UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics
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Transcriptional responses to Piscine orthoreovirus (PRV) and stress hormones in Atlantic salmon (*Salmo salar*) red blood cells

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A dissertation for the degree of Doctor of Philosophy

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"I love science and it pains me to think that so many are terrified of the subject, or feel that choosing science means you cannot also choose compassion, or the arts, or be awed by nature. Science is not meant to cure us of mystery, but to reinvent and reinvigorate it."

- Robert Sapolsky, *Why Zebras Don't Get Ulcers, 3rd ed. 2004*

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Acknowledgements

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Abbreviations

A		
	α-MSH	Alpha-melanocyte stimulating hormone
	AC	Adenyl cyclase
	ACTH	Adrenocorticotrophic hormone
	ASK	Atlantic salmon kidney
B		
	BANF	Barrier to autointegration nuclear assembly factor
C		
	CA	Carbonic anhydrase
	CATB	Cathepsin B
	CCR9	Chemokine (C-C motif) receptor 9
	CO_2	Carbon dioxide
	CRH	Corticotropin-releasing hormone
D		
	DAMP	Damage-associated molecular pattern
	DDIT4	DNA damage-inducible transcript 4
E		
	EIBS	Erythrocytic inclusion body syndrome
	ELOVL4	ELOV fatty acid elongase 4
F		
	FKBP5	FKBP prolyl isomerase
G		
	GC	Glucocorticoids
	GR	Glucocorticoid receptor
	GRE	Glucocorticoid response elements
H		
	HPI	Hypothalamic-pituitary-adrenal
	HSMI	Heart and skeletal muscle inflammation
I		
	IFN	Interferon
	IP	Intraperitoneal
	IPNV	Infectious pancreatic necrosis virus
	IQGAP	IQ motif containing GTPase activating protein 1
	IRF	Interferon regulatory factor
	ISAV	Infectious salmon anemia virus
	ISG	Interferon stimulated gene
K		
	KLF9	Krueppel-like factor 9
M		
	MDA5	Melanoma differentiation-associated protein 5
	MHC I	Major histocompatibility complex class I
	MRV	Mammalian reovirus
N		
	NLR	NOD-like receptor

	NF-KB	Nuclear factor κ-light-chain enhancer of activated B cells
O		
	OEC	Oxygen equilibrium curve
	O_2	Oxygen
P		
	PAMP	Pathogen-associated molecular pattern
	PCR	Polymerase chain reaction
	POMC	Proopiomelanocortin
	PRR	Pattern recognition receptor
	PRV	Piscine orthoreovirus
	PD	Pancreas disease
R		
	RBC	Red blood cells
	RD	Repressor domain
	RLR	Retinoid acid-inducible gene I-like receptors
	ROS	Reactive oxygen species
S		
	SAV	Salmonid alphavirus
	SGPV	Salmon gill pox virus
T		
	TBK1	TANK-binding kinase 1
	TRIF	TIR domain containing adaptor molecule 1
	TLR	Toll like receptors
	TRAF	Tumor necrosis factor receptor associated facto
U		
	uPAR	Urokinase receptor
V		
	VSHV	Viral hemorrhagic septicemia virus
W		
	Wpi	Weeks post injection

List of papers

Paper I:

Transcriptomics of early responses to purified *Piscine orthoreovirus*- 1 in Atlantic salmon (*Salmo salar* L.) red blood cells compared to non- susceptible cells lines

Thomais Tsoulia, Arvind Y.M. Sundaram, Stine Braaen, Jorunn B. Jørgensen, Espen Rimstad, Øystein Wessel and Maria K. Dahle

Published: *Frontiers in Immunology 15 (2024): 1359552*

Paper II:

Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of *Piscine orthoreovirus*

Thomais Tsoulia, Arvind Y.M. Sundaram, Marit M. Amundsen, Espen Rimstad, Øystein Wessel, Jorunn B. Jørgensen and Maria K. Dahle

Published: *Fish & Shellfish Immunology (Ref. YFSIM_FSIM-D-24-01278)*

Paper III:

Effects of glucocorticoid receptor activation on gene expression and antiviral response in Atlantic salmon red blood cells

Thomais Tsoulia, Arvind Y.M. Sundaram, Marit M. Amundsen, Martine J. Aardal, Maria E. Salvador Mira, Betty F. Ploss, Randi Faller, Ingvill Jensen, Mona Gjessing, Colin J. Brauner and Maria K. Dahle

Manuscript

Thesis summary

Fish red blood cells (RBCs) are nucleated and metabolically active with physiological and immunological properties. Salmonid RBCs are target cells of Piscine orthoreovirus (PRV), a double-stranded RNA virus with three known genotypes (PRV-1, 2 and 3). PRV-1 can give heart and skeletal muscle inflammation to farmed Atlantic salmon, which can lead to mortality in combination with stress, PRV-3 is non-pathogenic and cross-protective, whereas PRV-2 replicates less efficiently and protection is limited. The hypothesis is that RBCs respond differently to viruses depending on pathogenicity, that they respond to stress hormones, and that stress hormones inhibit their antiviral responses.

Paper I demonstrated that RBCs express genes involved in pathogen recognition, chemotaxis and regulation of antiviral immunity. Comparing RBC responses to PRV-1 *ex vivo* with non-susceptible Atlantic salmon cell lines, revealed that RBCs expressed a specific repertoire of genes associated with viral dsRNA sensing and non-canonical IRF1-signaling, which could be associated with increased susceptibility to PRV.

Paper II explored transcriptional differences in A. salmon blood cells post injection with different PRV genotypes. PRV-1 and PRV-3 replicated well in blood cells, but PRV-3 induced a potent antiviral response weeks earlier than PRV-1. This delay could increase the dissemination potential for PRV-1 and lead to increased pathogenicity. In contrast, PRV-2 and InPRV-1 primarily activated genes associated with intracellular signaling and protein trafficking.

Paper III showed that exposure of RBCs to dexamethasone *ex vivo* led to >100-fold increase in FKBP prolyl isomerase 5 (FKBP5) and DNA damage-inducible transcript 4 protein (DDIT4) gene expression, while suppressing genes involved in antiviral immunity and proteolysis. A comparison with cortisol-treated A salmon *in vivo* revealed that DDIT4 could be a putative stress biomarker.

These findings strengthen the notion that A. salmon RBCs are key mediators of antiviral and stress responses, while the specificity of their responses may have diagnostic potential.

Sammendrag av avhandlingen

Røde blodceller (RBC) i fisk har cellekjerne, metabolsk aktivitet, og både fysiologiske og immunologiske egenskaper. Hos laks er RBC målceller for Piscine orthoreovirus (PRV), et RNA-virus med tre kjente genotyper (PRV-1-3). PRV-1 kan gi sykdommen hjerte- og skjelettmuskelbetennelse (HSMB) i oppdrettslaks, en sykdom som i kombinasjon med stressende håndtering kan gi betydelig dødelighet. I motsetning til PRV-1 gir PRV-3 ikke patologi i laks. Hypotesen for avhandlingen er at genuttrykk i RBC kan avsløre hvorfor de er målceller for PRV, om viruset kan gi sykdom, og hvordan stress påvirker antiviral immunitet.

Det første arbeidet viser at lakse-RBC uttrykker gener med en rolle i patogengjenkjenning, kjemotakse, og regulering av antiviral immunitet. Sammenliknet med ikke-mottakelige laksecellelinjer uttrykker RBC andre gener involvert i virus-sensing og interferonsignaler (IRF1), noe som kan knyttes til økt mottakelighet for PRV.

I det andre arbeidet ble mRNA-responser på forskjellige PRV genotyper og inaktivert PRV sammenliknet i lakseblod. Etter to uker induserte kun PRV-3 en antiviral respons, selv om PRV-1 og PRV-3 replikerte like godt. Ved uke 5 responderte laksen likt på begge genotyper. Denne forsinkede responsen på PRV-1 kan være med på å øke virusets spredning til hjertet og dermed sykdom. I kontrast, aktiverte PRV-2 og inaktivert PRV-1 gener forbundet med intracellulær signaltransduksjon, proteintransport og antigenpresentasjon, men ikke antiviral respons.

Det tredje arbeidet viser at RBC eksponert for stresshormonreseptor-agonisten dexamethason og hydrokortison viste mer enn 100 gangers økning i uttrykket av FKBP prolyl isomerase 5 (FKBP5) og DNA damage-inducible transcript 4 protein (DDIT4) gener, mens gener involvert i antiviral immunitet og proteolyse ble signifikant hemmet. En sammenlikning med blod fra kortisolbehandlet laks viste at DDIT4 kan være en mulig biomarkør for stress.

Funnene styrker hypotesen om at laksens røde blodceller medierer antivirale responser og responser på stress, og at spesifisiteten i disse responsene kan ha diagnostisk potensiale.

1 Introduction

1.1 Atlantic salmon aquaculture

Aquaculture industry is rapidly expanding to meet the growing global demand for sustainable seafood (1). Norway is at the forefront of Atlantic salmon (*Salmo salar L.*) production, with annual harvests exceeding 1.4 million tons since 2020 (2). Other major producers include Chile, Canada, the United Kingdom, Australia and the Faroe Islands. Together, these countries account for approximately 90% of global annual salmon production (3). While technological and operational advances in aquaculture systems, driven by financial forces, have raised the expectations of salmonid production rate, the faltering support of biological insights pose significant risks to fish welfare (2).

Welfare entails aspects related to the biological traits, natural environment and individual experiences of the fish as sentient animals (4). Modern farming strategies involve high stocking densities, short production cycles and repeated handling, which increase stress and infection pressure, leading to higher disease susceptibility among farmed populations (5,6). In this context, to optimize preventive healthcare and align farming conditions with the essential needs of the fish, current biosecurity measures and welfare indicators based on both animal and environmental factors are under evaluation and development (2). However, large-scale aquaculture operations often rely on stressful handling practices, such as mechanical and thermal delousing, which carry a high risk of injury and weaken fish immune defense against various pathogens (7). In 2023, the mortality rate of A. salmon during the sea water phase of production reached an estimated 16.7%, causing substantial socio- economic impacts. The leading causes of mortality were infectious diseases (6,4%) and injuries primarily caused by handling procedures (5,5%) (2) (Figure 1). Therefore, the development of molecular-based methodologies for monitoring fish performance and health status is becoming more and more relevant.

Stress is a frequent event and important challenge in aquaculture. Restoration of fish homeostasis- the ability to maintain a constant internal environment (8)- during stressful events and injury requires complex physiological responses primarily controlled by the endocrine system. These responses include acceleration of metabolic rate and energy mobilization via glucose, alteration of hydromineral balance in blood (e.g. plasma chloride, sodium, potassium and/ or osmolality) and increase in cardiovascular activity to enhance oxygen delivery to tissues (9). In nature, most stressors are considered acute, occurring over a short duration with high intensity and typically triggering a fight-or-flight response. In contrast, stressors in aquaculture are often chronic, characterized by low to moderate intensity but extended duration. These chronic stressors are commonly associated with repeated operational activities, such as crowding, transportation and exposure to suboptimal environmental conditions (e.g. poor water quality, as well as fluctuation in temperature and/or oxygen levels) (10). However, chronic stressors may have cumulative effects, potentially compromising essential functions, including immune

protection (10,11). Given that the degree of response depends on the severity (low-moderate-high) and duration (short-moderate-long) of the stimuli, the excessive energetic cost of adaptive endocrine/physiological mechanisms in response to chronic exposure to stressors can be detrimental to fish welfare and survival, especially when co-occurring with high infection pressure (10,12,13).

Viral infections represent a major threat in salmonid farming (2). In Norway, five RNA viruses of different families have significant impact on A. salmon aquaculture by causing diseases of high prevalence and/or mortality: Piscine orthoreovirus (PRV), piscine myocarditis virus (PMCV) and infectious pancreatic necrosis virus (IPNV) that possess double-stranded (ds) RNA genomes, along with salmonid alphavirus (SAV) and infectious salmon anemia virus (ISAV), that possess single- stranded (ss) RNA genomes (Figure 1) (2). RNA viruses are known for high mutation rates, which enable them to rapidly adapt to selective pressures. These adaptations often result in enhanced immune evasion, vaccination resistance and increased pathogenicity (14). Thus, despite ongoing advances in vaccine development, commercial vaccines are unavailable for some viruses, while those that are available offer varying levels of protection (2,5).

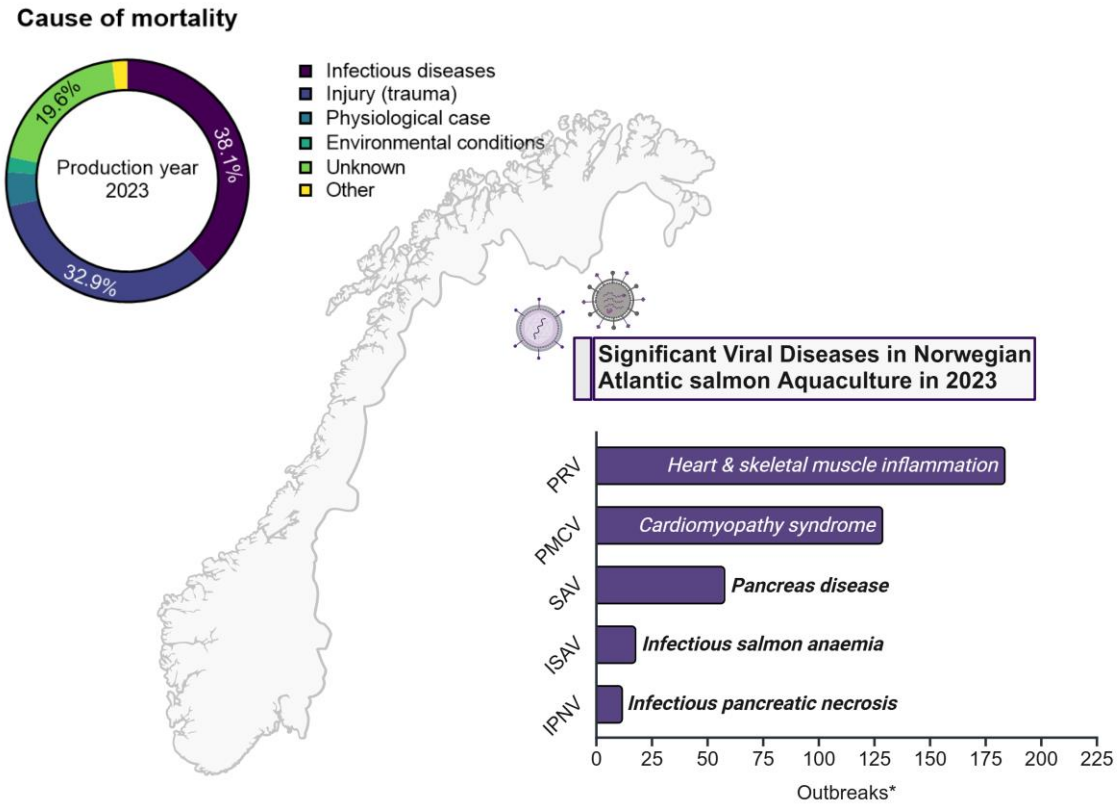

Figure 1. Overview of the main causes of mortality in Norwegian aquaculture in 2023 caused by the various categories, with a focus on the five most significant viral diseases and the number of outbreaks (*) with elevated mortality registered in 2023. The data were found in the Norwegian Fish Health Report (2023) (2).

Diagnosis of viral infections in aquaculture typically relies on a combination of clinical observations (e.g. loss of appetite, pale color, and bleedings), histopathological examination, and viral detection using RT-qPCR and/or immunochemistry (2). Thus, the successful control of diseases is often constrained by time limitations, while in most cases, fish sacrifice is inevitable. Although blood sampling, as a non-lethal alternative, and hematology analyses (e.g. hemoglobin content and hematocrit) could provide insights into fish health, particularly in the context of viral diseases affecting the circulatory system, their application remains limited (2,15). This limitation may largely be attributed to insufficient biological knowledge regarding the roles and functions of fish blood cells, including their responses to viral infections.

1.2 Fish red blood cells

Blood is composed of heterogeneous cell populations and plasma, with red blood cells (RBCs) accounting for over 70% of the total cells in the bloodstream (16,17). RBCs are typically flattened and ellipsoidal in shape, with variable size and longevity among vertebrate groups, according to their biological traits and adaptations to specific environments (Figure 2) (16,18). In mammals, erythropoiesis occurs in the bone marrow under the regulation of the erythropoietin hormone (17). In fish, the equivalent erythropoietic organ is the head kidney (16). During the final stages of erythropoiesis, mammalian RBCs shed their cellular organelles, resulting in irreversible transcriptional and translational arrest. In contrast, fish RBCs retain their nucleus and active transcriptional/translational machinery, allowing them to respond to internal and external threats through gene expression and protein synthesis (17,18). Although previous studies have shown that fish RBCs can undergo physiological alterations and employ innate immune mechanisms in response to systemic signals and pathogens (18,26–28), their transcriptional characterization in resting state and the extent of their contribution to physiological and immunological processes remain to be elucidated. Senescent RBCs are cleared by phagocytosis in the spleen and liver (16,17).

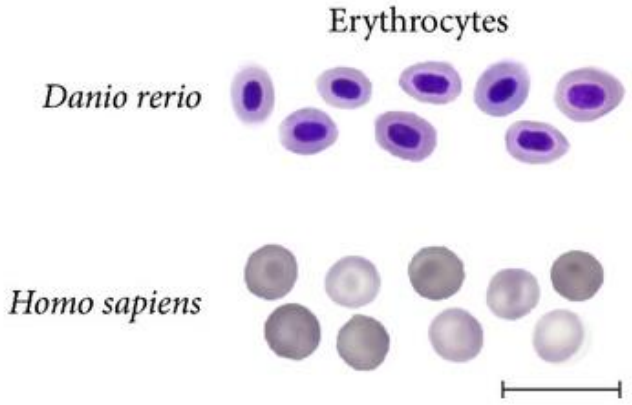

Figure 2. Comparative view of red blood cells (RBCs) from zebrafish (*Danio rerio*) and human (*Homo sapiens*). Modified image from (19).

Despite differences in morphology and function, vertebrate RBCs share some key physiological and immunological properties (17). They are primarily known for their role in gas exchange, a function mediated by the conserved respiratory pigment known as hemoglobin, crucial for the uptake and transport of oxygen and carbon dioxide during physiological respiration (17,20). Hemoglobin can also contribute to the innate immune response by stimulating the production of antimicrobial free radicals upon microbial invasion (17). RBC morphology, distribution and physiological characteristics can serve as indicators of health status in both fish and mammals (21,22). For instance, in mammals, variations in RBC shape and plasticity have been associated with increased oxidative stress and systemic inflammation (21). In fish, blood smears are commonly used to assess RBC shape and size, while alterations in total RBC count, hematocrit and hemoglobin content often serve as diagnostic tool in evaluating environmental stressors, including poor water quality, temperature fluctuations, and xenobiotic toxicity/pollution (22). While not routinely used for disease diagnosis, reduced hematocrit has also been linked to salmonid viral infections like infectious salmon anemia (2,23).

Salmonid RBCs are known to express β-adrenergic receptors, which respond to acute stress by alternating cells physiological characteristics (24,25). In addition, microarray analysis of A. salmon RBCs indicated the expression of a glucocorticoid receptor (GR) isoform typically induced in response to cortisol elevation (26). While RBCs may express stress hormone receptors, their capacity to initiate secondary transcriptional responses to stress remains unexplored.

1.3 Neuroendocrine responses to stress

The stress response is a well- conserved allostatic process that helps maintain internal stability in vertebrates. It occurs in both acute and chronic form, each associated with specific hormonal profile and distinct effects on physiological and immune functions (13,29). Catecholamines, such as adrenaline and noradrenaline (also referred to as epinephrine/ norepinephrine), are rapidly synthetized and cleared from the circulation, facilitating the immediate mobilization of glucose to cover stress- induced energy demands. These hormones are linked to acute stress responses (30). In contrast, the synthesis and release of glucocorticoids, particularly cortisol, occurs more slowly, with energy mobilization primarily driven through the activation of GR or mineralocorticoid receptor (MR) signaling pathways. In fish, cortisol is commonly used as a reliable stress indicator due to its elevation over longer time periods, including chronic stress (31). However, changes in blood plasma hormonal levels can only reflect the magnitude of a stress response within a specific time frame, but do not provide insight into how stress affects the ability of fish to cope with subsequent threats.

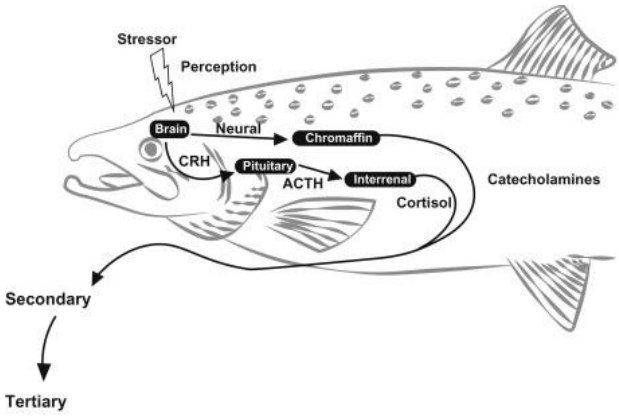

Figure 3. Activation of hypothalamus-pituitary-interrenal (HPI) axis and release of catecholamines and cortisol in response to stress in fish. Image was modified from the original by Shreck CB., et al. (2016) (133)

The primary endocrine responses to stress involve activation of the hypothalamus-pituitary- interrenal (HPI) axis, stimulating the synthesis and release of catecholamines and corticosteroids (13) (Figure 3). Stress is perceived by sensory cells, which send signals to the hypothalamus, prompting the release of corticotrophin-releasing hormone (CRH). CRH binds to the receptors on corticotropic cells in the anterior pituitary, initiating the synthesis of the proopiomelanocortin (POMC) hormone. POMC is then cleaved to produce its successor adrenocorticotropic hormone (ACTH), which enters the systemic circulation. POMC is also a precursor of alpha-melanocyte-stimulating hormone (a-MSH), and b-endorphins, both previously used as markers of acute stress in fish (9,30). Circulating ACTH binds to melanocortin 2 receptor (MC2R) in the interrenal steroidogenic cells of the head kidney, activating corticosteroid biosynthesis (10,30). In mammals, GRs are distributed in the cytoplasm of diverse cell types, bound to an HSP90 dimer. The GR-complex shows a modest affinity for corticosteroids and is only partially activated when cortisol levels in the bloodstream are low (32). During stress, cortisol diffuses through the cell membrane and binds to GR, promoting its translocation to the nucleus through disassociation of the complex chaperones. Once in the nucleus, GR/cortisol interacts with glucocorticoid response elements (GREs), leading to activation and/or repression of glucocorticoid- target genes (30). In fish, elevated cortisol levels in response to chronic stress have been associated with the suppression of both innate and adaptive immune functions, including disrupted cytokine signaling, induced apoptosis of T- and B- cells and reduced antibody production (10,31).

Catecholamines are produced in the chromaffin cells of the head kidney (interrenal tissue), with the neurotransmitter acetylcholine playing a regulatory role in this process (30). Adrenaline signal through β-adrenergic receptors, which belong to the g-protein coupled receptor (GPCR) family. These transmembrane receptors have a central role in the physiological response to stress in fish (29). One of the best characterized mechanisms in salmonids involves the regulation of Na^+/H^+ exchanger activity in red blood cells (RBCs), which affects hemoglobin- O_2 affinity and is crucial for maintaining O_2 transport efficiency under stressful conditions (20,33). Adrenaline binds to transmembrane β- adrenergic receptors, activating adenylyl cyclase (AC). This enzyme hydrolyzes ATP to produce cyclic AMP (cAMP), a secondary messenger that regulates diverse cellular processes by phosphorylating protein kinase A and subsequently activating cAMP response element binding protein (CREB). Although

adrenaline has been shown to positively affect immune protection in fish by enhancing innate immune responses (29), the stress-induced signaling pathways involved in immune-enhancement have not been fully elucidated.

Over the years, significant efforts have been made to identify molecular biomarkers of acute/chronic stress in fish, related to physiological processes, such as metabolism, growth and reproduction. These studies primarily focused on gene regulation and protein secretion in tissues such as liver, spleen and head kidney, and were largely based on mammalian models (30,34). Most fish welfare assessments continue to rely on physical signs of disturbance (swimming patterns/gill movements) and hormonal analyses, primarily cortisol levels. Detecting the secondary effects of stress hormones holds great potential for assessing fish health, but the regulatory gene networks modulated by cortisol and catecholamines have not been well-defined in salmonids.

1.4 Stress and immunity in fish

Viral infections, combined with repeated operational stressors in aquaculture, trigger physiological, endocrinological and immunological responses in fish that are essential for maintaining allostasis- the process of achieving stability through change (8)- and ensuring survival. These responses are orchestrated by a complex bidirectional communication network between the fish brain and immune system, involving hormones, neurotransmitters and cytokines. Although these molecules differ biologically, their functions are interrelated, often converging on common signaling pathways (e.g. JAK/STAT) (31). In addition, immune cells express adrenergic and glucocorticoid receptors, while neuroendocrine cells possess immune receptors, supporting the notion that handling of exogenous and/or endogenous threats involves synergy between immune and hormonal axes (10). From a physiological perspective, recovery from stress involves energy-demanding coping mechanisms that can detract from other essential biological functions, such as immune protection (30). Notably, some viral pathogens, such as IPNV and salmon gill pox virus (SGPV), have been detected in farmed population without initially causing disease, while the onset of pathogenesis and mortality has been linked to stress (35,36). Despite these observations, the mechanisms underlying stress- immune interactions, particularly in relation to immunosuppression and fish mortality, remain relatively uncharacterized.

1.5 Fish immunology

Teleost innate and adaptive immune system are closely interconnected, like in other vertebrates. The innate immune system is characterized by constitutive and inducible mechanisms, which provide immediate, host defense against foreign infectious agents or endogenous threats (37,38). In contrast, adaptive immunity is defined by slower, antigen-specific responses that are involved in the activation and differentiation of T and B lymphocytes, leading to long-term immunological memory (39).

1.5.1 Innate immune system

The innate immune system forms the front line of defense, primarily to stop, fight and prevent internal dissemination of pathogens, and to minimize disruptions of cellular homeostasis. Constitutive innate immune mechanisms have a broad range of actions, including physical and chemical barriers to block infection, such as skin, mucosal tissues and the humoral components with antiviral and antimicrobial properties that block pathogen entry and/or replication in these tissues (38,40). Important inducible mechanisms are pattern recognition receptor (PRR)- dependent and activated upon binding of pathogen-derived ligands (40). Germline- encoded PRRs recognize a wide range of pathogen- or damage-derived ligands and play a pivotal role in triggering innate immune responses (13,41). Some PRRs are distributed across the outer cell membrane, some within intracellular membranous compartments (e.g. endosomes) and some in the cytoplasm. The interaction of PRRs with pathogen-associated molecular patterns (PAMPs), such as foreign genomic material, or damage-associated molecular patterns (DAMPs), such as metabolites from apoptotic host cells, leads to potent innate immune responses that drive inflammation and promote activation of adaptive immunity (42). Some pathogens have evolved mechanisms to subvert detection by PRRs, either by camouflaging themselves with host- mimicking antigens or by altering receptor structural features, and thereby preventing the cells from binding to the pathogens (13,41).

In general, PRRs are classified into four main categories based on their protein domain homology: Toll like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) (38). Members of the class A scavenger receptor (SR-A) family have also been identified in some fish species, including zebrafish, rainbow trout and yellow croaker (43). TLRs are transmembrane homo- or heterodimers that exhibit high diversity in their ligand-binding properties, and therefore respond to a wide array of pathogen molecules. In fish, important PAMPs include viral ssRNA (interact with TLR8), dsRNA (interact with TLR3 and TLR22), bacterial components, such as lipopolysaccharides (LPS) (interact with. TLR1/TLR2 heterodimer, TLR5 and TLR2/TLR6), and CpG DNA motifs (interact with TLR9 and TLR21) (38). The RIG-like receptor (RLR) family consists of cytosolic DExD/H box RNA helicases that induce innate antiviral responses against dsRNA viruses (44). A limited number of intracellular NLRs and transmembrane CLRs have been identified fish, with their functions primarily associated with innate antibacterial immunity (42,45). Although each receptor varies in structure, cellular localization and ligand specificity, PRR- mediated signal transduction involves three major types of molecules- (a) protein kinases, (b) adaptor proteins and (c) transcription factors- that converge on several common signaling pathways. These pathways can stimulate the release of (pro-)inflammatory cytokines, chemotactic cytokines and/or activate antimicrobial and interferon (IFN)-mediated antiviral responses (46).

1.5.2 PRR- mediated antiviral pathways

Three major TLRs are involved in antiviral responses to RNA viruses in fish, the cell- surface TLR22 and the endosomal TLR3 and TLR8. TLR8 primarily recognizes small ssRNA, while TLR3 and TLR22 respond to viral dsRNA, including stimulation with the synthetic dsRNA analogue, poly(I:C) (43). TLR22 is unique to fish, and its signaling cascade resembles that of the well- conserved TLR3, which has been identified in both fish and mammals (47). In fish and mammals, most TLRs interact with MyD88 signaling adaptor, with the exception of TLR3 and TLR22 which signal through the TIR domain containing adaptor molecule 1 (TICAM, also referred to as TRIF). Following the recruitment of MyD88 and/or TRIF adaptors by TLRs, two major signaling pathways are activated, both involving specific mitogen- activated protein kinases (MAPKs), such as interleukin-1 receptor- associated kinase (IRAK) 1/4. One pathway results in the production of pro-inflammatory cytokines through engagement of transcription factor NF-kB, while the other induces type I IFNs through a MyD88-IRF1 and/or TNF receptor associated factor 6 (TRAF6)- IRF3/7- dependent mechanism (43) (Figure 4).

