 2011; Zhang et al., 2017). When fish are challenged with bacterial pathogens or relevant antigens, significant induction of antimicrobial peptides in response to these stimuli has been reliably observed (Ruangsri et al., 2013; Kitani et al., 2015; Furlan et al., 2018; Kitani et al., 2019). Additionally, in several cases fish AMPs have had synergistic effects when used in conjunction with other AMPs (Lauth et al., 2005) or when used with therapeutic drugs (Zahran & Noga,

2010) in laboratory settings. This is similar to what was observed in this thesis with PACAP when the presence of both bacteria and the AMP significantly enhanced the stimulation of pro-inflammatory transcripts. When used alone, PACAP was able to significantly stimulate pro-inflammatory transcripts of rainbow trout immune cells, indicating that this peptide has immunostimulatory activity similar to other AMPs (Chaly et al., 2000; Zhang et al., 2001; Niyonsaba et al., 2007). Interestingly, these stimulatory effects are contrary to what has been observed with PACAP in mammalian studies wherein anti-inflammatory responses have been primarily observed (Delgado et al., 1999a; Delgado et al., 1999b; Martinez et al., 2002).This emphasizes the importance of confirming the activity of these molecules in each model organism before assigning a canonical function. Additionally, as a member of secratin/glucagon/growth hormone-releasing hormone/vasoactive intestinal peptide superfamily (Miyata et al., 1989), PACAP has also been linked to improved growth in some fish species (Tao & Boulding, 2003; Lugo et al., 2008; Lugo et al., 2010a), thereby adding another potential benefit for farmers if used as an adjuvant and/or a therapeutic agent. Given the need for appropriate adjuvants in fish vaccines, the use of AMPs may provide an effective, environmentally friendly alternative that is naturally produced by the host species. Nevertheless, it is necessary to test the efficacy these small peptides, as described in the present thesis, because they may not always have the desired immunomodulatory effect.

7.5 The value of fish cell lines for immune analyses

Considering the diversity of teleostean species and their utility all over the world, there are relatively few cell lines available to represent the many members of this infraclass, especially when compared to mammals. The first permanent fish cell line, RTG-2, was established in 1962 from rainbow trout gonadal tissue (Wolf & Quimby, 1962). Since then, more fish cell lines have been established with greater than 300 developed from approximately 110 species of teleosts (reviewed by Fryer & Lannan, 1994; Lakra et al., 2011). This only accounts for 0.4% of the known 26,000 teleostean species. Many of these cell lines are derived from industrially relevant species but as aquaculture practises change to meet market demands/preferences, establishment of appropriate eukaryotic cell lines is

necessary. With the rising interest in Chinook salmon culture on the Pacific coast, the development of cell lines from relevant tissues will ensure that researchers have the necessary tools to explore various aspects of cellular function, physiology and immunity in this species.

Historically, the primary purpose of fish cell lines has been to isolate, propagate and study aquatic viruses. Though less common, these cultures can also provide a controlled, cost-effective environment to explore both the pathogenesis and host cellular immune response associated with bacterial pathogens. This has been observed with several aquatic bacterial pathogens including *Yersinia ruckeri* (Menanteau-Ledouble et al., 2018), *Francisella noatunensis* (Soto et al., 2017) and *Photobacterium damselae* (Lopez-Doriga et al., 2000) to name a few. These studies were able to significantly improve the understanding of the associated pathogens and helped to improve future *in vivo* trials. The data presented in this thesis focused on the establishment of a stromal cell line from the spleen of an adult Chinook salmon and its use to understand the host cytokine transcript response when exposed to *V. anguillarum*. There has been previous work with this bacterial pathogen and the embryonic Chinook salmon cell line, CHSE-214 (Krovacek et al., 1987; Ormonde et al., 2000) but these studies primarily looked at adhesion properties of the bacterium itself. With the establishment of CHST, it is now possible to study host immunity in response to many bacterial pathogens in cells obtained from an adult Chinook salmon. As previously shown by Semple and colleagues (2018d), developmental age of the animal can alter the immune defenses observed in isolated cell cultures. Considering that these cells were developed to help improve knowledge of host immunity for Pacific salmon aquaculture, using cells from an adult fish is much more applicable than the Pacific salmon cell lines currently available.

