Pituitary adenylate cyclase-activating polypeptide (PACAP) as a treatment for microbial infections in rainbow trout (*Oncorhynchus mykiss*) aquaculture

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. I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Chapter 2 - Tania Rodriguez-Ramos developed and optimized the quantitative ELISA for rainbow trout IL-1β and George Heath developed and optimized the quantitative ELISA for rainbow trout IFN-γ. Tania Rodriguez-Ramos and Xiaoqing Dang assisted with the performance of the IL-1β and IFN-γ ELISAs. George Heath originally cloned IL-6 and purified the anti IL-6 antibody used for Western blot.

Chapter 3 - Tania Rodriguez-Ramos and Janet Velazquez Perez assisted with the performance of the hemolytic assay. Tania Rodriguez-Ramos and Lowia Al-Hussinee assisted with the performance of the *in-vitro* assay in RTgill-W1.

Abstract

Diseases cause economic losses in aquaculture, and the widespread use of antibiotics in the industry has contributed to the development of antibiotic resistant pathogens, an issue of concern for its repercussions on the production system, the environment and human health. Antimicrobial peptides (AMPs) constitute a promising strategy to develop new drugs for prevention and treatment of diseases, due to their fast and efficient response against pathogens. Pituitary adenylate cyclase-activating polypeptide (PACAP) has shown strong antimicrobial activity and play a role as a regulator of the teleost fish immune system. In the present research, there was a significant increase in the transcription of PACAP splicing variants (PACAP and PRP/PACAP) in spleen, and in the transcription of VPAC2 receptor gene in spleen and head kidney of symptomatic rainbow trout naturally infected by *Y. ruckeri* compared to asymptomatic fish. Furthermore, the PACAP encoding gene is constitutively expressed in head kidney and skin of rainbow trout. Cytokines of interest in the immune response were also assessed and a significant increase in the transcription of IL-1β, TNFα, IL-6, IL-10 and IFN-γ was observed in spleen, head kidney and skin of *Y. ruckeri* naturally infected trout compared to clinically healthy trout, highlighting the importance of an active systemic and local immune response. We also studied the synthetic *Clarias gariepinus* PACAP-38 (PACAP 1) and three modified forms of the peptide (PACAP 2-4) to evaluate their direct antimicrobial function against bacterial pathogens of importance in rainbow trout aquaculture, safety in the host and immunomodulatory properties. The peptides were tested for cytotoxicity in a hemolytic assay using erythrocytes from diploid and triploid rainbow trout, and in a cell viability assay using an epithelial cell line from rainbow trout gills, RTgill-W1. The results showed that only concentrations of 40 and 50 µM of PACAP 4 caused a percentage hemolysis higher than 20%, and that none of the peptides at a concentration of 0.1 µM reduced the

cell viability of RTgill-W1. All active variants directly inhibit *Flavobacterium psychrophilum* and *Aeromonas salmonicida* growth by over 90% in a broth microdilution peptide assay (BMPA), but only PACAP 2 and 4 inhibited *Yersinia ruckeri* growth in more than 90% at the highest concentration of 50 µM. *In-vitro* trials with RTgill-W1 exposed to *F. psychrophilum* showed that a 24-hour pre-treatment with 0.1 µM of different PACAP variants significantly reduced bacterial growth, however, there was a lack of response of TNFα. Down-regulation in the expression of the IL-1β gene was observed in RTgill-W1 exposed to both PACAP alone and PACAP and *F. psychrophilum*, which was consistent with the lack of stimulation of the live pathogen alone on non-treated cells as well. These findings could be related to the nature of the cell line, as inflammation could impair the role of gills in respiration, so other mechanisms of the immune response may be involved. Overall, *C. gariepinus* PACAP-38 and variants have a direct antimicrobial activity and immunomodulatory properties against bacteria of importance in rainbow trout aquaculture, and PACAP 2 is a good candidate for future research due to its improved antimicrobial function and its limited cytotoxicity against host cells. Future i*n-vivo* studies are needed to determine PACAP function at the integral organism level.

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Dedication

To the universe and my ancestors, for guiding me on this journey of constant learning called life.

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List of Abbreviations

ADCYAP1	Adenylate Cyclase Activating Polypeptide 1
AMPs	antimicrobial peptides
BCIP	5-bromo-4-chloro-3'-indole-phosphate
BCWD	Bacterial Coldwater Disease
Blimp1	B lymphocyte-induced maturation protein-1
BMPA	broth microdilution peptide assay
cDNA	complementary deoxyribonucleic acid
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CFU	colony forming unit
COX-2	cyclooxygenase -2
CXCL-8	C-X-C Motif Chemokine Ligand 8
DNA	deoxyribonucleic acid
DNase I	deoxyribonucleic nuclease
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EF1a	elongation factor 1 alpha
FBS	fetal bovine serum
Fp	*Flavobacterium psychrophilum*
GALT	gut-associated lymphoid tissue
GIALT	gill-associated lymphoid tissue
GHRH	secretin/ growth hormone-releasing hormone
ICE	IL-1β converting enzyme
IL	interleukin
IFN-γ	interferon gamma
IFN-γrel	interferon gamma related

L-15	Leibovitz's L-15 medium
LPS	lipopolysaccharides
MALT	mucosal-associated lymphoid tissue
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger ribonucleuc acid
Mx	myxovirus resistance
NADPH	nicotinamide adenine dinucleotide phosphate
NALT	nasopharynx-associated lymphoid tissue
NBT	nitro-blue tetrazolium
NF-κB	nuclear factor kappa-B
NK	natural killer
NOS	nitric oxide synthase-derived metabolites
ROI	reactive oxygen intermediates
OD	optical density
$p47^{phox}$	neutrophil cytosol factor 1
PAC1	pituitary adenylate cyclase-activating polypeptide-selective receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline
PCR	polymerase chain reaction
poly(I:C)	polyinosinic:polycytidylic acid
proIL-1β	prointerleukin-1β
PRP/PACAP	pituitary adenylate cyclase-activating polypeptide-related peptide
qELISA	quantitative enzyme-linked immunosorbent assay
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid

rRNA	ribosomal ribonucleic acid
RTFS	Rainbow trout Fry Syndrome
RTgill-W1	epithelial cell line from rainbow trout gills
RTS11	monocyte/macrophage-like rainbow trout cell line
SALT	skin-associated lymphoid tissue
SDS	sodium dodecyl sulfate
SPC	standard plate count
TACE	TNFα converting enzyme
TAE	Tris Acetate-EDTA buffer
TBS	tris-buffered saline
Th1	T helper type 1
Th2	T helper type 2
TBS-T	tris-buffered saline with Tween-20
TLR3	toll-like receptor 3
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumour necrosis factor
TSA	trypticase soy agar
VHSV	viral haemorrhagic septicaemia virus
VIP	vasoactive intestinal peptide
VPAC1	vasoactive intestinal peptide receptor type 1
VPAC2	vasoactive intestinal peptide receptor type 2

Chapter 1: Literature review and thesis structure

1.1 Aquaculture and challenges in the industry

Aquaculture is a rapidly growing industry worldwide. In 2018, global fish production for human consumption reached 156 million tonnes, of which 82 million tonnes, valued at USD 250 billion, came from the aquaculture sector, which means that, currently, aquaculture provides 52% of the fish destined for human consumption. In addition to the economic importance of aquaculture industry, fish are one of the largest sources of animal protein. Fish provides the essential amino acids and micronutrients fundamental for a nutritious healthy diet at a cheap cost, which is essential mainly for densely populated countries with a low total protein intake (FAO, 2020). Within the industry, salmonids represent the most profitable species per unit of weight (Lucas & Southgate, 2012). Canada is one of the major producers of cold-water salmonids (FAO, 2020), and in Ontario, specifically, rainbow trout (*Oncorhynchus mykiss*) is the major species farmed, with a farm-gate value of 26.8 million dollars for 5 060 tonnes produced in 2016 (Moccia & Bevan, 2017). Peru is part of the top capture producer countries. Fishing and aquaculture production in 2018 was 7 273 thousand tons, of which less than 2% (104 000 tons) came from aquaculture (FAO, 2020). However, the sector has shown growth over recent years with rainbow trout being the main species harvested in 2018 with a volume of 64 372 tons (PRODUCE, 2019).

To accomplish this high productivity, the sector has developed common intensive animal production practices, which causes stress in the fish and leads to increased susceptibility to diseases caused by bacteria, viruses, fungi and parasites (Miranda *et al*., 2018; Pilco *et al*., 2013). Disease losses also enhanced by an aquatic environment where any physicochemical alteration such as an

abrupt change in temperature favors the outbreak of infectious diseases (Pilco *et al*., 2013). Microbes can survive in the aquatic environment independent of their host, acting as opportunistic pathogens when conditions are favorable (Madetoja *et al*., 2003; Wedemeyer & Nelson, 1977). Disease outbreaks are considered a major farm-level risk, and cost USD the industry 6 billion per year (Brummett *et al*., 2014). In aquaculture, antibiotics are used for the treatment of bacterial diseases, but can also be used for prophylactic and metaphylactic purposes, and even as growth promoters (Done *et al*., 2015; Pilco *et al*., 2013). The widespread use of antibiotics is a relevant challenge in the sector as this has contributed to the development of antibiotic resistance, issue of concern for its repercussions on the production system, the environment and human health (FAO, 2010; Done *et al*., 2015; Pilco *et al*., 2013).

Research that promotes the gaining of knowledge of the teleost fish immune response against bacterial diseases of importance could contribute to the development of better treatment and prophylactic measures. Moreover, the search for novel antimicrobial agents as an alternative or supplement to conventional antibiotics is important as antibiotic resistance is considered a priority problem in aquaculture. (Baltzer & Brown, 2011; Galdiero *et al*., 2015).

1.2 Bacterial pathogens of importance in rainbow trout aquaculture

Common and important bacterial agents in salmonid aquaculture include *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Aeromonas salmonicida* (Semple & Dixon, 2020). Mateo *et al*. (2017) identified *Yersinia ruckeri*, *Aeromonas salmonicida* and *Flavobacterium psychrophilum*

as the main bacterial agents associated with mortality and poor growth in rainbow trout in the Central Highlands of Peru, through microbiological analysis.

1.2.1 *Yersinia ruckeri*

Enteric Redmouth Disease or Yersiniosis is one of the main bacterial diseases in salmonids, whose etiological agent *Y. ruckeri* was first isolated in rainbow trout in the 1950s, in Hagerman Valley, Idaho, USA (Ross *et al*., 1966). Although this bacterial infection affects different species of fish, rainbow trout is the most susceptible (Furones *et al*., 1993). *Y. ruckeri* is widely distributed, it has been identified in North America, the Middle East, Europe, South Africa, China, Australia, New Zealand, India, Venezuela, Chile and Peru (Bravo & Kojagura, 2004; Kumar *et al*., 2015; Troncoso *et al*., 1994). *Y. ruckeri* is a facultative, gram-negative rod with rounded ends, belonging to the Enterobacteriaceae family. It has been subdivided into four serotypes with different subgroups: Serotype O1 subdivided into 2 subgroups O1a (serovar I) and O1b (serovar III), serotype O2 (serovar II) subdivided into 3 subgroups O2a, O2b and O2c, serotype O3 (serovar V) and serotype O4 (serovar VI), being the O1a serotype associated with most outbreaks (Romalde *et al*., 1993). Clinical signs of disease include lethargy, swimming near the surface, loss of appetite, exophthalmia, darkening of the skin (melanosis), distended abdomen, and subcutaneous hemorrhages in the oral cavity and body surface (head, base of fins and along lateral line) (Tobback *et al*., 2007). The characteristic hemorrhages in and around the oral cavity gave it the name of redmouth disease, however, this sign is not apparent in all affected fish and its absence does not imply that there is no infection, so the disease has also been referred to as Yersiniosis (Sirvas *et al*., 2011; Tobback *et al*., 2007). Studies have detected signs of redmouth with relatively low infection rates (Sierralta *et al*., 2013), however, severe acute infections are associated with the

presence of hemorrhages in the oral cavity (Carson & Wilson, 2009). Internally, the presence of petechiae can be observed on the surface of the liver, pancreas, intestine, swim bladder and lateral muscles, while an enlarged and darkened spleen can also be seen along with an inflamed intestine containing an opaque and purulent fluid (Kumar *et al*., 2015). The severity of this disease is associated with the virulence of the strain and the degree of stress (Tobback *et al*., 2007).

1.2.2 *Flavobacterium psychrophilum*

F. psychrophilum is the causative agent of two diseases of importance in salmonid aquaculture, Bacterial Coldwater Disease (BCWD) and Rainbow trout Fry Syndrome (RTFS) (Nematollahi *et al*., 2003), with RTFS being an acute bacteremia that affects the sac fry to early-feeding rainbow trout developmental stages, and BCWD a chronic disease more commonly observed in larger fish (Starliper, 2011). In 1948, Borg first described and named current *F. psychrophilum* from an outbreak in coho salmon (*Oncorhynchus kisutch*) from the Pacific Northwest in the United States (Barnes & Brown, 2011; Starliper, 2011). *F. psychrophilum* are strictly aerobic gram-negative, slender, weakly refractile, flexible rods (Pacha, 1968) and are currently classified into the Flavobacteriaceae family. This pathogen has been identified in salmonids and other fish species, but juvenile rainbow trout and coho salmon are especially susceptible to the disease (Barnes & Brown, 2011; Nematollahi *et al*., 2003). Its geographic distribution is broad, including North America, almost all Europe, Japan, Korea, Turkey, Australia, Chile and Peru (Barnes & Brown, 2011; Starliper, 2011). Disease occurs in water temperatures under 16˚C but is stronger at 10˚C and below (Starliper, 2011). Studies have reported at least three main serotypes (Lorenzen & Olesen, 1997). RTFS acute clinical signs include lethargy, often bilateral exophthalmia, dark skin pigmentation and pale gills, kidney and liver (Starliper, 2011). BCWD is characterized by erosion

of tissue mainly at the caudal peduncle or caudal fin that progresses to necrosis. Other clinical signs include lethargy, exophthalmia, scleritis, blindness, spiral swimming behavior, skin ulcerations in the lower jaw and musculature, pale or necrotic gills, increased mucus production and pigmentation ("black tail"), ascites, anemia, enlarged spleen, intestinal inflammation, pale liver and kidney, nervous disorders, spinal deformities, and a hemorrhagic and protruding anus (Barnes & Brown, 2011; Starliper, 2011).

1.2.3 *Aeromonas salmonicida*

A. salmonicida is the etiological agent of Furunculosis, a disease that was first reported in 1894 in Germany by Emmerich and Weibel (Miyata *et al*., 1996). Although infection by this pathogen has been recognized mainly in salmonids, it can affect many different species of fish. At present, *A. salmonicida* is considered endemic in most of the world (Dallaire-Dufresne *et al*., 2013; Menanteau-Ledouble *et al*., 2016). *A. salmonicida* is a facultative gram-negative rod belonging to the Aeromonadaceae family. *A. salmonicida* is divided into five subspecies (*salmonicida*, *achromogenes*, *masoucida*, *pectinolytica* and *smithia*), being *A. salmonicida* subsp. *salmonicida* considered the typical strain associated with Furunculosis, and the rest of the subspecies are considered atypical (Gudmundsdóttir *et al*., 2003). Disease can be presented in peracute, acute and chronic forms. In the peracute form, commonly in young fish, a few clinical signs are shown such as darkening of the skin (melanosis), exophthalmia and distention of the abdomen, in some cases. In the acute form, anorexia, and presence of petechiae and hemorrhages, mainly at the base of the fins, are observed in addition to the aforementioned clinical signs. Pathognomonic furuncal lesions can be found in the chronic form of the disease (Menanteau-Ledouble *et al*., 2016).

1.3 Immune response in teleost fish

Bony fish such as salmonids are included in the teleost clade and, like mammals, their immune system can be separated into innate and adaptive branches, however, due to limitations on the adaptive immune system, the innate immune system plays an important role as the first line of defense against pathogens (Semple & Dixon, 2020).

1.3.1 Lymphoid organs

Fish lack bone marrow and lymph nodes. Their primary lymphoid organs include the thymus, where T lymphocytes differentiate and are selected; and the head kidney, an important organ in hematopoiesis and also where many macrophages and B lymphocytes are found. Secondary lymphoid organs include the spleen, where antigen presentation occurs and the adaptive immune response is induced; and mucosal-associated lymphoid tissue (MALT), which has components of the innate and adaptive immune system (Vega-Ramírez *et al*., 2010; Zapata *et al*., 1996). MALT comprises gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT), skin-associated lymphoid tissue (SALT), and nasopharynx-associated lymphoid tissue (NALT). These tissues are of vital importance in fish since the aquatic environment has a rich and diverse microbiota, theses epithelial barriers have constant exposure to different pathogens while they also have to tolerate the commensal microbiota (Gomez *et al.*, 2013; Salinas, 2015).

1.3.2 Cytokines

Cytokines are small proteins that act as extracellular mediators. They have an important role in the interaction between cells and are essential linkers between the innate and adaptive immune

response. Cytokines can perform in autocrine, paracrine and endocrine fashion, regulating haematopoiesis, inflammation, immune cell activation and cell migration (Semple & Dixon, 2020; Zhang & An, 2007).

The inflammatory response is essential for combatting infections through the many different components that are important in this response (Martin & Leibovich, 2005), however, key pro-inflammatory cytokines, Interleukin 1β (IL-1β), Interleukin 6 (IL-6) and Tumour necrosis factor (TNFα) play a crucial role in the initiation and persistence of inflammation (Semple & Dixon, 2020; Zhang & An, 2007). IL-1β is a potent activator of the humoral immune response and is produced by a wide variety of cells, mainly monocytes and macrophages (Bird *et al*., 2002). IL-1β was the first interleukin characterized in teleost and cartilaginous fish, as it is a regulator of inflammation that is also conserved in fish (Secombes *et al*., 2011). It also modulates the expression of members of the IL-17 family, which are important in the rapid response against bacterial infections (Kono *et al*, 2011). IL-1β induces the expression of pro-inflammatory genes such as TNF-α, IL-1β, IL-6, IL-8, IL-34 and cyclooxygenase -2 (COX-2) in macrophages and primary leukocytes (Zou & Secombes, 2016), it attracts leukocytes to the site of infection by stimulating chemokine production (Chen *et al*., 2013), and studies have shown that it stimulates the production of antibodies when administered together with bacterial vaccines as an adjuvant (Yin & Kwang, 2000).

TNFα is a pro-inflammatory cytokine mainly produced by activated monocytes / macrophages, which exerts a variety of immunological functions on the regulation of inflammation and the

cellular immune response. TNFα is an important mediator in the immune response against infections caused by gram negative bacteria (Manning & Nakanishi, 1996; Zou *et al*., 2003). In fish, TNFα is one of the most rapidly expressed immune genes in the event of infection. Like mammals, fish TNFα plays a key role in the regulation of inflammation and shows overlapping functions with IL-1β (Zou & Secombes, 2016). TNFα also stimulates the phagocytic activity of leukocytes increasing their ability to kill microbes (Zou *et al*., 2003), stimulates the expression of several immune system inflammatory genes such as IL-1β, IL-8, IL-17C, TNFα, COX-2 and genes involved in the antimicrobial response. It has been suggested that TNFα is involved in leukocyte proliferation and migration in fish, increasing macrophage migration in a dose-dependent manner (Zou & Secombes, 2016). It also activates chemokine expression in local cells such as endothelial cells (Roca *et al*., 2008), and exerts a role over the activity of cytotoxic cells (Praveen *et al*., 2006). It has been described in rainbow trout and several other fish species (Zou *et al*., 2003).

IL-6 is mainly produced by monocytes and macrophages at the site of inflammation (Heinrich *et al*., 2003), and it is associated with an early inflammatory response and acquired immunity as it is the main mediator of the acute phase reaction and septic shock. Its production is stimulated by bacterial endotoxins, IL-1 and TNFα, and it also promotes B cell proliferation (Manning & Nakanishi, 1996; Tizard, 2009). In fish, IL-6 plays a central role in the immune response since it acts as a pro-inflammatory agent promoting acute phase reactions, hematopoiesis, and differentiation of immune cells against bacterial or parasitic infections (Zante *et al*., 2015). Abós *et al*. (2016) showed that, in trout, the effects of IL-6 on spleen B cells include proliferation, NF-kB activation, increased of IgM secretion, increased in Blimp1 transcription, and decreased in the expression of surface MHC-II, showing that it can be a differentiation factor for IgM-secreting

cells. *In-vitro* studies have shown that recombinant IL-6 is a macrophage growth promoter and that it increases the gene expression of antimicrobial peptides (AMPs) (Costa *et al*., 2011).

The resolution of immune responses and repair of tissues after inflammation is an essential component of the inflammatory response as persistent inflammation can lead to tissue damage (Serhan & Savill, 2005). Anti-inflammatory cytokine Interleukin 10 (IL-10), produced by a variety of leukocytes including dendritic cells, macrophages, T cells, natural killer cells, and B cells, exerts a key function in maintaining homeostasis as it targets both the innate and the adaptive immune responses to control inflammation (Ouyang & O'Garra, 2019). IL-10 has been identified in different teleost species and its function resembles that of mammals, acting as a suppressor of the immune response (Inoue *et al*., 2005; Zou & Secombes, 2016). A single copy gene has been described for most fish species, but two IL-10 paralogues have been identified in rainbow trout (Harun *et al*., 2011). In goldfish monocytes exposed to heat-killed *Aeromonas salmonicida*, it was found that recombinant IL-10 reduced the expression of TNFα1, TNFα2, IL-1β1, IL-10, CXCL-8, and NADPH oxidase component, $p47^{phox}$ (Grayfer *et al*., 2011). Also, in carp macrophages, IL-10 showed an inhibitory effect in the expression of genes involved in MHC antigen presentation (Zou & Secombes, 2016).

Other cytokines that are mainly involved in the adaptive immune response include Interleukin 2 (IL-2) and Interferon gamma (IFN-γ) (Wang & Secombes, 2013). IL-2, which is produced by $CD4^+$ T cells after being activated by an antigen, induces T cell growth, increases NK cytolytic activity, mediates activation-induced cell death and has a major function in the differentiation of $CD4^+$ T

helper subsets and $CD4^+$ T regulatory cells (Liao *et al*., 2011; Wang & Secombes, 2013). In teleosts, IL-2 has been identified in several fish species, and in salmonids the presence of two divergent IL-2 paralogs has been reported by Wang *et al*. (2018). Rainbow trout IL-2 isoforms promoted peripheral blood leukocyte (PBL) proliferation and up-regulated the proliferation of CD4 and CD8 cells suggesting their function as T cell growth/survival factors is conserved. Also, recombinant IL-2 up-regulated key Th1 and Th2 cytokines, cytokine and chemokine receptors, and the antimicrobial peptide cathelicidin-1 in PBL (Wang *et al*., 2018). IFN-γ is the only type II IFN and it is produced by NK cells in the innate immune response and by Th1 cells in the adaptive immune response (Wang & Secombes, 2013). Even though it was first described for its antiviral activity, it exerts an essential role in the regulation of the innate and adaptive immune response and also controls infectious disease (Boehm *et al*., 1997). IFN-γ is crucial for the initiation of a Th1 response (Wang & Secombes, 2013). Two duplicated copies of IFN-γ have been described in salmonids including rainbow trout. Trout IFN-γ functions are similar those of mammals and the functions of the teleost specific IFN-γ related (IFN-γrel) gene are still being studied (Zou & Secombes, 2011). The treatment of trout macrophages with recombinant IFN-γ demonstrated an increase in MHC-II gene expression showing its role in enhancing antigen presentation (Zou *et al*., 2005). IFN-γ in fish stimulates the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNFα, and CXC chemokine γ -IP (CXCL9/10/11), which acts on T cells (Wang & Secombes, 2013), increases the production of nitric oxide and reactive oxygen intermediates (ROI), and enhances phagocyte activity in several species (Zou & Secombes, 2016).