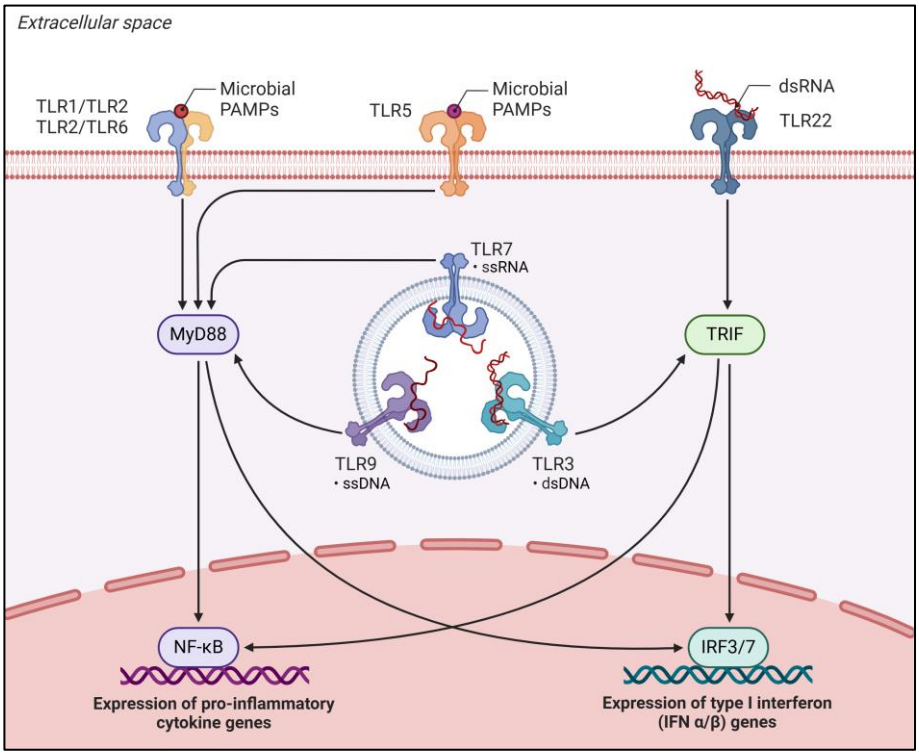

Figure 4. Toll like receptors (TLR) signaling pathways. In fish, TLRs signaling pathways involve interaction with the myeloid differentiation primary response protein 88 (MyD88) adaptor. However, exceptions include TLR3 and TLR22, which signal through the TIR domain containing adaptor molecule 1 (TRIF). Upon interaction with MyD88 or TRIF factors, downstream signaling promotes the expression of pro-inflammatory cytokines and type I interferon (IFN) through activation of a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB)- or IFN regulatory factor(IRF)- dependent pathways, respectively (43). The figure was created on BioRender.

The RLR family consists of three known members: RIG-I (also referred to as RLR1), melanoma differentiation-associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2, also

referred to as RLR3). RIG-I and MDA5 share similar structure, each featuring a DexD/H helicase domain in the middle, two caspase activation and recruitment domains (CARD) at the N-terminus, and a C-terminal domain (CTD). RIG-I also possess a repressor domain (RD), exhibiting self- inhibitory functions in a resting, uninfected cell (43,46). RLR3 differs structurally from RIG-I and MDA5 by the lack of CARD, while its specific role in antiviral signal transduction in fish remains to be fully understood (43). Upon ligand recognition, RIG-I and MDA5 bind to the adaptor mitochondrial antiviral-signaling protein (MAVS, also referred to as IPS-1). Activated MAVS interacts with the transcription factor TRIF3 to initiate synthesis of type I IFN through the TANK- binding kinase 1 (TBK1)- IRF3/7 signaling pathway (Figure 5). Although the signal transduction mechanism of RLR3 is not well-characterized, studies in both fish and mammalian cells suggest that RLR3 is implicated in dsRNA recognition and signals in a manner similar to RIG-I and MDA5 (43,46,48).

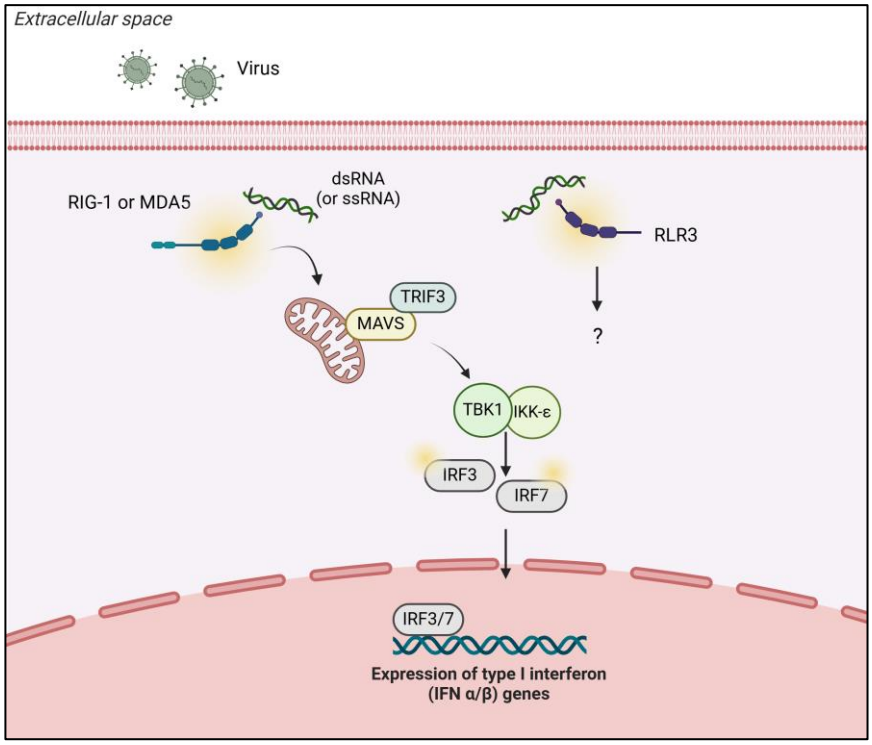

Figure 5. Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) signaling pathways. In fish, the RLR family members RIG-I (also referred to as RLR1) and melanoma differentiation-associated factor 5 (MDA5) contain a DExD/H box helicase domain (DEXDc), a helicase C-terminal domain (HELICc), a regulatory domain (RD) and two caspase activation and recruitment domains (CARDs) at the N-terminal region. In contrast, the laboratory of genetics and physiology 2 (LGP2, also referred to as RLR3) lacks CARDs. Upon sensing of double stranded RNA, RLR1 and MDA5 interact with mitochondrial antiviral signaling protein (MAVS). MAVS, in turns, through interaction with TRAF3 and TBK1 leads to the activation of interferon (IFN) regulatory factor 3 (IRF3) and IRF7. These factors translocate to the nucleus to initiate the expression of type I IFN genes. The signaling pathway of RLR3 has not been fully elucidated in fish (43,46,48). The figure was created on BioRender.

Fish type I IFNs are categorized into group I and group II based on their cysteine composition and sequence homology. Members of both groups are essential in inhibiting viral replication and dissemination by inducing an antiviral state, primarily through the activation of IFN-stimulated genes (ISGs) (49). Various immune mechanisms, mediated by different IRFs, modulate IFN responses in fish, ensuring that the antiviral response is tightly regulated (50–52). These mechanisms include repression of IRF3/IRF7- signaling and IFN induction by IRF10 (42). Secreted IFNs from virus- infected cells are recognized by membrane-bound heterodimeric type I IFN receptors (IFNAR) on neighboring cells. Upon receptor binding, Janus tyrosine kinases JAK1 and TYK2 are activated and phosphorylate the signal transducer and activator of transcription (STAT) 1 and STAT2. STAT heterodimers form a multimeric complex with IRF9, known as STAT1/STAT2/IRF9 complex. This complex translocate into the nucleus, where it binds to IFN-sensitive response elements (ISREs), thereby stimulating the transcription of ISGs, such as MX, viperin (also referred to as RSAD2) and ISG15 (43,53). In salmonid, in addition to IRF3, IRF7 and IRF9, eight other members of the IRF family have been identified (i.e. IRF1-IRF11), all linked to the regulation of interferon activity (51). Although IRF3 has been emerged as the most potent inducer of IFN responses (50), other factors such as IRF1 and IRF2 may also contribute to type I IFN activation (50,51).

1.6 Piscine orthoreovirus

Piscine orthoreovirus (PRV) is a non- enveloped reovirus, classified in the order *Reovirales*, family *Spinareoviridae*, genus *Orthoreovirus*. The viral particle consists of a double- shelled icosahedral capsid approximately 70 nm in diameter, that encloses ten linear segments of a double- stranded (ds) RNA genome (54,55). These genomic segments encode at least eleven proteins; eight structural proteins in the virus capsid and three non-structural proteins only present in the infected host cells. Based on studies conducted for MRV, each protein is predicted to play a distinct role in the viral life cycle, associated with (i) transcription and replication of the viral genome, (ii) formation of the integral parts of the particle, and (iii) virus- host interactions during infection (56). To date, the specific functions of μ1, σ3, p13 and μNS have been characterized for PRV (57–60). A summary of the structural and non- structural proteins of PRV, and their associated functions are shown in Figure 6 and described in Table 1.

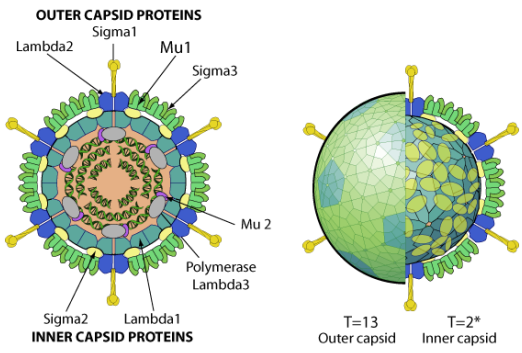

Figure 6. Orthoreovirus proteins and viral symmetry. Source: https: //viralzone.expasy.org, Swiss Institute of Bioinformatics.

Table 1. Structural and non- structural proteins encoded by PRV and their main functions. The table was modified based on the original by Dahle, Wessel and Rimstad (2022) (7).

Protein	Function
Outer capsid	
μ1	Likely to involved in endosomal membrane penetration
σ1	Cellular attachment protein
σ3	dsRNA binding properties (61)
Inner capsid	
λ1	RNA helicase properties
λ2	Involved in viral mRNA capping
λ3	RNA- dependent RNA polymerase for mRNA and dsRNA transcription
μ2	Polymerase- associated protein
σ2	dsRNA binding properties
Non- structural	
μNS	Organization of viral factories (62)
σNS	Formation of viral factories
p13	Cytotoxic, non-fusogenic integral membrane properties (60,63)

1.6.1 PRV genotypes and pathogenicity

Phylogenetic analysis of PRV genomes have revealed three distinct genotypes, PRV-1, PRV-2 and PRV-3. The comparison of PRV-3 to PRV-1 showed approximately 80% nucleotide identity in coding regions, and 90% amino acid (aa) sequence identity. In contrast, when comparing PRV-2 to both PRV-1 and PRV-3, nucleotide and aa sequence homology was approximately 70% and 80%, respectively. Hence, based on genome and protein alignment sequencing, PRV-1 and PRV-3 are more closely related (64).

PRV genotypes exhibit preference towards their primary hosts, but can also cross-infect different salmonid species, causing different pathogenicity (55,65–67). The main host species for PRV-1 is A. salmon. However, PRV-1 variants have also been detected in Pacific salmonid species, including coho (*Oncorhynchus kisutch Walbaum*), Chinook (*Oncorhynchus tshawytscha Walbaum*), pink salmon (*Oncorhynchus gorbuscha Walbaum*) and rainbow trout (*Oncorhynchus mykiss*) (68). PRV-1 is the causative agent of HSMI (55), one of the most prevalent viral diseases in A. salmon aquaculture in Norway (2). Disease outbreaks typically occur during the marine phase of production, with cumulative mortality reaching up to 20% of the infected population. HSMI is characterized by signs of circulatory disturbance and typical histopathological lesions, including extensive inflammation in all layers of the heart ventricle, initiated in the epicardium. The disease progress to extensive myocarditis, myocardial necrosis, and inflammation and necrosis of the red skeletal muscle (69). PRV-1 is ubiquitous in A. salmon aquaculture, and is often detected in farmed and wild populations without any clinical

symptoms. This asymptomatic prevalence is attributed to the persistence of the virus in salmon healed from disease, and also viral isolates with low levels of virulence (70,71). Stress may be a critical factor in the onset of the disease and the HSMI- mediated mortality (72). Notably, post- smolts affected by HSMI have demonstrated low tolerance to experimental hypoxia (73).

PRV-3 primarily targets farmed rainbow trout and causes heart inflammation and severe anemia (66). PRV-3 has also been detected in farmed coho salmon in Chile and wild brown trout (*Salmo trutta*) in central Europe, in association with jaundice syndrome (74), and in wild brown trout (*Salmo trutta*) in central Europe with proliferative darkening syndrome (PDS) (75,76). However, the causative role of PRV-3 in these diseases remains unconfirmed. Although PRV-3 is widespread in rainbow trout aquaculture in Europe, disease outbreaks have primarily been reported from hatcheries in Norway (77) and recirculating aquaculture systems in Denmark (78).

PRV-2 is the causative agent of erythrocytic inclusion body syndrome (EIBS) in farmed coho salmon in Japan. Unlike PRV-1- mediated HSMI, individuals affected by EIBS are severely anemic, with mortality often related to secondary infections by other pathogens (67). Recently, PRV-2 was also detected in wild coho salmon in North America that exhibited myocardial degeneration, mononuclear infiltration of the spleen and anemia (79).

Despite the high prevalence of PRV-1 in A. salmon aquaculture, there are no official eradication strategies or commercially available vaccines (2). In Norway, vaccination trials with an adjuvanted, inactivated PRV-1 vaccine (InPRV-1) has shown some efficacy against HSMI in A. salmon (65,80). Recently, immunization of A. salmon with three PRV genotypes, revealed that PRV-3 can efficiently block subsequent PRV-1 infection and HSMI, while PRV-2 and an inactivated PRV-1 vaccine provided only partial protection (65). However, the molecular mechanisms and specific cellular responses to each PRV genotype, and the drivers of protection against PRV-1 and HSMI are poorly understood.

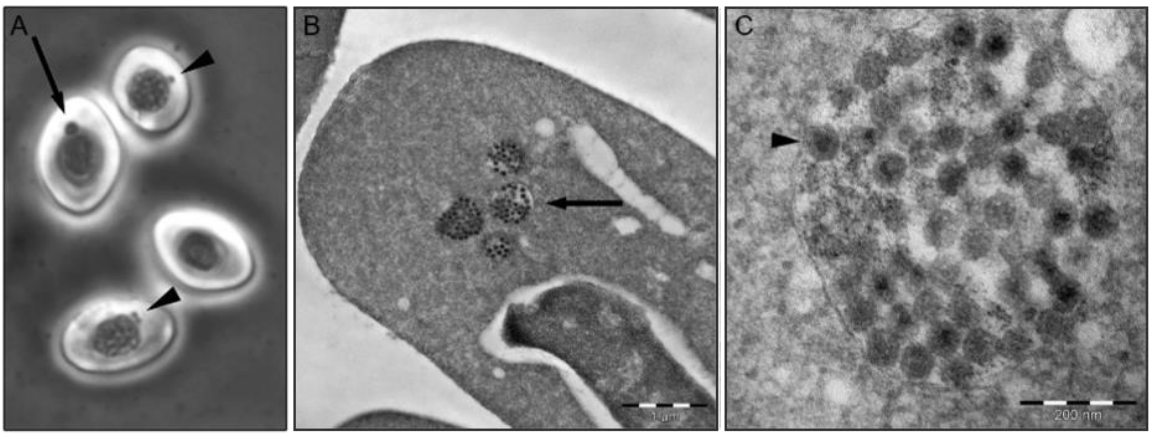

Figure 7. PRV in red blood cells. (A) Cytoplasmic inclusions (arrows) in blood smear of infected fish (pinacyanol chloride staining). (B) and (C) Electron microscopy of PRV inclusions in red blood cells cytoplasm. Modified images from (81).

1.6.2 The PRV infection dynamics

PRV infection and pathogenesis can be divided into distinct phases: (i) viral entry, (ii) replication in RBCs, (iii) dissemination to the heart, (iv) heart and skeletal muscle inflammation, (v) heart and skeletal muscle regeneration, and (vi) persistence. While PRV genotypes share similar systemic replication and dissemination patterns (65–67), pathogenesis depends on the specific traits of each genotype and the infected salmonid species (56). In addition, while PRV-2 and PRV-3 are cleared from their respective primary hosts after infection, PRV-1 demonstrates long-lasting persistence in A. salmon despite robust host antiviral responses (65–67).

PRV, similar to its mammalian counterpart MRV, enters the bloodstream of naïve individuals through the gastrointestinal tract (82). Nucleated red blood cells (RBCs) are the primary target cells during the initial stage of infection (66,67,81). While PRV internalization in RBCs likely occurs through receptor-mediated endocytosis, as observed in the MRV infection model, the specific proteins involved during endosomal uptake remain unknown (83). It has previously been shown for MRV that upon acidification of endosomes, the outer capsid of MRV proteins μ1/σ3 undergo proteolysis, allowing the viral core with the dsRNA genome to pass the membrane into the cytoplasm (83). In the cytoplasm, multiple copies of capped mRNA are synthesized from the genome within the core, and the released mRNAs are translated by the host cellular machinery (84). Both for MRV and PRV, the viral non-structural μNS proteins are important for the formation of globular cytoplasmic clusters, known as "viral factories", where viral progeny are assembled (Figure 7) (55,58,62). The mechanisms of virus- host interaction during early encounter, and how these interactions determine a pathological outcome, remain largely unknown.

During the peak of infection, high levels of viral RNA and proteins can be detected within RBCs and in blood plasma (55,62). This phase is when potent antiviral immune responses are typically observed in blood cells (26,62). In the early phase of infection, increasing viral RNA load within the RBCs is positively correlated with increased expression levels of key antiviral effectors, such as ISG15, viperin and protein kinase R (PKR) (also referred to as eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2)) (26,62,72). For PRV-1, this phase is also associated with virus dissemination to the cardiomyocytes before the onset of HSMI (26,85). PRV-1 infection is not associated with severe anemia, despite a decrease in hemoglobin content observed in PRV-1-infected fish (70,73). While some lysis may occur, the mechanism by which the virus exits the infected RBCs has not been determined (61,81).

Following the peak of plasma viremia, PRV-1 can be detected in several other cell types in A. salmon, including cardiomyocytes, macrophages and hepatocytes (85). Subsequent infection of heart and spleen has also been reported for PRV-3 in rainbow trout (66) and PRV-2 in coho salmon (86). However, whether PRV genotypes can replicate in these secondary target tissues remains unclear. The replication potential of PRV-1 has previously been tested *ex vivo* in various cell lines, including Atlantic salmon

head kidney (ASK & SHK-1) and heart endothelium (ASHe) cells (87); however, only primary RBCs were found to support viral amplification *ex vivo* (88).

2 Aims

The main objective of this study was to explore the transcriptional responses of A. salmon RBCs to PRV and cortisol, to determine their role as sensors of stress and infection. We hypothesized that RBC gene expression pattern can tell us more about RBC functions and biological implications of viral infection and stress. The following sub-goals were addressed:

Sub goals:

1. Characterize basal functions of A. salmon RBCs based on gene expression
2. Compare the transcriptional responses in A. salmon RBCs early after PRV-1 encounter with non-susceptible cell lines *ex vivo*
3. Compare the transcriptional responses in blood cells of A. salmon infected by pathogenic PRV-1 and non-pathogenic PRV-2 and PRV-3 genotypes, linked also to their different cross-protection potential against HSMI
4. Identify target genes specific to chronic stress responses in A. salmon RBCs
5. Characterize the effects of chronic stress hormones on the antiviral responses of A. salmon RBCs *ex vivo*

3 Methodological considerations

3.1 Isolation of red blood cells

In papers I and III, we used an *ex vivo* stimulation model of purified A. salmon RBCs to study their transcriptional responses under exposure to PRV-1, poly (I:C) and/ or stress hormones, without interference from other cell types, like leukocytes.

Isolation procedures of heterogeneous blood cell populations in mammalian and fish health research commonly involve Ficoll and Percoll density gradient centrifugation (81,89,90) (Figure 8). In papers I and III, we isolated RBCs from whole blood of healthy A. salmon using Percoll (51%) density gradient and low speed centrifugation. This method, similar to Ficoll, allows RBCs sedimentation, while unwanted cells and cellular debris with lower density settle at higher levels of the gradient.

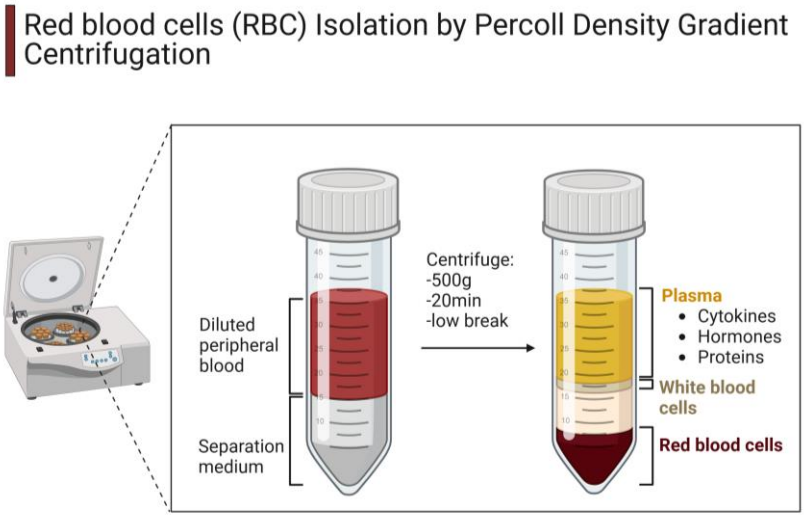

Figure 8. Red blood cell isolation procedure by Percoll (or Ficoll) density gradient. Template figure from fcslaboratory.com was modified in BioRender.

The purity of RBC cultures was evaluated using microscopy, based on the easily distinguishable oval and biconvex morphology of the cells compared to leukocytes. A culture purity threshold of 99% was deemed acceptable. RBC isolation using Ficoll density gradient centrifugation was also tested, yielding a culture purity comparable to Percoll (> 99%). However, the cells were found less resilient over long-term culturing and stimulation. Comparative studies of Ficoll and Percoll density gradient centrifugation of peripheral blood mononuclear cells in mammals have also reported significantly higher cell loss during Ficoll- based isolation procedures (89).

During transcriptome data analysis, the low or absent transcriptional level (< 5 median normalized transcript reads) of typical lymphocyte markers, such as CD3, CD4 and CD8, provided further

confirmation of RBC isolation quality. For the samples in paper III, relative expression of CD4 and CD8 was also evaluated using RT-qPCR prior to RNA-seq.

Isolation of RBCs from whole blood and head kidney has previously been performed using single- cell sorting (91). While this method may yield optimal cell separation and culture purity, it affects cell robustness and viability, factors with significant impact on transcriptional responses to the tested conditions. In addition, cell sorting is a laborious and costly procedure, criteria that often set limitations in the number of biological replicates in the experimental design. Thus, Percoll density gradient isolation procedure was deemed the most suitable for our experimental settings.

3.2 *Ex vivo* stimulation of RBCs and kidney cells with purified PRV-1

In paper I, RBCs, along with ASK and SHK-1 cells, were exposed to purified PRV-1. Viral purification was performed using cesium chloride (CsCl) density gradient ultracentrifugation of pelleted blood cells from previously infected fish (55). In this process, high-density supercoiled molecules, such as DNA from the infected blood cell suspension, move to high-density gradient (bottom layers), while smaller molecules, such as proteins, settle to low- density fractions (top layers). Intact virions, which typically exhibit a density of 1.36 g/cm^3, settle in a distinct "virus band" often visible in the intermediate layers of the gradient. Proper separation of whole virions from empty viral particles and debris is critical for maintaining the quality of the purified material and ensuring consistent interpretation of viral challenge results. However, it should be noted that during the *ex vivo* stimulation of RBCs, ASK and SHK-1, minor amounts of viral degradation products and structural fragments may still be present.

ASK and SHK-1 are large adherent cells, cultured at an optimal density of approximately 80% confluence under static conditions (Figure 9). In contrast, RBCs are maintained as suspensions under constant agitation, thriving at densities of 1- 2·10^7 cells/mL. Given their lower RNA content relative to the proliferating kidney cells, the high culture density of RBCs promotes the extraction of sufficient cellular nucleic acids during analysis. In addition, incubation under rotation likely enhances cell-virus contact, potentially facilitating their interaction (Figure 9).

Since PRV cultivation has not been possible in any cell line (87), determining viral infectious units via plaque-forming unit or 50% tissue culture infectious dose assays is not feasible. Instead, the viral genome copy number in purified PRV batches is determined using absolute quantification RT-PCR. Due to the limited availability of purified virus, viral input had to be carefully adjusted to ensure an adequate number of identical biological replicates for cell- pathogen interaction experiments across all cell types. The number of viral particles per cell (referred to as multiplicity of infection (MOI)) was set at 1 for RBCs and 10 for kidney cells. The primary objective was to identify transcriptional changes of

specific antiviral factors triggered during early viral encounter distinct for RBCs, ASK and SHK-1, rather than to compare the overall response potency between cell types.

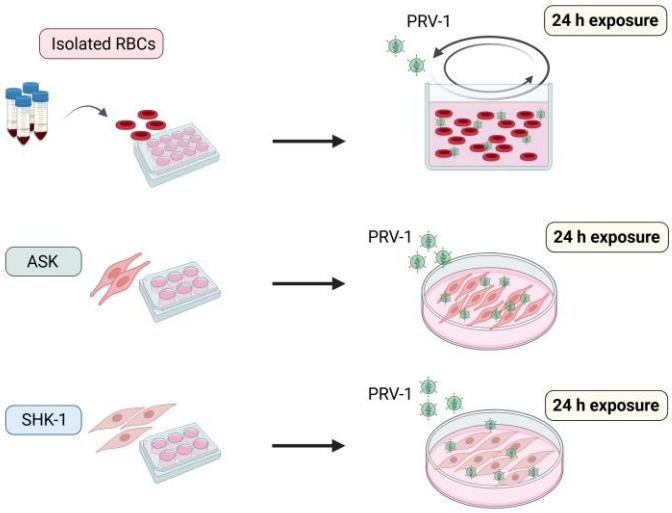

Figure 9. Cell *ex vivo* exposure to PRV-1 in paper I. Atlantic salmon RBCs, along with two kidney cell lines, ASK and SHK-1, were exposed to PRV-1 for 24 hours.

3.3 Comparison of glucocorticoid- mediated effects on RBCs antiviral responses to PRV-containing blood lysate and poly(I:C)

In paper III, we investigated the effects of glucocorticoids on the antiviral responses of A. salmon RBCs *ex vivo*. In particular, purified RBCs, pre-treated with dexamethasone or cortisol for 24 h, were subsequently exposed to poly(I:C) for an additional 72 h. The glucocorticoid incubation period was determined to mimic previous *in vivo* studies in fish, showing that plasma cortisol levels typically returns to baseline within 48-72 h post exposure to a stressor (92). Given that dexamethasone has generally higher binding affinity for GRs across tissues (93,94) and is more stable (longer plasma half- life) compared to cortisol (95), their functionality/effects may differ. Therefore, to ensure comparable effects on gene expression in A. salmon RBCs, both glucocorticoids were tested.

Preliminary stimulations of A. salmon RBCs with over one, three and seven days were performed. A three- day exposure to 50 µg/mL poly(I:C) was found to sufficiently induce potent antiviral responses, with MX1 mRNA levels increasing > 20-fold and ISG15 > 50-fold compared to unstimulated controls.

In addition to poly(I:C), PRV-1 containing blood lysate were used to induce antiviral responses in A. salmon RBCs, aiming to further investigate whether the glucocorticoid- mediated immunosuppression exhibits similar effects. RBCs exhibited strong antiviral responses, with MX1 and ISG15 relative expression levels substantially higher compared to those observed in poly(I:C)- treated cells (Figure 10). This difference may related to the origin of the infectious material, which in addition to a high viral

load, likely contained endogenous molecules with immunostimulatory actions, such as interferons and cytokines. Interestingly, co-stimulation of RBCs with dexamethasone and PRV-containing blood lysate led to immunosuppression in only some samples. This variability may reflect the complex interactions between endocrine and immune systems, where cytokines can modulate stress responses (13,31); thus, the effects observed may be influenced by immune-related molecules in the infected blood lysate. These results were not included in the published papers.