7.6 Final conclusions and future directions

The results of this thesis provide evidence that selective breeding or outbreeding will result in variable survival when challenged with live bacterial pathogens. Though there were interesting trends, identification of specific immune markers that could be linked to increased survival were not identified in either of the model systems explored. When analyzing rainbow trout that were resistant/susceptible

to *F. psychrophilum*, this may have been due to the age of the fish as they were assessed for resistance/susceptibility when they were much smaller in size. Future studies should focus on repeating these results in younger fish to determine if more obvious functional trends can be observed. When exploring the pathogenesis of *F. psychrophilum*, extracellular products produced by the pathogen significantly inhibited the functionality of immune cells *in vitro*. As it appears that something produced by the pathogen is necessary for its pathologic cycle, an interesting experiment would be to inject fish with the *Fp*CM to determine whether (a) an immune response to these extracellular products could protect the animals from future re-infection and (b) if the *Fp*CM alone would be enough to produce the muscle degradation as well as immune activation that is characteristic of *F. psychrophilum* infection. Finally, the antimicrobial peptide PACAP was shown to stimulate immune function and have direct bactericidal activity on *F. psychrophilum*. As this was completed in an *in vitro* model, an obvious next step would be to expose rainbow trout to PACAP via different routes and determine whether this could protect the animals during a live infection challenge with *F. psychrophilum*. Though there is still much to learn regarding BCWD in rainbow trout aquaculture, the data presented in this thesis was able to provide important information that could be used to enhance current vaccination and/or therapeutic efforts.

Despite the increasing demand for Chinook salmon aquaculture, there is still limited research devoted to understanding their immune function. In an effort to make domesticated stocks more resilient, outbred Chinook salmon were challenged with live *V. anguillarum* so that their immune responses could be assessed and compared. Significant differences were observed between the groups in survival, immune gene expression and serum IgM. Follow-up research should use the highest and lowest performing crosses to make a second generation and assess whether the observed immune differences are a consistent trend/are heritable. To make further study of the cellular immune response of this species possible, a novel stromal cell line derived from the spleen of an adult Chinook salmon was created and characterized. This cell line was exposed to heat-killed *V. anguillarum* so that cytokine transcripts could be assessed. Similar studies have been performed in other salmonid cell lines, but this

was the first using a cell line derived from an adult Chinook salmon. As a stromal cell line, future studies should utilize this tool to determine if cytokine production can be altered and influence the immune response of primary leukocytes from Chinook salmon. Additionally, the impact of various bacterial and viral pathogens can be analyzed using this cell culture to identify the permissiveness of Chinook salmon to certain pathogens. Answers regarding immune function at both the whole animal and cellular level will help to make well-informed choices regarding stock improvements for Chinook salmon. This will hopefully improve domestication efforts of this species.

Currently, the most valuable and prevalent salmonid species grown for global aquaculture is Atlantic salmon (FAO, 2018). Like all anadromous salmonids, Atlantic salmon has two distinct phases throughout development: the freshwater phase and the saltwater phase. For the three to five months following hatch (depending on light cycle and temperature manipulations), Atlantic salmon develop in freshwater (FAO, 2009). During this period, any pathogens that the fry/parr are exposed to will be of freshwater origin. Because BCWD/RTFS is largely a condition of young salmonids (reviewed by Cipriano & Holt, 2005), this disease has had a significant impact on the Atlantic salmon industry during freshwater development of this species (Nilsen et al., 2011a; Fredriksen et al., 2016). Before entering the saltwater phase of their life parr undergo a process called smoltification. This entails diverse physiological changes necessary to survive in marine environments, after which the fish can be transported to saltwater net pens (Johansson et al., 2016; FAO, 2009). The new marine environment exposes the fish to a variety of saltwater pathogens. One such bacterial pathogen, *V. anguillarum*, has historically been problematic for Atlantic salmon culture and can result in large financial losses during outbreaks (Myhr et al., 1991; reviewed by Toranzo et al., 2005; Frans et al., 2011). Thus, aside from directly improving both rainbow trout and Chinook salmon aquaculture, the data presented in this thesis can also help to improve treatment options for pertinent bacterial diseases throughout all developmental stages of Atlantic salmon culture.