Many studies of gene expression related to the immune response have been carried out experimentally in salmonids such as rainbow trout to evaluate the response to challenges with

important bacterial pathogens in their culture (Chettri *et al*., 2012; Croisetiere *et al.,* 2008; Evenhuis & Cleveland, 2012; Henriksen *et al*., 2013). However, little research has been performed in natural infections.

1.3.3. Antimicrobial peptides

The properties of AMPs have been explored for their importance in producing fast and efficient innate defense responses against microbial pathogens (Baltzer & Brown, 2011; Galdiero *et al*., 2015; Gordon *et al*., 2005). AMPs are small molecules produced by host organisms from eukaryotes to plants and animals. These peptides can form amphipathic conformations and directly lyse bacterial membranes. Having a different mode of action than traditional antibiotics that simply breaks membranes, they are less likely to lead to microbial resistance. They are selective, act quickly and exhibit a natural broad spectrum of antimicrobial activities against not only gram positive and negative bacteria, but also fungi, enveloped viruses and parasites (Baltzer & Brown, 2011; Cruz *et al*., 2014; Galdiero *et al*., 2015). The design of synthetic peptides derived from native AMPs constitute a promising strategy to develop new drugs for prevention and treatment of bacterial diseases (Galdiero *et al*., 2015; Gordon *et al*., 2005).

Teleost fish rely mainly on their innate immune system to prevent and fight pathogens as they have limitations in their adaptive immune system. As opposed to mammals, the fish adaptive immune response develops slowly and displays a poor affinity maturation, which is why the efficacy of vaccines in fish is limited. On the other hand, the innate immune system is highly conserved in all species and acts rapidly in defense of the organism (Semple & Dixon, 2020). AMPs constitute an

important component of the innate immune response and have been widely studied as a possible treatment against diseases of interest in aquaculture due to their immunomodulatory and antimicrobial activity (Brunner *et al*., 2020; Masso-Silva & Diamond, 2014). Salmonid AMPs are hydrophobic diverse molecules with length ranges from 11 to 79 amino acids and a net charge from 0 to 30 (Brunner *et al*., 2020). For their direct antimicrobial activity, AMPs can disrupt membranes by inducing permeabilization or can be non-membrane disruptive and pass directly into the cell to disrupt intracellular targets. As part of their immunomodulatory properties, they can stimulate chemotaxis, promote differentiation of immune cells and the initiation of adaptive immunity, and also stimulate pro- inflammatory and anti- inflammatory cytokines (Semple & Dixon, 2020).

1.4 Pituitary adenylate cyclase-activating polypeptide (PACAP)

In the last few years, neuropeptides have been shown to play a role in the host defense, including immunomodulatory action and direct antimicrobial properties, in addition to their conventional role as neurotransmitters (Augustyniak *et al*., 2012). PACAP is part of the secretin/ growth hormone-releasing hormone (GHRH)/vasoactive intestinal peptide (VIP) family (Starr *et al*., 2018). Because of its remarkably conserved functions among vertebrates and its biomedical interest, the neuropeptide PACAP has been widely studied. In mammals, PACAP is encoded by the ADCYAP1 gene, exon 3 of which encodes PACAP-related peptide (PRP) and exon 4 encodes PACAP. Thus, this gene produces two biologically active peptide isoforms (Cardoso *et al*., 2020). PACAP-38 is the predominant full-length form and PACAP-27 is a truncated active form, both are C-terminally amidated peptides containing either 38 or 27 amino acids (Cardoso *et al*., 2020;

Starr *et al*., 2018). PACAP-38 share characteristics with canonical AMPs including a basic charge of +10 at neutral pH, a structurally amphipathic arrangement with a notable compliment of hydrophobic residues and shows a strong antimicrobial activity similar in mechanical action on bacterial membranes to that of synthetic and natural AMPs. PACAP 38 and analogs have a strong antimicrobial response against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, and the gram-positive bacteria *Staphylococcus aureus* (Starr *et al*., 2018).

In fish, the constitutive expression of PACAP transcriptional splicing variants (PACAP and PRP) and VIP/PACAP receptors have been demonstrated in different lymphoid organs of rainbow trout (Lugo *et al*., 2011) and brown trout (*Salmo trutta*) (Gorgoglione *et al*., 2015). Also, Gorgoglione *et al*. (2015) revealed, that in brown trout, the two PACAP splicing variants and their receptor genes are modulated in kidney and spleen during viral (Viral Haemorrhagic Septicaemia virus - VHSV) and bacterial (*Y. ruckeri*) septicaemic infections. Both PACAP and PRP demonstrated it physiological role in enhancing growth performance and food intake (Carpio *et al*. 2008; Lugo *et al*., 2010; Lugo *et al*., 2010), and just like in mammals, PACAP has been shown to play a role as a regulator of the teleost fish immune system (Carpio *et al*., 2008). After the administration of recombinant African catfish (*Clarias gariepinus*) PACAP by bath immersion, Carpio *et al*. (2008) saw an increase in lysozyme, nitric oxide synthase-derived metabolites (NOS) and antioxidant defenses in African catfish fry. Lugo *et al*. (2010) evaluated the response of the intraperitoneal injection of recombinant *C. gariepinus* PACAP on juvenile catfish (*C. gariepinus*) and tilapia (*Orechromis niloticus*) immune parameters and saw an increase in the concentration of NOS, lysozyme and total immunoglobulin M (IgM) in serum 24 hours post-administration of the neuropeptide. Also, PACAP immunoreactivity and mRNA expression was observed in peripheral

blood leucocytes of juvenile catfish and tilapia suggesting the presence of an autocrine or/and paracrine mechanism of regulation of the peptide (Lugo *et al*., 2010). Interestingly, Lugo *et al*. (2013) found that PACAP-like peptides are also present in the white shrimp (*Litopenaeus vannamei*), with a conserved role in growth promotion and immune regulation. It has been demonstrated that *C. gariepinus* PACAP-38 has a direct antimicrobial activity against bacteria of importance in aquaculture (Lugo *et al.*, 2019; Semple *et al*., 2019). *In vitro* studies in a monocyte/macrophage-like rainbow trout cell line showed that PACAP-38 exerts immunostimulatory activity on rainbow trout immune cells (Semple *et al*., 2019). Velasquez *et al*. (2020) reported the first evidence of PACAP antiviral activity against VHSV in rainbow trout fry that were treated with synthetic *C. gariepinus* PACAP-38. See **Figure 1.1** for a summary of reported functions of *C. gariepinus* PACAP-38 in fish. Overall, evidence suggests that PACAP-38, and possibly synthetic modified forms of the peptide, constitute a potential alternative for prevention and treatment of microbial infections in aquaculture due to its immunostimulatory and antimicrobial properties.

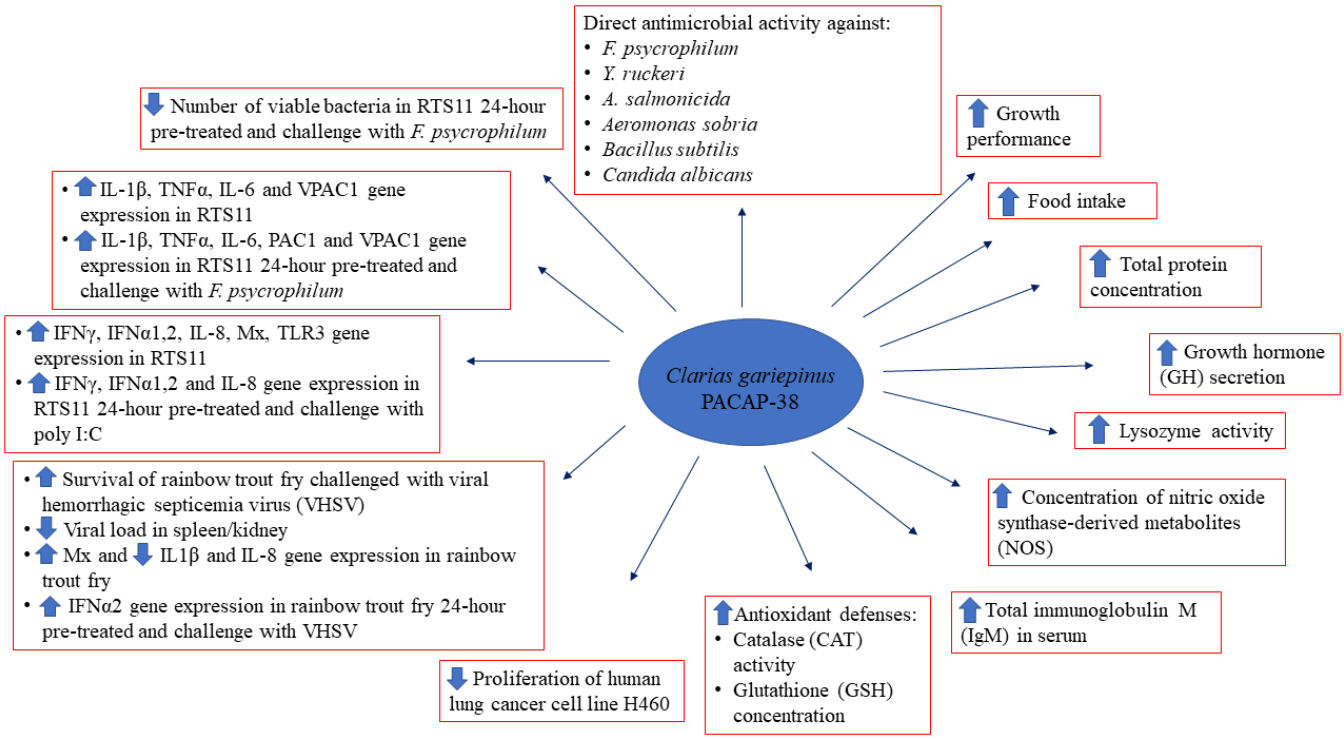

Figure 1.1. Synthetic *C. gariepinus* PACAP-38 reported functions. Summary diagram of reported functions of *C. gariepinus* PACAP-38 on fish growth and immune response (Data obtained from Carpio *et al*. 2008; Lugo *et al*., 2008; Lugo *et al*., 2010; Lugo *et al*., 2010; Lugo *et al*., 2019; Semple *et al*., 2019 and Velasquez *et al*., 2020).

1.5 Thesis structure

The search for novel treatments against bacterial diseases in aquaculture is a priority, with PACAP being a promising alternative. However, studies in fish are still scarce and the gaining of new knowledge that contribute to a better understanding of the mechanisms of pathogenicity and immunity in this commercially important group of animals is crucial. The thesis overall goal was to characterize PACAP's function as an effective antimicrobial and immunostimulatory agent against bacterial pathogens of importance in rainbow trout aquaculture. Thus, the first specific objective was to quantify the expression of PACAP splicing variants and receptors, and cytokines

of importance in the immune response (IL-1β, TNF-α, IL-6, IL-10, IL-2 and IFN-γ) in tissues of immune importance such as spleen, head kidney and skin of naturally infected and clinically healthy rainbow trout from an *Y. ruckeri* outbreak in the Central Highlands of Peru by qRT-PCR, and the second specific objective was to assess the direct antimicrobial properties of PACAP-38, and modified forms of the peptide, against bacterial pathogens of importance in rainbow trout aquaculture and their immunostimulatory properties using the epithelial cell line from rainbow trout gills (RTgill-W1) as a model. The hypothesis was that PACAP exerts a role in the immune response of rainbow trout, as its expression is stimulated in a natural infection by *Y. ruckeri*, and it demonstrates effective antimicrobial and immunostimulatory properties, both in its natural and modified forms, against important bacterial pathogens for this species, with modifications of the peptide improving its functions. The results will provide an insight into the immune response in fish affected by *Y. ruckeri* in a natural set-up and the PACAP's potential properties to prevent and fight bacterial diseases in rainbow trout aquaculture. Chapter 1 provides the literature reviewed, Chapter 2 describes the results of the immune response in *Y. ruckeri* naturally infected rainbow trout, Chapter 3 describes the results of the assessment of PACAP's properties using the RTgill-W1 cell line and Chapter 4 presents the conclusions and future directions of the research.

Chapter 2: Cytokines and PACAP splicing variants and receptors in the immune response of rainbow trout naturally infected with *Yersinia ruckeri*

2.1 Introduction

Rainbow trout is an important fish species for aquaculture in Latin America (Hernández-Rodríguez *et al*., 2001) and North America (Moccia & Bevan, 2017). However, disease outbreaks cause economic losses for the industry, including Enteric Redmouth Disease caused by *Y. ruckeri*, one of the critical diseases in rainbow trout farming (Kumar *et al*., 2015). This disease affects fish at all stages, but it is usually acute in fingerlings and chronic in adult fish (Tobback *et al*., 2007). Mortality after acute infections vary between 30-70% (Altinok, 2004; Horne & Barnes, 1999), and in conditions where *Y. ruckeri* is endemic, a low but persistent mortality can be observed, with losses varying between 10-15% in a productive cycle (Horne & Barnes, 1999). Yersiniosis is transmitted by direct contact between infected and non-infected fish, and the existence of carriers has been demonstrated since up to 25% of a population of rainbow trout can carry *Y. ruckeri* in the intestine, being able to release the bacteria under conditions of stress (Kumar et al., 2015).

Fish immunology is a field that is still being explored and understood. While many experimental studies have evaluated the expression of genes of importance in the immune response of rainbow trout infected with *Y. ruckeri* under controlled conditions (Chettri *et al*., 2012, Raida & Buchmann, 2008, Raida & Buchmann, 2009, Wiens & Vallejo, 2010), the immunological response against natural infections has not yet been described.

To investigate the response in natural infections caused by *Y. ruckeri*, samples from facilities with an outbreak were collected. Expression of cytokine genes including pro-inflammatory cytokines IL-1β, TNFα and IL-6, anti-inflammatory cytokine IL-10 and cytokines involved in adaptive immunity IL-2 and IFN-γ were assessed in relevant tissues such as spleen, head kidney and skin in order to gauge the immune response in symptomatic and asymptomatic individuals.

The neuropeptide PACAP has been shown to play an immunostimulatory role and has remarkably conserved functions in all vertebrates (Cardoso *et al*., 2020) and, similar to mammals, PACAP has been shown to play a role as a regulator of the teleost fish immune system (Carpio *et al*. 2008; Gorgoglione *et al*., 2015). Therefore, the present study also evaluated the expression of genes encoding PACAP splicing variants (PACAP and PRP/PACAP) and PACAP receptor VPAC2 in the natural infection by *Y.ruckeri*.

The hypothesis was that symptomatic rainbow trout naturally infected by *Y. ruckeri* display an increase in the expression of cytokine genes of importance in the immune response (IL-1β, TNFα, IL-6, IL-10, IL-2 and IFN-γ) and in the expression of PACAP and receptor genes, compared to asymptomatic fish. Studies aimed at understanding the immune response against diseases in fish are important to generate new knowledge related to pathogenesis and immunity against infections, also to develop more effective treatment and vaccination alternatives, which will contribute to the reduction of aquatic diseases and the impact they have on rainbow trout farming.

2.2 Materials and methods

2.2.1 Fish

Samples were taken from 67 rainbow trout with an approximate weight of 100-250 grams. These trout came from semi-intensive fish farms of the Central Highlands of Peru (Jauja, Huaraz, Huancayo, Huaura and Concepción provinces) that presented disease outbreaks suggestive of Yersiniosis during the period February - June 2018, summer - fall seasons. Temperature influences the development and severity of yersiniosis disease (Altinok, 2004), optimal water temperature to produce rainbow trout oscillates between 11 and 16°C, higher temperatures increase the risk of disease (PRODUCE, 2014). Fish were separated in two groups, the first group consisted of 32 trout naturally infected by *Y. ruckeri*, which presented clinical signs suggestive of Yersiniosis (lethargy, swimming near the surface, exophthalmia, melanosis, distended abdomen and subcutaneous hemorrhages in the oral cavity and base of fins), and whose molecular detection of the pathogen is detailed in section 2.2.3. The second group included 35 clinically healthy trout (without evidence of lesions or changes in behavior) from the same pools where disease outbreaks occurred.

2.2.2 Sample collection

Fish were euthanized with an overdose (100 mg/l) of tricaine methanesulfonate (MS-222, Sigma), followed by severing the spinal nerve to ensure mortality (AVMA, 2013; CCAC, 2010; Roberts & Syme, 2016). After an evaluation for the presence of external lesions (exophthalmia, melanosis, abdominal distension and subcutaneous hemorrhages in the oral cavity and base of fins), as shown in **Figure 2.1**, fish were cleaned with 70° ethanol before proceeding with the dissection according to the protocol described by Meyers (2009).

A **B**

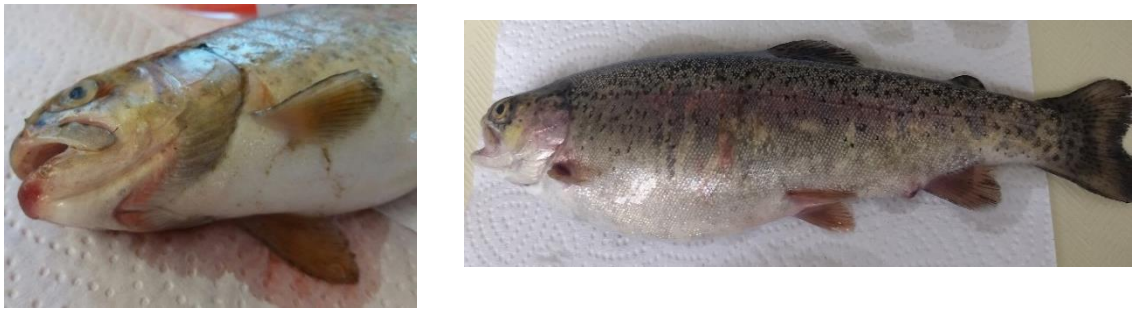

Figure 2.1. **Clinical signs related to *Y. ruckeri* infection**. Presence of subcutaneous hemorrhages in the oral cavity (**A**) and presence of abdominal distension (**B**).

Blood samples (1 ml) were collected from the caudal vein using a 21-gauge needle in a 3 ml syringe and transferred to 1.5 ml tubes. Fish with clinical signs of disease were assessed for internal lesions such as petechiae in internal organs, enlarged and darkened spleen, and intestinal inflammation, as shown in **Figure 2.2**. To isolate the bacteria in these fish, samples were taken aseptically from spleen and head kidney and cultured in trypticase soy agar (TSA). For all trout, tissue samples were obtained from spleen, head kidney and skin, and placed into 1.5 ml tubes containing 1 ml of RNA Later (Sigma) for future RNA and protein extraction. Samples were transported in ice to the Animal Parasitology Laboratory – Faculty of Veterinary Medicine and Zootechnics at Universidad Peruana Cayetano Heredia, Lima-Peru where the inoculated plates were placed in an IRE-160 incubator (RAYPA) and the tissue samples in RNA later were stored at -70 ° C. Blood samples were centrifuged at 3500 rpm, using a Spectrafuge ™ 16M High Speed microcentrifuge (Labnet International) to obtain the serum that was transferred and kept in new 1.5 ml tubes at -70^{o}C until further processing. This research was approved by the Institutional Ethics Committee for the Use

of Animals (CIEA) of the Universidad Peruana Cayetano Heredia as indicated in the certificate 049-09-18.

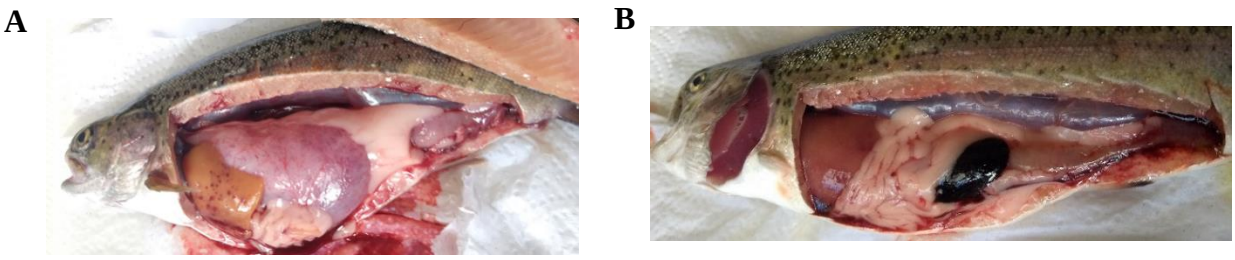

Figure 2.2 Internal lesions related to *Y. ruckeri* infection. Presence of petechiae in liver (**A**) and presence of enlarged spleen (**B**).

2.2.3 Identification of *Y. ruckeri*

Inoculated TSA plates were incubated at 25°C for 24 hours. Subsequently, the identification of the colonies was carried out considering their round shape, small size, bright cream color and defined regular borders, followed by gram staining (negative bacilli) and Catalase (+) and Oxidase (-) tests. A presumptive colony was taken for each sampled fish, they were enriched in brain heart infusion (BHI) broth at 25 ° C for 24 hours, followed by centrifugation to obtain a bacterial pellet. Bacterial DNA was extracted using the commercial Wizard® Genomic DNA Purification kit (Promega), according to the manufacturer's instructions. For molecular confirmation of *Y. ruckeri* infection, the polymerase chain reaction (PCR) protocol established by Gibello *et al*. (1999) for the 16S rRNA gene was followed, detecting the amplicon of the expected size of 575 bp (**Figure 2.3**). Each 20 µl PCR reaction included 1 µl of DNA, a concentration of 10 pmol for each primer, 2mM of each deoxynucleoside triphosphate (dNTP), 10 µl of Taq polymerase buffer, and 5 U/µl of Taq polymerase. Primers used are shown in **Table 2.1**. The Mastercycler ™ Nexus thermal cycler (Eppendorf) was used under the following conditions: initial denaturation for 5 minutes at 92^{o}C,

followed by 35 cycles for 1 minute at 92ºC, annealing at 60ºC for 1 minute, extension at 72ºC for 1 minute and final extension at 72ºC for 5 minutes. A positive control of *Y. ruckeri* ATTC® 29473 and a negative control constituted of DNase free water were included. Electrophoresis at 80 V/cm for 90 minutes in 1.2% agarose gel (Calbiochem) using buffer TAE 1X was carried out in the Enduro ™ Gel Systems and Power Supplies equipment (Labnet International), including the Perfect DNA™ 50 bp Ladder (Novagen). The gel was dyed with 0.5 μg/mL of ethidium bromide (Merck Millipore, Germany) for 20 minutes, and bands were observed in the MiniBIS transilluminator (DNR Bio-Imaging Systems).

Table 2.1 Primers used for the detection of 16S rRNA *Y. ruckeri* gene. Direction of the primers are presented as F and R representing forward and reverse primers, respectively.

Gene	Primer Sequence (5' to 3')	Amplicon Length (bp)	Accession Number	Reference
Y. ruckeri 16S rRNA	F: GCGAGGAGGAAGGGTTAAGTG R: GAAGGCACCAAGGCATCTCTG	575	EU401667	Gibello *et al.* (1999)

Figure 2.3. **Molecular identification of *Y. ruckeri* 16S rRNA gene by PCR.** Amplification products of 575 bp are shown. Lane 1 and 10: 50 bp DNA molecular weight marker. Lane 2 to 7: positive samples. Lane 8: positive control (*Y. ruckeri* ATTC® 29473). Lane 9: negative control (DNase free water).

2.2.4 RNA Extraction and cDNA Synthesis

Samples in RNA later stored in dry ice (-70°C) were sent to Dr. Brian Dixon's Laboratory at the Department of Biology – University of Waterloo - Canada, for the following procedures. Ten micrograms of tissue samples (spleen, head kidney and skin) were transferred to 5 ml eppendorf tubes containing 1 ml de TRIzolTM Reagent (Invitrogen) and the manufacturer's protocol was

followed. RNA samples were quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments). For the next step, 5 µg of RNA were treated with 10 units of DNase I (Thermo Fisher Scientific) to remove any genomic DNA that could be contaminating the sample at 25°C for 30 minutes, the enzyme was then removed using a column from an RNA purification kit (Norgen Biotek). RNA was quantified again and stored at −80◦C. Five hundred nanograms of total RNA were used to synthesize cDNA with a qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Synthesized cDNA samples were maintained at −80◦C until further use.