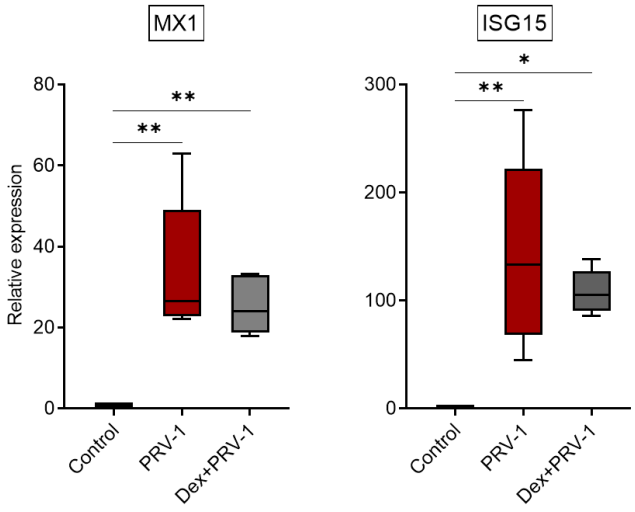

Figure 10. Effects of dexamethasone on the antiviral responses in RBCs induced by a PRV-1- containing blood lysate. Isolated RBCs were exposed to 100 μM dexamethasone, and after 24 h incubation under constant agitation, the cells were exposed to PRV-1- containing blood lysate for an additional 72 h. Expression of (A) MX1 and (B) ISG15 was measured by RT-qPCR, and is shown relative to the unstimulated controls (n=5). Data were analyzed using two-way ANOVA Tukey's multiple comparison test compared to the unstimulated controls. *: $p < 0.05$; **: $p < 0.01$

3.4 RNA-sequencing and differential expression analysis

RNA-sequencing (RNA-seq) provides a comprehensive snapshot of gene expression levels from a biological sample at a specific time point (96). The RNA-seq workflow involves several key steps, including RNA extraction and quality assessment, library preparation and two stages of data analysis: pre-processing and downstream analyses after alignment to a reference genome (96,97). We used RNA-seq to investigate gene expression patterns in A. salmon (red) blood cells, comparing individuals subjected to infection models with PRV (paper I and II) or poly (I:C), and chronic stress hormones (paper III) to healthy controls. Unlike qPCR and microarray technologies, which rely on predefined primers and probes, RNA-seq does not require prior sequence knowledge. This enables the identification of both known and novel genes, making it a well- suited method for the investigation of biomarker candidates in our study (paper III). In addition, RNA-seq facilitates simultaneous identification of variants and isoforms across multiple target regions, with the high sequencing depth allowing for

detection of subtle changes in gene expression that may have been missed by other quantification methods (98,99).

Total RNA isolation was performed using two different protocols: RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) in paper I, and automated MagNA Pure 96 Cellular RNA Large Volume Kit (Roche) in papers II and III. Both methods included genomic DNA elimination and resulted in high-quality RNA purification, evaluated by 260/280 and 260/230 absorbance ratios. RNA quality was also assessed via automated electrophoresis, with an RNA Integrity Number (RIN) of 8 (on a scale from 1 to 10) set as the minimum acceptable threshold. Samples with high RIN scores are more likely to maintain RNA integrity throughout the library preparation and sequencing process. However, RNA degradation may still occur, leading to low alignment rates to the reference genome and variability among samples within the same experimental group. This was observed in samples used for paper II, where RNA from blood of uninfected controls at week five, despite exhibiting A260/280 and A260/230 ratios and RIN values within the recommended thresholds, may have been more prone to degradation during the RNA-seq workflow. Therefore, these samples had to be excluded from downstream analysis.

The isolation of total RNA yields a mixture of coding and non-coding RNA molecules. During library preparation, the messenger RNA (mRNA) was enriched using poly-A selection, allowing RNA-seq to target only the protein coding transcriptome. While mRNA- seq covers only a small fraction of the transcriptome (~2%), it provides greater sequencing depth and requires less starting material compared to total RNA-seq, rendering it a more cost- effective option (97). It is worth noting that in cases where transcriptional activity is completely blocked, such as during exposure to UV radiation, mRNA-seq may limit the scope of the analysis (97). This is because expression data from ribosomal RNA (rRNA) and long non- coding RNA (lncRNA), both of which are involved in transcription regulation, are omitted from further analysis (97). An overview of the mRNA-seq workflow and data analysis is shown in Figure 11, and discussed in following subsections.

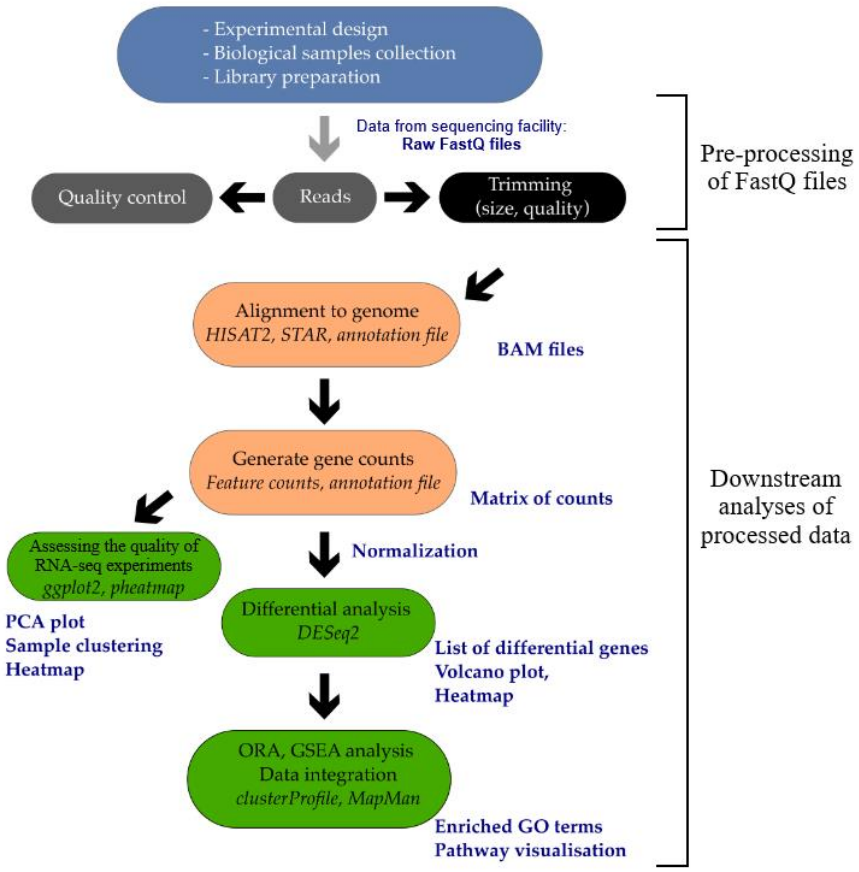

Figure 11. Overview of RNA-seq workflow and data analysis. The figure was modified from the original by Tijs et al., 2020 (100).

3.4.1 Pre-processing of raw RNA-seq data

Pre-processing of RNA- seq data involves three essential steps: adapter removal, trimming of low quality ends, and filtering out short reads (< 20 base pairs). Adapters are short oligonucleotides attached to the sequence of interest that facilitate the binding to the DNA linkers on the flow cell, enabling the sequencing process. Adapter remnants were removed from the RNA-seq data (paper I-III) to prevent result misinterpretation (97).

Low quality bases refer to nucleotides that have a high probability of being inaccurately assigned in the predicted sequence (101). As sequencing progresses, the quality of bases often decreases toward the 3' end of the read, primarily due to the gradual reduction in fluorescent signal emitted by the fluorophores with each amplification cycle (100). Trimming these low quality bases generally improves the alignment

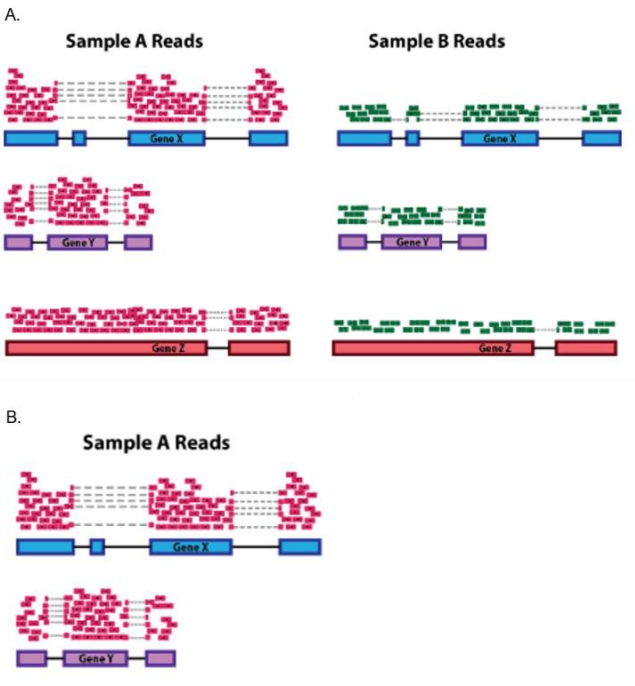

Figure 12. .Considerations for gene count normalization. The pink and green lines indicate reads aligned to genes. (A) Differences in sequencing depth can lead to inconsistences in the number of reads aligned to the same genes across different biological replicates. (B) Gene length bias, as a longer genes (e.g. Gene X) are likely to accumulate more reads compared to short genes (e.g. Gene Y), even when their expression levels are comparable (100).

rate to the reference genome. Short reads, often a byproduct of adapter removal and trimming, may align to multiple incorrect locations in the reference genome, introducing noise into the data (100). To enhance the overall quality and accuracy of the sequencing results, such reads were removed from our datasets (papers I-III).

The quality of the pre-processed data was assessed using several metrics, including Phred score, GC content percentage and amount of overrepresented sequences. Then, high quality data were aligned to previously published A. salmon genomes (ENSEMBL ICSASG_v2 in paper I and II, NCBI GCF Ssal_v3.1 in paper III). The selected reference genome in each paper corresponded to the most updated version available at the time of the analysis. Although an older version of the reference genome was used in paper I compared to the more recent versions used in papers II and III, A. salmon has been extensively studied over many years, therefore only minor discrepancies were anticipated, mostly related to gene annotations/IDs (e.g. IFIH instead of MDA5).

3.4.2 Data normalization and differential gene expression analysis

Since RNA-seq quantifies transcriptional responses in absolute rather than relative terms, differences in sequencing depth and gene length may lead to data misinterpretation, as raw transcript counts across different samples are not directly comparable (Figure 12) (96,100). To minimize these technical inconsistencies, we normalized the raw transcriptome data using DESeq2 v.1.34.0, both within and across experimental conditions prior to performing differential gene expression analysis (102). Despite normalization, biological replicates of the same experimental condition may still exhibit significant variability, for instance, due to the high individual variability in genes expression of RBCs in different maturation stages (103). Principle component analysis was performed in paper I-III, revealing notable outliers particularly in paper II, where PRV-1-3 infected A. salmon blood cells were analyzed.

Clustering of differentially expressed genes (DEGs) into functional groups through Gene ontology enrichment analysis and signaling pathways through Kyoto Encyclopedia of Genes and Genomes

(KEGG), was performed using ShinyGO v0.77 and STRING v12.0 in paper I and papers II and III, respectively. Although both tools allow manual customization of analysis parameters and provide comparable output regarding the biological processes affected by each experimental condition, they differ in their complexity. In paper I, the simplified scope of our objective- exploring the basal responses of unstimulated RBCs- and the low number of the differentially expressed genes between PRV-1-exposed cells and unexposed controls, allowed result interpretation using the more user- friendly ShinyGO software. In contrast, papers II and III involved more complex transcriptional responses and functional interactions across several experimental conditions, such as the impacts of different PRV genotypes over time in blood cells and the interplay between stress and infection in RBCs. For these studies, STRING software, with its detailed gene/protein interaction platform, was better- suited to address our hypotheses.

4 Results

4.1 Summary of papers

Paper I:

Transcriptomics of early responses to purified Piscine orthoreovirus- 1 in Atlantic salmon (*Salmo salar* L.) red blood cells compared to non- susceptible cells lines

Thomais Tsoulia, Arvind Y.M. Sundaram, Stine Braaen, Jorunn B. Jørgensen, Espen Rimstad, Øystein Wessel and Maria K. Dahle

Salmonid red blood cells (RBCs) are nucleated, and while primarily recognized for their role in respiratory processes, they also mediate antiviral activities. Atlantic salmon RBCs are the main target cells for Piscine orthoreovirus genotype 1 (PRV-1), a double- stranded RNA virus belonging to the *Spinareoviridae* family. PRV-1 is the etiological agent of heart and skeletal muscle inflammation (HSMI) in farmed A. salmon, a disease with significant impact on Norwegian aquaculture. Although PRV-1 can be detected in various tissues of infected fish, including blood, heart and spleen, viral replication has only been demonstrated *ex vivo* in RBCs. In this study, we examined the basal and PRV-1 induced gene expression in A. salmon RBCs at 24 hours post viral exposure, compared to Atlantic salmon kidney (ASK) cells and Salmon head kidney (SHK-1) cells. ASK and SHK-1 cells do not support PRV-1 infection. This may suggest that the ability of PRV-1 to replicate in RBCs may be linked to characteristics of the early transcriptional response to the virus, compared to non- susceptible cell lines. Transcriptional analysis showed that RBCs express a broad array of genes involved in innate immunity, including viral-specific pattern recognition receptors (PRRs), cytokine receptors and RNA helicases, some of which have not previously been reported in A. salmon. In addition, the expression of certain chemokine receptors in RBCs may suggest a putative role in chemotaxis during inflammation. PRV-1 exposure triggered moderate antiviral responses in RBCs. In contrast, SHK-1 cells activated a potent antiviral response, whereas ASK cells exhibited a non-typical immune response. Notably, the RIG-I-like receptor 3 (RLR3) gene was significantly upregulated in all PRV-1 exposed cells. Differences in the transcriptional profile of interferon regulatory factors (IRF) across cells may also contribute to virus-host specificity. In particular, IRF1 was only induced in RBCs, while IRF3/IRF7 were upregulated in SHK-1 cells. The divergent expression and regulation of PRRs and IRF genes may provide an explanation as to why viral replication is restricted to RBCs.

Paper II:

Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of Piscine orthoreovirus

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Piscine orthoreovirus (PRV) genotypes 1-3 induce different pathogenicity in different salmonid host species. PRV-1 causes heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*), PRV-2 has been linked to erythrocytic inclusion body syndrome (EIBS) in coho salmon (*Oncorhynchus kisutch*) in Japan, and PRV-3 causes HSMI-like disease in farmed rainbow trout (*Oncorhynchus mykiss*). All PRV genotypes exhibit similar systemic dissemination, with red blood cells (RBCs) being the primary targets during the early phase of infection. In a previously published trial aiming to explore immunization strategies against HSMI, A. salmon was injected with PRV-2, PRV-3 and an adjuvanted inactivated PRV-1 vaccine (InPRV-1). PRV-3 provided full protection against subsequent PRV-1 exposure and HSMI. In contrast, PRV-2 infection and InPRV-1 vaccine provided suboptimal protection. In the current study, we analyzed blood from fish infected with PRV-1-3, or InPRV-1 vaccinated controls, two and five weeks post injection, to investigate genotype-specific transcriptional responses. We aimed to explore responses that could be related to differential infection outcomes and/or cross-protective potential. PRV-1 and PRV-3 exhibited similar replication levels in blood cells of A. salmon in the first two weeks. However, PRV-3 induced a more potent antiviral immune response two weeks post injection compared to PRV-1. By week five, the transcriptional profile of blood cells infected by PRV-1 and PRV-3 was nearly identical. A few genes diverged between PRV-1 and PRV-3 responses, such as IQ motif containing GTPase activating protein 2 (IQGAP2) and cathepsin B (CATB), which may be involved in virus- host adaptations mechanisms. In addition, the genes urokinase plasminogen receptor (uPAR) and barrier-to-autointegration factor b (BANFB) showed the strongest transcriptional response to PRV-1, particularly at week five, compared to the other PRV genotypes, vaccinated and uninfected controls. These genes, emerging as promising biomarker candidates for PRV-1 infection, warrant future investigation. In contrast to PRV-3, PRV-2 and the InPRV-1 vaccine induced a different set of genes. Overall, the delayed antiviral responses to PRV-1 may facilitate viral dissemination to the heart of A. salmon, ultimately contributing to HSMI development.

Paper III:

Effects of glucocorticoid receptor activation on gene expression and antiviral responses in Atlantic salmon red blood cells

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Cortisol is the primary circulating glucocorticoid in fish, released from the interrenal tissue in response to several biotic and abiotic stressors. The hormonal stress response typically induces physiological adjustments aimed at restoring homeostasis. Intensified farming practices in salmonid aquaculture have been associated with fish welfare deterioration and increased susceptibility to viral infections. In this study, we treated Atlantic salmon red blood cells (RBCs) with synthetic and endogenous glucocorticoids (dexamethasone and hydrocortisone, respectively) *ex vivo* to investigate whether RBCs modulate (chronic) stress responses through activation of glucocorticoid receptor (GR)- signaling. RBCs were found to express GRs genes, and the three-day exposure to dexamethasone resulted in over 200-fold upregulation of FKBP propyl isomerase 5 (FKBP5) and Krueppel like factor 9 (KLF9) genes, implicated in GR signaling regulation both in fish and mammals. FKBP5 expression remained significantly elevated compared to unstimulated controls up to 14 days post dexamethasone exposure. An approximate 300-fold increase in FKBP5 expression was observed in RBCs stimulated with varying doses of dexamethasone or cortisol within physiological levels, suggesting gene activation in response to natural stressors. The effects of cortisol on gene expression in A. salmon blood cells *in vivo* were limited and did not resemble the patterns found in the dexamethasone-treated RBCs *ex vivo*. Only DDIT4 was significantly induced both *ex vivo* and *in vivo*.

We also explored the effects of dexamethasone on dsRNA-mediated immune responses in RBCs. Dexamethasone suppressed the poly(I:C)-induced antiviral responses, including genes associated with dsRNA recognition (e.g. RLR1 and MDA5), IRF signaling (e.g. IRF3 and IRF1) and IFN-mediated immunity (MX1 and RSAD2). In addition, genes involved in ubiquitin-dependent proteolysis via proteasomes and major histocompatibility complex (MHC) class I antigen presentation were significantly inhibited.

Altogether, RBCs responded to glucocorticoids with pronounced effects on antiviral innate immunity and protein degradation pathways. While DDIT4 and FKBP5 emerge as putative biomarker candidates for assessing stress-mediated responses in blood, further investigation is required.

4.2 Unpublished work

During the second semester of my PhD project, I undertook a five-month research stay at University of British Columbia (UBC), Vancouver, Canada. Under the supervision of Prof Colin Brauner, my research focused on investigating the effects of prolonged elevated cortisol levels on the antiviral responses of A. salmon RBCs *in vivo*. I also investigated the effects cortisol and/or poly(I:C)-mediated antiviral immunity on the respiratory processes of RBCs. Our primary hypothesis was that cortisol and poly(I:C), mimicking the responses to chronic stress and dsRNA viral infection, would compromise hemoglobin (Hb)-oxygen (O_2) carrying capacity in RBCs, as previously observed in PRV-1- infected fish under hypoxia (73).

A total of 32 A. salmon with average weight of 2 kg were divided into four experimental groups: (i) Untreated controls (Ctrl), (ii) intraperitoneally (IP) injected with 1 mg/kg poly(I:C) (P(I:C)), (iii) cortisol administrated through diet (200 μg/g feed) (HC), and (iv) cortisol fed and poly(I:C) injected (HCP). Fish per experimental group (n=8) were distributed in two tanks (n=4 fish per tank) to both avoid unnecessary handling stress and to minimize the working load, since all the analyses required fresh material. Briefly, fish pellets were spread in a single layer and sprayed with hydrocortisone dissolved in 100% ethanol. 24h post hydrocortisone coating, the pellets were stored at -20°C until further use. Cortisol diet was given to HC and HCP group for 10 days with the last feeding on day 10 being 12h before sampling. Saline (Ctrl and HC groups) and poly(I:C) IP injection (P(I:C) and HCP groups) took place 3 days before sampling. Upon sampling, heparinized blood was centrifuged to separate plasma and blood pellet. Plasma was collected for hormonal measurements and the blood pellet was re-suspended in sterile dPBS. Part of this suspension was used to isolate RBC and generate oxygen equilibrium curves (OECs).

Cortisol diet was given to achieve consistently elevated plasma cortisol levels without having to handle the fish, as previously demonstrated for rainbow trout and goldfish (*Carassius auratus*) (92,104) However, the plasma cortisol levels of HC and HCP groups were unexpectedly low and close to the P(I:C) group that was not fed cortisol pellets (Figure 13A). Desensitization of HPI axis to release cortisol or impaired recognition of cortisol by GR, leading to disrupted signaling initiation, could be potential explanations (105). Two individuals from the control group were excluded due to high cortisol levels (≥ 85 ng/mL) that may occur due to stress prior to sampling.

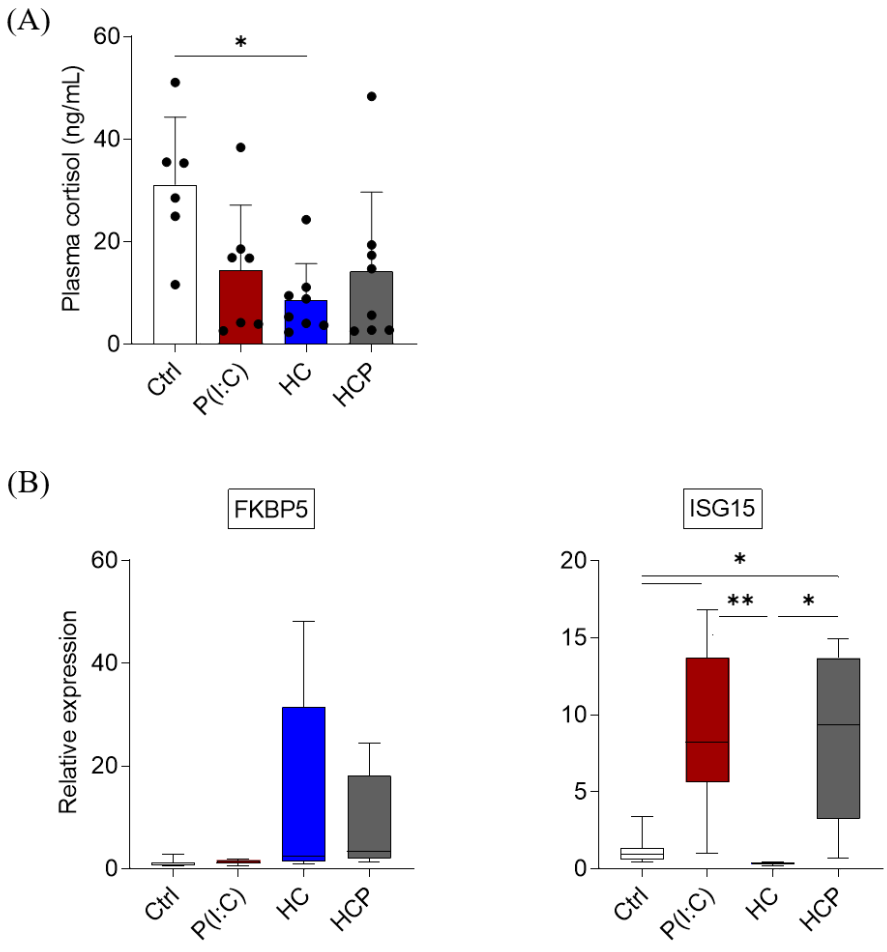

Figure 13. Effects of cortisol feed on the antiviral responses to poly(I:C) in A. salmon RBCs. (A) Plasma cortisol levels in A. salmon fed with cortisol- (HC and HCP) or regular diet (Ctrl and P(I:C)). Significant differences were calculated with one-way ANOVA Tukey's multiple comparison test. *$p < 0.05$, n=8 except control where n=6). (B) Expression of the FKBP5 gene (left) and ISG15 antiviral effector gene (right) was measured by RT-qPCR (n = 8). Significant differences of each experimental group compared to unstimulated controls were calculated with two-way ANOVA Dunnett's multiple comparison test. *: $p < 0.05$; **: $p < 0.01$

Poly(I:C) injection induced significant antiviral responses in RBCs. However, no immunosuppression was observed in HCP group, in contrast to RBCs exposed to dexamethasone and poly(I:C) *ex vivo* (Paper III) (Figure 13B). The low cortisol levels may be a possible explanation. In line with this, the cortisol responsive FKBP5 gene was induced in some individuals from the cortisol-fed groups (HC and HCP) (Figure 13B).

The OEC measurement showed that poly(I:C) injection and/or cortisol diet had no effect on the O_2 carrying capacity of Atlantic salmon RBC. However, a significant drop in RBC carbonic anhydrase (CA) activity, which regulate RBCs pH and facilitate O_2 delivery under stress, was observed in the P(I:C) group (Figure 14).

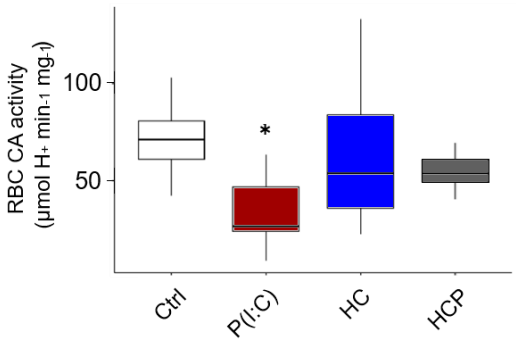

Figure 14. Effects of poly(I:C) and cortisol diet on RBC carbonic anhydrase (CA) activity in A. salmon. Poly(I:C) injection caused a significant decrease ($p < 0.05$; 51% drop) in RBCs CA activity.

5 Discussion

Fish RBCs are nucleated and transcriptionally/translationally active, exhibiting additional physiological and immunological properties compared to their enucleated mammalian counterparts (17). Primarily recognized as key mediators of gas exchange, salmonid RBCs have also been shown to play a crucial role in regulation of innate immunity, particularly against RNA viruses (106), as well as in physiological adaptations to stress through metabolic and morphological alterations (24,28). In this work, we investigated the transcriptional profile of A. salmon RBCs at rest, under stimulation with glucocorticoids and/or viral and synthetic dsRNA, using RNA-seq. We also explored the transcriptional differences induced in blood cells of A. salmon injected with PRV genotypes and an adjuvanted inactivated PRV-1 vaccine. An overview of our transcriptional data from the *ex vivo* and *in vivo* settings included in Papers I-III is shown in Table 2.

Table 2. Overview of the transcriptional analyses included in papers I-III.

Study	RNA-seq datasets
Paper I	A. salmon RBCs, along with ASK and SHK-1 cells, stimulated with purified PRV-1 for 24 h *ex vivo*
Paper II	Blood cells of A. salmon injected with PRV-1-3 genotypes or adjuvanted, inactivated PRV-1 vaccine two and five weeks post injection
Paper III	A. salmon RBCs stimulated with dexamethasone (4 d) and/or poly(I:C) (3 d) *ex vivo*
	Blood cells of A. salmon two and four days post cortisol injection *in vivo*

5.1 Putative roles of A. salmon RBCs in innate immunity

In fish, like in mammals, innate immune responses are initiated upon recognition of microbial PAMPs or viral genetic material by PRRs (38). Transcriptional analysis of A. salmon RBCs in resting state revealed the expression of transmembrane and cytosolic PRRs, underscoring their potential to detect a wide array of pathogens. These included the dsRNA-sensing TLR3 and two members of the RLR family, the RLR1 and RLR3 genes, as expected based on previous microarray analysis on purified A. salmon RBCs (26). The third member of the RLR family (i.e. the MDA5 gene), was also detected in our A. salmon RBC transcriptomes. Among the RNA- sensing PRRs, the ssRNA-specific TLR8 exhibited the highest basal transcriptional level. Notably, previous infection of A. salmon RBCs with infectious salmon anemia virus (ISAV) *in vivo* led to the upregulation of IFNa and MX1 genes (107,108); however, the signaling mediators driving this response were not fully characterized. In mammals, TLR8 expression was induced in response to ssRNA influenza virus A, a member of *Orthomyxoviridae* family like ISAV, leading to subsequent production of the inflammatory cytokine IL8 in modified HEK/TLR8

cells *ex vivo* (109). In this context, TLR8 could possibly interact with ISAV ssRNA, contributing to the antiviral response, a hypothesis warranting further investigation.

Interestingly, rainbow trout RBCs can manifest innate immune responses against the ssRNA viral hemorrhagic septicemia virus (VHSV) (90) and the dsRNA infectious pancreatic necrosis virus (IPNV) (110) *ex vivo*, without requiring direct infection. Thus, salmonid RBCs may act as potent sensors of viral threats, initiating antiviral responses independent of productive viral infection. Based on the insights from the current transcriptional analysis, which revealed the expression of receptors and signaling mediators involved in complete immune pathways, this aspect could also be extended to A. salmon RBCs. In particular, it may offer a novel perspective on their putative responses to viruses with significant economic loss in Norwegian aquaculture, such as the ssRNA salmonid alphavirus (SAV).