With the demand for salmonid aquaculture continually increasing, the results derived from this thesis provide valuable information regarding host immunity and how it relates to the pathogenesis of relevant bacteria. Based on the data presented, breeding strategies and future experimental design can be streamlined by focusing on, or disregarding, some of the immune markers that were explored here. By learning more about problematic bacterial pathogens and also what constitutes an effective immune response in the host, the results of this thesis can act as the basis of work that will enable the development of new and improved vaccines. This has the potential to significantly reduce a large portion of the $6 billion dollars of disease losses in the global aquaculture industry. Furthermore, with the negative atmosphere surrounding the culture of Atlantic salmon on the West coast of Canada, the Chinook salmon outbreeding results obtained here will improve the tenacity of this species for culture production. This will provide a much more environmentally friendly option for West coast aquaculture, regardless of whether BC fish farms are forced to move to land given the current political climate (Withers, 2019). Aquaculture is an important industry and as a result, studies such as these are invaluable for helping to determine the future direction of this expanding industry.

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Appendices

Appendix A1: Results of Mantel-Cox Test to compare survival curves between the 8 crosses when challenged with i.p. injection of live *V. anguillarum*. An adjusted alpha level of 0.0018 (p = 0.05/28) was used given the multiple pairwise comparisons among the eight populations. Significant differences when using this adjusted alpha level are outlined in red. This statistical analysis was completed using GraphPad Prism 8.1.0.

Crosses Compared	p-value
BQ and Punt	0.9401
BQ and YIAL	0.0003
BQ and Chil	0.0030
BQ and RC	0.0098
BQ and Cap	0.3449
BQ and Quin	0.0945
BQ and Nit	0.0016
Punt and Nit	0.0012
Punt and YIAL	0.0001
Punt and Chil	0.0017
Punt and Quin	0.0052
Punt and RC	0.0530
Punt and Cap	0.3254
Chil and RC	0.6682
Chil and YIAL	0.4152
Chil and Nit	0.8894
Chil and Quin	0.6444
Chil and Cap	0.1310
RC and Cap	0.3059
RC and Quin	0.8144
RC and Nit	0.6535
RC and YIAL	0.2198
Cap and Quin	0.5653
Cap and Nit	0.2246
Quin and Nit	0.5910
Quin and YIAL	0.2292
Nit and YIAL	0.6042
Cap and YIAL	0.0412

Appendix A2: Results of Mantel-Cox Test to compare survival curves between the 8 crosses when challenged with i.p. injection of sterile PBS. An adjusted alpha level of 0.0018 (p = 0.05/28) was used given the multiple pairwise comparisons among the eight populations. Significant differences are outlined in red. This statistical analysis were completed using GraphPad Prism 8.1.0.

Crosses Compared	p-value
BQ and Punt	0.2662
BQ and YIAL	0.4350
BQ and Chil	0.0544
BQ and RC	0.7146
BQ and Cap	0.1888
BQ and Quin	0.0119
BQ and Nit	0.0951
Punt and Nit	0.5983
Punt and YIAL	0.7741
Punt and Chil	0.3991
Punt and Quin	0.0003
Punt and RC	0.3813
Punt and Cap	0.0123
Chil and RC	0.0775
Chil and YIAL	0.2413
Chil and Nit	0.7498
Chil and Quin	<0.0001
Chil and Cap	0.0005
RC and Cap	0.0578
RC and Quin	0.0011
RC and Nit	0.1501
RC and YIAL	0.6344
Cap and Quin	0.1375
Cap and Nit	0.0024
Quin and Nit	<0.0001
Quin and YIAL	0.0010
Nit and YIAL	0.4213
Cap and YIAL	0.0319