2.2.5 Gene Expression by qRT-PCR

The relative expression of cytokines genes of importance in the immune response and PACAP splicing variants and receptor genes in *Y. ruckeri* naturally infected fish and clinically healthy fish was assessed. Each 10 µl qPCR reaction included 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water for cytokine genes and diluted 1:5 in RNase free water for PACAP splicing variants and receptor genes), 2x SYBR® Green qPCR Master Mix (Wisent Bioproducts), and the correspondent forward and reverse primers (Sigma-Aldrich) for a final concentration of 0.25 µM. Reactions were run in the LightCycler® 480 System (Roche). For each plate, all samples were run in triplicates, and a calibrator and a non-template control were included. The program consisted of pre-incubation for 2 minutes at 95◦C, 40 cycles of denaturation for 10 seconds at 95◦C, annealing for 5 seconds at 60◦C and finally an extension for 8 seconds at 72◦C. A melting curve for each plate was used to verify the amplification of a unique product, this was obtained by reading the fluorescence at each grade between 65 to 97◦C every 5 seconds. Elongation factor – 1 alpha (EF1α) was used as the reference gene, which CT values mean ± standard deviation of the mean

were 18.02 ± 0.8 for spleen, 17.88 ± 0.95 for head kidney and 19.35 ± 0.91 for skin. Each gene expression was normalized to the expression of the reference gene, and the levels of expression were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Data was expressed as fold change relative to the expression level in the clinically healthy trout. Primers used are shown in **Table 2.2**.

Table 2.2 qRT-PCR primers used in Chapter 2. Direction of the primers are presented as F and R representing forward and reverse primers, respectively.

Gene	Primer Sequence (5' to 3')	Amplicon Length (bp)	Accession Number	Reference
IL-1β	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	155	AJ298294.1	Semple *et al*. (2018)
TNFα	F: GTGCAAAAGATACCCACC R: CACTGCACGGTGTCAG	108	AJ278085.1	Semple *et al*. (2018)
IL-6	F: CTTCTACACGCTATCTCTCACTC R: CGTCTGTCCCGAGCT	128	NM_001124657.1	Semple *et al*. (2018)
IL-10	F: GCCTTCTCCACCATCAGAGAC R: GATGCTGTCCATAGCGTGAC	120	NM_001245099.1	Inoue *et al*. (2005)
IL-2	F: AGGAGAACAGCACAACGGAC R: CTTAGACGCTTTGCAGCATGA	124	NM_001164065.2	Diaz-Rosales *et al*. (2009)
IFN-γ	F: GAAGGCTCTGTCCGAGTTCA R: TGTGTGATTTGAGCCTCTGG	119	NM_001124620.1	Chaves-Pozo *et al*. (2010)
PACAP	F: AAATTGCTATAAGAAGTCCCCCATC R: GTATTTCTTGACTGCCATTTGCTTT	182	AF343977	Lugo *et al*. (2011)
PRP/PACAP	F: CTGGGTCAGTTATCAGCAAGAAAT R: TGTCTATACCTTTTCCCAAGGACTG	200	AF343976	Lugo *et al*. (2011)
VPAC2	F: CTCACTGTGACACGACAGTGATTCC R: GCTTTTCAGTTTCACCTCAACTTGT	135	AY706217	Lugo *et al*. (2011)
EF1α	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	134	NM_001124339	Semple *et al*. (2018)

2.2.6 Protein Isolation

For protein isolation of tissue samples, 80-100 mg of each tissue was transferred to a 5 ml Eppendorf tube containing 1 ml of T-PER (Thermo Fisher Scientific) complemented with protease inhibitor (Thermo Fisher Scientific) for posterior homogenization with the Omni Tissue Homogenizer (Omni International). Next, samples were centrifuged at 10,000 g for 5 minutes and the supernatant was collected and quantified with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. For serum samples, serums were diluted 1:3 in phosphate buffered saline (PBS, Gibco) and two extractions with chloroform (1: 1, V: V) were carried out. Following centrifugation, the upper and aqueous phase was stored. Protein samples were maintained at -80°C until further use.

2.2.7 Protein Expression Profile of Cytokines by Western Blot

Protein samples were mixed with 4× Laemmli sample buffer (BioRad) with β-mercaptoethanol, according to the manufacturer's instructions. Samples were subjected to electrophoresis in a 15% acrylamide gel to separate the protein, including the PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific). After this procedure, proteins were transferred to a nitrocellulose membrane through electroblotting at 0.15 Amps for 35 minutes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membrane was stained with Ponceau Stain (Sigma) for 5 minutes to verify equal protein loading per gel lane. After this, 3 washes were carried out with Tris Buffered Saline Solution with Tween (TBS-T) for 5 minutes each followed by membrane incubation in 5% skim milk in TBS-T at room temperature for one hour to block the protein-free spaces in the membrane. Blots were washed three times for five minutes each using TBS-T and then incubated in rotation overnight at 4 °C with an appropriate dilution of the

correspondent primary antibody for IL-1β, TNFα or IL-6 in 5% skim milk in TBS-T solution. For the serum samples, the anti IL-1β antibody made in chicken (Somru Bioscience) 1/300 was used as the primary antibody for IL-1β, the anti TNFα antibody made in rabbit (Cedarlane) 1/500 was used for TNFα, and for IL-6, the anti IL-6 antibody made in chicken (Somru Bioscience) 1/300 was used. For the spleen samples IL-1β blot, the anti IL-1β antibody made in chicken (Cedarlane) 1/1000 was used as the primary antibody. At the next day, blots were washed three-times for 5 minutes with TBS-T and incubated for one hour at room temperature with the correspondent secondary antibody raised against the appropriate animal and conjugated to alkaline phosphatase enzyme diluted in 5% skim milk solution. Subsequently, the membranes were washed three times with TBS-T and incubated with a detection solution containing the substrates of the alkaline phosphatase enzyme: nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indole-phosphate (BCIP) (Sigma) to visualize the proteins detected by the specific antibodies. Upon visualizing the expected bands, the enzymatic reaction was stopped by removing the detection solution and rinsing with distilled water. The membranes were allowed to dry at room temperature and then were digitized in the ChemiDoc imaging equipment (BioRad).

2.2.8. Quantifying Cytokines Using a Sandwich ELISA Assay

Capture and detection antibodies to specific cytokines IL-1β and IFN-γ previously validated via western blot and sandwich ELISA in Dr. Dixon's Laboratory were used for cytokines quantification. The protocol was performed as follows: 100 µl of capture antibody against rainbow trout cytokines diluted in carbonate/bicarbonate coating buffer were incubated for four hours at room temperature in the test wells of a 96 well plate. For IL-1β, the anti IL-1β antibody made in chicken (Cedarlane) 1/300 was used, and for IFN-γ, the anti IFN-γ antibody made in chicken

(Cedarlane) 1/1000 was used. After a three-time wash with TBS-T, a blocking solution of 300 µl of 5% skim milk in TBS was added to each well and the plate was incubated at 37ºC for one hour. After another three-time wash with TBS-T, 75 µl of a 1% PEG in General assay diluent solution (Immunochemistry Technologies LLC) was added to each well, followed by the addition of 100 µl of each sample (diluted 1:3 in PBS), standard or blank in triplicate in their respective wells for an overnight incubation at 4ºC. The next day, the plate was washed again three times with TBS-T before 100 µl of the detection antibody against rainbow trout cytokines (IL-1β or IFN-γ) in a 1% PEG 8000 diluted in 5% skim milk solution was added. The plate was incubated for 3 hours at room temperature and washed three times with TBS-T, followed by the incubation with 100 µl of the corresponding secondary antibody conjugated to biotin in a 5% skim milk solution for one hour in the dark. Wells were washed three times with TBS-T and 100 µl of a solution of Streptavidin-HRP diluted in 5% skim milk was added and incubated for one hour in dark. After a last four-time wash with TBS-T, 100 µl of TMB sens was be added to each well and the plate was incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 100 µl of 0.3 M of H_2SO_4 and the optical density was read at a wavelength of 450 nm using the Synergy H1 plate reader (BioTek Instruments).

2.2.9 Data Analysis

Data handling and graphic representation were performed using Office Excel 2010 (Microsoft Corporation) and GraphPad Prism version 6 (GraphPad Software Inc.), respectively. All statistical analyses were performed in the statistical software Statistica version 7 (StatSoft). A normal distribution and variance homogeneity were verified, followed by an unpaired two tailed Student's t-test to determine statistically significant differences in gene expression between infected and

clinically healthy trout. When variances were significantly different, a Mann–Whitney U test was performed. Data was shown as mean ± standard deviation, and differences between groups were considered significant, very significant and highly significant when the probability values resulting from the respective test were less than or equal to 0.05, 0.01 and 0.001 respectively.

2.3 Results

2.3.1 Cytokine expression in *Y. ruckeri* naturally infected trout and clinically healthy trout

Relative expression of cytokine genes of importance in the immune response including pro-inflammatory cytokines IL-1β, TNFα and IL-6, anti-inflammatory cytokine IL-10 and cytokines involved in the adaptive immunity IL-2 and IFN-γ in *Y. ruckeri* naturally infected fish and clinically healthy fish were determined by qRT-PCR.

Pro-inflammatory cytokines IL-1β, TNFα and IL-6 showed highly significantly up-regulation ($p \leq 0.001$) in all three tissues assessed in *Y. ruckeri* naturally infected trout compare to the clinically healthy trout. The greatest increases were observed in the relative expression of the genes encoding IL-6 and IL-1β in spleen, with a 318-fold and 264-fold increase, respectively, in *Y. ruckeri* naturally infected trout over the clinically healthy trout.

Pro-inflammatory cytokine gene IL-1β showed significantly up-regulation in all three tested tissues, with 264-fold, 253-fold and 53.5-fold increase in spleen, head kidney and skin of *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout, respectively, as shown in **Figure 2.4**. Moreover, western blots were performed to determine the presence of IL-1β cytokine protein in serum and spleen using four samples from *Y. ruckeri* naturally infected trout

and four samples from clinically healthy trout. As shown in **Figure 2.5A,** two protein bands of approximate 25 kDa in size were found in the serum of *Y. ruckeri* naturally infected trout that showed clinical signs of disease, which could correspond to the precursor IL-1β protein. These bands were not seen in the clinically healthy trout serum. The western blot of spleen samples (**Figure 2.5B**) showed protein bands between 25-35 kDa present in *Y. ruckeri* naturally infected trout, and not in clinically healthy trout, that could correspond to precursor IL-1β protein. Also, bands of 15 kDa approximately were also present in *Y. ruckeri* naturally infected trout, and not in clinically healthy trout, which could correspond to the mature IL-1β protein. To quantify the protein concentration, an ELISA for IL-1β in spleen was executed, showing a very significantly increase of IL-1β in *Y. ruckeri* naturally infected trout with a mean of 392 pg/mL compared to the clinically healthy trout with a mean of 23 pg/mL (**Figure 2.5C**), which agrees with the results at the transcript level.

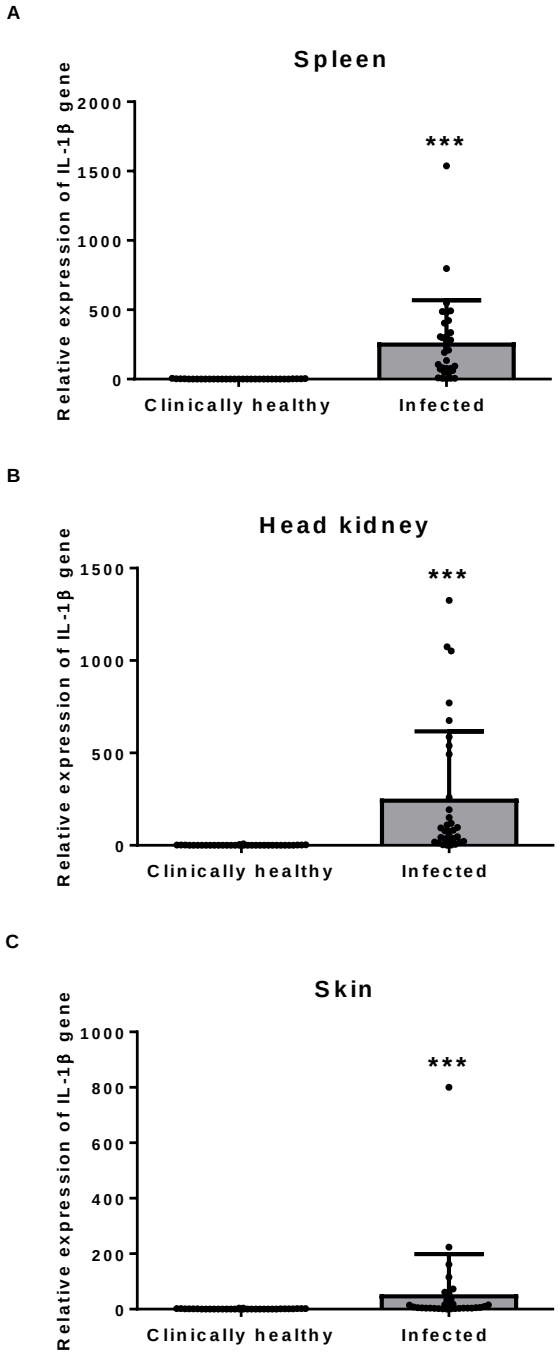

Figure 2.4. Relative expression of IL-1β cytokine gene in spleen, head kidney and skin tissues. Relative expression of IL-1β gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = 32 infected and a minimum of 33 clinically healthy trout.

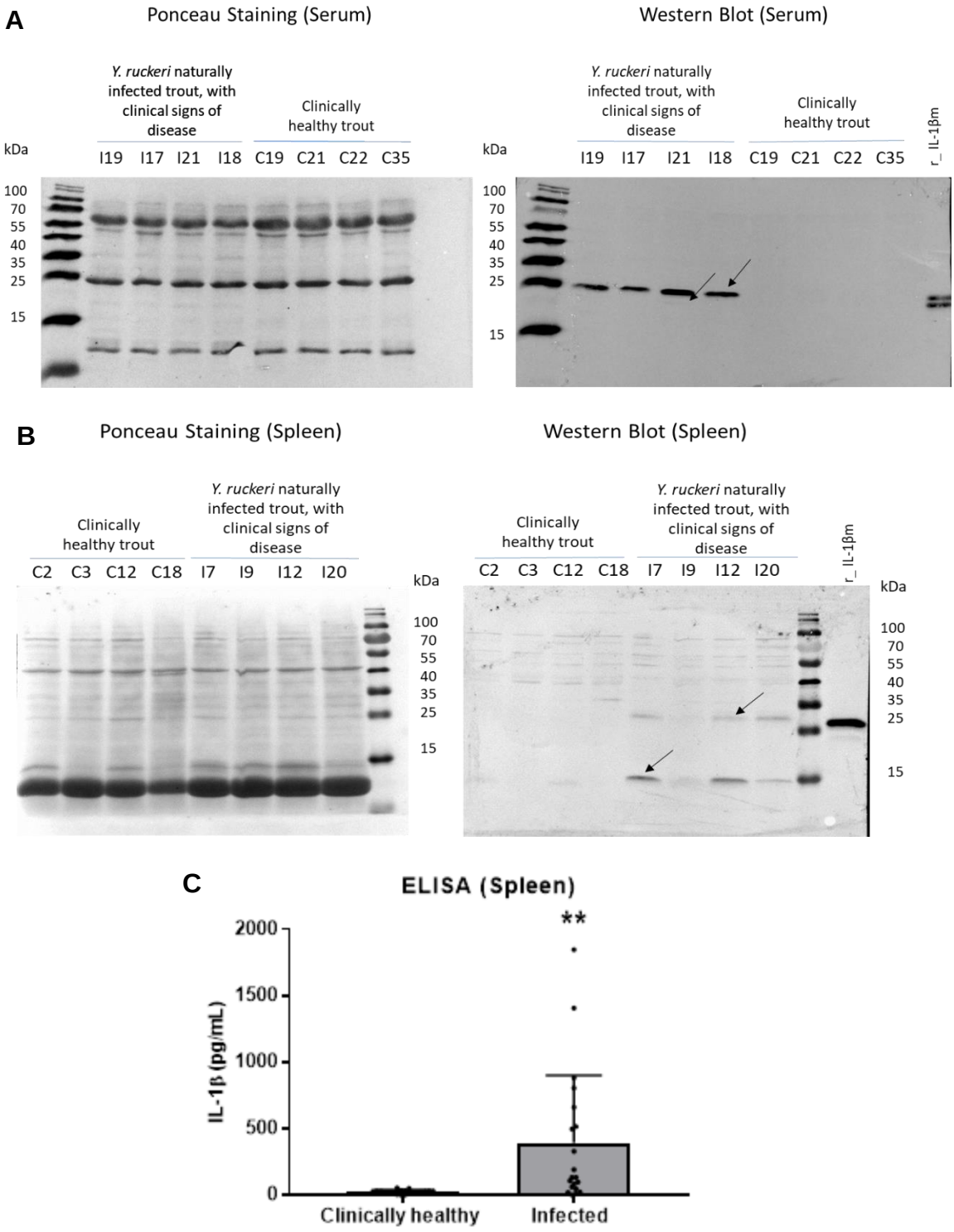

Figure 2.5. IL-1β protein presence and quantification. Determination of IL-1β protein presence in serum samples of trout naturally infected with *Y. ruckeri* that showed clinical signs of disease by Western blot (A). Determination of IL-1β protein presence in spleen samples of trout naturally infected with *Y. ruckeri* that showed clinical signs of disease by Western blot (B). Membrane stained with Ponceau stain shows the same protein load per lane was observed. A recombinant

fragment of IL-1β of 22.4 kDa was used as a positive control in the last lane. Quantification of IL-1β protein in spleen of *Y. ruckeri* naturally infected trout and clinically healthy trout by ELISA. Values are shown as the mean ± standard deviation of the mean. N = 20 samples per group (C).

For the gene encoding TNFα, up-regulation was also found in spleen with a 13.5-fold increase, followed by skin and head kidney with seven-fold and five-fold increase, respectively, in *Y. ruckeri* naturally infected trout relative to clinically healthy trout (**Figure 2.6**). A western blot was also performed to determine the presence of TNFα cytokine in serum using four samples from *Y. ruckeri* naturally infected trout and four samples from clinically healthy trout. As shown in **Figure 2.7**, indicated by the molecular weight marker, two bands greater than 25 kDa and lesser than 35 kDa were observed in the serum of trout naturally infected with *Y. ruckeri* that showed clinical signs of disease, which could correspond to the precursor protein. Likewise, bands with a size below 15 kDa were also obtained that could correspond to the mature protein. These bands were not seen in the serum of clinically healthy trout.

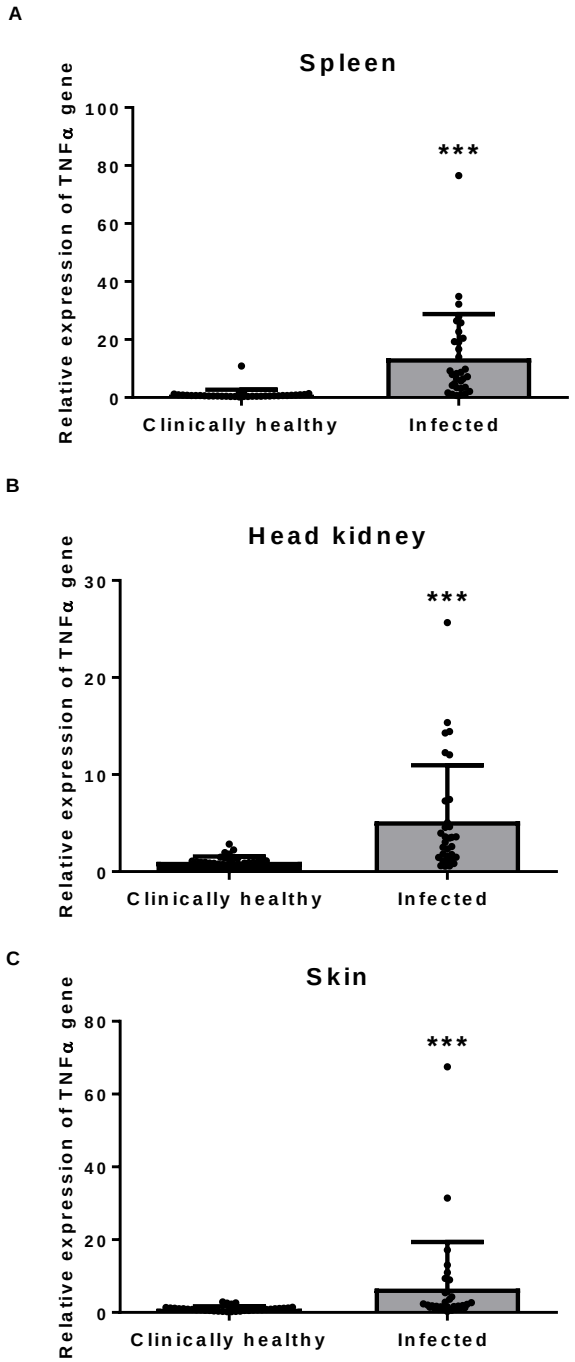

Figure 2.6. Relative expression of TNF*α* cytokine gene in spleen, head kidney and skin tissues. Relative expression of TNFα gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (fold change) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = 32 infected and a minimum of 34 clinically healthy trout.

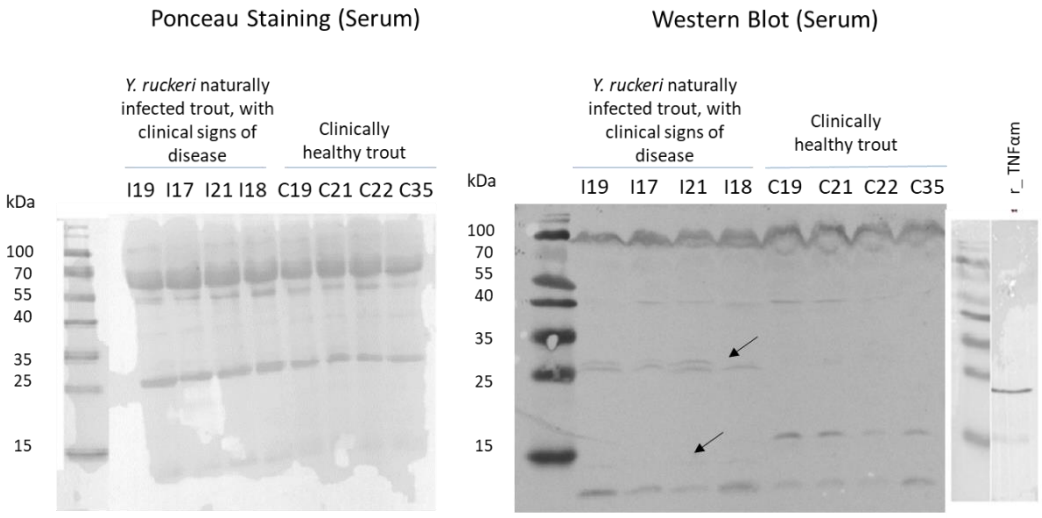

Figure 2.7. TNFα protein presence determination by Western blot. Determination of TNFα protein presence in serum samples of trout naturally infected with *Y. ruckeri* that showed clinical signs of disease by Western blot. Membrane stained with Ponceau stain shows the same protein load per lane was observed. A recombinant fragment of TNFα of 21.7 kDa was used as a positive control in the last lane.