Some PRR genes encoding NLRs, including NLRC3-like and NLRC5, as well as the TLR2 and TLR6, which primarily recognize bacterial components (111,112), were also expressed in A. salmon RBCs. TLR2 typically forms heterodimers with TLR1 and/or TLR6, initiating MyD88- dependent signaling pathways that lead to the production of pro-inflammatory cytokines (43,113). In mammals, TLR2 has also been shown to dimerize with additional TLRs, such as TLR10 and TLR4, potentially expanding its range of detectable pathogens (113). Although NLR-C signaling in fish has been modestly characterized, a previous study in common carp (*Cyprinus carpio*) indicated the involvement of IRAK protein kinases and NF-KB complex components in NLRC3 signal transduction (114), and these genes were also expressed in the RBC transcriptome. Thus, our findings suggest that A. salmon RBCs may have broader immunological functions that extend to bacterial detection, an aspect of their immune role that remains relatively unexplored in salmonids.

Activation of PRR signaling pathways typically lead to secretion of cytokines, which mediate cell-to-cell communication regulating innate and adaptive immune responses (38). In A. salmon RBCs, several cytokine receptor genes were expressed, while the expression of cytokine genes was limited. Thus, RBCs may primarily act as a passive participant in immune cell communication, responding to cytokines secreted by other cells to modulate immune responses, rather than actively producing cytokines. An exception is the expression of the IL15 gene. IL15 is a member of the IL-2 cytokine family with its function linked to CD+8 T-cell generation and proliferation in mammals (115) and CD4+ T-cell survival in rainbow trout (116). The IL15 mRNA level (3128 normalized median count reads) was comparable to the elongation factor 1 alpha (EF1a) housekeeping gene isoforms (fluctuating from 1416 to 7460 normalized median count reads), indicating that this cytokine may serve an important role and suggest that RBCs act as facilitators of T cell responses, potentially by enhancing the survival, differentiation and/or proliferation.

In addition, A. salmon RBCs are found to express some chemokine receptors, such as CCR9 and CXCR4, as well as the c-c motif chemokine chemokine 4-like (CCL4-like) gene, implying their potential involvement in chemotaxis, either by migrating toward the CCR9- and CXCR4- ligand-secreting tissues or by recruiting immune cells to the site of inflammation.

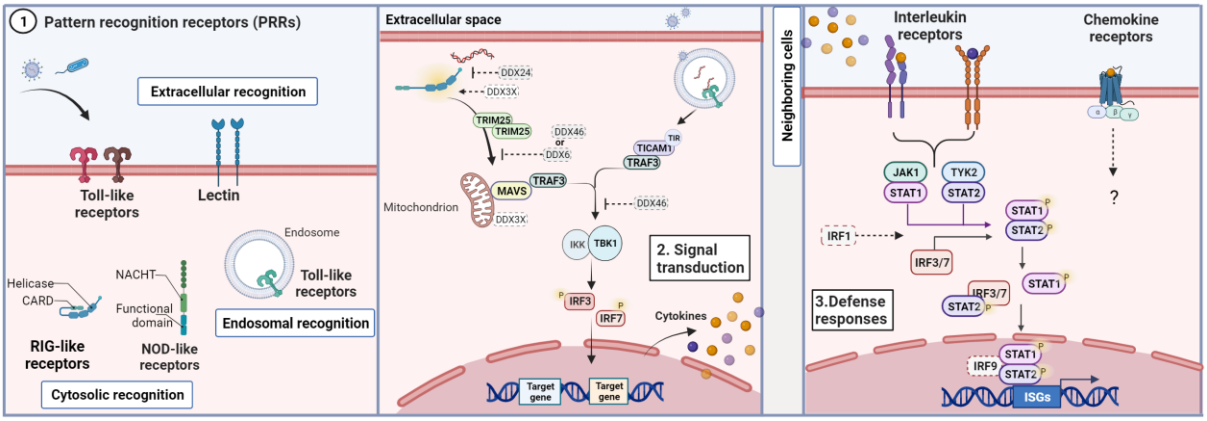

Figure 15. Examples of RBC genes involved in innate immune responses identified in A. salmon RBC (paper I). Short description of the pathways relevant for genes expressed in RBC and listed in the tables above. Elements drawn in dash have not been characterized in teleost, and their roles were based on mammalian models. Step 1. Pattern recognition receptors (PRRs). Step 2. Signaling mediators and interferon regulatory factors acting downstream of PRR binding, leading to secretion of IFNs and pro-inflammatory cytokines. Step 3.Pathways induced when secreted IFNs and cytokines bind to receptors, leading to expression of several innate immune effectors.

5.2 Transcriptional responses of A. salmon RBCs to poly(I:C) and PRV-1

In paper III, the expression patterns of genes involved in RLR- and IFN- mediated signaling pathways that were induced in A. salmon RBCs after a three-day stimulation with poly (I:C), closely resembled those previously reported for PRV-1 seven weeks post infection *in vivo* (26). The response included upregulation dsRNA-sensing PRRs (e.g. RLR1 and RLR3), IRFs (e.g. IRF1 and IRF3), and ISGs (e.g. RSAD2, MX1 and ISG15). In addition, genes encoding proteasome components (e.g. PSMBs), transporter proteins (TAPs) and MHC regulatory molecules (e.g. MR1) were significantly induced by poly(I:C) *ex vivo* and PRV *in vivo*, underscoring RBCs role in proteolytic degradation and presentation of intracellular antigens. This may lead to the generation immunological memory against PRV-1 and other dsRNA viruses (26).

In paper I, A. salmon RBCs, and the non-susceptible ASK and SHK-1 cells exhibited substantially different gene expression patterns after 24 hours of exposure to purified PRV-1 (Figure 16). RBCs elicited typical dsRNA-mediated responses, though weaker compared to those observed in SHK-1 cells. While ASK cells have been shown to induce ISGs, such as MX and ISG15, after a 24 hour stimulation with poly(I:C) (117), they did not demonstrate typical antiviral responses to PRV-1. This suggests that dsRNA recognition and activation of IFN-mediated responses may differ between viral and naked

dsRNA. SHK-1 cells exhibited potent antiviral responses, akin to those observed in RBCs five to seven weeks after PRV-1 infection *in vivo* (26), or following a three-day exposure to poly(I:C) *ex vivo* (Paper III). Interestingly, RLR3 gene was upregulated across all PRV-1- exposed cell types, but RBCs additionally induced MDA5, whereas SHK-1 cells induced several dsRNA-sensing PRRs, including MDA5, RLR3 and TLR3 (Figure 16A). These differences may reflect a delay in viral RNA recognition, as also discussed in the comparison between PRV-1 and PRV-3 responses in paper II.

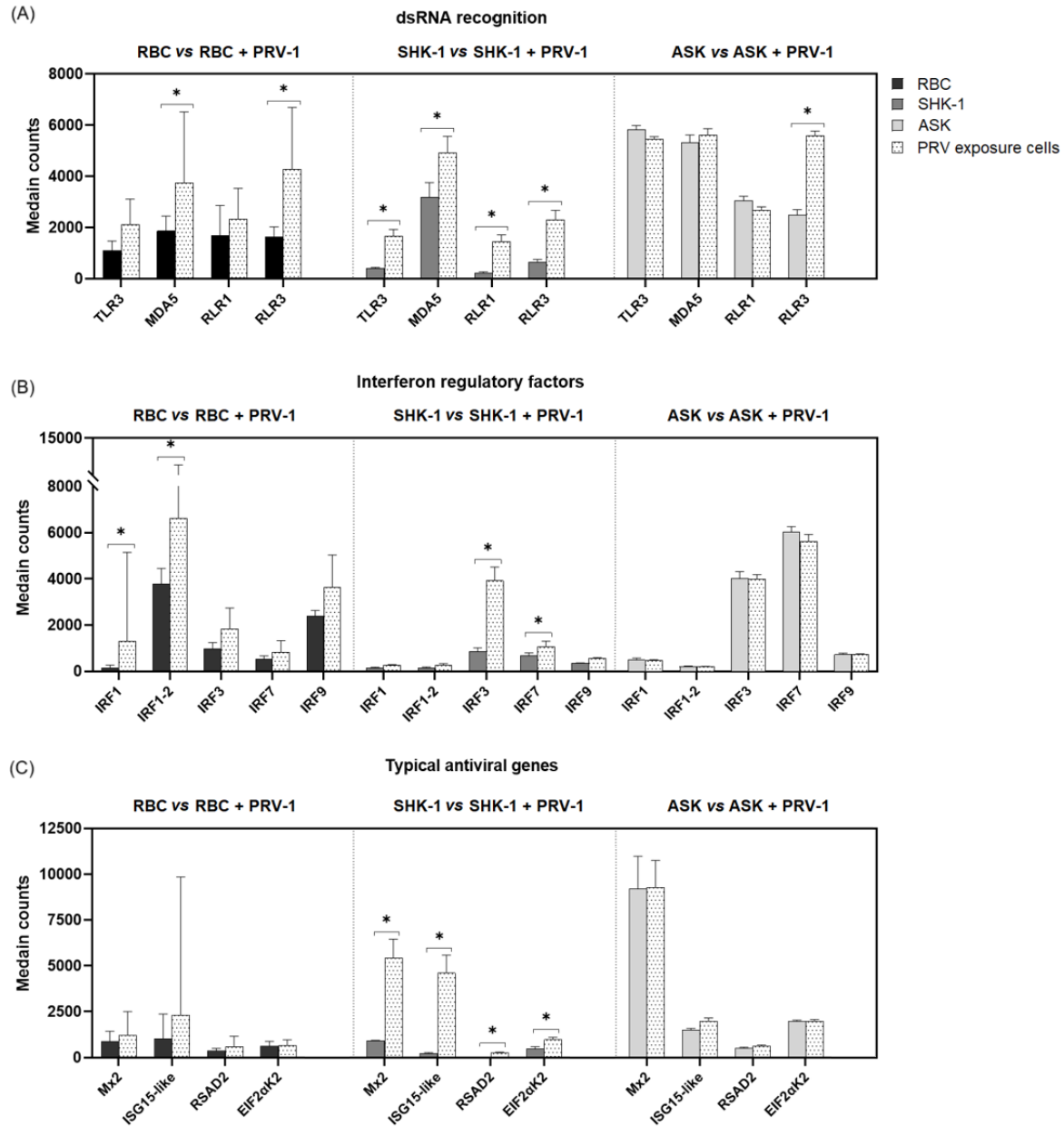

Figure 16. Comparison of the transcriptome responses linked to selected innate antiviral genes in RBCs, SHK-1 and ASK exposed to PRV-1 (RBC vs RBC + PRV-1, SHK-1 vs SHK-1 + PRV-1 and ASK vs ASK + PRV-1, respectively) (paper I). Regulation of (A) dsRNA pattern recognition receptors, (B) interferon regulatory factors, (C) genes involved in IFN-signaling pathway activation and (D) IFN-inducible antiviral effectors. RBC vs PRV-1, n=6, SHK-1 vs PRV-1 and ASK vs PRV-1, n=3. *p<0.05.

Furthermore, RBCs and SHK-1 cells induced a different set of IRF genes in response to PRV-1. In particular, RBCs expressed IRF1, while SHK-1 expressed IRF3, IRF7 and IRF9 (Figure 16B). Given that only RBCs support PRV-1 replication *ex vivo* (87,88), the modulation of antiviral immunity by different IRFs may also play critical role in determining the infection outcome. Although IRF1 can promote IFN- stimulated responses independently of IRF3/IRF7 activation both in fish and mammals, it is considered a secondary IFN inducer, less potent than IRF3 in initiating antiviral signaling (50,118). In this sense, the weak antiviral immune responses in RBCs to PRV-1 after 24 hours, characterized by nearly any ISG induction, could be a result of limited viral RNA recognition through MDA5 and RLR3, followed by IRF1-mediated signaling. This limited or delayed antiviral responses in RBC, influenced by differences in viral dsRNA recognition by PRRs and IRF signaling, may allow PRV-1 replication.

5.3 Transcriptional responses of A. salmon RBCs to pathogenic compared to non-pathogenic PRV genotypes

Although A. salmon is the natural host for PRV-1, recent findings indicated that infections with PRV-2 and PRV-3 can also be established after intraperitoneal (IP) injection, leading to different outcomes in replication efficacy and cross- protection. Notably, while PRV-3 provided efficient protection against PRV-1 and HSMI, immunization with PRV-2 or an inactivated adjuvanted PRV-1 (InPRV-1) vaccine conferred only partial protection (65). Given that RBCs are the primary targets of PRV during the initial phase of infection (66,67,81), we hypothesized that the transcriptional responses induced by each PRV genotype and the InPRV-1 vaccine in RBC could enlighten the observed differences in the infection outcome.

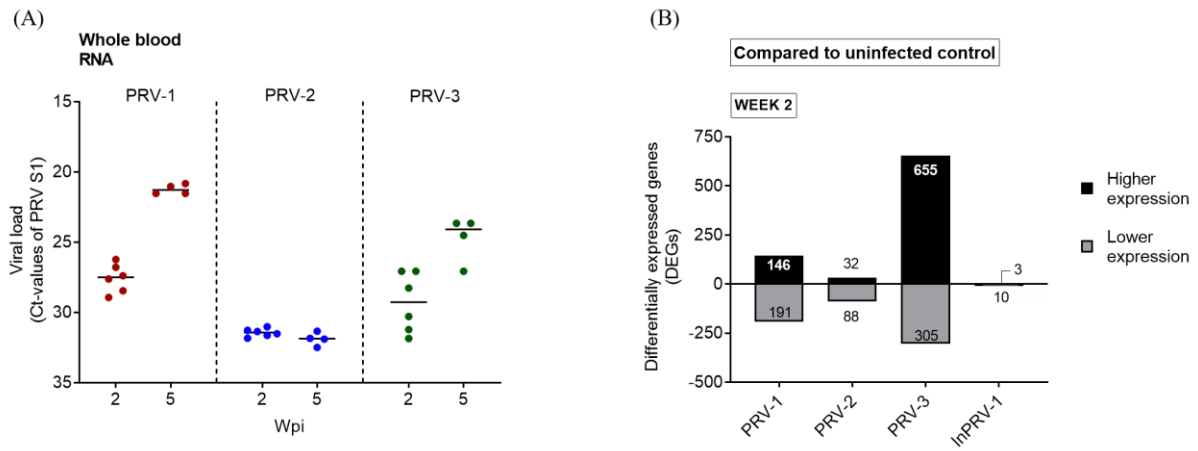

Figure 17. PRV genotypes can infect Atlantic salmon blood cells (paper II). (A) RNA loads of PRV-1-3 whole blood were measured two- and five-weeks post injection using RT-qPCR assays targeting virus-specific parts of the S1 genome segment. Virus levels are presented as Ct-values for each individual and as average (n= 6/group in week two and n= 4/group in week five). PRV-1 levels colored red; PRV-2 levels colored blue; PRV-3 levels colored green. (B) Number of significantly regulated genes (DEGs) in whole blood of A. salmon two weeks after infection, compared to uninfected controls. Cutoff ≥ 2- fold (higher) and ≤ 0.5- fold (lower), and a cutoff of normalized median read counts ≥ 10 were applied.

While PRV-1 and PRV-3 exhibited similar replication efficacy, the timing of immune responses in blood differed between the two genotypes (Figure 17), with genes involved in antiviral defense and adaptive immunity upregulated earlier in PRV-3- compared to PRV-1- infected blood (Figure 18A). The onset of the innate immune responses as a determinant of host pathology/mortality has also been demonstrated in studies on ISAV isolates. In particular, low virulent ISAV triggered immune responses more rapidly than the high virulent strain, which, despite exhibiting slower replication peaks, ultimately caused higher mortality (119). It has previously been shown for MRV that amino acid (aa) polymorphisms in μ2 and λ1 proteins influence viral recognition by RLR1 and the subsequent activation of IFN signaling (120). Thus, despite the high similarity of PRV-1 and PRV-3 in μ2 and λ1 core proteins, with 88.7% and 96.7% aa identity, respectively (64), some divergences in their aa sequences could explain the more rapid onset of antiviral innate immune responses observed in PRV-3- infected blood. It is worth noting that the low aa identity between MRV and PRV μ2 (20%) and λ1 (32%) proteins (60) rendered the identification of related polymorphisms between the two viruses both challenging and unlikely.

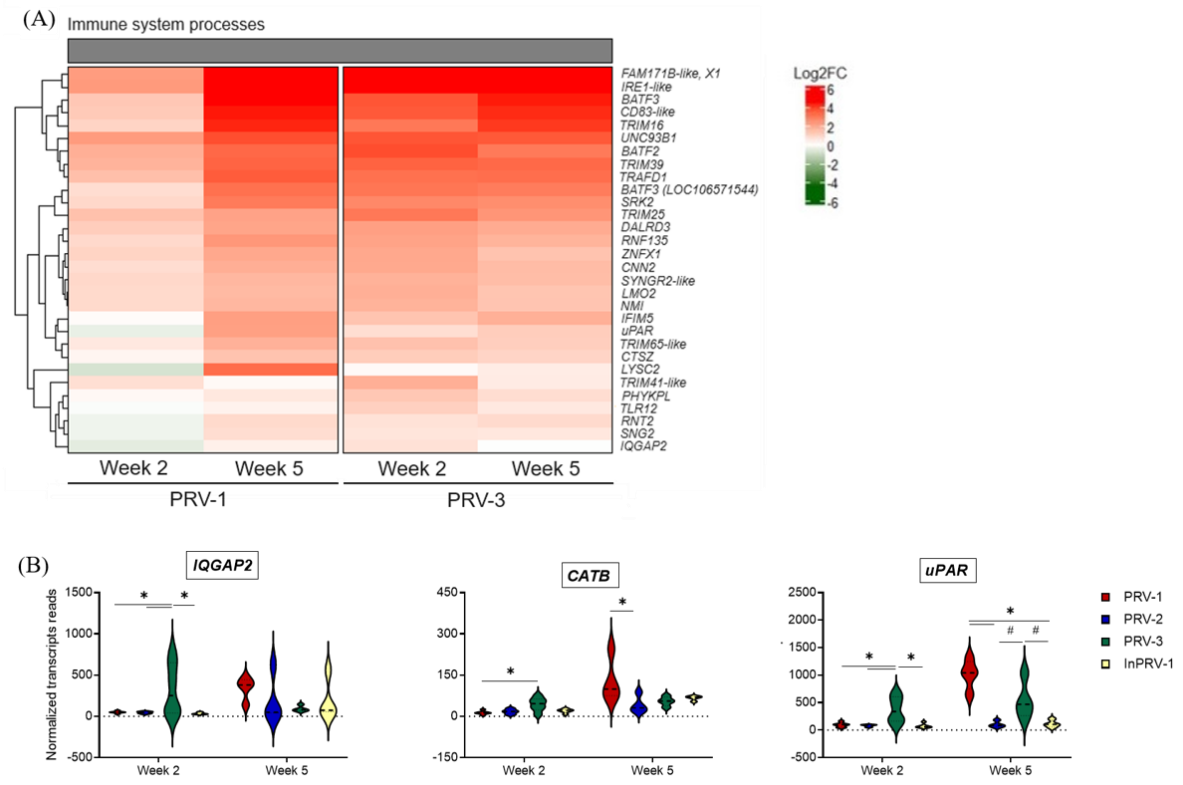

Figure 18. Transcriptional responses in whole blood of A. salmon injected with PRV-1 and PRV-3 (paper II). (A) Gene expression pattern of differentially expressed genes (DEGs) between PRV-1 and PRV-3, involved in immune system processes, compared to uninfected fish at week zero. Log2-fold change of the selected DEGs compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five compared to week zero; Green: Lower expression level at week two/five compared to week zero; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (B) Expression levels of IQ motif containing GTPase activating protein (IQGAP2), cathepsin B (CATB) and urokinase plasminogen activator receptor (uPAR) genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes or vaccinated. *: $p \leq 0.01$.

Although PRV-3 replication persisted up to 18 weeks post injection, the virus indicated a limited capacity to infect A. salmon heart compared to the pathogenic PRV-1 (65). This limitation may be attributed to less PRV-3 disseminated into blood plasma from RBCs, which could be a result of potent antiviral responses. In contrast, PRV-1 is more evolutionary adapted to A. salmon, and may have evolved to escape or dampen these mechanisms, enabling its efficient dissemination and infection within the host. For instance, genes associated with initiation of IFN signaling, such as the IQ motif containing GTPase activating protein (IQGAP2) (121) and cathepsin B (CATB) (122), and inhibitors of viral release, such as urokinase plasminogen activator receptor (uPAR) (123), were significantly induced in PRV-3- compared to PRV-1- infected blood at week two, supporting this hypothesis (Figure 18B). Given that the functional role of these genes has primarily been explored in mammals, further investigation is needed to assess their relevance and activity in fish.

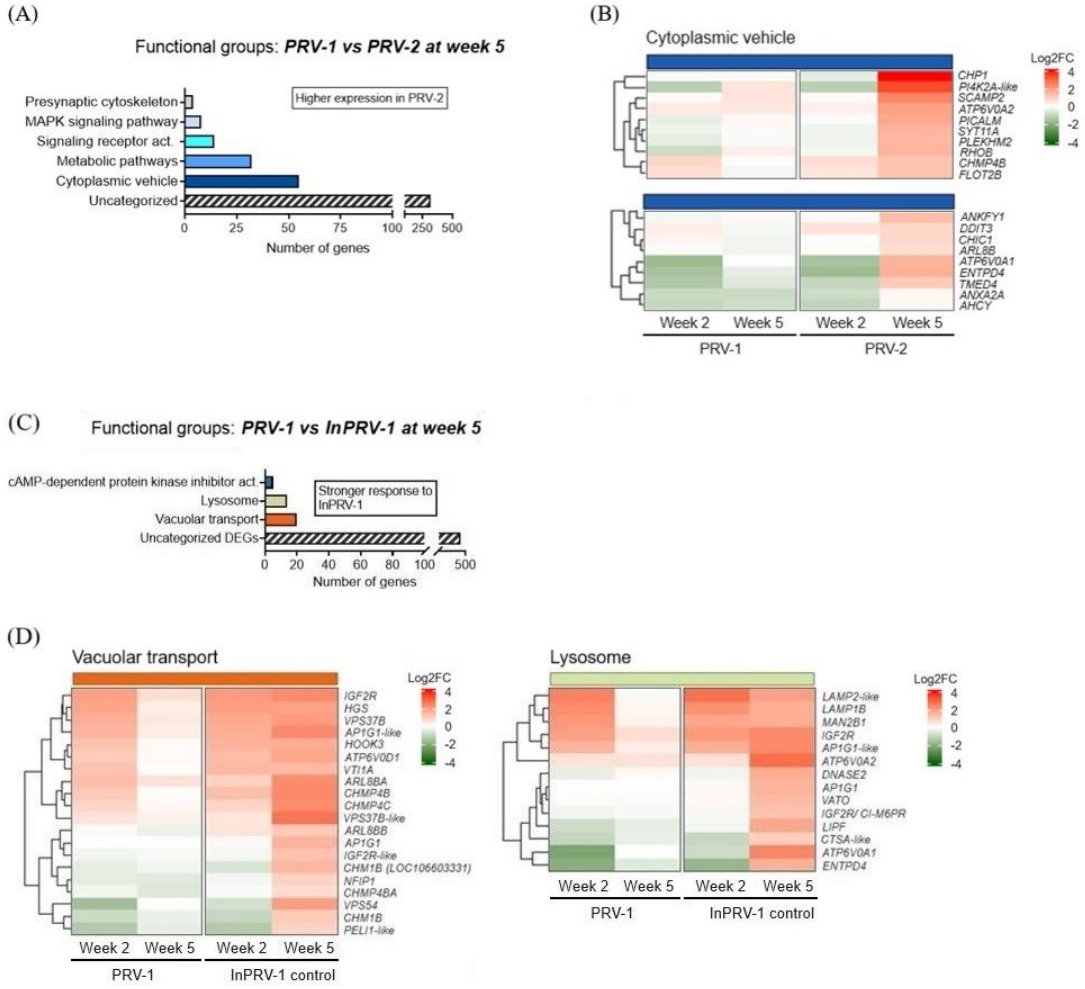

Figure 19. Gene expression profile in whole blood of A. salmon injected with low/non-replicating PRV genotypes (paper II). DEGs with fold- change > 2 (higher expression induced by PRV-1) and < 0.5 (lower expression induced by PRV-1) were included in the analysis. Log2-fold change of the selected DEGs were compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five; Green: Lower expression level at week two/five; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A)

Functional groups of DEGs with higher expression in PRV-2 compared to PRV-1 at week five. (B) Transcriptional profile of DEGs between PRV-1 and PRV-2 of the functional group “Cytoplasmic vehicle”. (C) Functional groups of DEGs with higher expression in inactivated PRV-1 (InPRV-1) vaccine compared to PRV-1 at week five. (D) Transcriptional profile of DEGs between PRV-1 and InPRV-1 of the functional groups “Vacuolar transport” and “Lysosome”.

PRV-2 exhibited low replication in blood, displaying transcriptional responses in blood cells similar to those observed in InPRV-1 vaccinated fish (Figure 17). While the expression levels of genes related to innate and adaptive immunity were relatively low compared to replicating PRV genotypes, several genes involved in cell signaling, membrane protein trafficking and substrate degradation in cellular compartments responded significantly only to PRV-2 and InPRV-1 five weeks post infection (Figure 19). These transcriptional differences between blood cells exposed to replicating and low/non-replicating PRV genotypes may be associated with a lacking ability of PRV-2/InPRV-1 viral particles to escape endosomes and expose dsRNA to PRRs. In this sense, genes involved in lysosomal and autophagic activity were induced over time, possibly as a result of viral degradation in endo/lysosomes. In the context of the partial protection observed against subsequent PRV-1 infection in some fish, a synergistic effect of putative antigen presentation together with alterations of basal cellular functions through the disruption of cAMP and MAPK signaling and/or apoptosis, may play a role.

5.4 RBCs as mediators of stress response through genomic signaling

In paper III, A. salmon RBCs were found to express GRs and GR response elements (GREs), indicating their potential to interact with glucocorticoids, such as cortisol, and regulate stress responses through GR-mediated signaling pathways. In this context, stress-induced transcriptional responses in RBCs could serve as an indicator of fish health and welfare, both physiological and immunological, providing a complementary approach to the often inconsistent results of hormonal screening in plasma (30).

In mammals, prolonged stress can alter morphological and physiological properties of RBC, reducing their plasticity (i.e. RBCs become rigid) and oxygen transport efficiency by affecting hemoglobin conformation (124,125). In addition, stress may trigger the production of reactive oxygen species (ROS), leading to lipid peroxidation and disruption of membrane integrity (126). Rainbow trout RBCs have been shown to change their shape in response to thermal stress (28), though the physiological aspect of this alteration is unknown. At a transcriptional level, A. salmon RBCs exposed to dexamethasone in paper III did not exhibit significant differences in the expression of genes related to oxidative stress response or hemoglobin formation. However, the ELOVL fatty acids protein 4 (ELOVL4) gene, which is involved in lipid biosynthesis (127), was significantly upregulated. Although this strong induction may suggest a potential role of ELOVL4 in cellular coping mechanisms under stress, its exact function in salmon RBCs remains unexplored.

Previous studies on glucocorticoid-mediated responses in zebrafish and mammals have identified FKBP propyl isomerase 5 (FKBP5) as a crucial modulator of GR signaling, with its expression increasing in response to elevated circulating glucocorticoid levels (128,129). In glucocorticoid-treated A. salmon RBCs *ex vivo*, the significant induction of FKBP5, along with its related transcription regulator KLF9 (130), supported our hypothesis that RBCs actively mediate glucocorticoid-driven stress responses. FKBP5 expression was initiated at low doses of dexamethasone and cortisol, and remained significantly elevated compared to untreated controls for up to 14 days under high dose treatment (Figure 20).

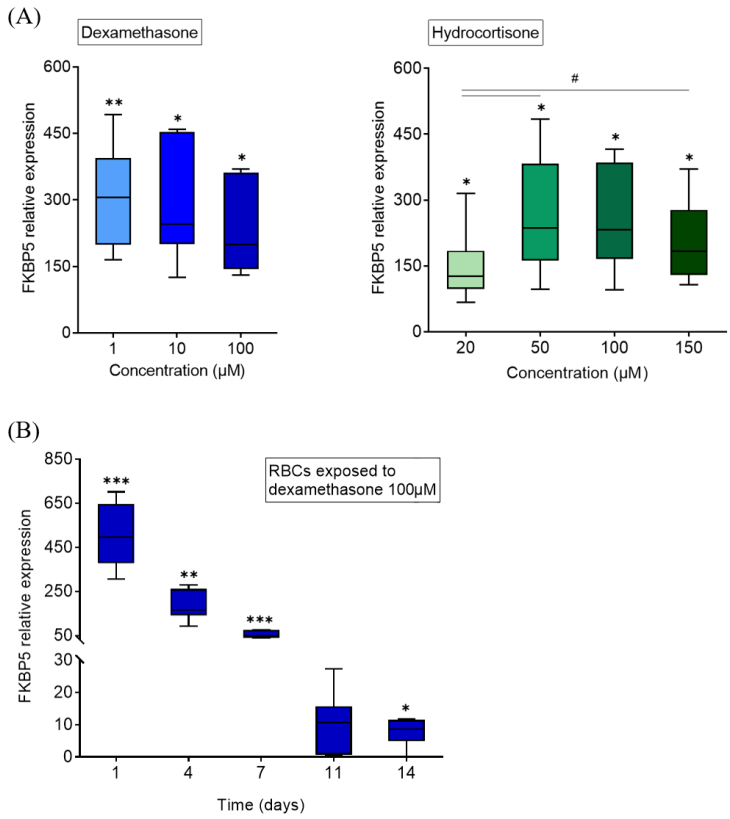

Figure 20. Effects of dexamethasone and hydrocortisone on the transcriptional profile of FKBP propyl isomerase 5 (FKBP5) in Atlantic salmon red blood cells (RBCs). Expression of FKBP5 was measured in purified RBCs by RT-qPCR (n= 6). (A) Relative expression of FKBP5 in A. salmon RBCs four days post stimulation with dexamethasone at concentrations 1, 10, 100 µM, and hydrocortisone at concentrations 20, 50, 100, 150 µM. Data were analyzed using one-way ANOVA Tukey test. *: $p < 0.05$ relative to the control; **: $p < 0.01$ relative to the control; #: $p < 0.05$ between the experimental conditions. (B) Relative expression of FKBP5 in A. salmon RBCs stimulated with 100 µM dexamethasone over 14 days. Data were analyzed using paired t-test for the treated RBCs of each day, compared to its respective untreated controls (n= 6). *: $p < 0.05$ relative to the control; **: $p < 0.01$ relative to the control; ***: $p < 0.0005$.

Although these *ex vivo* findings suggested that FKBP5 could serve as a putative indicator of secondary stress-mediated effects in RBCs, its expression in blood cells from cortisol-injected or cortisol-fed fish *in vivo* was not consistently induced (Figure 21C) (Paper III and unpublished data). This discrepancy may be attributed to the blockage of some cortisol effects due to eugenol-based anesthetics used during sampling (131) or to earlier activation of FKBP5 expression in both treated fish and untreated controls

due to cumulative exposure to other stressors during handling procedures. However, the DNA damage-inducible transcript 4 (DDIT4) gene was significantly upregulated in both *ex vivo* and *in vivo* studies, displaying a graded response to glucocorticoid dynamics, peaking at the highest cortisol levels and returning to baseline as cortisol normalized (Figure 21A-B). Altogether, FKBP5 may reflect long-term responses to stressors of varying intensity, while DDIT4 act as an immediate and dynamic indicator of cortisol fluctuations. Thereby, despite the limitations of using FKBP5 and DDIT4 as secondary biomarkers of stress exposure, their combined analysis in RBCs may present a promising diagnostic approach.

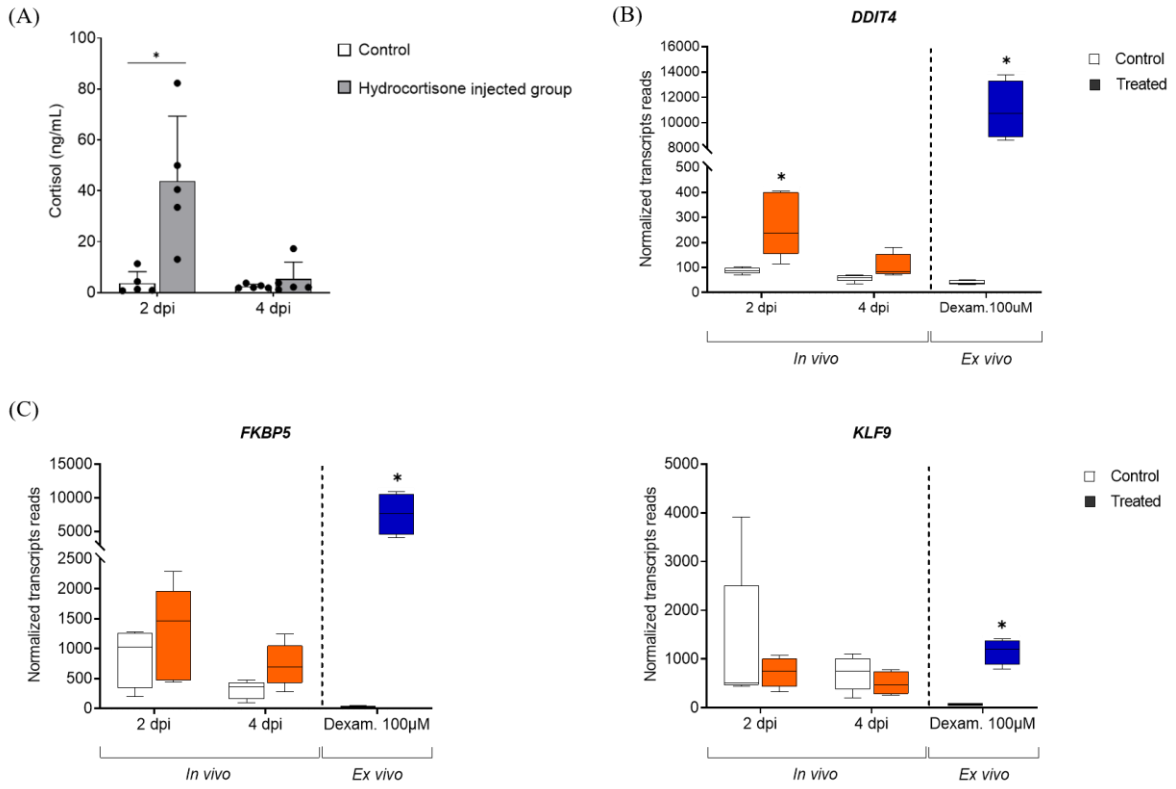

Figure 21. Transcriptional responses in whole blood of Atlantic salmon two and four days post- hydrocortisone injection (paper III). (A) Cortisol levels in blood plasma of A. salmon detected two and four days post- injection using ELIZA competitive enzyme immunoassay kit, as shown by Thoen et al. (2020) and Amundsen et al. (2021) (35,132). (B) Transcriptional profile of the DNA damage-inducible transcript 4 protein (*DDIT4*) gene in blood cells of A. salmon two and four days post-hydrocortisone injection *in vivo* and in purified RBCs four-days post stimulation with dexamethasone *ex vivo*. RBCs *ex vivo*: n= 4. Whole blood *in vivo*: n= 5. *: $p < 0.05$ compared to the controls, estimated during the transcriptional analysis using DESeq2 v.1.34.0. (C) Transcriptional profile of the FKBP prolyl isomerase 5 (FKBP5) and Krueppel-like factor 9 (KLF9) genes in blood cells of A. salmon two and four days post- hydrocortisone injection *in vivo* and in purified RBCs four-days post stimulation with dexamethasone *ex vivo*. RBCs *ex vivo*: n= 4. Whole blood *in vivo*: n= 5. *: $p < 0.05$ compared to the controls, estimated during the transcriptional analysis using DESeq2 v.1.34.0.

5.5 RBC physiological and transcriptional changes under stress and dsRNA-mediated antiviral immunity

Farmed fish frequently encounter prolonged or repeated stressors associated with rearing practices, which can suppress immune function and thereby impair disease resistance (13). For instance, previous studies on A. salmon injected with cortisol prior to infection with IPNV or salmon gill pox virus (SGPV) demonstrated significant inhibition of antiviral effectors, such as MX1 and ISG15, in mucosal and lymphoid tissues (36,132). In the IPNV-infected fish, repression of the MHC class I gene was also observed (36). Similarly, addition of GR agonists prior to poly(I:C)- treatment of A. salmon RBCs both attenuated the expression of genes involved in IFN-mediated antiviral immunity, and entirely blocked genes involved in ubiquitin-dependent degradation by proteasomes (e.g. PSMB7 and PSMB8) and MHC I antigen presentation (e.g. transporter proteins TAP1 and TAP2) (Figure 22). Fish infected by PRV-1 have been shown to exhibit higher mortality rates when subjected to stressors, such as hypoxia (73). Thus, in the context of PRV-1 infection in RBCs, this increased susceptibility may be linked to a diminished capacity of the cells to effectively eradicate the virus through antiviral responses, and to degrade the viral proteins for presentation of antigenic peptides, essential for activating cytotoxic T cells responses.

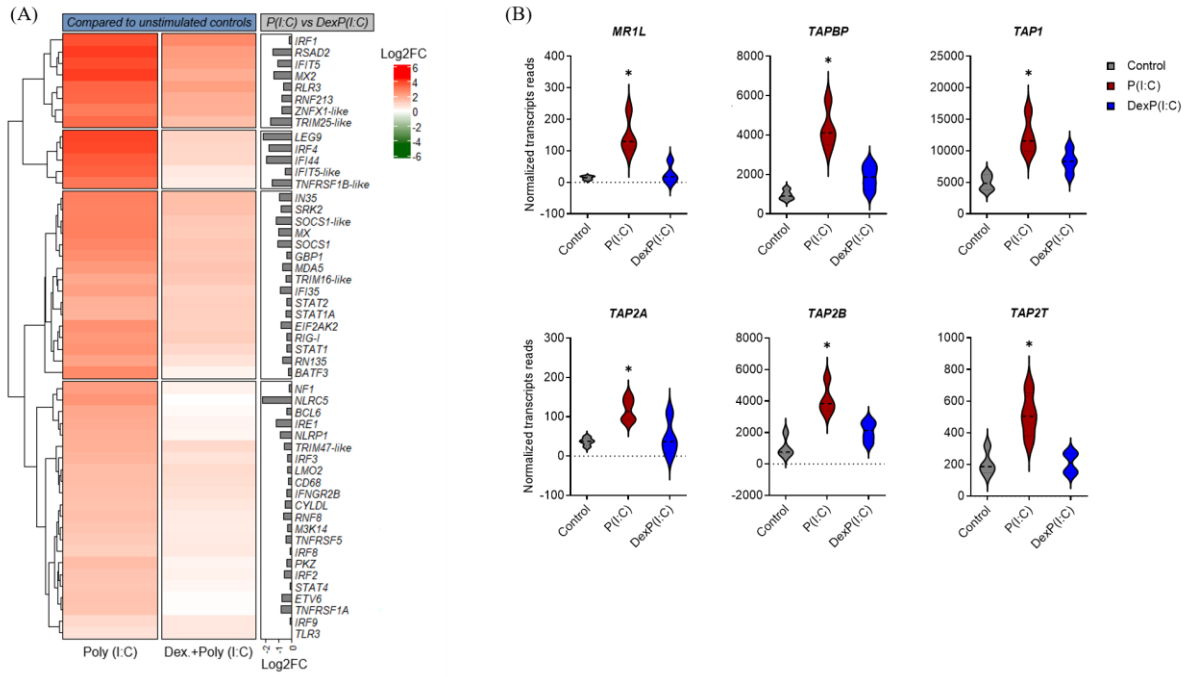

Figure 22. Transcriptional analysis of A. salmon RBCs stimulated with poly (I:C) or dexamethasone and poly (I:C), compared to unstimulated controls (paper III). (A) Gene expression profile in RBCs of A. salmon stimulated with either poly (I:C) or dexamethasone and poly (I:C), compared to unstimulated controls. Log2-fold change (Log2FC) of selected DEGs involved in immune system processes, compared to unstimulated controls (heatmap). Log2FC of selected DEGs is also provided from the comparison of poly (I:C)- with dexamethasone and poly (I:C)- stimulated RBCs (grey bar plot). Red: Higher expression level in stimulated RBCs compared to unstimulated controls; Green: Lower expression level in stimulated RBCs compared to unstimulated controls; White: No expression difference between stimulated and unstimulated RBCs. The darker the color, the

stronger the regulation (higher or lower). (B) Selected genes involved in MHC class I antigen processing and presentation with significantly different expression patterns between RBCs stimulated with poly (I:C) alone and those treated with dexamethasone and poly (I:C), compared to unstimulated controls. *$p \leq 0.01$ in poly (I:C)- stimulated RBCs compared to unstimulated controls; # $p \leq 0.01$ in dexamethasone and poly (I:C)- stimulated RBCs compared to unstimulated controls.

From a physiological standpoint, fish with HSMI have previously demonstrated significantly reduced hemoglobin levels in RBCs, potentially contributing to lower tolerance to hypoxia (73). In both fish and mammals, carbonic anhydrase (CA) is essential for facilitating rapid CO_2 hydration and regulation of RBC pH, thereby promoting oxygen release to metabolically active tissues (i.e. Bohr effect) (20). In addition, salmonid RBCs enhance oxygen transport under stress via activation of Na+/H+ exchange (β-NHE), a process triggered by the binding of secreted catecholamines, such as adrenaline (epinephrine), to adrenoreceptors in their membrane (20,24). Given the reduced hemoglobin content in RBCs of PRV-1 infected fish (73), their hemoglobin- oxygen carrying capacity may also be compromised. However, our experiments involving cortisol-fed and poly(I:C)-injected fish yielded inconclusive results in this aspect (unpublished work-section 4.2). Notably, impaired CA activity observed in poly(I:C)-injected fish may be linked to hypoxia-related mortality in PRV-1 infected fish, likely affecting oxygen delivery; a hypothesis representing an interesting field for study.

6 Main conclusion

This study demonstrated that A. salmon RBCs display antiviral responses and responses to glucocorticoids, indicating an active role in immune and physiological processes.

RBCs demonstrate antiviral responses:

- RBCs express genes encoding viral- and bacterial-sensing receptors, indicating recognition of a broad range of pathogens, as well as cytokine/chemokine receptors, indicating communication with immune cells
- Responses to PRV-1 in RBCs indicate sensing of dsRNA, but are limited compared to responses in the non-susceptible SHK-1 cell line, and characterized by non-canonical IRF expression. This may favor viral replication in RBCs
- Antiviral responses to PRV-1 in RBCs are delayed, compared to PRV-3 responses. This delay may be a determining factor for PRV-1 dissemination to the heart, a critical step for HSMI development

RBCs demonstrate responses to stress:

- RBCs express high mRNA levels of GR, and respond to glucocorticoids. Glucocorticoid pre-treatment leads to compromised transcriptional responses to dsRNA, in particular affecting innate immune responses and expression of proteolytic proteins
- Gene expression of DDIT4 and FKBP5 are putative biomarkers of glucocorticoid effects on RBCs. DDIT4 was the best biomarker candidate *in vivo*

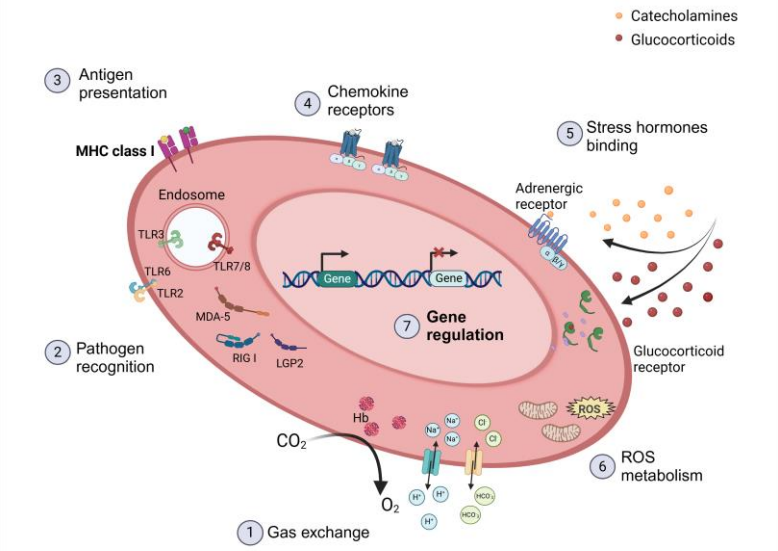

Figure 23. Overview of the physiological and immunological properties in A. salmon RBCs.

7 Future perspectives

Over the past decade the definition of fish red blood cells (RBCs) exclusively as gas exchangers has been challenged, revealing their multiple roles in innate immunity, metabolic regulation and the stress response. Atlantic salmon RBCs have been found to support viral infection, respond strongly to RNA viruses that invade the cells and express genes involved in RNA sensing and interferon-mediated signaling. Current transcriptional analysis of A. salmon RBCs in resting state has further showed the expression of genes encoding a wide array of pattern recognition receptors (PRRs), immune signaling modulators and cytokine receptors. Thus, RBCs may also be involved in immune protection against a range of pathogens. Pathogen clearance generally occur through the activity of many different immune cells in concert, requiring communication between the initial pathogen-sensing cells (like RBCs), and immune cells via cytokines and chemokines. To date, the potential contributions of salmonid RBCs to both innate and adaptive immune functions have been largely inferred from transcriptional data, with limited functional analyses conducted. The role of RBCs as non-specific scavengers of diverse foreign molecules coordinating responses with other immune cells warrants further investigation.

PRV-1 infection remains one of the most significant challenges in A. salmon aquaculture in Norway, with stress potentially exacerbating fish susceptibility and increasing heart and skeletal muscle inflammation (HSMI)-mediated mortality. Following virus internalization through the gastrointestinal tract, PRV-1 infects RBCs, exploiting their transcriptional/translational machinery to support its replication. However, the mechanisms underlying viral entry, exit and dissemination in the host have not fully elucidated. A better understanding of PRV- RBCs interactions and their influence on viral dissemination, as well as the different infection outcomes between PRV genotypes in A. salmon and between PRV-1 isolates with varying pathogenicity, could be key in predicting the onset of severe HSMI outbreaks. Such insights could also support the development of control strategies, such as minimizing stressful procedures in a sensitive phase, and develop effective vaccines. In the second paper, we showed a delayed response of blood cells to PRV-1 compared to PRV-3, highlighting some genes with significant transcriptional differences between the two genotypes, such as IQGAP2 and CATB. Whether the regulation of these genes contributes to an immune evasion mechanism for PRV-1, favoring its replication and dissemination to the heart, remains to be explored.

In fish, primary responses to stress are characterized by activation of the HPI axis and ultimately release of catecholamines and cortisol into the blood stream. Despite hormonal coexistence in circulation, earlier research has largely focused on the effects of the different hormones alone. RBCs express both adrenergic and glucocorticoid receptors. Exposure of RBCs to dexamethasone and cortisol suppressed the expression of genes involved in antiviral responses and protein degradation. However, the effects of catecholamines, such as adrenaline/noradrenaline, on the transcriptional profile of RBCs, particularly concerning immune and physiological functions, is not fully understood. In addition, whether these

effects are altered or overshadowed in presence of other hormones (e.g. cortisol) requires further exploration.

Abnormalities in the morphology and membrane protein conformation of RBCs in freshwater fish species have previously been reported in response to pollution by xenobiotics such as lead, cadmium chloride and copper sulphates. Similarly, changes in RBCs shape, hemoglobin concentration and cytoplasmic density have been observed following *ex vivo* exposure of rainbow trout RBCs to short-term thermal stress (i.e. 25 °C for three days). Given their sensitivity to such exogenous factors, evaluating RBCs physiological characteristics could serve as a promising diagnostic tool for assessing fish health status, particularly after intensive handling procedures, such as delousing with thermal treatment, or under conditions of poor water quality. Furthermore, exploring transcriptional changes in RBCs in response to waterborne toxins, harmful plankton and/or jellyfish sting cells- factors often linked to fish pathologies like complex gill disease- may provide an early-warning approach for detecting environmental hazards and (eventually) preventing impending mortality.

Fish RBCs are the most abundant cells in the circulatory system, and this together with their diverse physiological and immunological properties may underscore both their biological significance and great potential for establishing RBC-based diagnostic applications. Currently, diagnostics methods are typically conducted on tissues obtained from euthanized fish. In contrast, RBC collection could be performed non-lethally, offering a non-invasive approach for research and diagnostics. Hematology analyses, including cortisol measurements in blood plasma, RBC total counts, hematocrit and hemoglobin content, are commonly employed to assess fish physiology, pathology and toxicology under environmental changes and stressful rearing conditions. While these techniques are cost-effective and easy to implement, they often lack accuracy and sensitivity required for in-depth assessments. In this context, analyzing transcriptional responses in salmonid RBCs offers a promising approach to gain a holistic view of internal health, monitoring stress status through putative biomarkers like DDIT4 and FKBP5, and detecting early responses to viral infections.

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9 Scientific papers I-III

Paper I

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Transcriptomics of early responses to purified *Piscine orthoreovirus*-1 in Atlantic salmon (*Salmo salar* L.) red blood cells compared to non-susceptible cell lines

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Piscine red blood cells (RBC) are nucleated and have been characterized as mediators of immune responses in addition to their role in gas exchange. Salmonid RBC are major target cells of Piscine orthoreovirus-1 (PRV-1), the etiological agent of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*). PRV-1 replicates in RBC *ex vivo*, but no viral amplification has been possible in available A. salmon cell lines. To compare RBC basal transcripts and transcriptional responses to PRV-1 in the early phase of infection with non-susceptible cells, we exposed A. salmon RBC, Atlantic salmon kidney cells (ASK) and Salmon head kidney cells (SHK-1) to PRV-1 for 24 h. The RNA-seq analysis of RBC supported their previous characterization as pluripotent cells, as they expressed a wide repertoire of genes encoding pattern recognition receptors (PRRs), cytokine receptors, and genes implicated in antiviral activities. The comparison of RBC to ASK and SHK-1 revealed immune cell features exclusively expressed in RBC, such as genes involved in chemotactic activity in response to inflammation. Differential expression analysis of RBC exposed to PRV-1 showed 46 significantly induced genes (≥ 2-fold upregulation) linked to the antiviral response pathway, including RNA-specific PRRs and interferon (IFN) response factors. In SHK-1, PRV induced a more potent or faster antiviral response (213 genes induced). ASK cells showed a differential response pattern (12 genes induced, 18 suppressed) less characterized by the dsRNA-induced antiviral pathway. Despite these differences, the RIG-I-like receptor 3 (*RLR3*) in the family of cytosolic dsRNA receptors was significantly induced in all PRV-1 exposed cells. IFN regulatory factor 1 (*IRF1*) was significantly induced in RBC only, in contrast to *IRF3/IRF7* induced in SHK-1. Differences in IRF expression and activity may potentially affect viral propagation.

KEYWORDS

piscine orthoreovirus, red blood cell, Atlantic salmon, salmon kidney cell line, transcriptome

1 Introduction

Red blood cells (RBC) are primarily known for their physiological role in respiratory processes, where intracellular heme and hemoglobin molecules regulate the uptake and transport of oxygen and carbon dioxide (1). In addition to this, a diverse range of physiological and immunologic properties have been attributed to vertebrate RBC, including redox homeostasis, hemoglobin antimicrobial activity and pathogen binding (2, 3). While mammalian RBC are enucleated and lack transcription/translation machinery, teleost RBC have retained their nucleus and organelles in the cytoplasm, essential for intracellular signaling, gene expression and protein production in response to stimuli (2, 4, 5). Previous studies of teleost RBC have shown their ability to react by innate immune responses and physiological differentiation in response to viral infections and systemic signals, respectively (2–4, 6–8). Unlike mammalian RBC, where the nucleus and cellular components are extruded during erythropoiesis to ensure efficient gas exchange (3, 9), transcriptome analyses of teleost RBC has revealed the expression of a complex set of genes involved in virus sensing, antiviral defense and antigen presentation (5, 8, 10, 11). However, the scale of RBC contribution to innate and potentially adaptive immunity is not fully understood.

Viral infections represent a major threat for the piscine aquaculture industry, and efficient prevention remains challenging. Heart and skeletal muscle inflammation (HSMI) is one of the most common viral diseases in farmed Atlantic salmon (*Salmo salar* L.) in Norway (12). The disease is characterized by extensive heart and muscle inflammation with infiltration of immune cells in the epi-, endo- and myocardium, myositis and necrosis in the red skeletal muscle (13–15). The causative agent of HSMI is Piscine orthoreovirus-1 genotype (PRV-1) (14, 16), a member of the order *Reovirales*, family *Spinareoviridae*, genus *Orthoreovirus.* This genus also contains the mammalian and avian orthoreoviruses (MRV and ARV, respectively). PRV-1 has a ten-segmented, double stranded RNA (dsRNA) genome packed in a double-layered icosahedral protein capsid, and was the first orthoreovirus reported in fish (14, 17).

Salmonid RBC are the main target cells of PRV-1 in the primary phase of infection (18). Comparative *in silico* studies with MRV indicate that PRV-1 may use the same infection mechanism, and further studies have indicated that the virus replication occurs in globular neo-organelles referred to as viral factories in the cytoplasm (16, 17, 19, 20). During the peak of infection, high loads of viral RNA and protein are produced within the cells and virus is released into plasma (16, 20). The peak in antiviral responses to PRV-1 has been associated with a decrease in plasma viremia and reduction in viral protein production in RBC (6, 16, 20), along with suppression of some RBC functions, such as hemoglobin production, and expression of metabolic genes (16, 21). Even though the impacts of PRV-1 infection on A. salmon RBC gene expression have been partly characterized *in vivo* and *in vitro* (6, 8, 22), the regulation of genes in RBC shortly after PRV-1 encounter has not been explored in detail.

In the present study, we compared the transcriptomic responses of A. salmon RBC to those of two A. salmon kidney cell lines at resting state, and 24 h after PRV-1 exposure. Atlantic salmon kidney cells (ASK) (23) and Salmon head kidney cells (SHK-1) (24) have been screened and characterized as non-supportive for PRV-1 propagation earlier, showing no evidence of virus replication (25). Here, we report the similarities and differences observed between A. salmon RBC, ASK and SHK-1 before and after PRV-1 exposure, focusing on pathways of the innate immune system.

2 Materials and methods

2.1 Blood sampling

Six A. salmon pre-smolts (30-50g) were euthanized using benzocaine chloride (1g/5L water) for 5 min, and peripheral blood from the caudal vein was collected in heparinized vacutainers (Vacutest, Sarstedt). The blood was used for isolation of red blood cells.

2.2 Isolation of RBC

RBC were isolated from the heparinized blood diluted 1:10 in sterile phosphate buffered saline (dPBS) and laid on top of a Percoll (GE healthcare, Uppsala Sweden) gradient (bottom layer 49%; top layer 34%) which was centrifuged (500 x G, 4°C, 20 min), washed with dPBS and collected as previously described (18). The cells were counted, and their viability was assessed using Countess (Invitrogen, Eugene, Oregon, USA) and resuspended to a concentration of 3×10^7 cells/mL in Leibovitz's L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with fetal calf serum (2%) (Sigma- Aldrich) and gentamicin (50 µg/mL- Lonza Biowhittaker, Walkersville, USA). The isolated RBC were inspected by light microscopy in three areas (approximately 100 cells/area, ≥ 300 cells in total) to ensure a maximum of two cells without typical RBC morphology (99% culture purity) (8) The cultures were placed at 15°C under constant agitation (225 rpm).

2.3 Atlantic salmon cell line cultures

The A. salmon kidney (ASK) cell line and the Salmon head kidney (SHK-1) cell line, were routinely split (1:2) once a week and cultivated at 20°C in Leibovitz's L15 medium supplemented with 4 mM L-glutamine (Life Technologies, Carlsbad, CA, USA), fetal bovine serum (10%) (Sigma- Aldrich), 40 µM 2-mecaptoethanol and gentamicin (50 µg/mL- Lonza Biowhittaker, Walkersville, USA). The cells were kept at 15°C during culturing and experiments.

2.4 Preparation of purified piscine orthoreovirus-1

Purified PRV-1 was used as inoculum in the *ex vivo* stimulation experiment. The virus was a variant of high virulence (NOR2012) (16), that had been purified from a blood cell pellet of infected fish using cesium chloride density gradient as described previously (16) and stored in Dulbecco's PBS with 15% glycerol at -80°C. The copy

number was determined using absolute quantification RT-qPCR as previously described (16).

2.5 *Ex vivo* stimulation

RBC isolated from six fish were plated in NuncTM non-treated 24-well plates with flat bottom (Thermo Fisher) (5×10^6 RBC per well, in 0.5 mL medium). RBC cultures were kept at 15°C under constant agitation (225 rpm) using an Ecotron incubation shaker (Infors HT, Basel Switzerland) to ensure a homogenous suspension. The virus exposure setup included six wells (one per fish) exposed identically to purified PRV-1 (5×10^6 virus particles per well/multiplicity of infection (MOI) of 1) and six control wells (one per fish). Following 24 h of incubation, exposed and control cells were harvested by centrifugation in Eppendorf tubes, media removal and lysis in RT buffer (Qiagen, Hilden, Germany) for RNA isolation.

ASK and SHK-1 experiments were performed at three separate time points (3 parallels). Each time, cells were counted and seeded in 6-well plates with flat bottom (4.5×10^4 cells in 1 mL medium-approx. 80% confluent) (Thermo Fisher) and kept at 15°C in brand incubator. The cultivation setup each time included three wells exposed identically to purified PRV-1 and 3 control wells. Briefly, the cells in the wells were washed three times with dPBS and 4.5×10^5 virus particles (MOI of 10) was added per exposed well. After 24 h of incubation, the cells were washed with dPBS and lysed with RT buffer (Qiagen, Hilden, Germany) for RNA isolation and subsequent RT- qPCR analysis to assess whether PRV-1 was associated with the cells.

2.6 RNA isolation and sequencing

Lysed cells were homogenized using 5 mm steel beads and TissueLyser II (Qiagen). Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Isolated RNA was eluted in 50 µL Rnase-free distilled water. RNA was quantified using NanoDrop ND- 1000 spectophotometer (Thermo Fiscer Scientific, Wilmington, DE, USA). RNA quality (RIN >8) was ensured using Agilent 2100 Bioanalyser (Agilent, USA) before being sent for sequencing.

Six biological replicates of the exposed and control RBC (12 samples in total), along with three experimental replicates of the exposed and control kidney cells (6 samples for ASK and 6 samples for SHK-1, respectively) were sent to Norwegian Sequencing Centre (NSC). Library preparation was performed using strand- specific TruSeq RNA Library Prep kit (Illumina, CA, USA). Libraries were subsequently sequenced on Illumina HiSeq to obtain 150 bp paired end reads.