As shown in **Figure 2.8**, the IL-6 cytokine encoding gene showed the highest up-regulation with a 318-fold increase in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout. However, in the case of head kidney and skin, although expression of the gene of interest was obtained in the samples from *Y. ruckeri* naturally infected trout, the same did not occur in the clinically healthy trout so a CT value of 35 was assigned to these trout in order to use the $2^{-\Delta\Delta CT}$ method for comparisons. With this value, significant up-regulation of the cytokine gene was observed in head kidney and skin tissues (97-fold and 199-fold increase, respectively) of *Y. ruckeri* naturally infected trout compared to clinically healthy trout. In a western blot carried out for IL-6 cytokine using serum samples from four *Y. ruckeri* naturally infected trout and four samples from clinically healthy trout, two protein bands between 10 and 15 kDa in size were observed in the

serum of trout with natural infection by *Y. ruckeri* that showed clinical signs of disease, which could correspond to IL-6 protein, although these do not correspond to the expected predicted size of 22.36 kDa for IL-6 cytokine in rainbow trout according to the limited literature on the subject (Iliev *et al*., 2007). These bands were not present in the serum of clinically healthy trout.

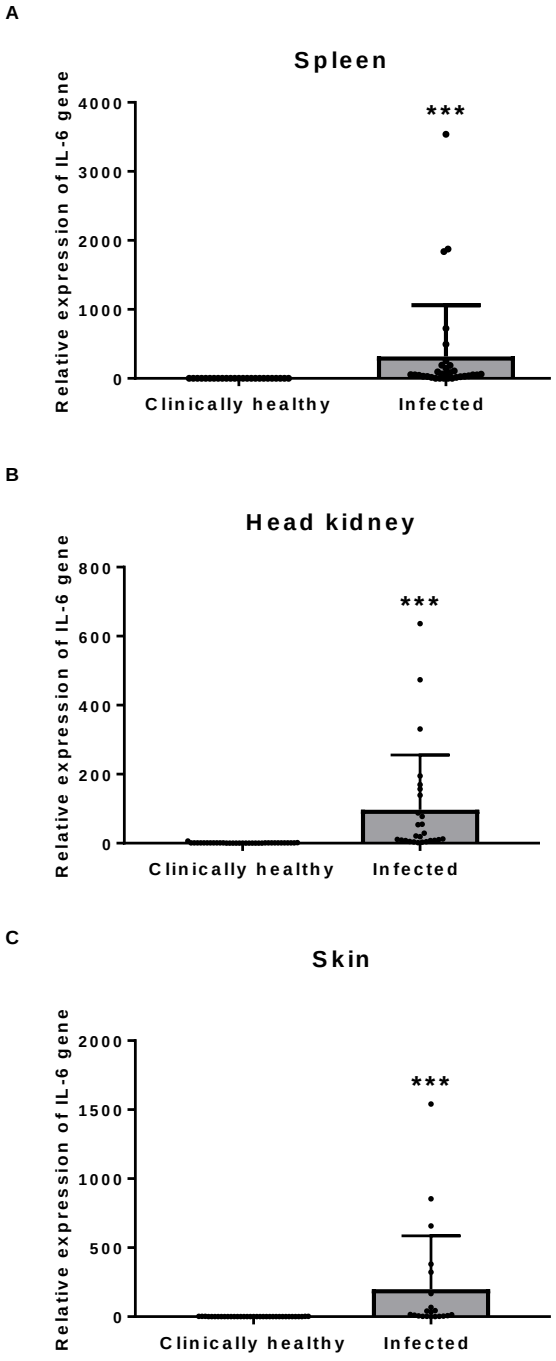

Figure 2.8. Relative expression of IL-6 cytokine gene in spleen, head kidney and skin tissues. Relative expression of IL-6 gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. For head kidney and skin tissues in clinically healthy trout, no expression of the gene was detected so CT values of 35 were given to each sample for the analysis.

Values are shown as the mean ± standard deviation of the mean. N = A minimum of 21 infected and a minimum of 25 clinically healthy trout.

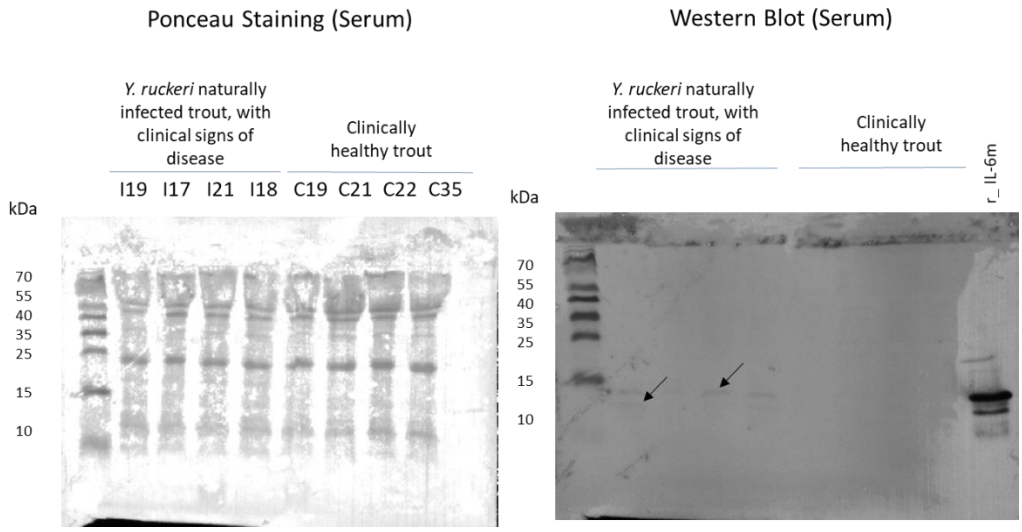

Figure 2.9. IL-6 protein presence determination by Western blot. Determination of IL-6 protein presence in serum samples of trout naturally infected with *Y. ruckeri* that showed clinical signs of disease by Western blot. Membrane stained with Ponceau stain shows the same protein load per lane was observed. A recombinant fragment of IL-6 of 19 kDa was used as a positive control in the last lane.

Relative expression of the gene encoding anti-inflammatory cytokine IL-10 was also assessed, showing also highly significantly up-regulation ($p \leq 0.001$) in all three tissues examined in *Y. ruckeri* naturally infected trout compare to clinically healthy trout, with an increase of 113-fold, 147-fold and five-fold in spleen, head kidney and skin, respectively (**Figure 2.10**).

For the gene encoding IL-2, as shown in **Figure 2.11,** no significant difference was found in the gene expression in trout naturally infected with *Y. ruckeri* in relation to clinically healthy trout in any of the tissues evaluated.

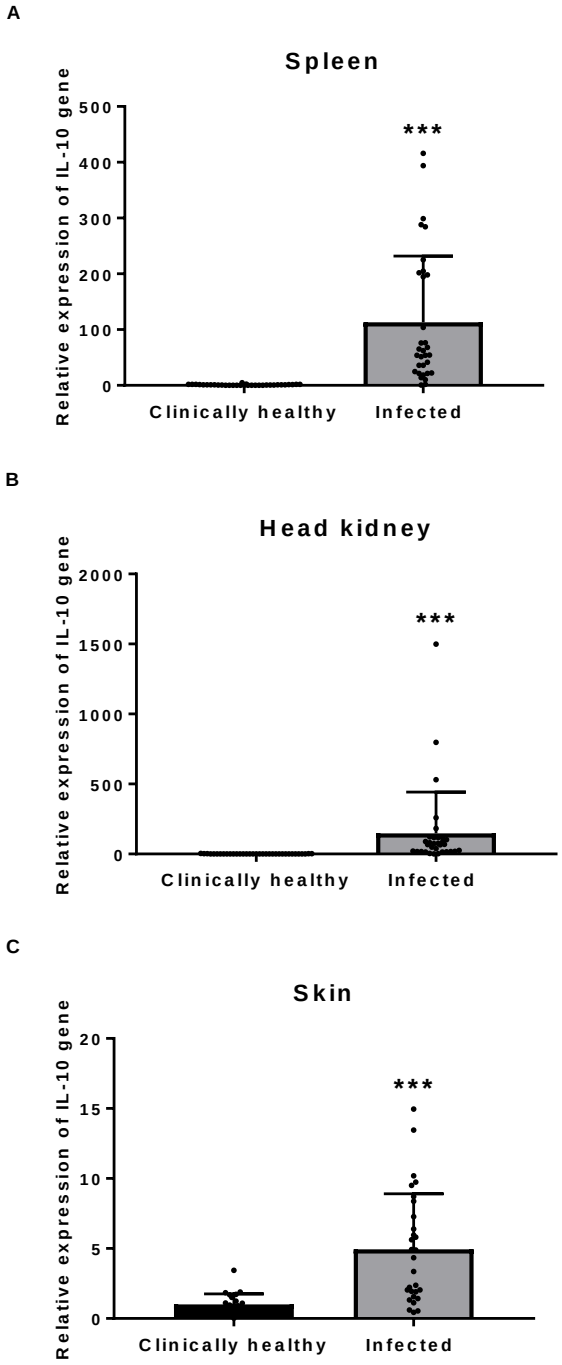

Figure 2.10. Relative expression of IL-10 cytokine gene in spleen, head kidney and skin tissues. Relative expression of IL-10 gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = A minimum of 29 infected and a minimum of 23 clinically healthy trout.

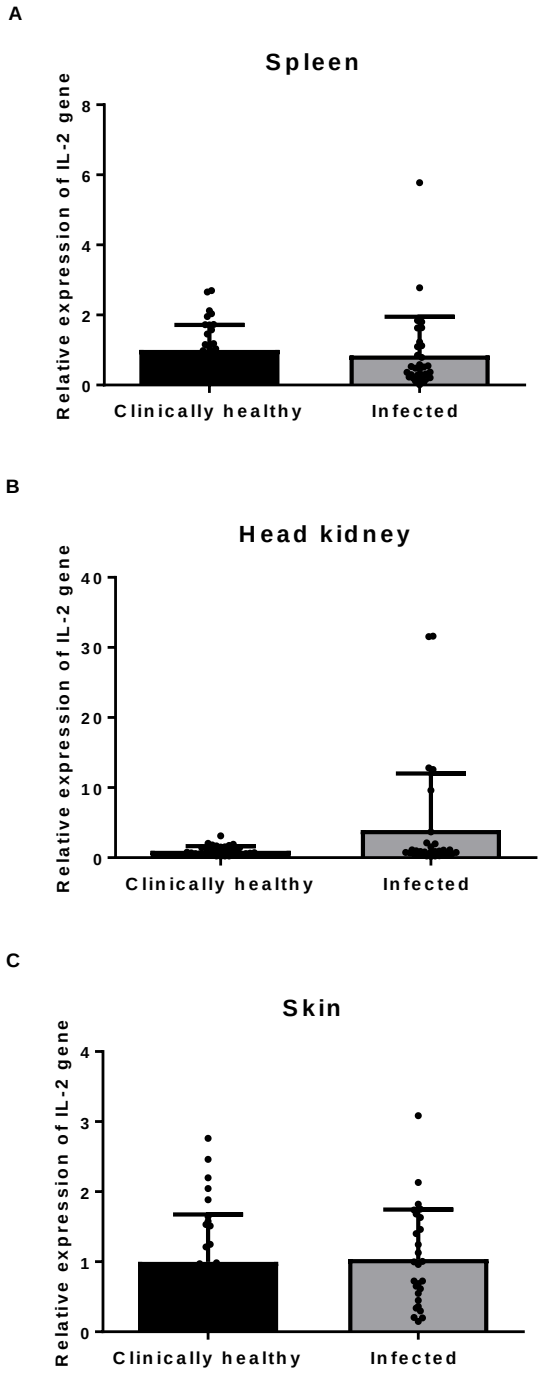

Figure 2.11. Relative expression of IL-2 cytokine gene in spleen, head kidney and skin tissues. Relative expression of IL-2 gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = A minimum of 27 infected and a minimum of 34 clinically healthy trout.

As shown in **Figure 2.12**, the IFN-γ cytokine encoding gene also showed highly significantly up-regulation ($p \leq 0.001$) in all three evaluated tissues, with seven-fold, 43.5-fold and four-fold increase in spleen, head kidney and skin, respectively, in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout. Moreover, a qELISA for IFN-γ in spleen was performed to quantify the protein concentration, showing a very significantly increase of IFN-γ in *Y. ruckeri* naturally infected trout with a mean of 122 pg/mL compared to the clinically healthy trout with a mean of 50 pg/mL (**Figure 2.13**), which agrees with the results at the transcript level.

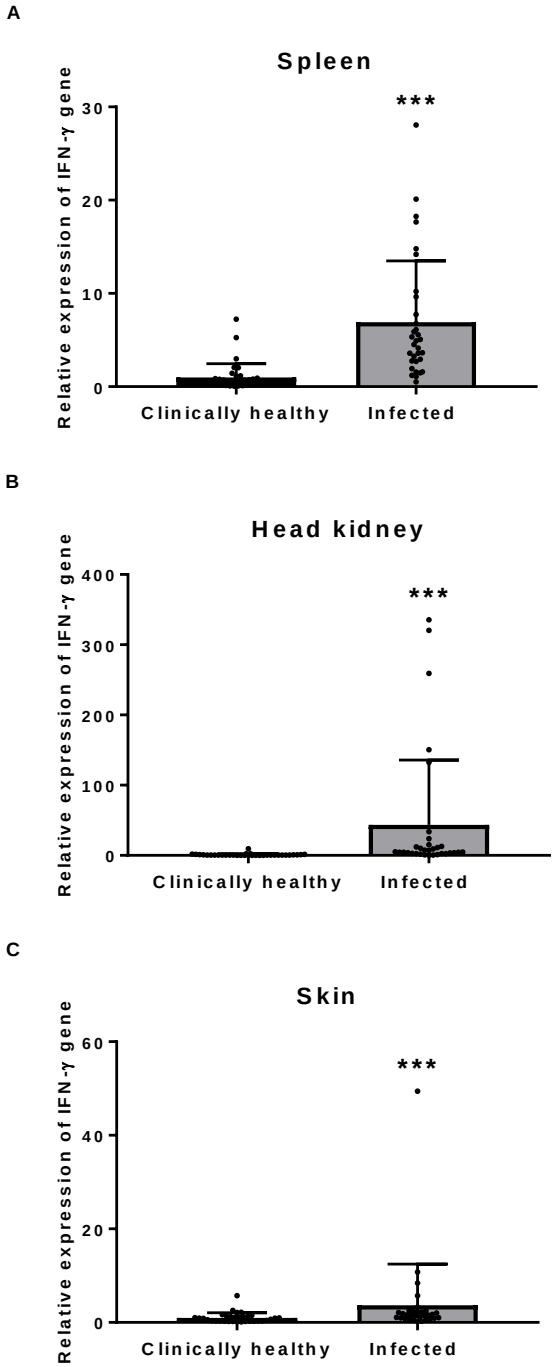

Figure 2.12. Relative expression of IFN-γ cytokine gene in spleen, head kidney and skin tissues. Relative expression of IFN-γ gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = A minimum of 31 infected and a minimum of 33 clinically healthy trout.

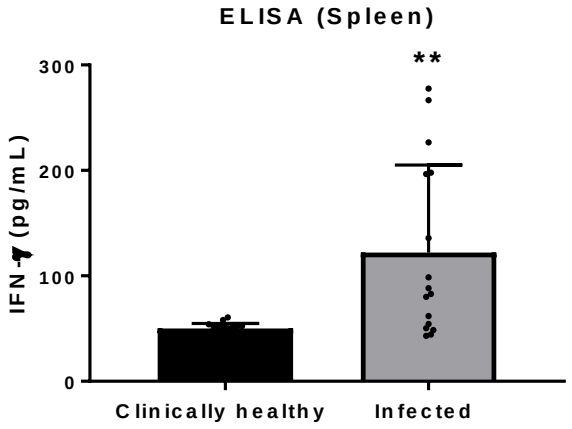

Figure 2.13. IFN-γ protein quantification. Quantification of IFN-γ protein in spleen of *Y. ruckeri* naturally infected trout and clinically healthy trout by qELISA. Values are shown as the mean ± standard deviation of the mean. N = 16 infected and 17 clinically healthy trout.

2.3.2 PACAP splicing variants and receptor gene expression in *Y. ruckeri* naturally infected trout and clinically healthy trout

Relative expression of genes encoding PACAP splicing variants (PACAP and PRP/PACAP) and PACAP receptor VPAC2 were also assessed in *Y. ruckeri* naturally infected fish and clinically healthy fish by qRT-PCR. PACAP gene showed highly significantly up-regulation in spleen with a 36-fold increase in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout (**Figure 2.14A**). No significant differences were found in head kidney (**Figure 2.14B**) and skin (**Figure 2.14C**). For the gene encoding PRP/PACAP, as shown in **Figure 2.14D**, very significant up-regulation was seen in spleen with an increase of 11-fold, however, no expression of the gene was found in head kidney and skin of *Y. ruckeri* naturally infected fish and clinically healthy fish.

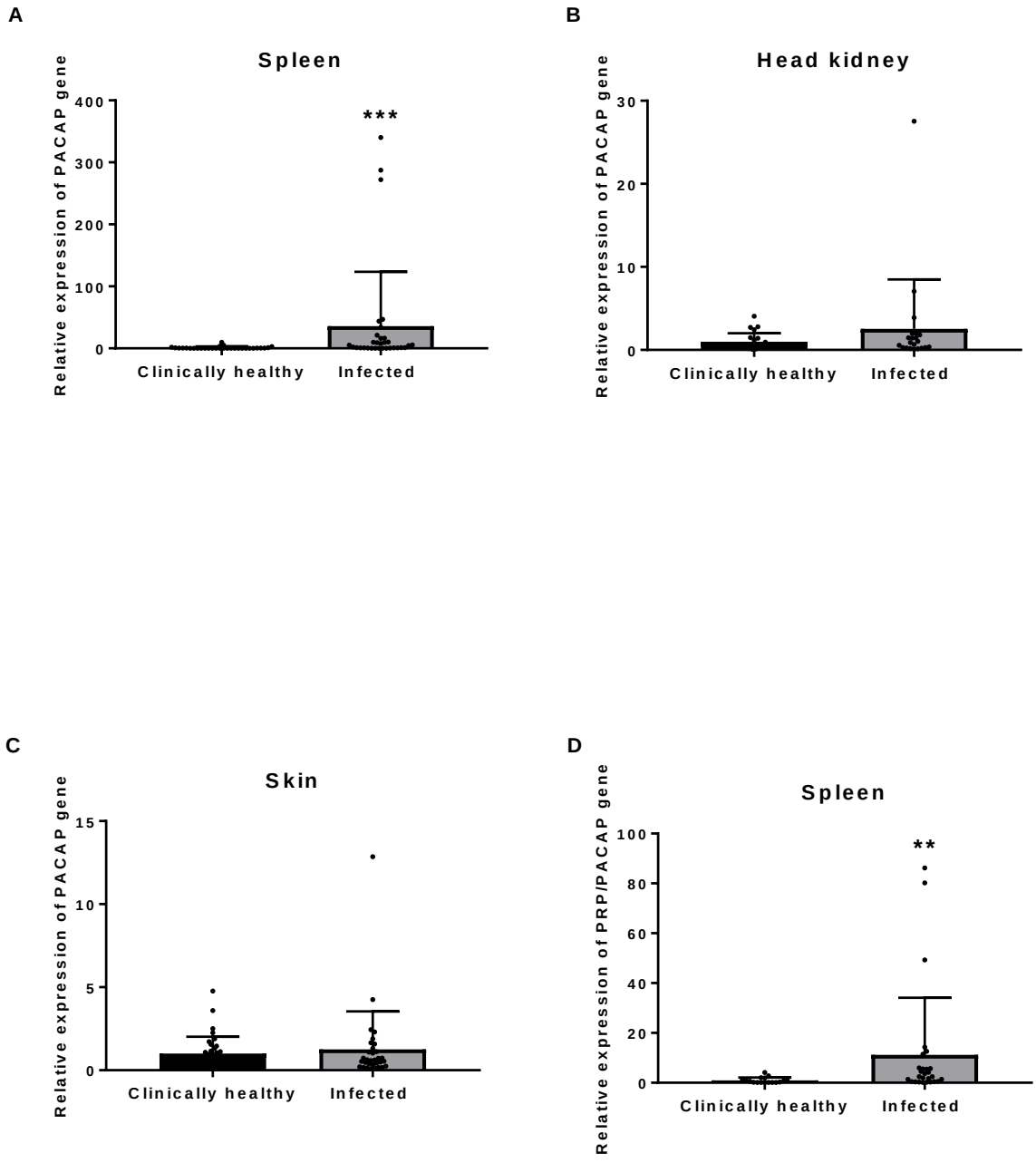

Figure 2.14. Relative expression of PACAP and PRP/PACAP genes in spleen, head kidney and skin tissues. Relative expression of PACAP gene in spleen (A), head kidney (B) and skin (C) and PRP/PACAP gene in spleen (D) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = A minimum of 21 infected and a minimum of 17 clinically healthy trout.

Relative expression of the gene encoding the VCAP2 receptor was significantly up-regulated in spleen and head kidney with an increase of four-fold and 83-fold, respectively, in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout (**Figure 2.15A-B**). No significant differences were found in skin for this gene (**Figure 2.15C**). .

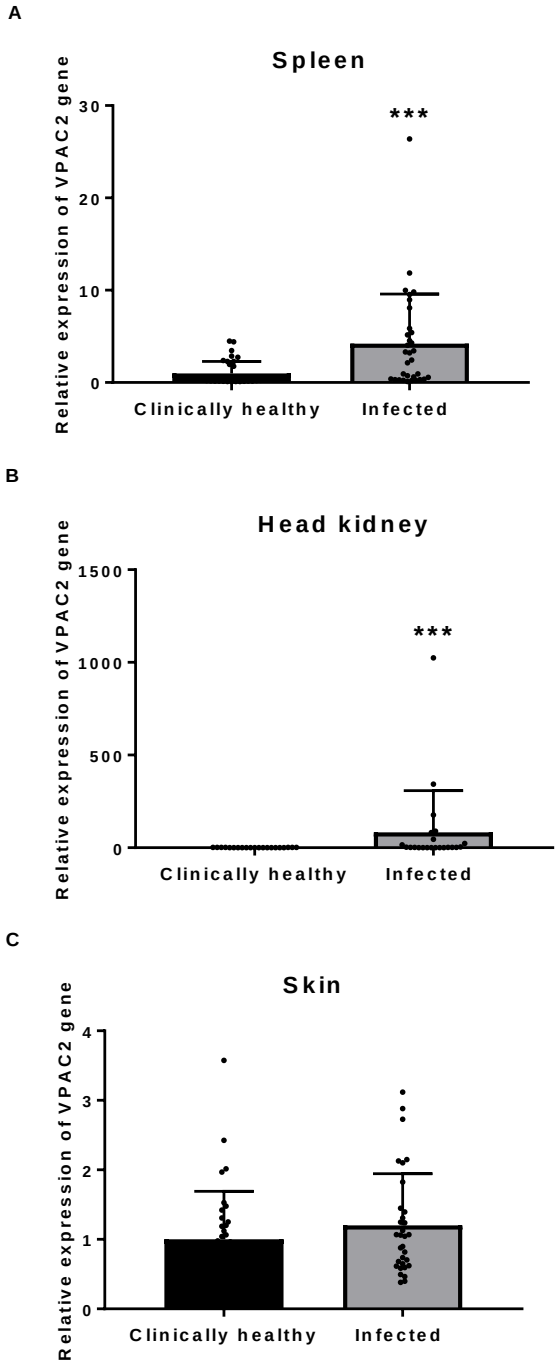

Figure 2.15. Relative expression of VPAC2 gene in spleen, head kidney and skin tissues. Relative expression of VPAC2 gene in spleen (A), head kidney (B) and skin (C) of *Y. ruckeri* naturally infected trout and clinically healthy trout. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. In the clinically healthy samples, the relative expression value was adjusted to 1 to calculate the increase in number of times (*fold change*) in the infected samples. Values are shown as the mean ± standard deviation of the mean. N = A minimum of 22 infected and a minimum of 21 clinically healthy trout.