2.7 Bioinformatics and statistics

Fastq files of reads from RNA-seq were cleaned (trim/remove adapter and low quality sequences) using BBDuk tool in BBMap v38.22 suite (parameters: ktrim=r, k=23, mink=11, hdist=1, tbo, tpe, qtrim=r, trimq=15, maq=15, minlen=36, forcetrimright=149) (26). Cleaned reads were further mapped to the A. salmon genome (ENSEMBL ICSASG_v2) using the HISAT2 v.2.2.1 (parameters: –rna-strandness RF) (27). FeatureCounts v.1.4.6-p1 (parameters: -p -s 2) was used for estimating the number of reads and aligning against the reference genes in ENSEMBL r104 GTF annotation (28). Initial data analysis was performed using the Bioconductor packages in R, including DESeq2 v.1.34.0 (29) and the SARTools v.1.7.4 (30). Normalization and differential expression analysis were conducted for the cells exposed to the virus against their unexposed controls using DESeq2. The annotation tables were cleaned using median count reads ≥ 10 as a cut off, to get rid of genes with zero or low counts. Subsequently, adjusted p-value (padj) was calculated using Benjamin- Hochberg (BH) correction and gene with padj below 0.05 were considered as differentially expressed genes (DEGs). ShinyGO v0.77 (31) was used for both gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with FDR cutoff 0.05. Pathview R package was used to draw KEGG pathway maps (32, 33).

3 Results

3.1 Transcriptome analysis of Atlantic salmon RBC and kidney cell lines in resting state

Information on total sequenced reads and alignment rate of mapping of all biological conditions is provided in Supplementary File A, Table 1. Normalized RNA- seq data were compared to identify features that are differentially expressed between RBC and kidney cell lines, ASK and SHK-1, at the unexposed resting state. The variability of the biological conditions within the experiment was assessed with a principal component analysis (PCA) (Supplementary File A, Figure 1). This analysis showed low variability within the biological (RBC) and experimental (ASK, SHK-1) replicates of each cell type, confirming consistency in the data, while the distribution of the clusters against the two first principal components indicated that SHK-1 and ASK are more closely related.

3.2 Transcriptional profiling of Atlantic salmon RBC and kidney cell lines, ASK and SHK-1

The original dataset consisted of 55819 features (genes). After filtering out 16989 genes with zero normalized median count reads, the differences and similarities in the expression profile of RBC, ASK and SHK-1 were assessed using an upset plot, including 38830 features (referred to as analyzed dataset) (Figure 1). A cutoff ≥ 10 counts was applied, and 24962 genes were found transcribed in RBC, 27518 genes in ASK and 27461 in SHK-1. In the three cell types, 24559 common genes were expressed. ASK and SHK-1 were sharing 2769 expressed genes (ASK & SHK-1 cutoff ≥ 10 median

counts, RBC = 0 median counts), verifying their highest level of similarity as indicated by PCA. A subset of 346 genes were exclusively expressed in RBC, while 44 genes were only expressed in RBC and ASK, and 13 genes were only expressed in RBC and SHK-1 (Figure 1).

To identify the processes in which the genes of each subset are involved, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. The lists of gene functional groups found in the enrichment analysis are provided in Supplementary File B. RBC, ASK and SHK-1 appeared to all share genes related to fundamental cellular processes, such as endocytosis, protein processing in ER and ubiquitin mediated proteolysis. Two KEGG pathways associated to cellular responses activated by viral and bacterial invasion, "Herpes simplex virus 1 infection" and "Salmonella infection" respectively, showed the greatest representation of shared genes (456 and 410 genes, respectively) between RBC, ASK and SHK-1. This indicated that RBC possess immune functions similar to ASK and SHK-1 and are able to respond to viral and bacterial pathogens. The KEGG pathways named "Herpes simplex virus 1 infection" and "Salmonella infection" were first described in mammals in response to these pathogens but have also been identified in teleost (33). In this study, the official KEGG nomenclature is used even if they refer to pathogens not relevant for this study.

3.2.1 Gene ontology enrichment analyses for the genes exclusively mapped to RBC

The subset of genes mapped exclusively in RBC consisted of 346 features. To identify biological processes that may be regulated by these genes, gene ontology (GO) enrichment analysis on Biological Process (GO : BP) was performed. Most genes were involved in "Cell surface-" and "G protein-coupled receptor" signaling pathways, whereas only a few appeared to contribute to physiological processes, such as gas transport and respiratory burst. Regarding the immune characteristics of the cells, genes involved in chemotaxis (e.g. C-C chemokine receptor type 9 (*CCR9*) and C-C motif chemokine 4 (*CCL4*) –like), phagocytosis (e.g. coronin-1A-like) and innate immune response pathway [e.g. interferon regulatory factor 4 (*IRF4*) and interleukin-1 receptor type II (*IL1R2*)] were represented. The detailed GO : BP categories along with the list of the 346 genes are provided in Supplementary File B. KEGG pathway enrichment analysis was considered inconclusive for such a small input.

3.3 Identification of differentially expressed genes between Atlantic salmon RBC and kidney cell lines, ASK and SHK-1

Differential gene expression analysis was performed to estimate differences in gene expression patterns between RBC and each kidney cell line (ASK and SHK-1). Filtering out low count genes (cutoff ≥ 10 median counts), the comparison of RBC against ASK and SHK-1 resulted in 14493 and 14397 differentially expressed genes (DEGs), respectively (Supplementary File A, Figure 2). In both comparisons, approximately 7500 DEGs indicated higher expression levels in RBC (thus, lower expression levels in ASK and SHK-1). Accordingly, approximately 6800 DEGs indicated lower expression level in RBC (thus, higher expression levels in ASK and SHK-1). ASK vs SHK-1 resulted in 10018 DEGs, 5041 with higher expression levels in SHK-1 and 4977 with higher expression level in ASK. The lists of DEGs emerging from the comparison of RBC vs SHK-1, RBC vs ASK and ASK vs SHK-1 are provided in Supplementary File C.

To determine the pathways to which DEGs of RBC vs ASK and SHK-1 belonged, KEGG pathway enrichment analysis was performed. The analysis was performed for DEGs with normalized median counts ≥ 10 and fold- change ≤ 0.5 for the

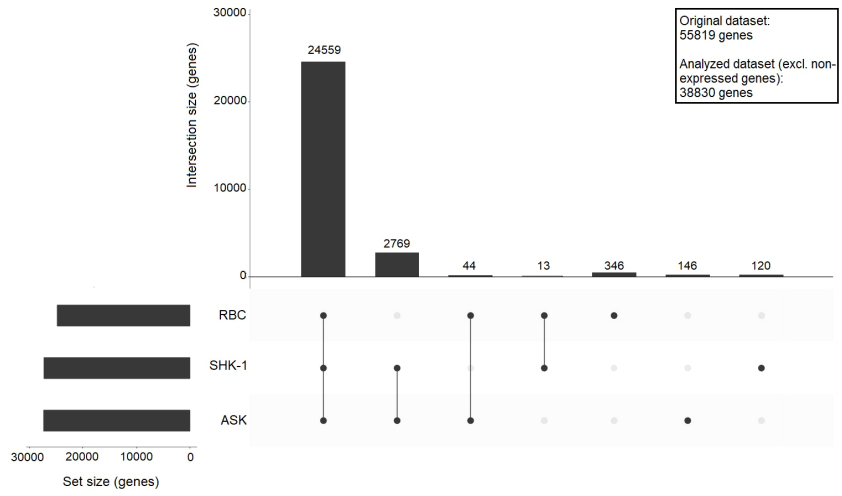

FIGURE 1
Upset plot showing sharing and unique gene expression for ASK, SHK-1, and RBC. A cutoff ≥ 10 counts was applied to define genes as expressed, and 0 counts required to define genes as not expressed in a cell type; The bars show the number of shared expressed genes between the indicated motifs: RBC vs SHK-1, RBC vs ASK and ASK vs SHK-1, or unique for a specific cell type. The analysis was performed using a dataset of 38830 genes (analysed dataset).

downregulated genes in ASK and SHK-1 compared to RBC (i.e "Higher expression compared to RBC" group of genes) and ≥ 2 for the upregulated genes in ASK and SHK-1 compared to RBC (i.e"Lower expression compared to RBC" group of genes). The majority of DEGs with higher expression in RBC compared to both ASK and SHK-1 were involved in innate immune processes related to viral sensing (KEGG nomenclature "Herpes simplex virus 1 infection") (119 and 126 genes, respectively), as shown in Figures 2, 3 in detail. Several genes with significantly higher transcripts in RBC were also involved in pathways associated with cellular functions like "Endocytosis", "Autophagy" and "Ubiquitin mediated proteolysis" (Figure 2). RBC DEGs belonging to KEGG groups, "MTOR-" and "FoXO" signaling pathways were only reported in the comparison of RBC vs ASK (Figure 2A, top), while "Ribosome" and "Basal transcription factors" in RBC vs SHK-1 (Figure 2B, top).

The majority of DEGs with lower expression in RBC were primarily involved in processes of cytoskeleton and paracellular communication ("Reg. of actin cytoskeleton" and "Tight junction") and host defense against bacterial invasion ("Salmonella infection"). Several genes were grouped within KEGG categories related to cellular senescence, metabolism and oxidative phosphorylation (Figure 2). Genes involved in ribosome biogenesis were more highly expressed in SHK-1 compared to RBC, indicating that RBC are less active in protein production (Figure 2B, bottom). Results from RBC vs ASK showed that genes linked to cell cycle events were more highly expressed in ASK (Figure 2A, bottom), which is expected for a continuous cell line.

To better understand the role of RBC in modulating functions of the innate immune system, we focused on signaling pathways involved in viral sensing and infection. These are included in the KEGG category referred to as "Herpes simplex virus 1 infection-sasa05168" pathway that consisted of the largest amount of DEGs with significantly higher expression levels in RBC. Figure 3A was extracted from the original pathway sasa05168 as established by Kanehisa Laboratories (2020). The detailed modified pathway is provided in Supplementary File A, Figure 3.

RBC expressed genes involved in toll-like receptor (TLR) and RIG-I-like receptor (RLR) signaling. Several signaling mediators in these pathways, such as interleukin 1 receptor associated kinase 1

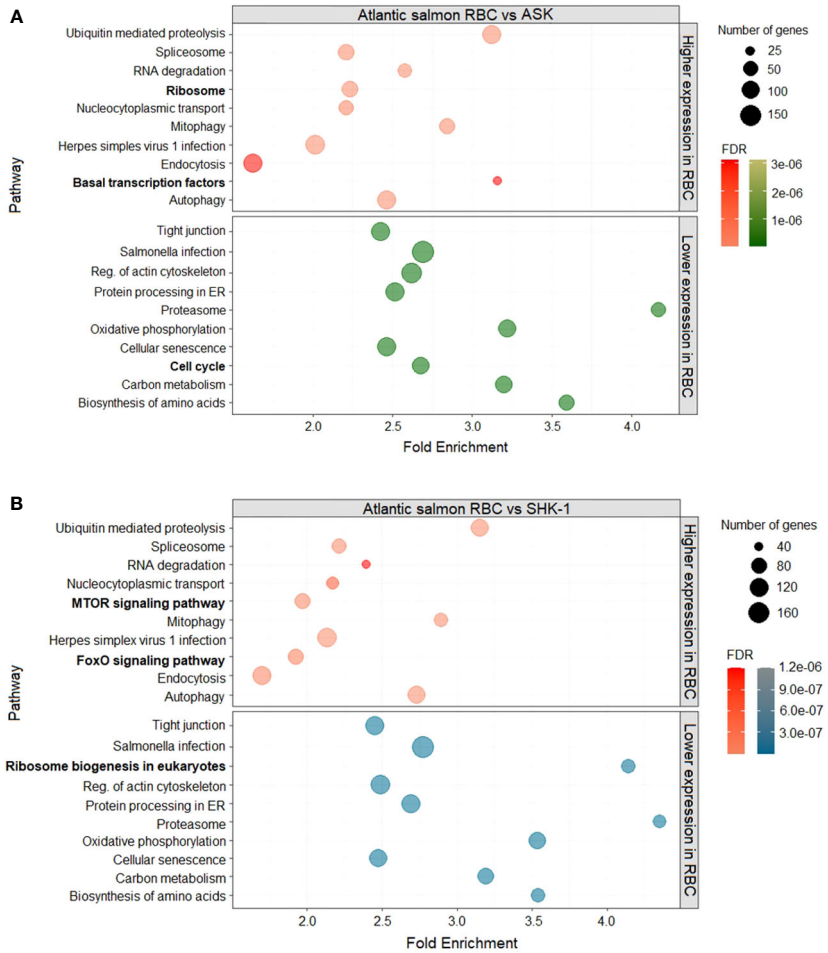

FIGURE 2
DEGs of RBC (red) compared to the kidney cell lines, **(A)** ASK (green) and **(B)** SHK-1 (blue). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was further analysed in ShinyGO 0.76 for FDR cutoff ≤ 0.05 and DEGs with fold-change ≥ 2 and ≤ 0.5.

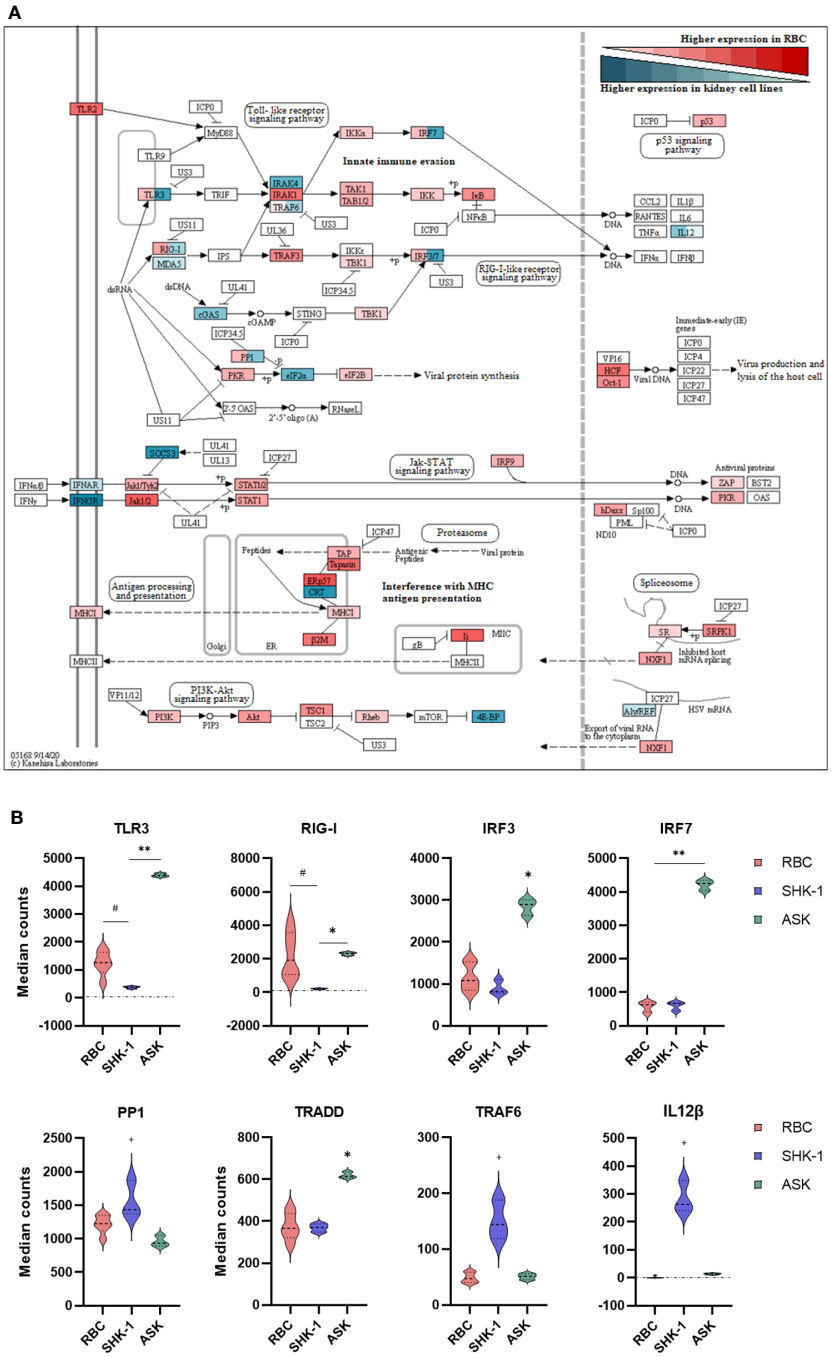

FIGURE 3
Differential expression analysis of selected genes associated with innate immunity in RBC, ASK and SHK-1. **(A)** Signaling pathways triggered by viral invasion. Red: Significantly higher normalized counts in RBC; Cyan: Similar and Significantly higher normalized counts in ASK and SHK-1. Red and cyan: Significantly different expression levels between ASK and SHK-1, and also with RBC were colored in both red and cyan. This figure was modified from the "Herpes simplex virus 1 infection" pathway- sasa05168 in KEGG, to include only immune pathways of interest. **(B)** Selected genes with significantly different expression pattern between the kidney cell lines, ASK and SHK-1, and RBC. $^{\#}p \leq 0.05$ in RBC vs ASK and SHK-1; $^{*}p \leq 0.05$ in ASK vs RBC and SHK-1; $^{**}p \leq 0.05$ in ASK vs RBC and SHK-1; $^{+}p \leq 0.05$ in SHK-1 vs ASK and RBC.

(*IRAK1*) and TNF receptor associated factor 3 (*TRAF3*), showed a higher expression level in RBC compared to ASK and SHK-1 (Figure 3A). However, the basal expression levels of pattern recognition receptors (PRRs), *TLR3*, melanoma differentiation-associated protein 5 (*MDA5*) and *RLR1* (also referred to as *RIG-I or DDX58)*, and interferon regulatory factors (*IRF*) 3 and 7 were significantly higher in ASK. Several components essential to antigen processing and presentation (MHCI pathway), inhibition of viral production (*PKR* regulation and *Jak-STAT* signaling pathway) and regulation of apoptosis and viral propagation (*PI3K- Akt* pathway)

showed significantly higher transcripts in RBC than ASK and SHK-1 (Figure 3A). While ASK and SHK-1 indicated similar expression patterns overall, a few genes related to cell cycle and immune cell differentiation were expressed significantly higher in SHK-1 (Figure 3B).

3.4 Identification of innate immune function genes in Atlantic salmon RBC

RBC have traditionally been characterized exclusively as gas exchangers expressing hemoglobins (3). As expected, several hemoglobin (Hb) subunits were found among the most highly expressed genes in RBC in the dataset (Table 1), also indicating culture purity. Expression levels of iron storage ferritins and mediators of heme biosynthetic pathway (such as *BLVRB* and *ALAS2*), which typically function in blood/RBC (34, 35), were also among the highest expressed genes. Also, MHC class I-related gene protein-like and thymus-specific serine protease (*TSSP*) antigen processing components were among the most highly expressed genes in salmonid RBC (Table 1). To further assess the purity of the RBC culture, transcripts of typical T cells and B cells markers were sought and evaluated. While many were not identified in our datasets, such as *CD3* and *CD34*, a few typical T cell and B cell markers such as *CD4* and *CD8* (36), showed near- zero count reads (Table 1).

To assess the contribution of RBC to innate immunity, we focused on identifying components associated with pathogen recognition, cell-to-cell communication, activation of the innate immune system and host defense. The detection of infectious agents is mainly mediated by (germline-encoded) PRRs. PRRs are highly conserved among vertebrates and the main families described in

TABLE 1 Transcript counts of the 20 most highly expressed genes in A. salmon RBC compared to ASK and SHK-1.

Gene	Description	Ensembl ID	RBCs (counts)	ASK (counts)	SHK-1 (counts)
HBAA2	Hemoglobin subunit alpha-4	*ENSSSAG00000044737*	797987	157	172
-	Ferritin heavy subunit	*ENSSSAG00000049977*	671668	112424	70642
HBB1	Hemoglobin subunit beta-1-like	*ENSSSAG00000044957*	579951	130	137
-	Hyperosmotic glycine rich protein	*ENSSSAG00000068063*	421881	200118	163430
HBA4	Hemoglobin subunit alpha-4	*ENSSSAG00000065254*	321654	89	87
HBB	Hemoglobin subunit beta-like	*ENSSSAG00000045065*	321344	83	92
HBA	Hemoglobin subunit alpha	*ENSSSAG00000065229*	244043	75	68
HSPA8	Heat shock protein 8	*ENSSSAG00000049191*	213336	55203	37017
HBB	Beta globin	*ENSSSAG00000065233*	210828	44	47
HBB1	Hemoglobin subunit beta-1-like	*ENSSSAG00000065315*	187925	106	117
FRIH	Ferritin, heavy polypeptide 1-1	*ENSSSAG00000051567*	156074	32006	99410
HBBA2	Hemoglobin subunit beta-1-like	*ENSSSAG00000065226*	150808	45	44
NRK2	Nicotinamide riboside kinase 2-like	*ENSSSAG00000077245*	142822	9601	2613
TSSP	Thymus-specific serine protease	*ENSSSAG00000053130*	136772	32	38
BLVRB	Biliverdin reductase B	*ENSSSAG00000069097*	117596	965	1314
ALAS2	5'-aminolevulinate synthase 2	*ENSSSAG00000068428*	106223	28	30
-	Major histocompatibility complex class I-related gene protein isof. X1	*ENSSSAG00000077419*	87427	29250	54093
MIBP2	Nicotinamide riboside kinase 2-like	*ENSSSAG00000068654*	79622	4905	3683
WBP4-like	WW domain-binding protein 4-like	*ENSSSAG00000077000*	78270	434	278
5NTC	Cytosolic purine 5-nucleotidase	*ENSSSAG00000045618*	67967	23	21
Cd4	***S. salar* T-cell surface glycoprotein CD4**	***ENSSSAG00000076595***	**1**	**6**	**0**
Cd8a	***CD8- alpha***	***ENSSSAG00000065860***	**0**	**0**	**0**
Cd8b	***CD8- beta***	***ENSSSAG00000045680***	**1**	**0**	**0**
Cd34	***CD34* molecule**	***ENSSSAG00000079346***	**0**	**952**	**589**
MME	**Neprilysin- like**	***ENSSSAG00000042374***	**5**	**0**	**1**

Transcript counts of five distinct T cells and B cells markers (in bold) were also included to assess RBC culture purity. The expression levels of the genes were measured as median normalized count reads (counts). All listed genes indicated significantly higher expression in RBC ($p \leq 0.5$).

fish include toll-like receptors (TLRs), nucleotide oligomerization domains (NOD) -like receptors, retinoid acid-inducible (RIG) -like receptors (RLRs), C-type lectin receptors (CLRs) and scavenger receptors (SRs) (37). A wide repertoire of PRRs from all five families was found in RBC. TLRs, RLRs and NLRs were the most abundant PRRs in the cells and those with the highest transcript levels are listed in Table 2. RLRs, which primarily recognize double- stranded (ds) RNA oligonucleotides, showed collectively the highest expression. *TLR3*, previously identified in salmonid RBC and known to bind dsRNA, was detected in high transcript numbers (8). *TLR8*, which recognizes single- stranded (ss) RNA, showed the highest expression among the TLRs (38, 39). Several NLRs, which primarily have been characterized in mammals as sensors of bacterial components, such as lipopolysaccharides (LPS) and peptidoglycans (PGNs) were identified in RBC. Variants of NLR family CARD domain containing 3- like (*NLRC3L*) showed the highest expression (45). In addition, *NLRC5* and *NOD1/NOD2* were detected. Their role and functionality in teleosts are modestly studied.

The majority of the signaling regulators and effectors which interact with TLRs and RLRs, along with various non-RLR DEAD/DEAH box RNA helicases with diverse roles in innate immunity, were identified in RBC, as shown in Figure 4 (top). Indicatively, *DHX37* showed the highest expression level, however details about its function have not been determined in either fish or mammals. *IRF1* (isoform 2), known to regulate the induction of interferon (*IFN*) and IFN-stimulated genes, and *IRF9*, associated with antiviral immunity (46), were highly expressed in the RBC transcriptome. Several cytokine receptors were found in our dataset, but only a few cytokines (interleukins and chemokines) were expressed in RBC, including interleukin 15 and 34 (*IL15* and *IL34*), and *CCL4- like* chemokine (Figure 4). Common IFN stimulated antiviral effector genes, such as IFN stimulated gene 15 (*ISG15*) like (*UBIL*) and myxovirus resistance (*Mx2*), known to be induced by IFNs, were also identified in RBC in high transcript numbers.

3.5 Differential expression analysis of RBC and kidney cell lines exposed to PRV-1

To identify the antiviral responses in RBC at early PRV-1 exposure (24 h) compared to non- susceptible cell lines, normalized RNA-seq data of the samples exposed to the virus were compared to unexposed controls through differential expression analysis (DESeq2). Information on total sequenced reads and alignment rate of mapping, along with principal component analysis (PCA) are provided in Supplementary File A, Figure 4. Differential expression analysis of RBC exposed to PRV-1 vs the unexposed controls showed a set of 46 significantly induced genes (≥ 2-fold upregulation) and 1 significantly suppressed (≤ 0.5-fold downregulation) gene (Figure 5). In contrast, 213 genes were significantly induced and 10 genes were significantly suppressed in

TABLE 2 Pattern recognition receptors (PRRs) identified in A. salmon RBC.

Gene	Ensembl ID	RBC (counts)	Ligands	Reference in teleost
Toll-like receptors (TLRs)				
TMSB4X (or TLR8)	*ENSSSAG00000076485*	2060	ssRNA	(38)
TLR3	*ENSSSAG00000040910*	1244	dsRNA	(8)
TLR2	*ENSSSAG00000003781*	50	LPS	(39)
TLR19	*ENSSSAG00000042328*	31	Non specified	(39)
Retinoic acid-inducible gene (RIG)-like receptors (RLRs)				
MDA5	*ENSSSAG00000078885*	2264	dsRNA	(40)
DDX58	*ENSSSAG00000045391*	2232	(ds)RNA	(41)
DHX58	*ENSSSAG00000037858*	1824	ssRNA; dsRNA	(40)
Nucleotide oligomerization domains (NOD)-like receptors (NLRs)				
NLRC3L1	*ENSSSAG00000005336*	1461	DNA and RNA oligonucleotides	(42)
	ENSSSAG00000056446	1177		
	ENSSSAG00000046213	1033		
NLRC5	*ENSSSAG00000068298*	233	Bacterial components	(43)
NOD1	*ENSSSAG00000053537*	170	Bacterial PGNs	(44)
NOD2	*ENSSSAG00000076025*	26	Bacterial PGNs	(44)

The majority of mapped PRRs were categorized in 3 major groups: toll-like receptors (TLRs), retinoic acid inducible gene (RIG)- like receptors and nucleotide- oligomerization domain (NOD)-like receptors. The basal expression levels of the genes were measured as median normalized count reads (counts). Only genes with transcripts ≥ 10 (cutoff ≥ 10 median counts) were included in the analysis. LPS, lipopolysaccharides; PGNs, peptidoglycans.

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Non- RLR DEAD/H box helicases	Counts	Signaling adaptors	Counts	IFN regulatory factors	Counts
DHX37	23590	*IPS* (or *MAVS*)	1026	*IRF9*	5548
DDX46	5431	*TRAF3*	513	*IRF1 (isof. 2)*	4517
DDX3X (isof. X2)	2714	*IKBKγ* (or *IKK*)	316	*IRF3*	1152
DDX6	2552	*TRIM25-like*	242	*IRF7*	574
DDX24	2183	*TBK1*	266	*IRF1*	234
DHX33	1782	*TICAM1*	35	*IRF4*	72

Interleukins & interleukin receptors	Counts	Chemokines & chemokine receptors	Counts	IFN pathway activators & IFN-inducible genes	Counts
IL15	3588	*CCL4- like*	197	*STAT1*	14545
IL6R β (isof. X2)	1500		40	*STAT2*	8350
IL34	152	*CCR9*	598	*JAK1*	1018
IL15Rα	728		294	*EIF2αK2*	42
IL6Rα	177	*CXCR4*	266	*UBIL*	1820
I10R2	172	*CXCR4β*	42	*MX2*	970

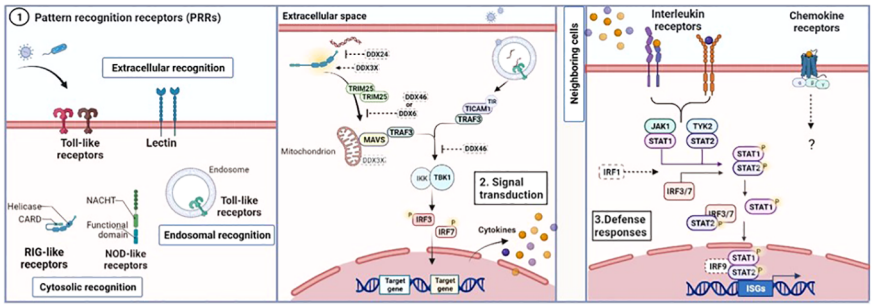

FIGURE 4
Examples of RBC genes involved in innate immune responses identified in A. salmon RBC. Transcripts of non- RLR DEAD/DEAH box helicases, signaling adaptors and interferon regulatory factors (IRFs) (table on top). Transcripts of interleukins (ILs) and interleukin receptors (ILRs), chemokines (C-C and C-X-C motifs) and chemokine receptors and interferon (IFN) pathway activators and IFN- inducible genes (table on bottom). The expression levels of the genes were measured as median normalized count reads (counts) (RBC n= 6). Only genes with transcript reads ≥ 10 (cutoff ≥ 10 median counts) were included in the analysis. Short description of the pathways relevant for genes expressed in RBC and listed in the tables above. Elements drawn in dash have not been characterized in teleost, and their roles were based on mammalian models. Step 1. Pattern recognition receptors (PRRs). Step 2. Signaling mediators and interferon regulatory factors acting downstream of PRR binding, leading to secretion of IFNs and pro-inflammatory cytokines. Step 3. Pathways induced when secreted IFNs and cytokines bind to receptors, leading to expression of several innate immune effectors.