2.4 Discussion

2.4.1 Up-regulation of pro-inflammatory cytokine gene expression in *Y. ruckeri* infected trout

The results presented here show that there is significant up-regulation in the relative expression of the pro-inflammatory cytokine genes IL-1β, TNFα and IL-6 in the spleen, head kidney and skin of rainbow trout naturally infected with *Y. ruckeri*, which suggest that there is an active immune response against this bacterial infection. These results prove that the responses in naturally infected rainbow trout are similar to the ones found in experimental studies, where there is up-regulation in the expression of these pro-inflammatory cytokine genes after challenge with *Y. ruckeri* (Chettri *et al*., 2012, Raida & Buchmann, 2008, Raida & Buchmann, 2009, Wiens & Vallejo, 2010).

The IL-1β gene showed significant up-regulation in the three evaluated organs (264-fold increase in spleen, 253-fold increase in head kidney and 53.5-fold increase in skin) of rainbow trout naturally infected with *Y. ruckeri*, relative to the expression level in clinically healthy trout. Some bacterial components induce the transcription of IL-1β by leucocytes (Secombes *et al*., 1999), and these results demonstrate the importance of this process in inducing early cytokines in the systemic and local immune response against this disease.

Our study has similarities with previous experimental research. Studies that evaluated the expression of IL-1β gene in rainbow trout after *Y. ruckeri* infection by intraperitoneal injection showed a marked up-regulation in the spleen at 24, 48 and 72 hours post injection, with a peak of almost 1000-fold increase at 48 hours post challenge (Harun *et al*., 2011), a significant up-regulation in the spleen at eight hours, day one and day three (peak of 78-fold increase) post

challenge (Raida & Buchmann, 2008), and significant up-regulation in a dose dependent manner in spleen at day one, three, five and seven post infection, with a peak of 322-fold increase at the third day post challenge in rainbow trout infected with 5000 CFU (Wiens & Vallejo, 2010), compared to PBS injected control fish.

In the case of the TNFα encoding gene, previous experimental studies like the one of Harun *et al*. (2011), showed significant up-regulation of over 10-fold in the spleen of rainbow trout at 24, 48 and 72 hours post infection with *Y. ruckeri* compared to PBS injected control fish. In a similar way, Wiens & Vallejo (2010) found that after a *Y. ruckeri* challenge, an up-regulation in the expression of TNFα gene was seen in rainbow trout spleen at day one, three, five and seven, with a peak (776-fold increase, compared to the expression in non-infected trout) in fish infected with 50,000 CFU at day one post challenge. This study demonstrated that significant up-regulation in the expression of TNFα and IL-1β genes in the spleen occurred at early time points and at low infectious doses of *Y. ruckeri* (Wiens & Vallejo, 2010).

In the present study a highly significant up-regulation in the relative expression of TNFα gene (13.5-fold change in spleen, five-fold change in head kidney and seven-fold change skin) was seen in trout naturally infected with *Y. ruckeri*, compared to the clinically healthy fish. This gene is usually expressed at early time points of infection, in our case the increase in its transcription might be due the continuous exposure to the pathogen in field conditions causing reinfections in new animals.

In our study, IL-6 gene showed the highest expression up-regulation with 318-fold increase in the spleen of naturally *Y. ruckeri* infected trout, compared to the clinically healthy trout. Harun *et al*. (2011), also showed up-regulation of over 100-fold in the relative expression of IL-6 gene in rainbow trout spleen at 24, 48 and 72 hours post challenge with *Y. ruckeri* compared to PBS injected fish, and Raida & Buchmann (2008) found a significant up-regulation of IL-6 gene expression in the spleen of rainbow trout experimentally infected with *Y. ruckeri*, at day one (10-fold increase) and day three (21-fold increase) post injection with the pathogen, compared to the control group.

The lack of detection of expression of IL-6 gene in in head kidney and skin of clinically healthy trout may be due to the extremely low constitutive expression levels for this gene in head kidney and skin cells, due to the inactivity or resting state of cells like dendritic cells in the case of skin. Raida & Buchmann (2008) highlighted in their work that an almost silent expression of IL-6 gene was observed in their control fish spleen. Despite this, the fact that the expression of the gene that encode this cytokine was only detected in samples of infected fish, suggests an up-regulation in the expression of this gene in head kidney and skin (showed in the graphs with the assumption of CT values of 35 for clinically healthy trout) produced by the bacterial infection. A future experiment in the laboratory would be necessary to verify the basal expression levels of IL-6, as well as the increased expression levels after exposure to *Y. ruckeri*.

Raida & Buchmann (2008) found that at day seven, 14 and 28 post-challenge there was no difference in the relative expression of pro-inflammatory cytokine genes IL-1β and IL-6 in the spleen of rainbow trout challenged with *Y. ruckeri* compared to the PBS injected control fish. The

present study used infected specimens in a natural environment, therefore, determining the infection time was not possible, however, when associating our findings with those of experimental studies, it may be that the infection time was less than 14 days. Nevertheless, as shown in the figures, a lot of variation in seen in individuals, this could be related to the stage of infection or to biological variability where many individuals respond only a little, a few respond in a regular manner, and some showed an over-response.

Is worth noting the marked up-regulation in the expression of the three studied pro-inflammatory cytokines (IL-1β, TNFα and IL-6) in the spleen, similar to experimental infections (Harun *et al*. 2011, Raida & Buchmann, 2008, Wiens & Vallejo, 2010). This could be related to the bacterial replication and flux of immune cells in the spleen during Yersiniosis, which is considered a major secondary immune organ in fish (Wiens *et al*., 2006).

The size of the spleen can also be associated with disease progression and to the response against disease, Wiens & Vallejo (2010) found a significant and positive correlation between spleen size and *Y. ruckeri* load in this organ (CFU per mg of tissue). The authors observed that the spleen showed increase in its size since day three post infection, and this was influenced by the dose of injection. Also, a significant and positive correlation was seen between spleen size and pro-inflammatory genes IL-1β and TNFα transcription. In the present study, we found splenomegaly as one of the principal findings in fish infected with *Y. ruckeri*, but studies of correlation with the bacterial load, using qPCR to titre the bacteria, would be necessary to demonstrate this association. On the other hand, genetic factors, independent of the disease, could be associated with the spleen size too (Hadidi *et al*., 2008).

There are studies about expression of pro-inflammatory cytokine genes in other organs against infection with *Y. ruckeri* in rainbow trout. Raida & Buchmann (2009) found a peak in the up-regulation of IL-1β gene (67-fold increase), TNFα (13-fold increase) and IL-6 (1100-increase) in liver of rainbow trout at day three post challenge via intraperitoneal injection with *Y. ruckeri*, compared to their expression in non-infected rainbow trout. Chettri *et al*. (2012) showed a significant increase in the expression of IL-1β and TNFα genes in the internal organs (mixture of spleen, liver, kidney, gills, intestine, etc.) of fry infected with *Y. ruckeri* by immersion bath, compared to control group. IL-1β gene showed increase in its expression at 24-96 hours post infection, where there was a peak of expression of 21-fold compared to the control group. The expression of TNFα gene was up regulated at 72 and 96 hours post infection, with respect to controls. At 25 days post infection there was no significant difference in the relative expression of neither of the two cytokines related to the control group.

About research concerning MALT, Evenhuis & Cleveland (2012) found a significant increase in the expression of TNFα gene (almost two-fold) in the gut of rainbow trout at day nine post immersion challenge with *Y. ruckeri*, compared to controls. Harun *et al*. (2011), on the other hand, showed increase in the transcription of TNFα in the gills of rainbow trout after only 6 hours post intraperitoneal injection with *Y. ruckeri*, compared to non-infected trout. At 24 hours post challenge, authors saw an increase in the expression of IL-6 and TNFα genes, and at 48 hours post infection an up-regulation in the expression of IL-1β, TNFα and IL-6 genes was seen on the gills, compared to the control group.

Research on the response in vaccinated rainbow trout against *Y. ruckeri* and challenged with the pathogen showed a significant increase in the expression of IL-1β, TNFα and IL-6 genes in spleen compared to PBS injected controls, however, the expression was much lower compared to non-vaccinated challenged trout. Moreover, the response was observed earlier in vaccinated trout, the expression of IL-1β and IL-6 genes was increased in the spleen at only six hours post challenge, compared to the controls and by 48 hours there was no significant difference in its expression compared to the control group. In the case of the gills, the opposite was observed, by 48 hours the transcription of these genes was higher in fish that were both vaccinated and challenged, so authors inferred that the adaptative response may vary according to the place and stage of infection (Harun *et al*., 2011).

On the other hand, research that involved re-infection in fish that survived a primary infection with *Y. ruckeri* showed that pro-inflammatory cytokine gene (IL-1β, TNFα and IL-6) expression is significantly increased after a first infection but not after a second infection, proposing that the development of the adaptative immune response would explain the quick elimination of the pathogen and the little pronounced activation of innate cytokines (Raida & Buchmann, 2008; Raida & Buchmann, 2009).

According to this, it can be suggested that the variation seen at the individual level in the present study could be related to the presence of some symptomatic fish that had have a prior infection with the pathogen showing a minimum pro-inflammatory response.

2.4.2 Up-regulation of other cytokine genes of interest expression in *Y. ruckeri* infected trout

The relative expression of other cytokine genes of interest in the fish immune response were also assessed in *Y. ruckeri* naturally infected trout, including the anti-inflammatory cytokine IL-10 and cytokines involved in the adaptive immunity IL-2 and IFN-γ.

It is well-known that inflammation needs to be regulated and this results from the balance in the activity of pro- and anti-inflammatory cytokines, diminishing possible damage in the host due to an excessive response (Raida & Buchmann, 2008). In our study, relative expression of the gene encoding IL-10 showed highly significantly up-regulation in all three tissues examined (113-fold increase in spleen, 147-fold increase in head kidney and five-fold increase in skin) in *Y. ruckeri* naturally infected trout compare to clinically healthy trout. This suggests that an anti-inflammatory response occurs systemically and locally in *Y. ruckeri* naturally infected trout, which is consistent as pro-inflammatory cytokine genes were highly up regulated in all three tissues as well.

Harun *et al*. (2011) found a significant over 10-fold up-regulation of the expression of IL-10 gene in the spleen of rainbow trout at 24, 48 and 72 hours post infection with *Y. ruckeri* compared to PBS injected control fish. Raida & Buchmann (2008) showed a significantly up-regulation of IL-10 gene with a 396-fold increase in the spleen of rainbow trout at day three post challenged with *Y. ruckeri* compared to the control group. Interestingly, no pro-inflammatory cytokines were found up-regulated in later time points. Relative expression assessed in gills showed a significant up-regulation of IL-10 gene at 24 (peak of over 1000-fold increase) and 48 hours in trout infected with *Y. ruckeri* compared to PBS injected control fish (Harun *et al*., 2011). Chettri *et al*. (2012)

observed a significant increase in the expression of IL-10 gene in the internal organs (mixture of spleen, liver, kidney, gills, intestine, etc.) of fry infected with *Y. ruckeri* by immersion bath, compared to the control group, at 72 hours and 96 hours (peak of over 5-fold) post infection. At 25 days post infection, authors found a significant down-regulation in the expression of the gene.

In the present study no significant difference was found in the gene expression of IL-2 in *Y. ruckeri* naturally infected trout compared to clinically healthy trout. This was not the case in the experimental study performed by Harun *et al*. (2011), where the authors found a significant up-regulation over five-fold in the expression of IL-2 gene in the spleen of rainbow trout at 24 and 48 hours post infection with *Y. ruckeri* compared to PBS injected control fish. The same research showed that in gills, IL-2 encoding gene transcription was significantly up-regulated at 24 hours post infection and down-regulated at 48 and 72 hours, compared to PBS injected fish (Harun *et al*. 2011). Based on the findings seen with these results, it could be inferred that the infection time in the evaluated fish exceeded 48 hours.

In contrast, for the IFN-γ cytokine encoding gene, highly significantly up-regulation was seen in all three evaluated tissues (seven-fold increase in spleen, 43.5-fold increased in head kidney and four-fold increased in skin) in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout. In experimental studies, Wiens & Vallejo (2010) observed a significantly up-regulation in the relative expression of IFN-γ gene in the spleen of rainbow trout at high doses of infection at day one and three post challenge with *Y. ruckeri*, with a peak (20-fold increase, compared to the expression in non-infected trout) in fish injected with 50,000 CFU at day one post

challenge. Also, it is worth nothing that IFN-γ gene expression was significantly correlated with IL-1β and TNFα gene expression. Harun *et al*. (2011) found a significant up-regulation in the expression of IFN-γ gene in the spleen of rainbow trout at 24 and 48 hours post infection with *Y. ruckeri* compared to PBS injected control fish. Similarly in gills, IFN-γ encoding gene was significantly up-regulated at the same time points in fish infected with *Y. ruckeri*. In gut, Evenhuis & Cleveland (2012) showed a significant increase in the expression of IFN-γ gene (almost two-fold) in rainbow trout at day three post immersion challenge with *Y. ruckeri*, compared to controls. Raida & Buchmann (2008) found a significant increased of 22-fold in the expression of the IFN-γ encoding gene in rainbow trout spleen at day three post primary infection with *Y. ruckeri* compared to the control group, and in contrast, a significantly down-regulation of the gene transcript was observed at day three and seven post PBS injection in the non-infected control group after re-infection.

As discussed previously, cytokines expression patterns and extent vary regarding the tissue evaluated. IL-2 and IFN-γ are cytokines involved in the Th1 type adaptive immune response (Wang & Secombes, 2013; Zou *et al*., 2005). Raida & Buchmann (2008) detected an increase in IL-1β followed by a doubling in CD4 expression associated with an increase in IFN-γ expression in the primary infection of rainbow trout with *Y. ruckeri*, suggesting that the immune response in fish is similar to that of mammals being elicited by cytokines that activate lymphocytes to initiate the adaptive immune response.

Y. ruckeri is considered an intracellular facultative pathogen for its ability to survive and propagate in macrophages (Ryckaert *et al*., 2010) so a Th1 type response could be expected. Although our *Y. ruckeri* naturally infected trout showed up-regulation of IFN-γ but not of IL-2, it could be possible that a Th1 response was being elicited, however, more extensive research examining a wider range of cytokines needs to be done.

2.4.3 Expression of cytokine genes in head kidney and skin tissues

According to our knowledge there are no previous studies available on the effect of *Y. ruckeri* infections on the transcription of pro-inflammatory cytokine genes in head kidney and skin of fish. However, other researchers have studied the effect on these genes after infections with other gram-negative bacteria of importance in aquaculture. This is the case of a study developed by Henriksen *et al*. (2013) that shows how immersion with *F. psychrophilum* induced a significant up-regulation in the expression of IL-1β gene (almost four-fold increase, compared to the control group) at four hours post challenge, and a significant down-regulation of IL-10 (three-fold decrease, compared to the control group) at 48 hours post infection, in head kidney of rainbow trout fry. No changes were found for IL-6.

Wu *et al*. (2015) found a significant increase in the transcription of IL-1β gene in head kidney of the large yellow croaker, with a peak at 12 hours (14-fold increase, compared to controls) post intraperitoneal infection with *Vibrio alginolyticus*. After challenge with formalin-killed atypical *A. salmonicida* in Atlantic cod, Feng *et al*. (2009) found significant up-regulation of IL-1β gene in

fish infected compared to the control group of fish injected with PBS, the higher expression took place at six hours post infection (684-fold increase in spleen and 356-fold increase in head kidney).

After the experimental infection with *Photobacterium damselae* subsp. *piscicida* in cobia, Tran *et al*. (2019) showed increase in the expression of TNF-α gene (11-fold increase) at three hours post challenge, up-regulation in the transcription of IL-6 gene at three hours and a peak of expression at 48 hours (12-fold increase) post injection, and increase in expression of IL-1β gene at 48 hours (45-fold increase) and 96 hours (33-fold increase) post infection, in head kidney; compared to the controls. For the IFN-γ gene, an up-regulation was seen at 3, 24 hours (peak of 5.6-fold increase) and 48 hours in the head kidney of fish infected compared to controls. In the case of IL-10 encoding gene, significant up-regulation was observed at 8 and 24 hours (peak of 14-fold increase) in fish infected compared to controls (Tran *et al*., 2019). Orieux *et al*. (2013) demonstrated that IL-1β gene is overexpressed in kidney, spleen, liver and gills of rainbow trout naturally infected with *F. psychrophilum*, compare to apparently healthy trout, and suggested this gene as a good disease immune marker for Flavobacteriosis.

The head kidney is a primary lymphoid organ in teleost fish with abundance of macrophages, consider the principal producers of IL-1β, TNFα and IL-6 (Heinrich *et al*., 2003; Manning & Nakanishi, 1996; Zapata *et al*., 1996). Our results show a significant increase in the relative expression of IL-1β, TNFα, IL-6, IL-10 and IFN-γ genes in head kidney of *Y. ruckeri* naturally infected trout respect to clinically healthy fish, which emphasize the systemic response against this bacterial infection.

Chaves-Pozo *et al*. (2005) suggested that the recruitment and activation of lymphocytes following infection occurs in the spleen, while head kidney mainly acts as a hematopoietic tissue that provides and replace cells during bacterial infections, however, they did not exclude that this organ would be associated with the immune response too. Raida & Buchmann (2007) observed that the expression of the gene that encodes IL-1β was higher in the spleen than in the head kidney after intraperitoneal vaccination of rainbow trout with an *Y. ruckeri* bacterin. This is coherent with our results that showed a higher increase in the relative expression of the genes that codify IL-1β, TNFα and IL-6 in spleen, compared to the head kidney.

The immune response in skin has been mainly studied against parasitic infection. (Chettri *et al*., 2014; Forlenza *et al*., 2008; Zhang *et al*., 2018). Muñoz-Atienza *et al*. (2019), demonstrated that in the case of natural infection caused by *F. psychrophilum* in rainbow trout, the local immune response is vital because they found significant up-regulation in the expression of different immune response related genes including IFN-γ in skin lesions of symptomatic fish, compared to asymptomatic fish. On the other hand, there was no significant difference in the expression of those genes in the spleen of symptomatic fish respect to the controls. In the case of skin, an organ selected for its role as a lymphoid tissue and the first barrier of defense against pathogens, as well as the lack of studies associated to its response against *Y. ruckeri*, our results show a significant up-regulation in the expression of IL-1β, TNFα, IL-6, IL-10 and IFN-γ genes in rainbow trout naturally infected with *Y. ruckeri* respect to clinically healthy trout, this demonstrates the importance of SALT and local immune response against this pathogen.

Tobback *et al*. (2009) reisolated *Y. ruckeri* from gills, skin, gut, liver, kidney, and spleen of rainbow trout previously challenge by immersion with this pathogen; finding higher bacterial load in the gills immediately post infection. They suggested the gills as the portal of entrance for *Y. ruckeri*, saying, although, that these bacteria could colonize other fish surfaces like skin and gut to get into the host. The increase in the expression of cytokine genes of importance in skin, observed in the present study, might suggest that this is another route of entry of the pathogen, however, more studies are needed in this subject.

2.4.4 Cytokine protein determination and quantification

IL-1β is synthesized as a precursor protein that lack a signal peptide and need to be process for its later release as an active protein. In mammals, different proteases such as caspase 1, elastase and cathepsin G recognize specific sequences in the central region of proIL-1β, separating the mature peptide from the precursor. Caspase 1, also known as IL-1β converting enzyme (ICE), is the principal protease for processing IL-1β. For this mechanism to occur, the assembly and activation of a large multiprotein complex known as inflammasome is required, which function is to produce mature IL-1β through the activation of caspase 1 (Ogryzko *et al*., 2014; Zou & Secombes, 2016). Even though fish IL-1β orthologues lack an identifiable ICE cute site, emergent evidence has proved that teleost IL-1β can be cleaved by caspases and the processing of proIL-1β to its active form can involve canonical and non-canonical activation of effector caspases 1 and 8 on the inflammasome. Likewise, different cut sites in a small central region of proIL-1β had been reported for diverse species (Zou & Secombes, 2016). Zou *et al*. (1999) predicted a precursor IL-1β protein with a size of 29 kDa and a mature IL-1β protein with a size of 18.7 kDa. Hong *et al*. (2004)

detected a native precursor IL-1β protein of an approximate size of 29 kDa that matched previous theoretical size, and a mature IL-1β protein of approximate 24 kDa, using the cellular line RTS-11 of rainbow trout macrophages.

The results obtained on the IL-1β western blot on serum samples showed the presence of two bands of protein of approximately 25 kDa of size on rainbow trout naturally infected with *Y. ruckeri* that displayed clinical signs of disease, which could correspond to the precursor IL-1β protein. A similar pattern was found in the spleen samples were protein bands between 25-35 kDa appeared in *Y. ruckeri* naturally infected trout which could correspond to the precursor protein, however, bands of 15 kDa approximately were also present in *Y. ruckeri* naturally infected trout which could correspond to mature IL-1β. These bands were not present on the serum or spleen of clinically healthy rainbow trout. Moreover, quantification results by ELISA supported the response seen in spleen at the transcript level into the protein level, showing a very significantly increase of IL-1β in *Y. ruckeri* naturally infected trout with a mean of 392 pg/mL compared to the clinically healthy trout with a mean of 23 pg/mL, which confirms the real behavior of the active protein. Protein sequencing of the bands needs to be performed to further confirm these findings.

Interestingly, unlike the IL-1 family of mammals which has expanded from a single locus, it is presumed that during the duplication of fish genome, the IL-1 locus duplicated itself generating multiple IL-1 genes in various fish species. There is evidence from sequence alignment, phylogenetic and syntenic comparison, to support the conclusion that two distinct paralogues IL-1β protein in fish exist (Ogryzko *et al*., 2014), which may explain the two slightly different bands seen in our serum western blot.

In the case of the TNFα protein, the release of its soluble form requires the removal of the precursor region, the process of which is facilitated by the TNFα converting enzyme (TACE) that anchors proTNF-α at the specific site. Unlike IL-1β, there is a predicted cleavage site for TACE in all TNFα sequences in fish, suggesting that the processing and release mechanism for TNFα is conserved in all vertebrates (Zou & Secombes, 2016). Three TNFα proteins paralogues have been described in teleost fish; TNFα1, whose precursor form has a size of 27.11 kDa and the mature form a predicted size of 17.83 kDa (Laing *et al*., 2001); TNFα2, with a size of 28.17 kDa in its precursor form and a predicted size of 17.89 kDa in its mature form (Zou *et al*., 2002); and TNFα3, with a size of 26.78 kDa in its precursor form and a predicted size of 19.35 kDa in its mature form (Hong *et al*., 2013). The western blot performed for TNFα in this study showed the presence of two protein bands slightly greater than 25 kDa in size in the serum of trout with natural infection by *Y. ruckeri* that presented clinical signs of disease, which could correspond to two of the three protein isoforms in their precursor form. It is also possible that some of the detected bands contained more than one protein and that the separation method was not sensitive enough to separate and visualize bands of very close sizes. Furthermore, a band close to 15 kDa in size was visualized that could correspond to the mature protein. This protein would be smaller than those predicted in the literature. However, the site of the proteolytic cleavage of TNFα in fish has not been experimentally studied and has only been predicted theoretically by sequence alignment with mammalian TNFα (Hong *et al*., 2013). These bands were not seen in the serum of trout without clinical signs of disease; however, further studies are required for their confirmation.

Very little is known about the function and signaling pathways of IL-6 in fish. The IL-6 protein from rainbow trout has a predicted molecular weight of 22.36 kDa (Iliev *et al*., 2007). However, as in other fish cytokines, the IL-6 family could have multiple paralogs (Zou & Secombes, 2016). Although in our results the bands that were found were close to 15 kDa in size, the protein patterns were different for sick fish and clinically healthy fish serum, so further studies are required to identify the identity of these bands.

For IFN-γ cytokine, quantification of the protein was determined by ELISA and supported the response seen in spleen at the transcript level, showing a very significant increase of IFN-γ in *Y. ruckeri* naturally infected trout with a mean of 122 pg/mL compared to the clinically healthy trout with a mean of 50 pg/mL. Function and signaling pathways of IFN-γ in fish have yet to be elucidated. It has been demonstrated that IFN-γ biologically active form is a noncovalent homodimer (Schroder *et al*., 2004) and that the precursor molecule of rainbow trout IFN-γ is a 180-aa protein with a predicted 24-aa signal peptide (Zou *et al*., 2005). Our results confirm the importance of the active protein in the immune response against a natural infection with *Y. ruckeri*, but it is necessary to examine other cytokines to determine the possible type of adaptive response.

2.4.5 Up-regulation of PACAP splicing variants and receptor genes expression in *Y. ruckeri* infected trout

Similarly, as in mammals, PACAP exerts an immunostimulatory action in fish, however, little is known about its expression and distribution in specific tissues (Carpio *et al*., 2008). In rainbow

trout, both PACAP splicing variants (PACAP and PRP/PACAP) genes have been discovered to be constitutively expressed in brain, intestine, and spleen. Only PACAP gene have been found to be constitutively expressed in gills, and no expression of the genes have been found in head kidney, skin, blood, liver, gonads or in RTS11 (trout monocyte/macrophage) cell line. Interestingly, immunoreactivity was detected in spleen lymphocyte-like treated with PACAP antiserum (Lugo *et al*, 2011). PACAP expression have not been detected in the kidney of other species such as channel catfish (Small & Nonneman, 2001) and grass carp (Sze *et al*., 2007).

Gorgoglione *et al*. (2015) did found detectable constitutive expression of PACAP and PRP/PACAP encoding genes in gills, thymus, intestine, spleen, liver and kidney of brown trout. Moreover, authors conducted an infection trial with *Y. ruckeri* and demonstrated a significantly increase in the transcription of PACAP encoding gene at day one, three, seven and 14 in spleen (peak of 132-fold increase at day one), and at day one and 14 in kidney (peak of over 8-fold increase at day 14) post challenge in infected fish compared to controls. PRP/PACAP encoding gene was also significantly up regulated at day one, three and 14 in spleen (peak of 29-fold increase at day one), and at day seven and 14 in kidney (peak of over 4-fold increase at day 14) post challenge in infected fish compared to controls. Bacterial burden was also positively correlated with PACAP expression in both tissues (Gorgoglione *et al*., 2015).

In our study, PACAP encoding gene was significantly up regulated in spleen with a 36-fold increase in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout. For the gene encoding PRP/PACAP, significant up-regulation was seen in spleen with an increase of 11-fold in *Y. ruckeri* naturally infected trout compared to the clinically healthy trout.

Even though no significant differences were found in head kidney and skin for PACAP, the gene was found to be constitutively expressed in this rainbow trout tissues. However, in the case of PRP/PACAP no expression of the gene was found in head kidney and skin of *Y. ruckeri* naturally infected trout and clinically healthy trout.

Little is known, also, about PACAP receptors (PAC1, VPAC1 and VPAC2) expression in fish (Fradinger *et al*., 2005; Wong *et al*., 1998). In rainbow trout, PAC1 gene have been discovered to be constitutively expressed in brain, gills, intestine, head kidney, spleen, blood, skin and in the RTS11 cell line; VPAC1 transcript, in brain, intestine, spleen, blood and in RTS11 cell line but not in gills, head kidney and skin; and VPAC2 encoding gene, in brain, gills, intestine, spleen and skin but not in head kidney, blood or RTS11 cell line. No receptors showed detectable expression in liver or gonads (Lugo *et al*, 2011). Lugo *et al*. (2011) inferred that the different distribution of PACAP and its receptors in the lymphoid tissues could be associated to different mechanisms of regulation in the immune response.

In brown trout, Gorgoglione *et al*. (2015) found detectable constitutive expression of all three PACAP receptors in gills, thymus, intestine, spleen, liver and kidney. After challenge with *Y. ruckeri*, gene encoding PAC1 showed significant down-regulation in spleen at day one in fish infected compared to controls, and a delayed significant up- regulation in kidney (over 16-fold increase) and spleen (over 4-fold increase) at day 14 in fish infected compared to controls. VPAC-1 gene did not show any significant difference in its expression in spleen or kidney after challenge. For VPAC2 encoding gene, significant up-regulation was seen in kidney from day one (over 32-fold increase) to day 14 (peak of over 64-fold increase) in fish infected compared to controls,

which was positively correlated with the bacterial burden in that tissue. No differences were found in spleen (Gorgoglione *et al*., 2015).

In the present study, gene encoding VCAP2 receptor was significantly up regulated in spleen (four-fold increase) and in head kidney (83-fold increase) in *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout, and no significant difference was found in skin, which is coherent with previous experimental studies. Our findings demonstrated that there is a stimulation in the expression of PACAP splicing variants and VPAC2 receptor encoding genes after the natural infection with *Y. ruckeri*.

Overall, an inflammatory response was observed in rainbow trout naturally infected with *Y. ruckeri*. The variability found between symptomatic individuals could be related to different response times, since the stages of infection in a natural environment are not the same for all fish. The response elicited in spleen, head kidney and skin of *Y. ruckeri* naturally infected trout compared to the clinically healthy fish highlights the importance of an active systemic and local immune response. Furthermore, PACAP splicing variants and VPAC2 receptor genes were shown to play a role in the response against natural infection by *Y. ruckeri*.

Chapter 3: Characterization of PACAP's function as an effective antimicrobial and immunostimulatory agent, both natural and modified forms, against bacterial pathogens of importance for rainbow trout.

3.1 Introduction

Intensive farming in aquaculture had led to economic losses due to diseases caused by bacterial pathogens (Miranda *et al*., 2018; Pilco *et al*., 2013), and the widespread use of antibiotics in the sector to solve this issue has contributed to the development of antibiotic resistant pathogens (FAO, 2010; Done *et al*., 2015; Pilco *et al*., 2013). Antibiotic resistance is a priority problem in aquaculture, so the search for novel antimicrobial agents as an alternative or supplement to conventional antibiotics is being encourage (Baltzer & Brown, 2011; Galdiero *et al*., 2015).

The neuropeptide PACAP has been widely studied for its role in immunity and antimicrobial activity (Cardoso *et al*., 2020). In fish, PACAP has been shown to have a physiological role in enhancing growth performance and food intake (Carpio *et al*. 2008; Lugo *et al*., 2010; Lugo *et al*., 2010), and to also play a role as a regulator of the teleost fish immune system (Carpio *et al*., 2008), however, research is still scarce, and more studies are needed to elucidate its function.

F. psychrophilum, *Y. ruckeri* and *A. salmonicida* are important bacterial pathogens in rainbow trout aquaculture (Mateo *et al*., 2017; Semple & Dixon, 2020). In particular, *F. psychrophilum* is capable of causing the highest mortalities. Reported mortalities are varied but can be as high as 90% (Barnes & Brown, 2011; Nilsen *et al*., 2011). *F. psychrophilum* is transmitted horizontally and reservoirs include carrier fish. The bacteria can survive for long periods in a variety of

environments and there is evidence that suggest it can also be vertically transmitted (Starliper, 2011).

Even though PACAP is a promising alternative to antibiotics, its use as a therapeutic agent could have some limitations due to its very short half-life in the circulation after systemic administration due to rapid proteolysis (Starr *et al*., 2018). To address this issue, Starr *et al*. (2018) studied the function of designed PACAP-38 analogs with varying degrees of modifications, finding an equal or improved antimicrobial activity compared to the original peptide. Taking as a base the analogs proposed by Starr *et al*. (2018) for human PACAP-38, Dr. Dixon's Lab worked in the design of three variants from synthetic *C. gariepinus* PACAP-38. In the present study, synthetic *C. gariepinus* PACAP-38 and four modified forms of PACAP-38, including the three designed variants and a scrambled sequence peptide used as a control, were assessed for their direct antimicrobial activity against bacterial pathogens *F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*, evaluated for cytotoxicity activity against red blood cells from diploid and triploid rainbow trout and epithelial cell line from rainbow trout gills (RTgill-W1), and finally tested in an *in-vitro* assay using RTgill-W1 cells to investigate the immunostimulatory effect of PACAP by evaluating the expression of pro-inflammatory cytokine genes IL-1β and TNFα after exposure with PACAP only or with PACAP and *F. psychrophilum.* The hypothesis was that PACAP-38 exerts effective antimicrobial and immunostimulatory properties, in both its natural and modified forms, against bacterial pathogens of importance for rainbow trout, and that modifications of the peptide would even improve PACAP functions.

3.2 Materials and methods

3.2.1 Peptides

Synthetic *C. gariepinus* PACAP-38 and four sequences of modified PACAP with a minimum of 95% purity were obtained from Bio Basic. The sequences of all PACAP peptides are shown in **Table 3.1**. These five peptides were used in *in-vitro* trials to determine the optimal forms of PACAP as an antimicrobial and immunostimulant agent. PACAP 5 (scrambled sequence) was used as a control peptide.

Table 3.1. Synthetic *Clarias gariepinus* PACAP-38 and the four modified PACAP sequences.

PACAP 1 corresponds to original synthetic *Clarias gariepinus* PACAP-38, PACAP 2-4 correspond to active PACAP variants and PACAP 5 (scrambled sequence) corresponds to the control peptide. PACAP forms will be named after the number provided in further descriptions.

Name	Sequence
1-PACAP38	HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
2-PIP3 PACAP38	HS-Pip-GIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
3-[N-acetyl-His1, Pip3]PACAP38	Ac-HS-Pip-GIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK
4-[Pip3, Aib16,28, Ala17, Lys34, D-Lys38] PACAP38	HS-Pip-GIFTDSYSRYRK-Aib-AAVKKYLAAVL-Aib-RRYRQKFRN(d-K)
5-PACAP 38-Random	AVLGIFTDSRVKYSRYRKQMAFRKYLAGRRYRQHSDNR

3.2.2 Bacteria

Bacteria of importance in rainbow trout aquaculture used for the in-vitro experiments *F. psychrophilum* was obtained from the bacterial stock at Dr. Dixon's Laboratory, and *Y. ruckeri* and *A. salmonicida* were obtained from the bacterial stock at Dr. Mark Fast's Laboratory in the University of Prince Edward Island.

3.2.3 Broth microdilution peptide assay (BMPA)

Direct effect of all PACAP variants on the growth of the most important bacterial agents in rainbow trout aquaculture worldwide (*F. psychrophilum, Y. ruckeri* and *A. salmonicida*) were determined. Each bacterial species was cultured from their respective glycerol stock, *F. psychrophilum* in cytophaga agar at 14◦C for four days, and *Y. ruckeri* and *A. salmonicida* in trypticase soy agar at 25◦C for one day. One single colony of each bacteria species were grown in 3 ml of cytophaga broth for three days at 14◦C for *F. psychrophilum*, or overnight at 25◦C for the others. After incubation, 1 ml of bacterial culture was centrifuged, and the pellet resuspended in 4 ml of fresh media to get an OD600 of 0.1-0.4. Then, the bacterial suspension was diluted in cytophaga broth to obtain an OD of 0.001. The BMPA was performed using a flat-bottom 96-well plate (Thermo Fisher Scientific). All wells were filled with 90 µl of bacterial suspension and 10 µl of PACAP variants at 10 different concentrations from one to 50µM (diluted in sterile PBS). PACAP was replaced with 10 µl of PBS (Gibco) in the positive control wells, and negative control wells contained 10 µl of PBS (Gibco) only and 90 µl of cytophaga broth. All samples were tested in triplicates. After three days of incubation at 14◦C for *F. psychrophilum* and overnight at 25◦C for the others, the absorbance was measured at 600 nm using a microplate reader (BioTek). Curves of bacterial growth inhibition were obtained and evaluated.

3.2.4 Hemolytic assay

PACAP variant's ability to lyse erythrocytes was determined using blood from rainbow trout (three diploid specimens and three triploid specimens). Each 5 ml fresh heparinized blood sample was centrifuged at 4°C for 5 minutes at 2000 × g, then washed with PBS (Gibco) until the supernatant was clear and re-suspended in 50 ml of PBS (Gibco) supplemented with glucose (0.2%, v/v) that was previously filtrated using a 0.2 μm filter. PACAP variants (10 μl) at eight different concentrations from one to 50 μM (diluted in PBS) were added to 90 μl of the erythrocyte suspension in a U-bottom 96-well plate (Thermo Fisher Scientific). The samples were incubated for 30 min at 25 °C and then centrifuged for 5 minutes at 2000 × g. Non-treated erythrocytes were included as control of basal hemolysis and samples with erythrocytes incubated with 0.1% of sodium dodecyl sulfate (SDS) were included as control of 100% hemolysis. For the next step, 70 μl of supernatant were transferred to a flat-bottom 96-well microtiter plate (Thermo Fisher Scientific), and the OD was determined at 405 nm. The percentage of hemolysis was calculated using the formula: % hemolysis = (absorbance units of the erythrocytes exposed to peptide × 100)/absorbance units of the erythrocytes exposed to SDS.

3.2.5 Maintenance of cell line

The epithelial cell line from rainbow trout gills (RTgill-W1) was used for in-vitro assays. Cells were maintained at 20°C in 75 cm2 plug-seal tissue culture treated-flasks (Biolite) containing 10 ml of complete media that included Leibovitz's L-15 medium (L-15, HyClone) supplemented with 100 U/ml penicillin (HyClone), 100 μg/mL streptomycin (HyClone) and 10% fetal bovine serum (FBS; Gibco). Cells were subcultured by washing with PBS (Gibco) followed by detachment with 0.25% trypsin-EDTA (Gibco) and split 1:2 every seven days.

3.2.6 Viability test: RTgill-W1 cell line exposure to PACAP

Viability of RTgill-W1 cell line was tested by exposure to all PACAP variants. Cells were seeded at 0.6 x 10^6 cells/well in 1 ml of L-15 media (HyClone) without antibiotics at 25◦C overnight, in 12-well tissue culture plates (Thermo Fisher Scientific). Cells were then exposed to the variants of PACAP at 0.1µM to a final volume of 2 ml per well. Control wells of L-15 media (HyClone) only were included. Incubation at 14◦C for 24 hours followed. On days two, three and four after single exposure, the supernatant was removed from the wells, wells were washed with 1 ml of PBS and the adherent cells were detached using 300 µl of 0.25% trypsin-EDTA (Gibco) for 3 minutes. Incubation was stopped with 10% FBS L-15 and mixed well. Cell viability after exposure to PACAP or L-15 as a control was determined using a trypan blue (Sigma) exclusion test, cell count was performed with a haemocytometer using a phase contrast microscope (Leica). Each sample was tested in triplicate.

3.2.7 RTgill-W1 cell line exposure to PACAP

Twelve-well tissue culture plates (Thermo Fisher Scientific) were seeded with 0.6 x 10^6 cells/well in 1 ml of L-15 media (HyClone) with no antibiotics and incubated overnight at 25◦C. Cells were exposed to all PACAP variants at a concentration of 0.1µM (and L-15 only as a control) to get a final volume of 2 mL per well, and then were incubated at 14◦C. On days one, two, three and four after single exposure with PACAP variants, the supernatant was removed from the wells, wells were washed with 1 ml of PBS and the adherent cells were detached using 300 µl of 0.25% trypsin-EDTA (Gibco) for 3 minutes. Incubation was stopped with 10% FBS L-15, mixed and transferred to a 15 ml tube to be centrifuged (5 min, 400 x g, 4◦C). Supernatant was discarded and cell pellet was stored at −80◦C for future RNA extraction. Each sample was tested in triplicate.

3.2.8 Bacterial in-vitro exposure trial in RTgill-W1 cell line with 24 hours PACAP pre-treatment

F. psychrophilum was selected for the bacterial trial for its worldwide importance in aquaculture and as all PACAP variants showed to inhibit it growth in the BMPA. Bacteria was cultured from a glycerol stock into cytophaga agar and grown for five days at 14◦C. After being checked for purity, 15-20 colonies were obtained to inoculate 4 ml of cytophaga broth and grown at 14◦C for four days. After the incubation period, the OD600 of the bacterial growth was measured. This procedure was repeated five times in the same conditions to get a consistent OD value that can be trusted to estimate the multiplicity of infection (MOI). For every bacterial culture, a standard plate count (SPC) was performed to confirm the anticipated CFU/ml.

Twelve-well tissue culture plates (Thermo Fisher Scientific) were seeded with 0.6 x 10^6 cells/well in 1 ml of L-15 media (Gibco) with no antibiotics and incubated overnight at 25◦C. Cells were exposed to all PACAP variants at a concentration of 0.1µM (and L-15 only as a control) to get a final volume of 2 ml per well, and then were incubated at 14◦C for 24 hours. On the next day, 0.1 ml of the correspondent diluted bacterial suspension (in L-15) was added to each well to expose the cells with a MOI of 0.5. Control wells with only L-15 media, that were not infected, were added as well. All plates were incubated at 14◦C. On days one, two and three post-infection (day two, three and four after PACAP exposure), in order to compare bacterial growth among treatments, 100 µl of cell culture supernatant were transferred in duplicates to a flat-bottom 96-well plate (Thermo Fisher Scientific) and placed at 14◦C for four days before measuring the absorbance at 600 nm using a microplate reader (BioTek). Extra supernatant was removed from the wells, wells were washed with 1 ml of PBS and the adherent cells were detached using 300 µl of 0.25% trypsin-EDTA (Gibco) for 3 minutes. Incubation was stopped with 10% FBS L-15,

mixed and transferred to a 15 ml tube to be centrifuged (5 min, 400 x g, 4◦C) to obtain a cell pellet that was stored at −80◦C for future RNA extraction. Each sample was tested in triplicate.

3.2.9 RNA Extraction and cDNA Synthesis

From the storage cell pellets, RNA was extracted using a RNeasy RNA Extraction Kit (Qiagen) following the manufacturer's protocol. An extra step was performed to remove any genomic DNA that could be contaminating the sample, a treatment with 6 units of DNase I (Thermo Fisher Scientific) per reaction. RNA was quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and was stored at −80◦C. Two hundred and fifty ng of total RNA were used to synthesize cDNA with a qScript cDNA Supermix (Quanta Biosciences) following the manufacturer's instructions. Synthesized cDNA samples were maintained at −80◦C until use.

3.2.10 Gene Expression by qRT-PCR

The relative expression of pro-inflammatory cytokine genes IL-1β and TNF-α were assessed after different respective stimulation. The volume of each qPCR reaction was 10 µl and consisted of 2.5 µl of cDNA (25 ng/µl diluted 1:10 in RNase free water), 2x SYBR® Green qPCR Master Mix (Wisent Bioproducts), and the correspondent forward and reverse primers (Sigma-Aldrich) for a final concentration of 0.25 µM. Reactions were run in the LightCycler R 480 II (Roche). For each plate, all samples were run in triplicates, and a calibrator and a non-template control were included. The program consisted of pre-incubation for 2 minutes at 95◦C, 40 cycles of denaturation for 10 seconds at 95◦C, annealing for 5 seconds at 60◦C and finally an extension for 8 seconds at 72◦C. A melting curve for each plate was used to verify the amplification of a unique product, this was obtained by reading the fluorescence at each grade between 65 to 97◦C every 5 seconds. Elongation

factor – 1 alpha (EF1α) was used as the reference gene, which CT values mean ± standard deviation of the mean were 16.77 ± 1.7 for RTgill-W1 cells exposed to PACAP variants only and 17.36 ± 1.79 for RTgill-W1 cells exposed to PACAP variants and challenged with *F. psychrophilum*. Each gene expression was normalized to the expression of the reference gene, and the levels of expression were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Data was expressed as fold change relative to the expression level in the day zero control group. Primers used are shown in **Table 3.2**.

Table 3.2 qRT-PCR primers used in Chapter 3. Direction of the primers are presented as F and R representing forward and reverse primers, respectively.

Gene	Primer Sequence (5' to 3')	Amplicon Length (bp)	Accession Number	Reference
IL-1β	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC	155	AJ298294.1	Semple *et al*. (2018)
TNFα	F: GTGCAAAAGATACCCACC R: CACTGCACGGTGTCAG	108	AJ278085.1	Semple *et al*. (2018)
EF1α	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	134	NM_001124339	Semple *et al*. (2018)

3.2.11 Statistical Analysis

Data handling and graphic representation were performed using Office Excel 2010 (Microsoft Corporation) and GraphPad Prism version 6 (GraphPad Software Inc.), respectively. All statistical analyses were performed in the statistical software Statistica version 7 (StatSoft). A normal distribution and variance homogeneity were verified, followed by a two-way ANOVA analysis

and a Fisher's least significant difference (LSD) or Tukey's (for qRT-PCR) post-hoc test. Data was shown as mean ± standard deviation, and differences between groups were considered significant, very significant and highly significant when the probability values resulting from the respective test were less than or equal to 0.05, 0.01 and 0.001 respectively.

3.3 Results

3.3.1 Direct antimicrobial activity of PACAP variants against bacterial pathogens of importance

The direct antimicrobial activity of PACAP variants against the three major bacterial pathogens of importance in rainbow trout aquaculture (*F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*) was assessed by BMPA. For *F. psychrophilum*, PACAP 4 showed the best performance inhibiting bacterial growth by more than 90% with a concentration of 15 µM, followed by PACAP 2 and 3 with a concentration of 25 µM, and PACAP 1 with 35 µM (**Figure 3.1A**). For *A. salmonicida*, PACAP variants showed inhibition of bacterial growth by more than 90% with even smaller concentrations, 2.5 µM for PACAP 2, 3 and 4 and 5 µM for PACAP 1 (**Figure 3.1B**). In the case of *Y. ruckeri*, only PACAP 2 and 4 showed to be effective at inhibiting bacterial growth by more than 90% at the highest concentration tested of 50 µM (**Figure 3.1C**). Considering these results and taking in account the importance of the pathogen, *F. psychrophilum* was chosen to be assessed for the following *in-vitro* live infection with RTgill-W1.

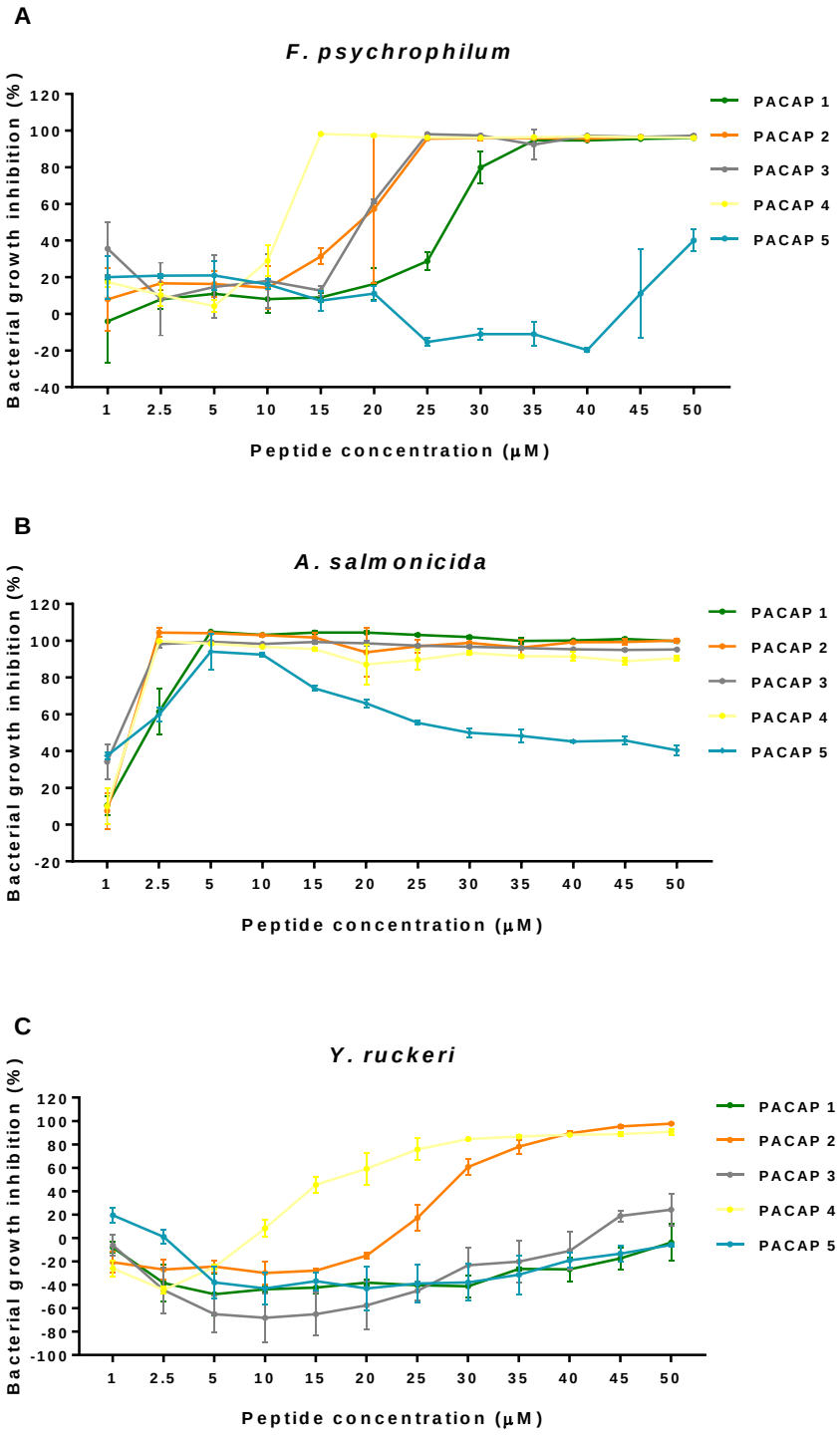

Figure 3.1. Direct antimicrobial activity of PACAP variants against bacterial pathogens of importance in rainbow trout aquaculture by BMPA. Direct microbial activity of five different PACAP peptide variants (four active variants and a control, scrambled peptide (PACAP 5)) on *F. psychrophilum* (A), *A. salmonicida* (B) and *Y. ruckeri* (C) at increasing concentrations from 1 to 50 µM. Values are shown as the mean ± standard deviation of the mean. N=3

3.3.2 Cytotoxic effect of PACAP variants by hemolytic assay

A hemolytic assay was performed to evaluate the potential cytotoxic effect of PACAP variants against red blood cells from both diploid and triploid rainbow trout using similar concentrations from those used to test the direct antimicrobial activity. For diploid rainbow trout, only PACAP 4 induced hemolysis of almost 20% with the highest concentration of 50 μM. PACAP 1, 2, 3 and 5 showed to induced hemolysis of up to 6% with 50 μM, and with the smaller concentrations the percentage of hemolytic activity was almost zero (**Figure 3.2A**). Similarly, for triploid rainbow trout, PACAP 4 induced hemolysis of over 20% with concentrations above 40 μM. PACAP 1, 2, 3 and 5 showed to induced hemolysis of up to 5% with 50 μM, and smaller concentrations showed almost no hemolytic activity (**Figure 3.2B**).

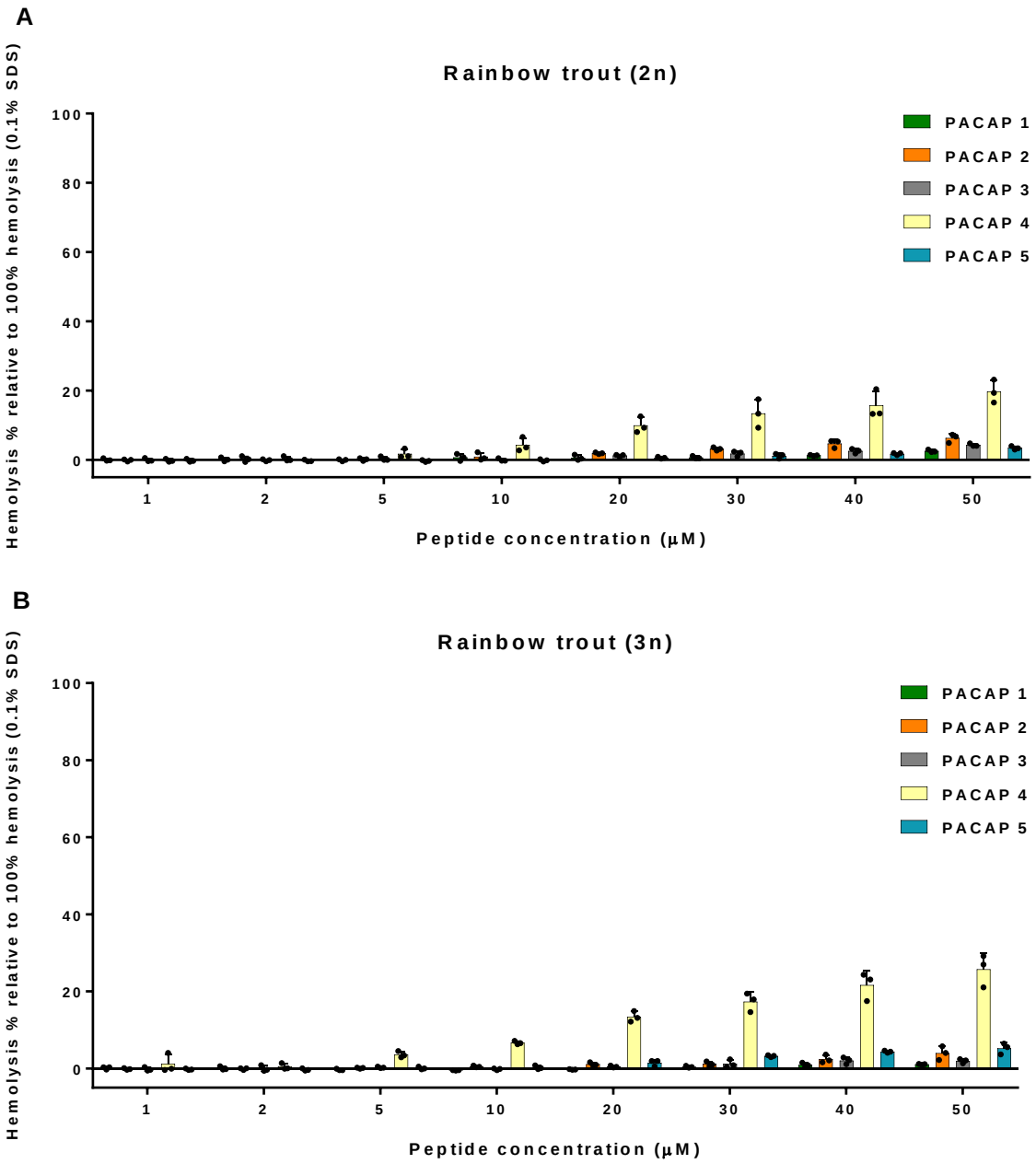

Figure 3.2. Hemolytic activity of PACAP variants on rainbow trout red blood cells. Hemolytic activity of five different PACAP peptide variants (four active variants and a control, scrambled peptide (PACAP 5)), at increasing concentrations from 1 to 50 µM, on red blood cells of diploid (2n) rainbow trout (A) and triploid (3n) rainbow trout (B). Values are shown as the mean ± standard deviation of the mean. N=3. Hemolysis percentage was defined relative to the 100% hemolysis obtained from red blood cells treated with 0.1% SDS.

3.3.3 Impact of PACAP variants in RTgill-W1 cells survival

Previously, Semple *et al*. (2019) determined that concentrations up to 0.1 µM of synthetic *C. gariepinus* PACAP-38 (PACAP 1) did not affect the viability of the monocyte/macrophage-like cell line RTS-11. In our study, a viability assay of RTgill-W1 exposed to all variants of PACAP at a concentration of 0.1 µM was also performed to determine if the peptides could negatively affect the viability of this rainbow trout epithelial cells. As seen in **Figure 3.3** below, none of the PACAP variants negatively impacted cell survival as there were no significant differences compared to the L15 only control at day 2, 3 and 4 post exposure.

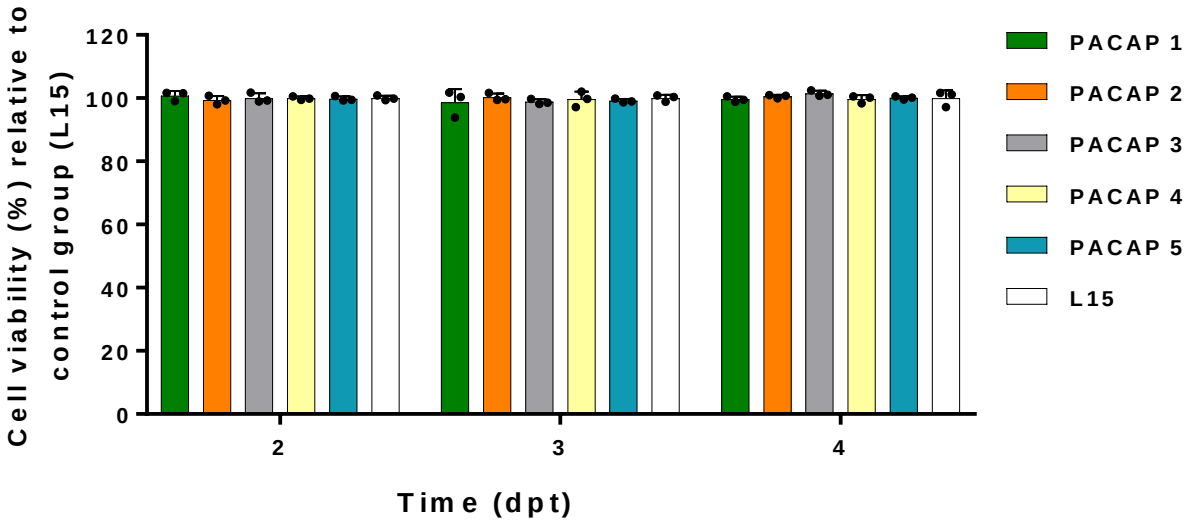

Figure 3.3. Cell viability of RTgill-W1 at day 2, 3 and 4 after treatment with PACAP variants. Cell viability of RTgill-W1 at day 2, 3 and 4 after exposure to 0.1 µM of five different PACAP peptide variants (four active variants and a control, scrambled peptide (PACAP 5)) using a trypan blue exclusion test. Values are shown as the mean ± standard deviation of the mean. N=3. Cell viability percentage was defined relative to the 100% viability obtained from L15 only control group.

3.3.4 Effect of pre-treatment with PACAP variants on *F. psychrophilum* bacterial growth in RTgill-W1 cell line challenge

Pre-treatment with PACAP variants at a concentration of 0.1 µM reduced bacterial growth in an RTgill-W1 infection challenge. At day 1 post infection (day 2 post PACAP treatment) no significant differences were seen. However, at day 2 post infection (day 3 post PACAP treatment), PACAP 3 significantly reduced bacterial growth compared to the L-15 only control, PACAP 5 (scrambled control peptide), PACAP 1 and PACAP 4, and at day 3 post infection (day 4 post PACAP treatment), PACAP 1, 2 and 4 significantly reduced bacterial growth compared to the L-15 only control (**Figure 3.4**).

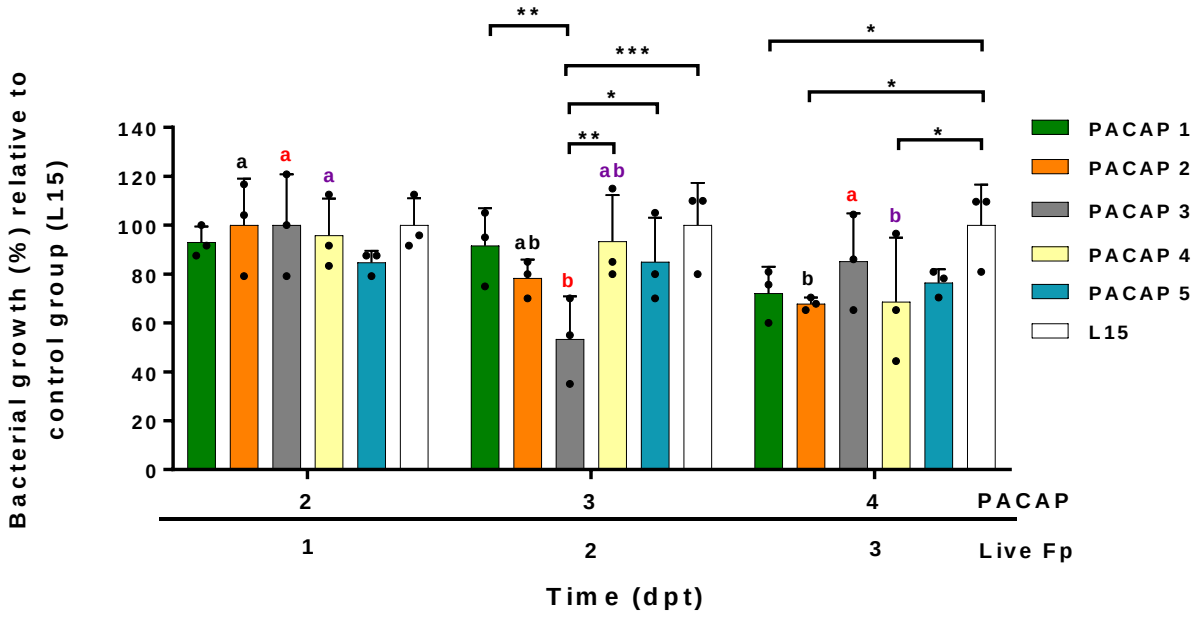

Figure 3.4. Bacterial growth (%) of RTgill-W1 cell culture media during live infection with *F.psychrophilum* (MOI 0.5) pre-treated with PACAP variants. RTgill-W1 cells were pre-treated with 0.1 µM of PACAP variants and challenged with live *F. psychrophilum* after 24 hours. Values were obtained from the absorbance measure at 600 nM. Bacterial growth percentage was defined relative to the 100% bacterial growth obtained from L15 only control group. Data are

shown as the mean ± standard deviation of the mean. N=3. Asterisks (*) denote significant differences between treatments on each day and lowercase letters denote significant differences between days for each specific treatment ($p < 0.05$).

3.3.5 Effect of PACAP variants on RTgill-W1 immune gene expression

RTgill-W1 cells were exposed to either PACAP variants alone or to PACAP variants for 24 hours prior to infection with *F. psychrophilum*. In both cases, to study the immunostimulatory influence of PACAP, the expression of pro-inflammatory cytokines genes of importance IL-1β and TNFα were assessed by qRT-PCR.

After exposure with 0.1 µM of PACAP variants only, RTgill-W1 expression of IL-1β cytokine gene seemed to be down-regulated by PACAP 2, 3, 4 and 5, however significant differences were only seen for treatments with PACAP 3 and 4 compared to PACAP 1 at day 1 post exposure, with PACAP 3, 4 and 5 compared to non-treated cells (L-15 only) control at day 2 post exposure, and with PACAP 3 compared to PACAP 1 and non-treated cells (L-15 only) control at day 4 post exposure (**Figure 3.5A**). In the case of TNFα cytokine encoding gene, up-regulation in its the expression was only seen at day 1 post exposure in cells treated with PACAP 2 compared to treatments with PACAP 4 and non-treated cells (L-15 only) control. Interestingly, PACAP 4 significantly increased RTgill-W1 expression of the TNFα cytokine gene in time, at day 4 compared to day 1 (**Figure 3.5B**).

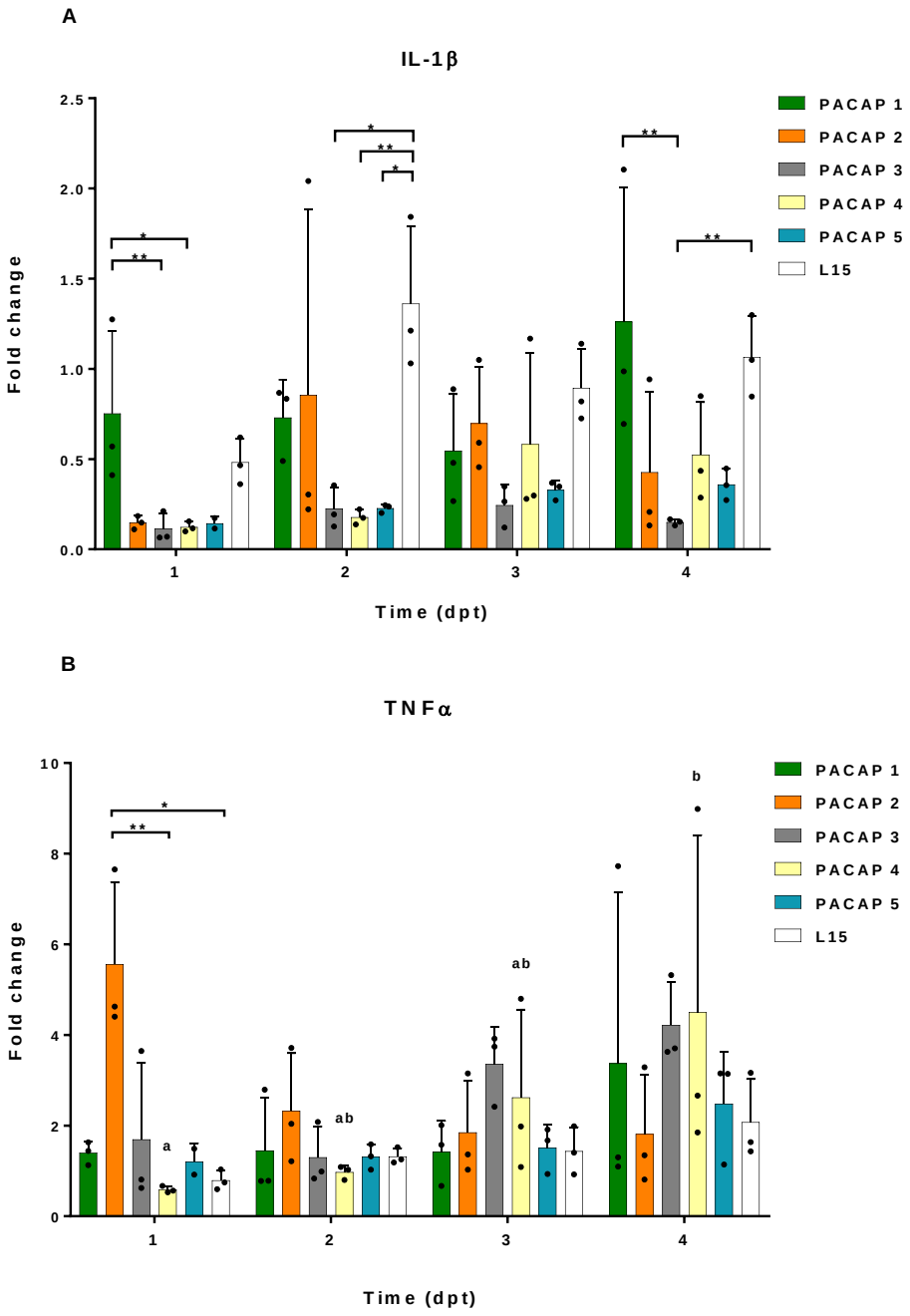

Figure 3.5. Effect of PACAP variants on RTgill-W1 pro-inflammatory cytokines expression at 1-4 days post peptide exposure. Transcript expression of pro-inflammatory cytokines IL-1β (A) and TNFα (B) were measured to assess the impact of 0.1 µM of PACAP variants alone on the immune function. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. Relative expression value at day zero was adjusted to 1 to calculate the increase in number of times (*fold change*) at the different time points. Values are shown as the mean ± standard deviation of the mean. N=3. Asterisks (*) denote significant differences between treatments on each day and lowercase letters denote significant differences between days for each specific treatment ($p < 0.05$).

After pre-treatment of RTgill-W1 with 0.1 µM of PACAP variants for 24 hours followed by live infection with *F. psychrophilum*, expression of the IL-1β cytokine gene was similar to the findings with PACAP variant exposure alone as there seemed to be down-regulation induced by PACAP 1, 2, 3, 4 and 5, however significant differences were only seen for treatments with PACAP 4 and 5 compared to non-treated cells (L-15 only) control at day 1 post infection (day 2 post PACAP treatment) and with PACAP 5 compared to non-treated cells infected (L15-Fp) and alone (L-15 only) controls at day 2 post infection (day 3 post PACAP treatment) (**Figure 3.6A**). For the gene encoding TNFα cytokine, no significant differences were found at any time point (**Figure 3.6B**).

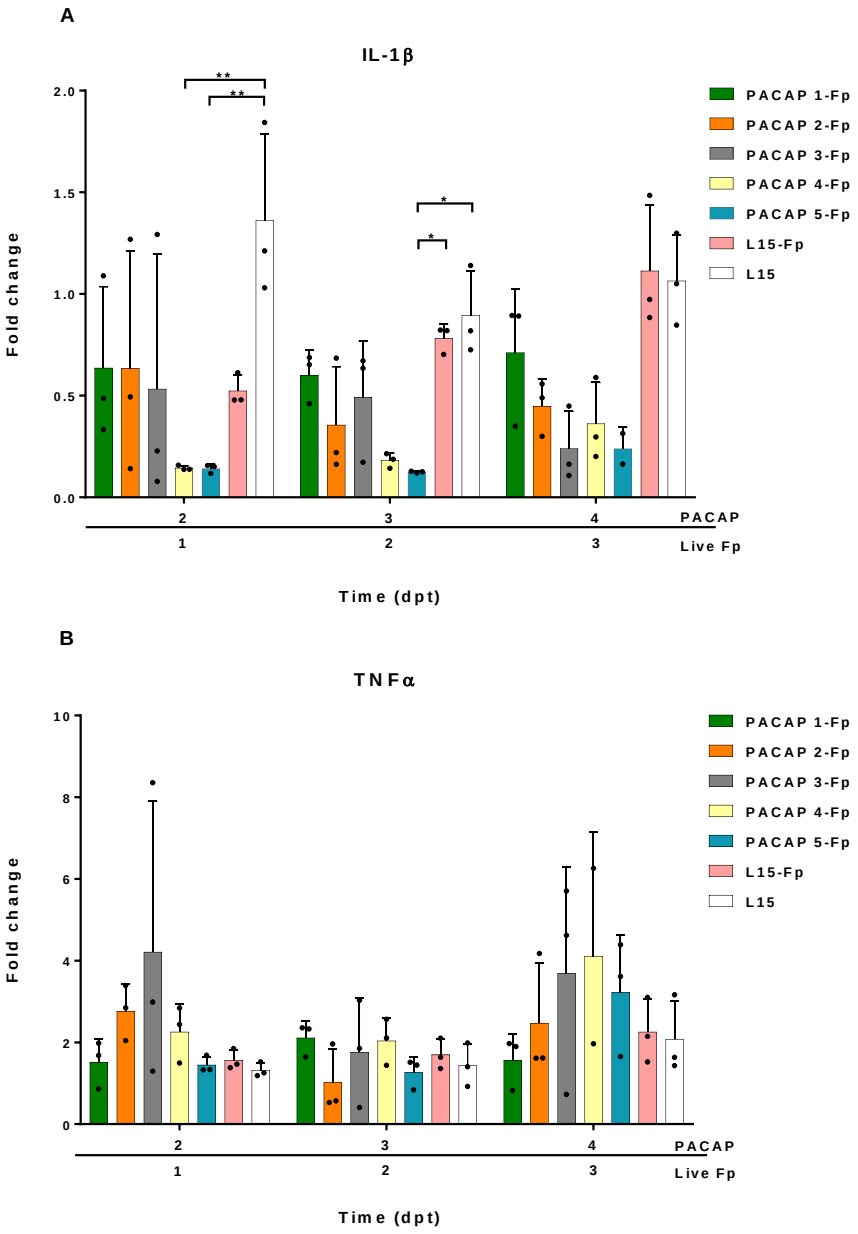

Figure 3.6. RTgill-W1 pro-inflammatory cytokines transcript expression when challenged with live *F. psychrophilum* after a 24-hour pre-treatment with PACAP variants. Transcript expression of pro-inflammatory cytokines IL-1β (A) and TNFα (B) were measured at day 1,2 and 3 post infection to assess the impact of the 24-hour pre-treatment with 0.1 µM of PACAP variants on the immune function after a live challenge with *F. psychrophilum*. RTgill-W1 not exposed to PACAP variants or bacteria was also included as a non-stimulated control. Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF1α was used as the reference gene. Relative expression value at day zero was adjusted to 1 to calculate the increase in number of times (*fold change*) at the different time points. Values are shown as the mean ± standard deviation of the mean. N=3. Asterisks (*) denote significant differences between treatments on each day and lowercase letters denote significant differences between days for each specific treatment ($p < 0.05$).

3.4 Discussion

3.4.1 Direct antimicrobial activity of PACAP against bacterial pathogens

Previous research has demonstrated the direct antimicrobial activity of human PACAP-38 and variants (Starr *et al*., 2018) and synthetic *C. gariepinus* PACAP-38 against gram-negative and gram-positive bacteria of biomedical importance, and of synthetic *C. gariepinus* PACAP-38 on bacteria of importance in aquaculture (Lugo *et al.*, 2019; Semple *et al*., 2019). The results obtained in the present study show that PACAP variants have a direct antimicrobial activity against three major bacterial pathogens in rainbow trout aquaculture *F. psychrophilum, Y. ruckeri* and *A. salmonicida*.

All active PACAP variants showed to inhibit *F. psychrophilum* growth in over 90% with concentrations of 15 (PACAP 4), 25 (PACAP 2 and 3) and 35 (PACAP 1 or original *C. gariepinus* PACAP-38) µM. This is consistent with the findings of Semple *et al*. (2019), which revealed that 100% inhibition of *F. psychrophilum* growth was achieved with 30 µM of synthetic *C. gariepinus* PACAP-38. Our results indicated that for *A. salmonicida,* concentrations of only 2.5 µM for PACAP 2, 3 and 4 and of 5 µM for PACAP 1 inhibited bacterial growth in more than 90%. In contrast, for *Y. ruckeri* only PACAP 2 and 4 showed to inhibit bacterial growth in more than 90% with the highest concentration of 50 µM. Lugo *et al*. (2019) studied the direct antimicrobial activity of synthetic *C. gariepinus* PACAP-38 against these two gram-negative bacteria using higher concentrations of the peptide and they found that PACAP-38 completely inhibited the growth of *A. salmonicida* and *Y. ruckeri* with a concentration of 50 µM. Our results suggested that *A. salmonicida* was more susceptible to PACAP-38 (PACAP 1) with a smaller concentration being

able to inhibit the bacteria and that PACAP 1 was not effective against *Y. ruckeri* in concentrations up to 50 μM, however these differences may be due to the strain and origin of the bacteria. Moreover, our findings suggest that modified forms of *C. gariepinus* PACAP-38 (variants 2 and 4) are more effective in their direct antimicrobial activity against the evaluated bacteria. Semple *et al*. (2019) associated the direct antimicrobial activity of synthetic *C. gariepinus* PACAP-38 on *F. psychrophilum* with the ability of the peptide to permeabilize the bacterial membrane. The mode of action of PACAP-38 on *A. salmonicida* and *Y. ruckeri* has not been studied, so more research is needed to elucidate PACAP antimicrobial mechanisms.

3.4.2 PACAP effect on viability of cells

To consider PACAP and its modified forms as possible prophylactic/therapeutic agents, is necessary to demonstrate that they are not cytotoxic for the host. So, to test the impact of PACAP variants on the host cells, a hemolytic assay using red blood cells from rainbow trout and a viability test for RTgill-W1 cell line were assessed. For the hemolytic assay, similar concentrations to the ones assessed for direct antimicrobial activity were tested in both diploid and triploid rainbow trout, finding that only PACAP 4 induced hemolysis of almost 20% with the highest concentration of 50 μM in red blood cells of diploid rainbow trout and hemolysis of over 20% with concentrations above 40 μM in red blood cells of triploid rainbow trout. Triploid fish cell nuclei are larger than the diploids as triploid cell nuclei contain 50% more DNA than diploid cell nuclei, with a resulting increase in cellular volume to maintain the diploid nucleus to cytoplasmic ratio (Benfey, 1999), the slightly increased hemolysis seen in red blood cells from triploid compared to diploid rainbow trout with PACAP 4 could be related to a more fragile nature of the cells due to their increased size. Lugo *et al*. (2019) reported that the hemolytic activity of *C. gariepinus* PACAP-38 reached

39% in tilapia red blood cells with the highest concentration tested of 300 μM, concentrations from 18.75 μM to 150 μM induced a hemolytic activity of less than 20%, and concentrations under 18.75 μM caused no hemolysis. PACAP variants, similarly to previous studies with PACAP-38, seem to exert limited cytotoxicity against red blood cells in fish. In the case of PACAP 4, which is the most modified form of PACAP-38, the addition of a-aminoisobutyric acid to the sequence of the original peptide could be causing the increased hemolysis, more studies are needed to elucidate if this variant could be consider a viable treatment.

The different nature of *in-vitro, ex-vivo* and *in-vivo* procedures has been studied, findings expected differences in the doses needed that are related to the more direct stimulation in a controlled *in-vitro* setting (Gotlieb *et al*., 2015). Semple *et al*. (2019) studied the effect of synthetic *C. gariepinus* PACAP-38 on the viability of RTS11 at concentrations ranging from 0.002 to 20 μM and found that concentrations of and above 0.2 μM had a significantly negative impact on the viability of this rainbow trout cell line at day 2 post exposure to the peptide. For the present study, the viability of RTgill-W1 cells exposed to all PACAP variants at a concentration of 0.1 μM was assessed and it was found that none of the PACAP variants negatively impacted cell survival at days 2, 3 and 4 post exposure, so the following experiments were performed with this concentration.

3.4.3 PACAP effect on bacterial growth in an RTgill-W1 live infection

Our results suggest that RTgill-W1 pre-treatment with 0.1 μM of PACAP variants (1-4) 24 hours before infection significantly reduced *F. psychrophilum* growth compared to non-treated cells at day 2 post infection for PACAP 3 and at day 3 post infection for PACAP 1,2 and 4. Results for

PACAP 1 (*C. gariepinus* PACAP-38) are consistent with the results of Semple *et al*. (2019) that showed that pre-treatment with PACAP-38 at concentrations of 0.002, 0.02, and 0.1 μM significantly reduced the growth of *F. psychrophilum* viable bacteria in the monocyte/macrophage-like rainbow trout cell line RTS11 at day 2 post infection, and that the two highest concentrations still significantly reduced bacterial growth at day 3 post infection. However, in our study only the absorbance measured at 600 nM was used to assess bacterial growth so a standard plate count (SPC) will be needed to confirm these results. Our findings could indicate that PACAP action is not only present over immune cells but also epithelial cells such as those of gills, which would highlight GIALT importance in the immune response against *F. psychrophilum.* Although skin, and mainly skin injuries are the most likely portal of entrance of the pathogen (Miwa & Nakayasu, 2005), all mucosal barriers are known to be constantly exposed to pathogens in fish (Salinas, 2015). In-vivo research by Velasquez *et al*. (2020) reported that pre-treatment for two weeks (6 doses) with synthetic *C. gariepinus* PACAP-38 administrated by immersion bath (200 μg/L, 5 L for 30 fish) in rainbow trout fry significantly increased the survival rate of fish when challenged with Viral Hemorrhagic Septicemia Virus (VHSV) and decreased the viral load in spleen and kidney. Antibacterial activity of PACAP *in-vivo* have not yet been studied in teleosts.

3.4.4 PACAP effect on immune gene expression

The expression of pro-inflammatory cytokines genes IL-1β and TNFα were assessed by qRT-PCR. For cytokine gene IL-1β, results showed that exposure of RTgill-W1 cells with a concentration of 0.1 μM of PACAP variants only, significantly induced down-regulation in the expression of this cytokine gene compared to non-treated cells (L-15 only) control at day 2 post exposure for

treatments with PACAP 3, 4 and 5, and at day 4 post exposure only for treatment with PACAP 3. PACAP 1 and 2 treatments were not different from non-treated cells (L-15 only) control at any time point. When cells were pre-treated with PACAP variants 24 hours prior to infection with *F. psychrophilum*, results were similar as significant down-regulation in the expression of IL-1β was seen with PACAP 4 and 5 compared to non-treated cells (L-15 only) control at day 1 post infection and with PACAP 5 compared to non-treated cells infected (L15-Fp) and alone (L-15 only) controls at day 2 post infection. Interestingly, challenge with the bacteria in non-treated cells (L15-Fp) showed no stimulation as the expression of the gene was not different from non-treated non-challenged (L-15 only) cells. In contrast, Semple *et al*. (2019) found that RTS11 cells that were exposed to 0.1 µM of *C. gariepinus* PACAP-38 (PACAP 1) significantly increased their expression of IL-1β gene at day 2 post exposure, and when cells were pre-treated with PACAP-38 before challenge with *F. psychrophilum*, a significantly up-regulation in the transcription of the gene was seen at day 3 post infection compared to RTS11 cells exposed to live pathogen only. Similarly, Wang *et al*. (2013) reported that grass carp PACAP recombinant protein induced the expression of IL-1β gene in head kidney leukocytes and head kidney of grass carp (*Ctenopharyngodon idella*) but had no extra effect in the expression of the cytokine gene in cells or fish stimulated simultaneously with LPS or *Aeromonas hydrophila*, respectively.

In the case of TNFα encoding cytokine gene, we found that exposure of RTgill-W1 cells with a concentration of 0.1 µM of PACAP 2 only, significantly up-regulate the expression of this cytokine gene compared to non-treated cells (L-15 only) control at day 1 post exposure. When cells were pre-treated with PACAP variants 24 hours prior to infection with *F. psychrophilum,* no significant differences were found with PACAP treatments compared to the control group. Similar to the

response of IL-1β gene, challenge with the bacteria in non-treated cells (L15-Fp) showed no stimulation in the expression of TNFα gene. Previously, Semple *et al*. (2019) reported that RTS11 cells that were exposed to 0.1 µM of *C. gariepinus* PACAP-38 (PACAP 1) significantly increased the expression of TNFα gene at day 2 post exposure, and when cells were pre-treated with PACAP-38 before challenge with *F. psychrophilum*, a significantly up-regulation in the transcription of the gene was seen at day 1, 2 and 3 post infection compared to RTS11 cells exposed to the live pathogen alone. Wang *et al*. (2013) also demonstrate that grass carp PACAP recombinant protein induced the expression of TNFα encoding gene in head kidney leukocytes and head kidney of grass carp but had no extra effect in the expression of the cytokine gene in cells or fish stimulated simultaneously with LPS or *A. hydrophila*, respectively.

Our results suggest that PACAP function may differ according to the cell model or location. In immune cells of fish, RTS11 and grass carp head kidney leukocytes, a pro-inflammatory response was induced (Semple *et al*., 2019; Wang *et al*., 2013), however, this study found almost no response for TNFα and down-regulation of IL-1β, and unexpectedly, no induction of pro-inflammatory genes expression in cells stimulated with the live pathogen alone. The lack of response could be related to the function of gills in respiration, to preserve oxygen up-take other mechanisms of the immune response may be involved instead of inflammation. Also, although induction of a pro-inflammatory response (Semple *et al*., 2019; Wang *et al*., 2013) and no anti-inflammatory response (Wang *et al*., 2013) has been found in fish, in mammals PACAP is known to stimulate an anti-inflammatory action in the immune response (Ganea & Delgado, 2002). In an *in-vivo* study by Velasquez *et al*. (2020), it was reported that *C. gariepinus* PACAP-38 treatment

significantly down-regulated the expression of IL-1β at day 14 post-treatment in rainbow trout, however, more research is needed to elucidate the role of PACAP in fish inflammatory response.

Overall, our results confirm that *C. gariepinus* PACAP-38 exerts a direct antimicrobial activity and has immunomodulatory properties against bacteria of importance in rainbow trout aquaculture. PACAP variants 2 and 4 appear to have an improved function as only they showed effective antimicrobial activity against *Y. ruckeri*, but as PACAP 4 demonstrated the highest hemolytic activity of around 20% in rainbow trout red blood cells and it is also highly modified, PACAP 2, which has slight modifications from the original peptide, should be further studied in future *in-vitro* and *in-vivo* experiments.

Chapter 4: Conclusions and future directions

The thesis overall goal was to study PACAP antimicrobial and immunomodulatory properties against bacterial diseases of importance in rainbow trout aquaculture, due to its promising role as a novel antimicrobial agent that could diminish the use of antibiotics in the industry.

To address this, in the first part of the study, the immune response in symptomatic and asymptomatic rainbow trout from a natural outbreak caused by *Y. ruckeri* was assessed. Our results showed that the expression of PACAP splicing variants PACAP and PRP/PACAP encoding genes was significantly up-regulated in the spleen of *Y. ruckeri* naturally infected trout compared to the clinically healthy trout. No expression of PRP/PACAP gene was detected in head kidney and skin tissues of *Y. ruckeri* naturally infected trout and clinically healthy trout, but in the case of PACAP encoding gene, although no significant differences were found in head kidney and skin between symptomatic and asymptomatic fish, we demonstrated for the first time that the gene is constitutively expressed in this rainbow trout important lymphoid organs. Gene encoding PACAP receptor, VCAP2, showed an increased transcription in spleen and head kidney of *Y. ruckeri* naturally infected trout relative to the expression level in clinically healthy trout, and no significant difference was found in skin. These findings indicate that there is a stimulation in the expression of PACAP splicing variants and VPAC2 receptor encoding genes after a natural bacterial infection with *Y. ruckeri*. The response of other PACAP receptors, PAC1 and VPAC1, in a natural setting need yet to be elucidated.

Also, the response of cytokines of importance were studied as well. Significantly increased in the transcription of pro-inflammatory cytokine genes IL-1β, TNFα and IL-6, and anti-inflammatory

cytokine gene IL-10 was observed in spleen, head kidney and skin of *Y. ruckeri* naturally infected trout compared to the clinically healthy trout, highlighting the importance of an active systemic and local immune response against this disease. Results suggest there is a strong inflammatory response against *Y. ruckeri* natural infection, but that inflammation is also being regulated by an anti-inflammatory response elicited by IL-10 gene transcription. In the case of cytokine gene IL-6, which was not detected in head kidney and skin of asymptomatic fish, further studies are needed to verify it basal expression levels in these tissues. Although results for the expression of IL-2 cytokine gene showed no significant difference between symptomatic and asymptomatic fish in the three tissues assessed that could be related to the time infection, significant up-regulation of IFN-γ encoding gene was seen in spleen, head kidney and skin of *Y. ruckeri* naturally infected trout compared to the clinically healthy trout, which could suggest a possible Th1 type response associated to the intracellular nature of the pathogen (Ryckaert *et al*., 2010), however, in future research a wider range of cytokines related to the adaptive immune response needs to be examine in order to elucidate this. Results for spleen were consistent with previous experimental research (Harun *et al*., 2011; Raida & Buchmann, 2008; Wiens & Vallejo, 2010), which is expected due to the importance of the spleen as a major secondary lymphoid organ in teleost fish where bacterial replication and flux of immune cells occur during Yersiniosis (Wiens *et al*., 2006). No previous studies have investigated the immune response in head kidney and skin of rainbow trout after challenge with *Y. ruckeri*, our results in head kidney, primary lymphoid organ of teleost fish, emphasize the systemic response against this bacterial infection in a natural setting, and for skin, our findings demonstrate the importance of SALT and local immune response against this pathogen suggesting this tissue as another possible route of entry of the pathogen. Further studies, including the response in gills and gut will be important to elucidate these results and describe the

Y. ruckeri route of infection. Results at the protein level were also evaluated for some of the cytokines tested, a different profile was found for *Y. ruckeri* naturally infected trout compared to the clinically healthy trout as bands that were close to the expected protein size were found in symptomatic-infected and not in asymptomatic fish serum for TNFα and IL-6, and in fish serum and spleen tissue for IL-1β. Moreover, for IL-1β and IFN-γ, protein quantification by optimized ELISAs showed significantly increase for both cytokines in spleen of *Y. ruckeri* naturally infected trout compared to the clinically healthy trout which confirms the real behavior of the active protein that are consistent with the findings at the transcript level. Moving forward, protein sequencing of the bands needs to be performed to confirm their identity, and development of qELISAs for the other cytokines tested to corroborate the results at a functional level would be interesting.

Is important to highlight the variation in response found in symptomatic fish with some individuals showing a minimum response, and others an over-response. As the present study used infected fish from a natural environment, the time of infection was not determined, however, when associating our findings with those of experimental studies (Raida & Buchmann, 2008; Wiens & Vallejo, 2010), it could be suggested that the infection time was less than 14 days, so the variation could be related to the stage of infection or to biological variability. Also, some symptomatic fish could have had a prior infection with the pathogen showing a minimum pro-inflammatory response. It is also important to keep in mind that while fish usually carry *Y. ruckeri*, disease only occurs when stress associated with intensive farming (poor handling, high densities, etc,), poor water quality and high temperatures overcome the immune defense in fish (Horne & Barnes, 1999). In Peru, the management conditions in trout farming are still deficient, which promotes the presence of this and other bacterial diseases such as Furunculosis caused by *A. salmonicida* and BCWD and RTFS

caused by *F. psychrophilum* in the production system. Natural infections are more complex and are necessary to investigate after knowing the response against experimental infection, so we can better understand the host-pathogen interaction.

In the second part of the study, synthetic *C. gariepinus* PACAP-38 (PACAP 1) which has previously showed to exert a role as a regulator of the teleost fish immune system (Carpio *et al*., 2008; Lugo *et al*., 2010; Lugo *et al.*, 2019; Semple *et al*., 2019; Velasquez *et al*., 2020), and three designed modified forms of the peptide (PACAP 2-4) were tested to evaluate their direct antimicrobial function against bacterial pathogens of importance in rainbow trout aquaculture, safety to be used in the host and immunomodulatory properties, with the objective of assessing PACAP-38 functions in the immune response and to determine is modifications could improve them. A scramble sequence peptide (PACAP 5) was also included as a control peptide.

Through a BMPA, synthetic *C. gariepinus* PACAP-38 (PACAP 1) and all active variants (PACAP 2-4) showed to inhibit *F. psychrophilum* growth in over 90% with concentrations from 15 (PACAP 4) to 35 µM (PACAP 1). For *A. salmonicida*, concentrations of only 2.5 µM for PACAP 2, 3 and 4 and of 5 µM for PACAP 1 inhibited bacterial growth in more than 90%. And for *Y. ruckeri*, only PACAP 2 and 4 showed to inhibit bacterial growth in more than 90% with the highest concentration of 50 µM. These findings suggest that modified forms PACAP 2 and 4 are more effective in their direct antimicrobial activity against the evaluated bacteria. The direct antimicrobial activity of *C. gariepinus* PACAP-38 on *F. psychrophilum* has been associated with the ability of the peptide to permeabilize the bacterial membrane (Semple *et al*., 2019), it will be important to elucidate the mechanisms of action against *A. salmonicida* and *Y. ruckeri*. To

investigate the safety of using PACAP as a possible prophylactic/therapeutic agent, the impact of PACAP-38 and variants on host cells were assessed. The hemolytic assay on red blood cells from diploid and triploid rainbow showed that only PACAP 4 induced the highest hemolysis of around 20% with concentrations above 40 μM. PACAP-38 and the rest of variants showed to induced hemolysis of up to 6% with concentrations of 40 and 50 μM, and smaller concentrations showed almost no hemolytic activity, so PACAP seems to exert limited cytotoxicity against rainbow trout red blood cells, however, more studies are needed to elucidate if PACAP 4, which is the most modified form of PACAP-38, should be consider a viable treatment. In order to proceed with the following *in-vitro* assays and as Semple *et al*. (2019) had previously reported that C. gariepinus PACAP-38 had a significantly negative impact on the viability of rainbow trout cell line RTS11 at concentrations of and above 0.2 μM, we evaluated the viability of the epithelial cell line from rainbow trout gills, RTgill-W1, when exposed to PACAP-38 and variants at a concentration of 0.1 μM, finding no significant negative impact on cell survival at days 2-4 post exposure.

To evaluate the immunomodulatory properties of PACAP, the expression of pro-inflammatory cytokines genes IL-1β and TNFα in RTgill-W1 after exposure to PACAP alone or PACAP and *F. psychophilum* were studied. After the exposure with 0.1 μM of PACAP only, IL-1β gene expression in RTgill-W1 cells showed to be significantly down-regulated at day 2 post exposure for treatments with PACAP 3, 4 and 5, and at day 4 post exposure only for treatment with PACAP 3 compared to non-treated cells (L-15 only) control. For TNFα encoding cytokine gene, only PACAP 2 showed to significantly up-regulate the expression of this cytokine gene compared to non-treated cells (L-15 only) control at day 1 post exposure. When cells were pre-treated with PACAP 24 hours prior to infection with *F. psychrophilum*, results showed again down-regulation

in the expression of IL-1β in treatments with PACAP 4 and 5 compared to non-treated cells (L-15 only) control at day 1 post infection and with PACAP 5 compared to non-treated cells infected (L15-Fp) and alone (L-15 only) controls at day 2 post infection, and for TNFα no significant differences were found with PACAP treatments compared to the control group at any time point. As previous studies have reported induction of a pro-inflammatory response (Semple *et al*., 2019; Wang *et al*., 2013) and no anti-inflammatory response (Wang *et al*., 2013) in fish immune cells, in contrast to the anti-inflammatory action that is known for PACAP in mammals (Ganea & Delgado, 2002), our results suggest that PACAP function may differ according to the cell model or location. The almost lack of response of TNFα and down-regulation of IL-1β genes could be related to the function of gills in respiration, to preserve oxygen up-take other mechanisms of the immune response may be involved instead of inflammation, which is consistent with the unexpected finding that no induction of pro-inflammatory genes expression was seen in cells stimulated with the live pathogen alone. For future research, a wider set of cytokine genes and assays in other epithelial and immune cells would help us elucidate the role of PACAP in the inflammatory response. Despite of this findings, RTgill-W1 pre-treatment with 0.1 µM of PACAP-38 and variants 24 hours before infection significantly reduced *F. psychrophilum* growth compared to non-treated cells at day 2 post infection for treatment with PACAP 3 and at day 3 post infection for treatment with PACAP 1, 2 and 4. However, our study only used the absorbance measured at 600 nM to assess bacterial growth so a standard plate count (SPC) will be needed to confirm the results. If this is confirmed, it would indicate that PACAP action is not only exert over immune cells but also epithelial cells such as those of gills that are in constant exposure to pathogens, which would highlight GIALT importance in the immune response against *F. psychrophilum.*

Overall, the hypothesis of the thesis is accepted, as we demonstrated that *C. gariepinus* PACAP-38 and variants have a direct antimicrobial activity and immunomodulatory properties against bacteria of importance in rainbow trout aquaculture, being the PACAP 2 variant, which has slight modifications from the original peptide, a good candidate for future research for its improved antimicrobial function and its limited cytotoxicity against host cells. Moving forward, *in-vivo* studies in rainbow trout and other fish species of importance in aquaculture will need to be developed to define a dosage that is safe and effective at the integral organism level. Oral administration of the peptide in feed would be the best form of administration to reduce handling stress in fish, so future research should evaluate this route. If the immunostimulatory properties of PACAP can be used to prevent disease outbreaks in the field, it could save the industry up to 6 billion USD per year (Brummett *et al*., 2014), and reducing the use of antibiotics would decrease the development of antibiotic resistant pathogens so that we can continue to use available antibiotics for human and animal health for years to come.

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