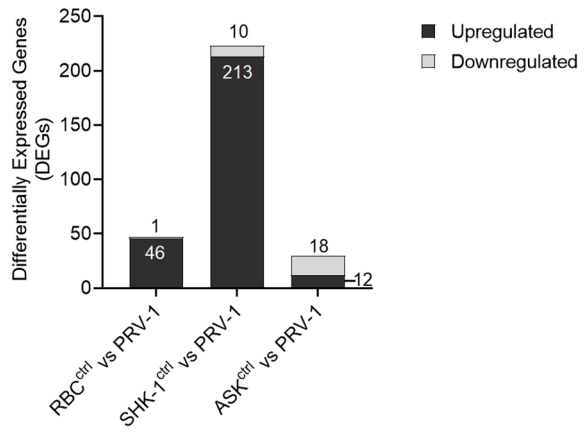

FIGURE 5
Differential gene expression analysis of A. salmon RBC, SHK-1 and ASK exposed to PRV-1 for 24h compared to their unexposed controls (RBC vs PRV-1, SHK-1 vs PRV-1 and ASK vs PRV-1, respectively). The analysis was performed on genes with median counts ≥ 10. Cutoff ≥ 2-fold change for upregulated DEGs and ≤ 0.5-fold change for downregulated DEGs was applied.

SHK-1. In ASK, 12 genes were significantly induced and 18 genes significantly suppressed. Thus, SHK-1 demonstrated the strongest and ASK the weakest responses to PRV-1.

3.6 GO and KEGG enrichment analysis for the DEGs of RBC, ASK and SHK-1 exposed to PRV-1

We performed GO and KEGG pathway enrichment analyses with an FDR (adjusted p value) cutoff of 0.05 for the upregulated DEGs (≥ 2-fold change) in RBC, ASK and SHK-1 to identify biological processes and signaling pathways activated in response to PRV-1 (Figure 6). As the significantly downregulated genes were too few, they were not subjected to these analyses. GO enrichment analysis for Biological Process (GO : BP) resulted in 9 GO terms for RBC, 6 for SHK-1 and 3 for ASK. Genes in RBC were mainly involved in four biological processes: “Response to biotic stimulus”, “Protein modification by small protein conjugation or removal”, “Defense response” and “Immune system process”. GO term “Immune system process” consisted of 6 genes, including *RLR3*

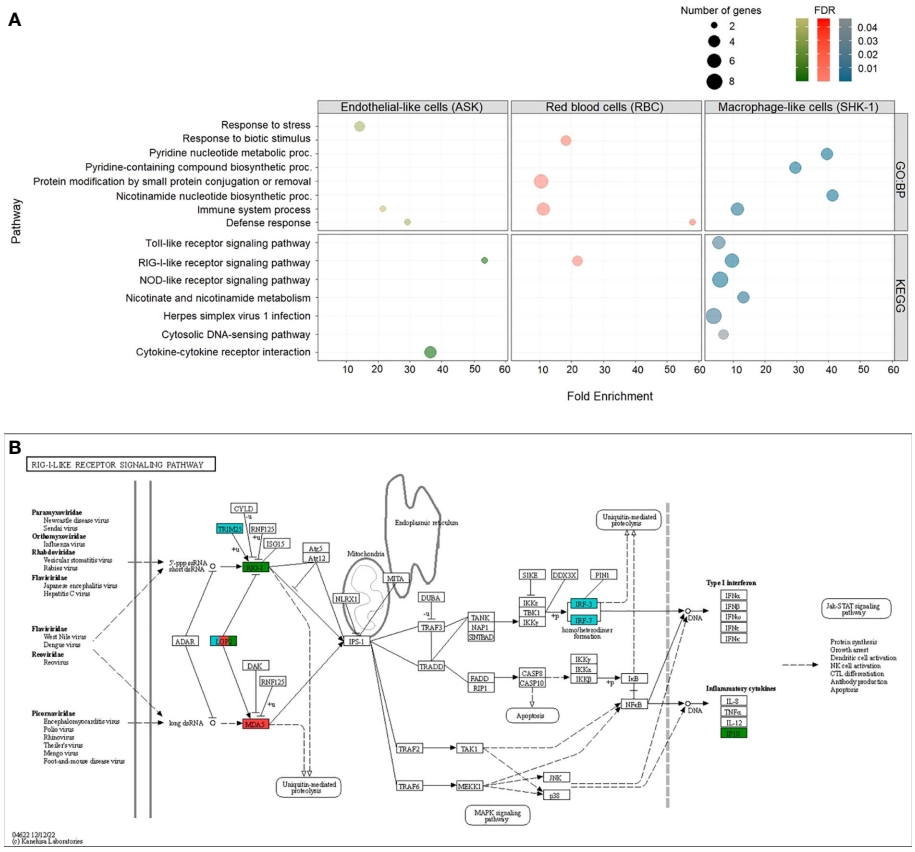

FIGURE 6
Up-regulated DEGs (cutoff ≥ 2-fold change) in ASK, RBC and SHK-1 exposed to PRV-1. Enriched Gene Ontology (GO) terms within the GO category "Biological Process" (GO : BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with FDR (adjusted p value) lower than 0.05 were considered significant. **(A)** GO : BP (top) and KEGG pathways (bottom) enriched in ASK (green), RBC (red) and SHK-1 (blue). **(B)** Representation of KEGG pathway:"RIG-I-like receptor signaling pathway"-sasa04622, as significantly enriched in RBC, ASK and SHK-1. Genes involved in pathway and significantly induced in RBC, ASK and SHK-1 exposed to PRV-1 were annotated in red, green and blue, respectively.

[also referred to as laboratory of genetics and physiology 2 (*LGP2*)], melanoma differentiation-associated protein 5 (*MDA5*) and transcription factors involved in *type I IFN*-pathway activation, *IRF1-2* and *IRF1*. From the GO terms that appeared for ASK, biological functions associated with response to stress showed the greatest representation, while groups "Immune system process" and "Defense response" consisted of only two significantly expressed genes, one of which was *RLR3*. Other significantly induced genes in SHK-1 were primarily involved in metabolic functions associated with the formation of nicotinamide-adenine dinucleotide phosphate, such as "Pyridine nucleotide metabolic process", "Pyridine-containing compound" and "Nicotinamide nucleotide" biosynthetic processes. The GO : BP term "Immune system process" was also significantly enriched for SHK-1, including genes such as the dsRNA receptors *RLR3* and *TLR3*, and the antiviral effectors, *UBIL* and *Mx2*. A detailed description of GO terms in RBC, ASK and SHK-1 is provided in Supplementary File D.

KEGG analysis revealed one category, "RIG-I-like receptor signaling pathway"- sasa04622, which was significantly enriched in RBC, ASK and SHK-1. This category consists of genes involved in immune pathways activated upon binding of dsRNA to RLRs, including the *RLR3* gene (referred to as *LGP2* in the pathway). The cytosolic dsRNA receptor *MDA5* gene was induced only in RBC, and the *RLR1* gene was induced only in ASK (Figure 6B). In SHK-1, the tripartite motif-containing protein 25 (*TRIM25*) gene, *IRF3* and *IRF7* in this pathway was also significantly induced (Figure 6B). In contrast to RBC, genes significantly induced in SHK-1 were categorized in five more groups, four of which are involved in innate immunity (such as "Toll like receptor" and "NOD-like receptor" signaling pathways), while significantly induced genes in ASK were categorized in one additional group, associated with cytokine- cytokine interaction (Figure 6A).

Given the outcome of the differential expression analysis, GO and KEGG pathway enrichment analyses, 24 h exposure of RBC to PRV-1 triggered the activation of PRRs that recognize viral dsRNA (*MDA5* and *RLR3* induction) and signaling factors that regulate the secretion of IFNs and pro-inflammatory cytokines. To better understand the immune responses occurring in RBC after PRV-1 exposure, compared to non- susceptible kidney cell lines, we focused on genes typically involved in dsRNA viral recognition, signal transduction, *IFN*-pathway activation, and virus eradication. The comparison of the immune transcriptome responses of RBC to SHK-1 showed that SHK-1 respond more potently to PRV-1 than RBC by significantly inducing the expression of a wider repertoire

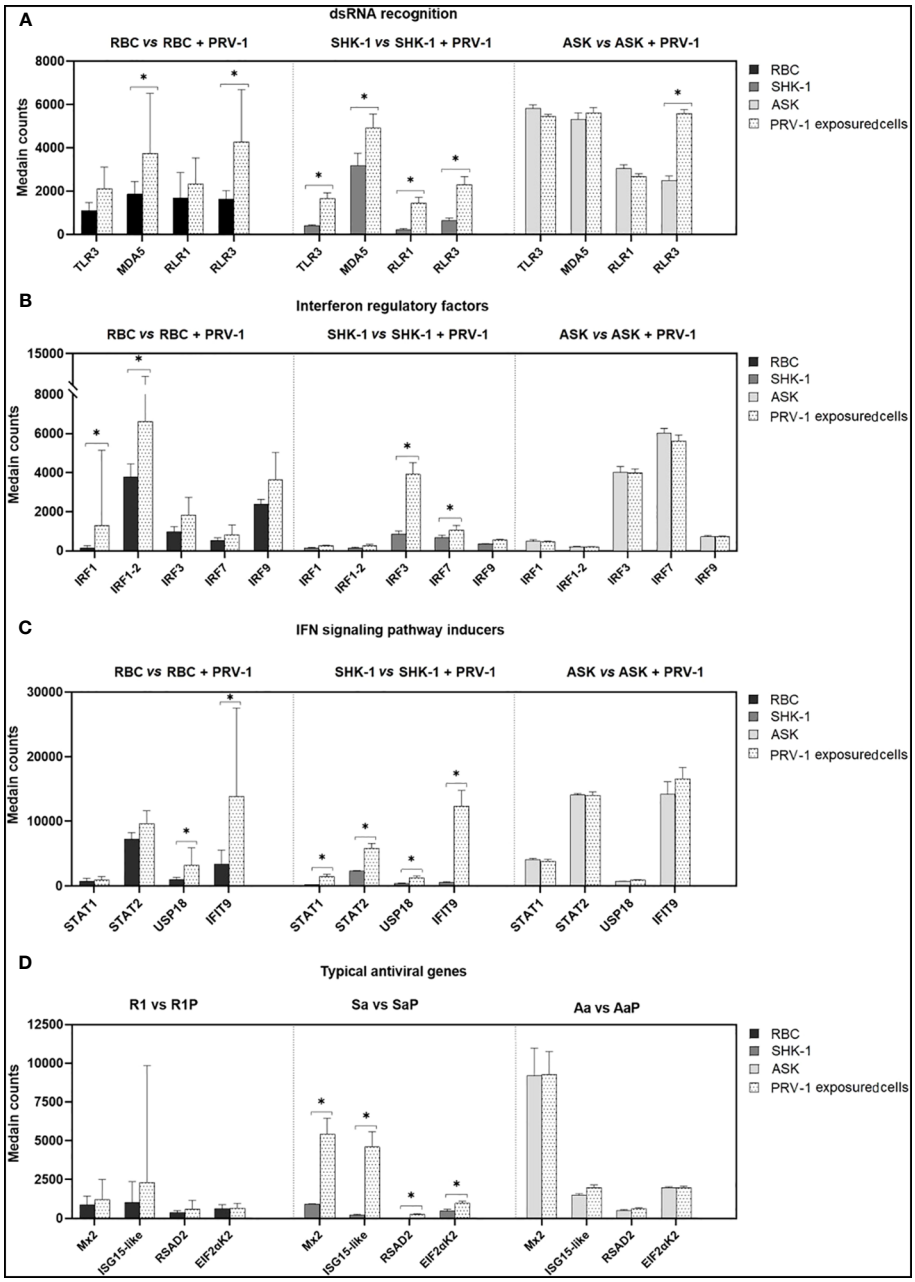

FIGURE 7
Comparison of the transcriptome responses linked to selected innate antiviral genes in RBC, SHK-1 and ASK exposed to PRV-1 (RBC vs RBC + PRV-1, SHK-1 vs SHK-1 + PRV-1 and ASK vs ASK + PRV-1, respectively). Regulation of **(A)** dsRNA pattern recognition receptors, **(B)** interferon regulatory factors, **(C)** genes involved in *IFN*-signaling patway activation and **(D)** IFN-inducible antiviral effectors. RBC vs PRV-1, n=6, SHK-1 vs PRV-1 and ASK vs PRV-1, n=3. *p<0.05.

of dsRNA pattern recognition receptors and typical antiviral genes. On the contrary, the comparison of RBC to ASK showed that ASK induced *RLR3*, while other typical antiviral responses were absent (Figure 7).

4 Discussion

The present transcriptional analysis showed that genes with the highest expression levels in RBC are primarily involved in respiratory processes, including multiple hemoglobins and mediators of heme biosynthesis. This is consistent with the traditional physiological characteristics of RBC as gas exchangers (3). Previous multi-omics analyses of salmonid RBC in response to viral infection revealed the expression of several genes involved in different aspects of immunity, including antigen presentation through MHC I and MHC II (8, 47). Current transcriptomic data indicated exceedingly high basal levels of the MHC I- associated protein- encoding genes, such as *UBA* and *UGA* genes, supporting A. salmon RBC role in innate immunity. Earlier characterization of

UBA and *UGA* genes in rainbow trout leukocytes and lymphoid organs showed induced gene expression in response to viral infection (48). However, this was not the case in A. salmon RBC exposed to PRV-1 for 24 h, for which the short period of exposure to the virus may be a possible explanation.

The number of genes expressed in RBC in resting state was at comparable level as in ASK and SHK-1 cell lines, indicating that RBC are multifunctional. Although sets of genes involved in regulation of cellular homeostasis and survival (e.g. RNA processing and protein biosynthesis) showed similar expression patterns in all three cell types, genes associated with physiological functions which promote intracellular transport (e.g. endocytosis and nucleocytoplasmic transport) and molecule degradation (e.g. ubiquitin mediated proteolysis and autophagy) appeared to be more highly expressed in RBC. In contrast, genes essential for cellular structural integrity and differentiation, such as keratins (type I or II), serpines and cofilins, showed low transcription levels in RBC, while being more prominent in both kidney cell lines. Entry of PRV-1 into RBC have been predicted to occur via receptor-mediated endocytosis through *in silico* comparison of PRV proteins with MRV, for which viral uptake mechanisms are well characterized (49). In this sense, higher expression levels of genes involved in intracellular transport in RBC compared to non-susceptible ASK and SHK-1, may be linked to differences in uptake mechanisms. No genes involved in endocytic processes in RBC were significantly induced in response to 24 h-exposure to PRV-1.

Genes involved in signaling pathways triggered by viral invasion were expressed in RBC as well as ASK and SHK-1, confirming that RBC possess innate immune functions, as previously published (2, 5, 8, 47). Notably, the basal expression of genes associated with antiviral defense was more distinguished in RBC, compared to genes involved in responses to bacteria, indicating that RBC exhibit higher sensitivity to viruses.

Innate immunity represents the first line of host defense against invading pathogens, the recognition of which is mediated by PRRs (36, 50). Interestingly, RBC express a wide repertoire of PRRs, some of which have not been reported in salmonid erythrocytes earlier and that are able to detect pathogen-associated molecular patterns (PAMPs) derived from viruses and other pathogens. *TLR8* and *NLRC3- like* receptor genes appeared among the most highly expressed. Earlier studies on PRR signaling in fish showed that *TLR8* and *NLRC3-like* receptors trigger inflammatory responses through *MyD88-* and *NOD1/RIP2-* dependent signaling pathways upon recognition of synthetic ssRNA oligonucleotides and bacterial cell wall components, respectively (38, 42). The ability of salmonid RBC to manifest innate immune responses has most extensively been studied in response to RNA viruses (8, 47, 51) and there are few reports that demonstrate their immune responses to bacterial and parasites (51, 52). Although the gene expression of microbial-specific PRRs alone should not be considered indicative for their functional role, it may strengthen the notion of RBC as contributors to innate immunity against a broad range of infectious agents.

It is worth noting that various DEAD/H- box RNA helicases, recently characterized for their diverse roles in antiviral immunity in fish and mammals, were largely detected in RBC transcriptome (41, 53). Herein, *MDA5* and *RLR3* are reported in A. salmon RBC for the first time. Together with *RLR1*, these genes belong to the RLR family. Teleost RLRs, like in mammals, bind dsRNA viruses, and subsequently induce the activation of *type I IFN* signaling pathway and secretion of pro-inflammatory cytokines (53, 54). Previous transcriptional studies reported significant upregulation of *RLR1* in PRV-1 infected A. salmon, and *MDA5* and *RLR3* in viral hemorrhagic septicemia virus (VHSV) infected rainbow trout RBC (8, 22, 47).

RBC express multiple transcriptional activators that are essential for dsRNA-PRRs signaling, including several IRFs. For instance, binding of dsRNA to the cytosolic RNA sensors *RLR1* or *MDA5* leads to the activation of interferon promoter stimulating protein- 1 (*IPS* or *MAVS*). This activator, in association with TNF receptor- associated factor 3 (*TRAF3*) and TANK-binding kinase 1 (*TBK-1*), phosphorylates/activates *IRF3/7*, which potentiate the transcription of pro-inflammatory cytokines and IFNs (46, 53, 54). *TLR3*, similar to RLRs, is known to interact with TIR domain-containing adaptor (*TRIF* or *TICAM1*) to regulate the secretion of IFNs through the nuclear factor kappa B (*NF-κB*)- and *IRF3/7* - dependent signaling pathways (55). In general, secreted *IFN* and cytokines, in turn, bind to transmembrane *IFN*/cytokine receptors, and trigger the expression of IFN- stimulated genes by means of recruiting kinases and transcription factors, such as *JAK*, *STAT1/2*, *IRF9* and/or *IRF1* (53–55). The identification of genes corresponding to such complete signaling pathways in RBC transcriptome not only reinforces RBC characterization as immune mediators, but also contributes to our original hypothesis that they regulate multiple immune functions through both well characterized and unexplored signaling pathways in salmonid RBC.

A rather intriguing finding was the expression of several interleukin (IL) and chemokine receptors in A. salmon RBC, but only a few of the corresponding cytokines were expressed. As in mammals, fish cytokines are secreted by many cell types and involved in cell-to-cell communication though an endocrine and/or paracrine manner (56, 57). The expression of pro-inflammatory IL receptor subunits, such as *IL6R* and *IL1R*, may imply immune activation of RBC upon binding to *IL1* and *IL6*, secreted by other immune cells. Fish and mammalian *IL10* and *IL10R* regulate anti-inflammatory functions, a feature that suggests involvement in mechanisms of viral persistence (58). Since RBC express *IL10R*, they may participate in processes related to such mechanism, for example in the persistent phase of PRV-1 infection (59). In contrast to rainbow trout RBC, which were shown to express *IL1β*, *IL8* and *IFNγ* in response to infectious hematopoietic necrosis virus (IHNV) and thermal stress, A. salmon RBC demonstrated high transcript levels of only *IL15* and *IL34* (7, 36). Studies on the characterization of *IL15* in rainbow trout suggested its involvement in CD4+ T cell survival, where it induces *IFNγ* through a *STAT5p*-dependent signaling pathway (60, 61). The function of *IL34* is modestly explored in salmonids. However, in recent studies in fresh water fish species such as Largemouth bass (*Micropterus salmoides*) and grass carp (*Ctenopharyngodon idella*), *IL34* was suggested to be involved in macrophage activation (62, 63).

The comparison of RBC to ASK and SHK-1 revealed sets of genes, which were exclusively expressed in RBC, and involved in

innate/adaptive immune processes and chemotaxis. This supports the multifunctional nature of RBC, while providing insight into their unique immunological features. Indicatively, among the wide assortment of IRFs identified in the total transcriptome, *IRF4* expression appeared only in RBC. Earlier characterization of IRFs in A. salmon showed that *IRF4*, similar to its mammalian counterpart, inhibits *IFN* production (64). Additional immunosuppressive effects on RBC may be mediated by *IL1R2*, which have been shown to compete with *IL1* for binding *IL1Ra* in seabream (*Sparus aurata*) and grass carp (*Ctenopharyngodon idellus*) (65, 66). In mammals, *CCR9* is distributed on the surface of intestine cells where it binds its specific ligand *CCL25*. In both mammals and teleost, upregulation of *CCL25* in gut has been associated with infiltration of *CCR9*- expressing inflammatory cells (67, 68). The expression of *CCR9* in RBC may indicate that, similarly to immature T-lymphocytes, they may migrate into tissues expressing *CCL25* ligand. Mammalian C-C chemokine 4 (*CCL4*) is commonly expressed in different antigen- presenting cells (APC), and *CCL4* regulation has only recently been studied in fish (57, 69, 70). Functional characterization of *CCL4* in orange- spotted grouper (*Epinephelus coioides*) showed that recombinant *CCL4* exhibits chemotactic activity, attracting leukocytes, such as macrophages and NK-cells, and stimulating lymphocyte differentiation (71); thus, the role of *CCL4* was suggested to be conserved in teleost and mammals (69, 71). Since A. salmon RBC express *CCL4*, they may be involved in inflammatory responses by recruiting macrophages and NK-cells and/or triggering lymphocyte differentiation. Although, it is hard to assume the role and involvement of these cytokines and cytokine receptors in the immune functions of RBC, hypotheses regarding the possible migration of RBC into inflammatory tissue like other circulating immune cells could represent an open and interesting field of study.

A few immune genes were significantly induced in RBC 24 h after PRV-1 encounter. Most DEGs are involved in dsRNA recognition and subsequent signal transduction via IRFs, but *type I IFN* and IFN- stimulated genes were not found induced. In contrast, several genes implicated in RNA virus recognition and antiviral defense were significantly expressed in SHK-1, while remaining at basal levels in ASK. Previous transcriptional analysis of ASK cells in response to synthetic dsRNA analogue, poly(I:C), revealed significant induction of the RNA-specific PRRs genes *MDA5* and *RLR3*, and antiviral effectors genes, such as *type I IFN* and *Mx1* and *ISG15*, 12 h post stimulation (72). This suggests that despite the ability of ASK to respond to naked dsRNA, the processes associated with ligand recognition and initiation of immune defense may differ in response to purified virus. To date, recognition of PRV-1 in RBC has primarily been associated with the induction of endosomal *TLR3* and cytosolic *RLR1* (6, 8). Our data, however, showed upregulation of the *MDA5* and *RLR1* genes after 24 h-exposure to the virus. Several putative PRR- genes for RNA viruses were significantly induced in SHK-1, including *TLR3*, *RLR1*, *MDA5* and *RLR3*, whereas only *RLR3* was induced in ASK. PRV-1 propagation is not supported by SHK-1, and the upregulation of many genes involved in a range of different antiviral pathways in response to virus, may be a possible explanation. Interestingly, the *RLR3* gene was significantly induced in all PRV-exposed cells. In contrast to *RLR1* and *MDA5*, the role of *RLR3* in antiviral immunity in fish cells is poorly understood. In mammals, *RLR3* is associated with both positive and negative contribution to antiviral signaling in a concentration- dependent manner. *RLR3*, when at low levels, functions synergistically with *MDA5*, and thereby enhance *MDA5*-mediated antiviral signaling. Oppositely, *RLR3* at high expression levels competes with *RLR1* and *MDA5* for dsRNA viral recognition and suppresses RLR signaling pathway by inhibiting receptor interaction with the *IPS* activator (73). In teleost, *RLR3* has mainly been associated with positive regulation of antiviral signaling; its expression was linked to significant induction of antiviral effectors, such as *Mx*, in rainbow trout, and decrease of grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV) titers in black carp (*Mylopharyngodon Piceus*) *in vitro* (40, 74). In contrast to mammals, functional characterization of *RLR3* in fish did not show suppression or synergy with *MDA5*, but rather a parallel function (40). Relative expression of *RLR3* in A. salmon RBC, ASK and SHK-1 in response to PRV-1 do not provide sufficient evidence for its putative function. However, its significant induction may indicate a pivotal contribution to viral recognition and the following antiviral events in the cell.

As mentioned above, *MDA5* activation is commonly followed by the transcriptional activity of *IRF3* and/or *IRF7* (53, 75). Induction of these IRF genes has previously been reported in salmonid erythrocytes at later stages of PRV-1 infection *in vivo* (8). Here, only *IRF1* was significantly upregulated, whereas there was not significant induction of *IRF3* and *IRF7* in response to PRV-1. *IRF1* has been shown to actively participate in induction of IFN and ISG transcription as a response to RNA viruses in mammals and fish (46, 76). As opposed to RBC, ASK and SHK-1 expressed low levels of *IRF1* both pre- and post- exposure to PRV-1. In contrast, the expression of *IRF3* and *IRF7* was significantly induced in SHK-1 after PRV-1 exposure, while expressed at constitutively high levels in ASK. Previous investigation of IRF involvement in antiviral defense in mammals revealed that *IRF1* may function independently of *IRF3/IRF7* (77). Considering that RBC is the only cell type susceptible to PRV-1, the low activation of *IRF3/7* and strong induction of *IRF1* in RBC could represent a difference associated with antiviral responsiveness to PRV-1.

The entry of PRV into RBC likely occur through endosomal uptake, as its mammalian counterpart MRV (78). This process leads to virion disassembly at late endosomes and release of transcriptionally active viral core particles into the cytoplasm that subsequently produce capped, but not poly- adenylated ssRNA copies (79). Interferon-induced protein with tetratricopeptide repeats 5-like (*IFIT9*, also referred to as IFIT5 in rainbow trout) and ubl carboxyl-terminal hydrolase 18-like (*USP18*) have been implicated in inhibition of VHSV replication and negative regulation of immune responses mediated by type I IFN, respectively (80, 81). Both *IFIT9* and *USP18* were significantly upregulated in RBC, which in correlation with the expression profile of PRRs and IRFs, may be indicative of viral status in the cells. Complementary to this, no induction of typical antiviral genes, such as *Mx*, interferon-stimulated gene 15- like (*UBIL*), *PKR* (referred to as *EIF2aK2*) and viperin-like (*RSAD2*), which have previously been found upregulated in PRV-1 infected RBC *in vivo*, was observed after a 24 h viral stimulation of

RBC (8). In contrast, SHK-1 responded to PRV-1 by inducing the expression of several IFN-inducible genes and their corresponding transcription factors (e.g. *STAT1/2* that regulates *Mx* and *ISG15* transcription) significantly. Typical antiviral response genes highly expressed in SHK-1 but not in RBC, such as *Mx2* and *ISG15-like*, may play a role in the successful eradication of the virus (72, 80, 82, 83). The comparison, however, of RBC to ASK showed that no typical antiviral responses were observed in ASK. Instead, pro-inflammatory cytokines *IL-11* and *CXCL10* were significantly induced. These findings may indicate that ASK cells lack viral uptake and sufficient sensing of viral RNA, whereas SHK-1 cells may take up PRV, respond, but inhibit viral replication more efficiently by strong antiviral responses. The antiviral response in RBC may be delayed compared to the SHK-1 response, which might favor the replication of the virus.

In conclusion, the present transcriptional analysis supports previous characterization of RBC as multifunctional cells with both physiological and immunological properties. In contrast to ASK and SHK-1 cells, RBC showed higher expression levels of genes related to endocytosis and intracellular transport and uniquely expressed *CCL4* and *CCR9* genes, suggesting putative chemotactic activity and an ability to recruit immune cells. Exposure of RBC to PRV-1 for 24 h induced a typical antiviral response of intermediate strength, stronger than in ASK cells, but possibly delayed compared to responses in SHK-1. A difference in IRF gene induction (*IRF1* in RBC, *IRF3/7* in SHK-1 cells) may affect the antiviral response pathway and allow onset of PRV-1 replication in RBC.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI SRA BioProject- PRJNA1028935, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1028935.

Ethics statement

The animal study was approved by Norwegian Animal Research Authority/Norwegian University of Life Sciences Aquatic facility. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TT: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Software. AYMS: Data curation, Formal Analysis, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Software. SB: Formal Analysis, Methodology, Validation, Writing – review & editing. JBJ: Investigation, Supervision, Validation, Visualization, Writing – review & editing. ER: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. ØW: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. MKD: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1359552/full#supplementary-material

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Paper II

Tsoulia, T., Sundaram, A.Y.M., Amundsen, M.M., Rimstad, E., Wessel, Ø., Jørgensen, J.B. & Dahle, M.K.

Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of *Piscine orthoreovirus*

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Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of *Piscine orthoreovirus*

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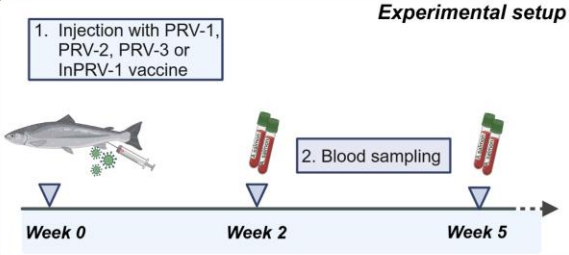
Abstract

Piscine orthoreovirus (PRV) infection is common in aquaculture of salmonids. The three known PRV genotypes (PRV-1-3) have host species specificity and cause different diseases, but all infect and replicate in red blood cells (RBCs) in early infection phase. PRV-1 is the causative agent of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*), PRV-2 causes erythrocytic inclusion body syndrome (EIBS) in coho salmon (*Oncorhynchus kisutch*), while PRV-3 induces HSMI-like disease in farmed rainbow trout (*Oncorhynchus mykiss*). PRV-3 can also infect A. salmon without causing clinical disease and has been shown to cross-protect against PRV-1 infection and HSMI, while PRV-2 or inactivated adjuvanted PRV-1 vaccine only partially reduced HSMI pathologic changes. In the present work, we studied the transcriptional responses in blood cells of A. salmon two- and five-weeks post infection with PRV-1, PRV-2, PRV-3, or post injection with inactivated PRV-1 vaccine. PRV-1 and PRV-3 replicated well in A. salmon blood cells, and both induced the typical innate antiviral responses triggered by dsRNA viruses. Two weeks post infection, PRV-3 triggered stronger antiviral responses than PRV-1, despite their similar viral RNA replication levels, but after five weeks the induced responses were close to equal. PRV-2 and the InPRV-1 vaccine did not trigger the same typical antiviral responses as the replicating PRV-1 and PRV-3 genotypes, but induced genes involved in membrane trafficking and signaling pathways that may regulate physiological functions. These findings propose that the protection mediated by PRV-3 against a secondary infection by PRV-1 occur due to a potent and early activation of the same type of innate immune responses. The difference in the timing of antiviral responses may give PRV-1 an evolutionary edge, facilitating its dissemination to A. salmon heart, a critical step for HSMI development.

Keywords: Atlantic salmon; *Piscine orthoreovirus*; mRNA transcriptome analysis; antiviral response

Comparison of transcriptome responses in blood cells of Atlantic salmon infected by three genotypes of *Piscine orthoreovirus* (PRV)

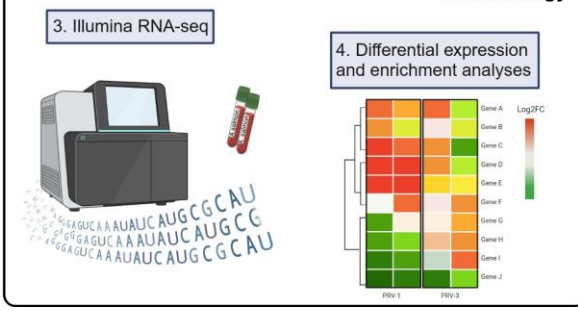
Experimental setup

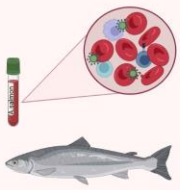


Results

- PRV genotypes induce different transcriptome responses in A. salmon blood cells
- PRV-1 and -3 replicate well in blood cells
- PRV-3 triggered strong antiviral responses at week 2, similar to PRV-1 at later stage of infection
- PRV-1 and -3 induced antiviral immune responses of equal strength by week 5
- Non-replicating PRV-2 and InPRV-1 vaccine induced genes involved in diverse physiological processes, but not antiviral defense

Methodology





The delayed onset of antiviral responses in PRV-1 infected blood cells may provide an evolutionary advantage for viral dissemination to the heart, a critical for HSMI development.

Potent antiviral responses in blood cells at early stages of PRV-3 infection may be linked to its protective effect against PRV-1 and HSMI.

1. Introduction

Unlike mammals, teleost red blood cells (RBCs) are nucleated and possess an active transcriptional/translational machinery essential for gene expression [1, 2]. *Piscine orthoreovirus* (PRV, family *Spinareoviridae*, genus *Orthoreovirus*), a virus with an icosahedral, double-layered capsid, and a segmented double-stranded RNA genome [3, 4], replicates in salmonid RBCs [5, 6]. PRV infection is associated with disorders of the circulatory system and is a significant threat in salmonid aquaculture [7]. There are three genotypes of the virus, PRV-1, PRV-2 and PRV-3, which demonstrate similar systemic dissemination but different pathogenicity in different salmonid species [8]. PRV-1 primarily targets farmed Atlantic salmon (*Salmo salar*) and causes heart and skeletal muscle inflammation [9], a prevalent viral disease in A. salmon aquaculture in Northern Europe [7, 10–12]. PRV-1 establishes a persistent infection, and is ubiquitous in the marine phase of farmed A. salmon [13]. Genetic viral reassortants have different virulence, and PRV-1 is also commonly detected in fish populations without clinical signs of disease [14, 15]. PRV-3 was first detected in Norway in 2013, in farmed rainbow trout (*Oncorhynchus mykiss*) with pathological lesions resembling HSMI [16]. A causative role of PRV-3 in heart inflammation in rainbow trout was experimentally confirmed in 2019 [17]. PRV-1 and PRV-3 genotypes have also been found and associated with jaundice syndrome in Chinook salmon (*Onchorynchus tshawytscha*) in British Columbia and coho salmon (*Oncorhynchus kisutch*) in Chile [18, 19]. PRV-2 is the etiological agent of erythrocytic inclusion body syndrome (EIBS) in Japanese coho salmon aquaculture [20] and has been found in wild coho salmon in Alaska [20, 21].

Previous transcriptional analyses of A. salmon RBCs have revealed expression of a wide repertoire of pattern recognition receptors (PRRs), interferon (IFN) transcription regulators and IFN inducible genes known to confer resistance to viral infections, several of which were strongly activated in response to *ex vivo* and *in vivo* exposure to PRV [2, 22, 23]. The PRV virion, akin to the infection mechanism described in mammalian orthoreovirus (MRV), is internalized into the host cell via receptor- mediated endocytosis. The outer capsid proteins undergo proteolytic degradation, facilitating penetration of viral core particles across the late endosomes [9, 24]. Although the membrane proteins implicated in PRV internalization remain unknown, viral recognition upon entry into host cells has been associated with the endosomal toll like receptor 3 (TLR3) and potentially ATP- dependent RNA helicase DHX58 (also referred to as retinoid acid-inducible (RIG)- like receptor 3) [22, 23]. In the cytoplasm, the viral assembly occur in globular neo- organelles, referred to as viral factories, which provide an environment conducive to viral replication, potentially evading detection by host cell innate immune system [9, 24], [25]. The peak of PRV infection in A. salmon RBCs typically occurs between two to five weeks post exposure, coinciding with high plasma viremia and antiviral responses [9, 22]. Both humoral and cellular responses are elicited, leading to infiltration of immune blood cells into the heart and production of PRV- specific antibodies [26–28].

Despite the significant impact of PRV infection on salmonid aquaculture, the absence of robust monitoring strategies and effective prevention measures remain a pressing concern [7, 29]. Experimental vaccines against HSMI have been developed and tested, including an inactivated whole virus vaccine based on virulent PRV-1 [30], and DNA vaccines encoding PRV non-structural proteins [31]. These vaccines only led to partial protection against HSMI. Recently, PRV-3 infection in A. salmon was shown to efficiently block consecutive PRV-1 infection and HSMI. In comparison, the injection of PRV-2 and inactivated adjuvanted PRV-1 vaccine did not protect from infection, and only partially reduced HSMI pathology. Only PRV-3 triggered PRV-1 specific antibody production [29], as demonstrated using a bead-based immunoassay [27]. Given the importance of developing effective vaccines in salmonid aquaculture against PRV-1 infection, understanding the link between initial responses in infected blood cells and the effective cross protecting potential of PRV-3 are of great interest. In the present study, we report on transcriptional differences and similarities in whole blood of A. salmon infected with PRV-1, PRV-2 and PRV-3 two and five weeks post- injection. We focused on factors that may be involved in the previously reported cross- protection mediated by PRV-3, but not PRV-2, and early responses that may explain why PRV-1 infection leads to a pathological outcome in A. salmon, while PRV-3 does not.

2. Materials and Methods

2.1. Experimental trial and blood sampling

Blood samples from A. salmon infected by either PRV-1, PRV-2 or PRV-3, immunized with an inactivated, adjuvanted PRV vaccine (InPRV-1), and mock controls originated from a previously published experimental trial [29]. Briefly, 300 fish of a mean weight of 41.3 g (+/- 5.8 g) were divided into five experimental groups and kept in freshwater (10 °C, 24:0 light: dark cycle, >90% O2). The experimental fish were injected intraperitoneally (IP) with 0.2 mL of the following materials. The PRV-1 infection material was based on an infected blood pellet (PRV-1 isolate NOR2012-V3621). The isolate had been passaged in previous experimental trials in A. salmon, all resulting in HSMI [9]. The PRV-3 infection material was prepared from a blood pellet harvested during a disease outbreak in 2014 (PRV-3 NOR2014) [16], that had been passaged in rainbow trout leading to HSMI-like pathology [17]. Mock blood lysate was obtained from non-infected A. salmon. Frozen blood pellets from PRV-1, PRV-3 and mock control samples were diluted 1:10 in L15-medium, sonicated, centrifuged, and the supernatant was collected. PRV-2 infection material originated from a frozen spleen sample from coho salmon [20]. The tissue sample was homogenized in L15 medium, sonicated and centrifuged. Inactivated PRV-1 material was prepared from purified PRV-1 particles (PRV-1 NOR2012, $5.35 \cdot 10^9$ copies/ mL) by PHARMAQ AS as described earlier [30]. Briefly, the batch was inactivated by formalin immersion and prepared as a water-in-oil formulation where the water phase (containing PRV antigens) was dispersed into a mineral oil continuous phase containing emulsifiers and stabilizers. Ten weeks post injection with PRV-1-3 infection material or InPRV-1 vaccine, the groups were infected horizontally by addition of PRV-1 infected shedder fish, and the full immunization trial lasted 18 weeks, reporting on the cross-

protective potential of the injected viruses and inactivated vaccine against subsequent PRV-1 infection and HSMI [29]. Additional details on the trial are presented in Malik & Teige, 2021 [29].

Eight fish were sampled prior to injection (week 0), and from each of the five experimental groups (PRV-1-, PRV-2- or PRV-3- infected fish, immunized fish with InPRV-1 vaccine and mock controls) at week 2 and 5 after IP injection. Blood was drawn from the caudal vein of the fish using BD Medical Vacutainer heparin-coated tubes (BD Medical, Mississauga, ON, USA). The samples were stored at 4 °C for a maximum of 6 h, centrifuged (3000 × g for 5 min at 4 °C), and plasma and blood pellets were separated into different microtubes and stored at -80 °C.

In the present study, blood samples from six fish per group sampled at week 2 and four fish per group sampled at week 5 were analyzed. In addition, blood samples from four fish sampled at week 0 were used as additional controls. The sample selection was based on RNA quality, to ensure optimal RNA-seq results.

2.2. RNA isolation and sequencing

Blood cell pellets of 20 µL were resuspended in MagNA Pure LC RNA Isolation Tissue (Roche) to a final volume of 400 µL and homogenized using 5 mm steel beads and TissueLyzer for 3 min at 25 Hz. MagNA Pure 96 Cellular RNA Large Volume Kit (Roche) was used for automated total RNA isolation following the manufacturer's protocol. RNA was quantified using Multiskan SkyHigh microplate spectrophotometer (Thermo Fiscer Scientific). RNA quality (RIN >8) was ensured using Agilent 2100 Bioanalyser (Agilent, USA) before being sent for sequencing.

Total RNA from 30 samples harvested week 2 (Mock control, n= 6; PRV-1 infected, n= 6, PRV-2 infected, n= 6; PRV-3 infected, n= 6; Inactivated PRV-1, n= 6), 20 samples harvested week 5 (Mock control, n= 4; PRV-1 infected, n= 4, PRV-2 infected, n= 4; PRV-3 infected, n= 4; Inactivated PRV-1, n= 4), and 4 samples from week 0, were sent to the Norwegian Sequencing Centre (NSC, Norway). Library preparation was performed using strand-specific TruSeq mRNA-seq Library prep kit (Illumina, CA, USA). The libraries were pooled and sequenced on one lane of Illumina NovaSeq S4 flow cell to obtain 150bp paired end reads. The raw sequencing data are available in NCBI SRA BioProject - PRJNA1148351.

2.3. RT-qPCR for PRV variants

RNA loads of PRV-1 and PRV-3 were assessed using Qiagen One-Step RT-qPCR kit (Qiagen). The input was standardized to 50 ng (10 µL of 5 ng/ µL) of total RNA per reaction and the samples were run in duplicates. Prior to RT-qPCR, the template was denatured at 95 °C for 5 min. The RT-qPCR reactions were performed under the following thermal conditions: 50 °C for 30 min, 94 °C for 15 min, and 45

cycles for 30 s at 95 °C and 1 min at 60 °C. To define a sample as positive, a cutoff of Ct < 35 was set. For PRV-2, a Quantitect SYBR Green RT-qPCR kit (Qiagen) was used according to manufacturer's instructions. A total of 50 ng RNA (5 µL of 10 ng/ µL) was denatured at 95 °C for 5 min and the samples were run in duplicates with the following thermal conditions: 50 °C for 30 min, 94 °C for 15 min, and 40 cycles for 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. To assess the specificity of the assay, melting curve analysis was performed. A cutoff of Ct < 35 was set, similar for PRV-1 and PRV-3. Probes and primer sequences are given in Supplementary File A, Table A1 [29].

2.4 Bioinformatic processing and statistical analysis

Raw sequence data (Fastq files) were processed to trim/remove adapter and low quality sequences using BBDuk tool in BBMap v.38.18 suite (parameters: ktrim=r, k=23, mink=11, hdist=1, tbo, tpe, qtrim=r, trimq=15, maq=15, minlen=36, forcetrimright=149) [32]. Cleaned reads were mapped to *Salmo salar* genome (ENSEMBL ICSASG_v2) using the HISAT2 v.2.2.1 (parameters: –rna-strandness RF) [33]. FeatureCounts v.1.4.6-p1 (parameters: -p -s 2) was used for estimating the number of reads and aligning against the reference genes in ENSEMBL r104 GTF annotation [34]. Initial raw data analysis was performed using SARTools v.1.7.4 and R v.4.1.1 [35, 36]. Normalization and differential expression between groups and against the control at week 2 and 5 were performed using DESeq2 v.1.34.0 [37]. The annotation tables were cleaned using median count reads > 10 as a cut off, to omit genes with zero or low counts. Adjusted p-value (padj) was calculated using Benjamin-Hochberg (BH) correction and gene with padj below 0.05 were considered as differentially expressed genes (DEGs). For gene regulation, upregulated features with less than 2-fold change and downregulated features with higher than 0.5- fold change in expression (0.5 < fold change < 2) were filtered out.

STRING Database v.12.0 was used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with 0.05 as p-value cutoff, BH adjusted [38]. In particular, DEGs were sorted into functional categories via KEGG pathway and gene ontology GO enrichment analyses. Potential functions of uncategorized genes were explored within databases primarily focused on mammalian genome and gene function, such as Reactome and InterPro, and were also considered. The magnitude of transcriptional similarities/ differences of each PRV genotype to PRV-1 was determined using heatmaps. To better understand how PRV immunization/injection affected gene regulation over time, heatmaps were constructed using Log2-fold changes (Log2FC) of a selected set of DEGs, compared to week zero (transcripts) as baseline reference.

3. Results

3.1. Overview of PRV immunization trial data

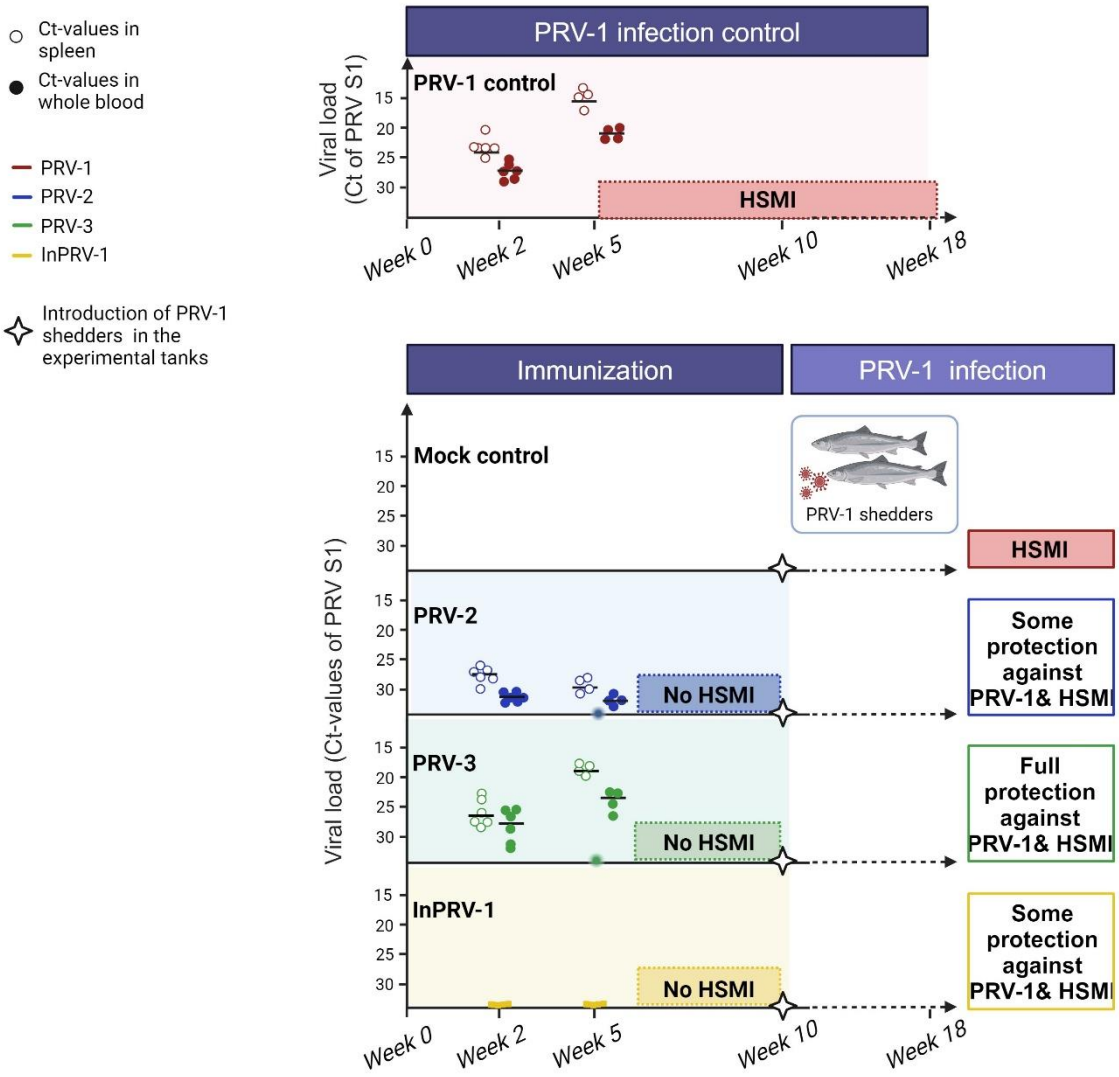


Figure 1. Experimental setup and results of the original Piscine orthoreovirus (PRV) infection trial [29]. Fish were allocated into five experimental groups injected intraperitoneally (IP) with blood lysate containing PRV-1 or PRV-3, spleen homogenate containing PRV-2, purified, inactivated and adjuvanted PRV-1 (InPRV-1 vaccine control) and blood lysate originating from uninfected healthy fish (Uninfected control). RNA loads of PRV-1-3 in spleen (open dots) and whole blood (filled dots) were measured two- and five-weeks post injection using RT-qPCR assays targeting virus-specific parts of the S1 genome segment. Virus levels are presented as Ct-values for each individual and as average (n= 6/group in week two and n= 4/group in week five). PRV-1 levels colored red; PRV-2 levels colored blue; PRV-3 levels colored green; InPRV-1 colored yellow. A PRV-1 cohabitation challenge took place 10 weeks post immunization, in the PRV-2-, PRV-3-infected groups and InPRV-1 vaccinated controls, along with the uninfected control group. The infection outcome and cross- protection conferred by the three PRV genotypes and InPRV-1 vaccine was assessed through histopathological analysis of HSMI in week 15 and 18, as mean of 8 individuals (score 0- no HSMI, score 3- full HSMI) by Malik and Teige [29].

The transcriptional analysis performed in this study further investigates key observations from a previous published work by Malik & Teige et al., 2021 [29]. Here, we measured the RNA load of PRV genotypes in whole blood of A. salmon two and five weeks post injection, to explore potential correlation between transcriptional responses and viral replication status. These results are shown in Figure 1, together with an overview of the original experimental setup and key findings reported by Malik & Teige et al., 2021 [29]. The trial consisted of two distinct parts; fish immunization (week 0-10) and secondary PRV-1 infection by cohabitation challenge (week 10- 18). RNA loads of PRV genotypes in spleen were similar in week 2 (~Ct 25), but diverged in week 5, where PRV-1 and PRV-3 levels increased, while PRV-2 levels decreased over time [29]. In whole blood, RNA loads of PRV-1 and PRV-3 showed the same increasing pattern over time as in spleen, whereas PRV-2 levels were lower both week two and five. These findings supported the ability of PRV-2 and PRV-3 to infect A. salmon when injected IP. However, in contrast to the original analyses in spleen, only PRV-1 and PRV-3 could be confirmed to replicate in whole blood.

The protection against secondary PRV-1 cohabitation challenge (week 10), and HSMI was shown by histopathological analysis of heart tissue at week fifteen and eighteen [29]. Infection by PRV-3 efficiently blocked secondary PRV-1 infection and HSMI (no individual developed pathology, HSMI mean score =0), while PRV-2 and InPRV-1 injection only partially protected against HSMI (PRV-2: 6 out of 8 fish, HSMI mean score =2, and InPRV-1: 2 out of 8 individuals, HSMI mean score =0,5) [29].

3.2. Transcriptional analysis of infected and uninfected Atlantic salmon whole blood

In the RNA-Seq data obtained from blood, the reads were mapped to a total of 55,819 features (genes) in the A. salmon genome (ENSEMBL ICSASG_v2/ ENSEMBL r104 annotation). Information on total sequenced reads and alignment rate of mapping of the biological groups in week zero, two and five is provided in Supplementary File B, Table A1 and A2. Most of the samples showed overall alignment rate > 75%, to the A. salmon genome.

Principal component analysis (PCA) was performed to assess the variability of samples from infected groups and uninfected controls at two and five weeks post injection (Supplementary File B, Figure A1 and A2). All biological groups showed wide dispersion at week two, but blood replicates from PRV-1 and PRV-2- infected fish tended to cluster in closer proximity. PCA at week five showed lower variability within the biological replicates of each infected group, while distribution of the clusters against the first principal component indicated that PRV-1 and PRV-3, along with PRV-2 and InPRV-1 are more closely related at week 5. Considering the increasing viral load of PRV-1 and PRV-3 from week 2 to 5, not found for PRV-2 and InPRV-1 in whole blood (Figure 1), PCA clustering may be in line with the replication status of each PRV genotype.

Two uninfected controls from week two were identified as outliers in the PCA plot. Three out of four controls from week five showed an overall alignment rate below 50%. Therefore, these samples were omitted from further analysis.

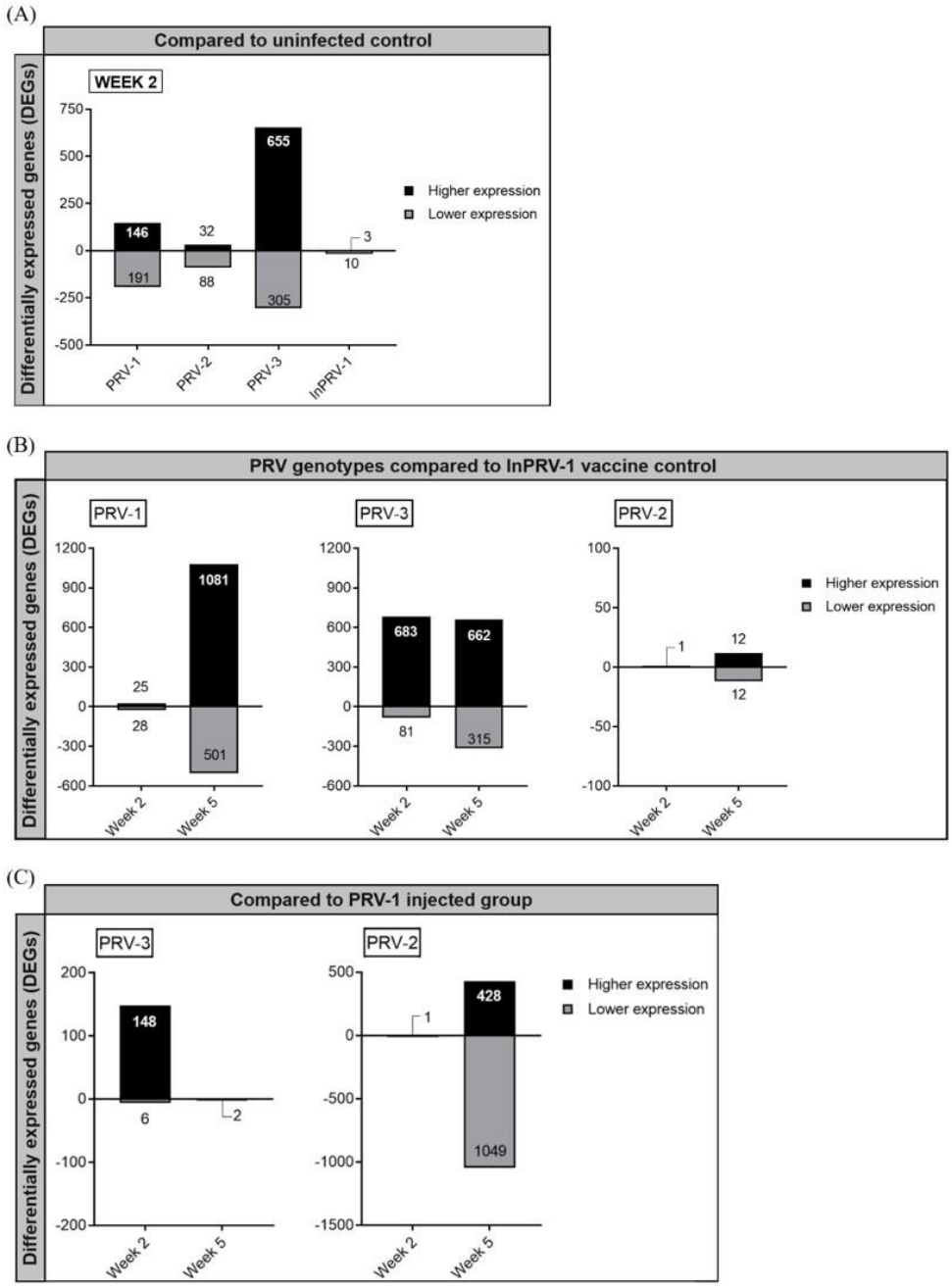


Figure 2. Number of significantly regulated genes (DEGs) in whole blood of A. salmon infected by PRV genotypes and the inactivated adjuvanted PRV-1 (InPRV-1) vaccinated control. (A) Number of DEGs in whole blood of A. salmon two weeks after infection, compared to uninfected controls. Cutoff ≥ 2- fold (higher) and ≤ 0.5- fold (lower), and a cutoff of normalized median read counts ≥ 10 were applied. (B) Number of DEGs in whole blood of A. salmon two and five weeks after infection compared to vaccinated with vaccinated controls. Cutoff ≥ 2- fold (higher) and ≤ 0.5- fold (lower), and a cutoff of normalized median read counts ≥ 10 were applied. (C) Number of DEGs in whole blood of A. salmon two and five weeks after infection compared to PRV-1 infected fish. Cutoff ≥ 2- fold (higher) and ≤ 0.5- fold (lower), and a cutoff of normalized median read counts ≥ 10 were applied.

3.3. Differentially expressed genes in whole blood of PRV- infected Atlantic salmon

Differential gene expression analysis was performed to assess differences in gene expression patterns between immunization groups (PRV-1, 2, 3 and InPRV-1) and compared to uninfected controls of whole blood of A. salmon at week two (Figure 2A). Whole blood of PRV-3 infected fish showed the greatest transcriptional differences compared to uninfected controls (655 genes with higher expression and 305 with lower expression level). PRV-1 triggered intermediate transcriptional differences, with more genes showing lower (191 genes) than higher (146 genes) expression compared to controls. Immunization with PRV-2 resulted in the fewest transcriptional differences out of the three PRV genotypes (88 higher and 32 lower expressed genes). For PRV-1 vaccine group, there were almost no expression differences compared to the uninfected controls. No such comparison was performed between infected and uninfected groups at week five, as week five control datasets were excluded.

To identify shared and/or unique expression patterns, we compared the whole blood transcriptional responses of all PRV- infected groups to each other both for week two and five (Figure 2B-C). Although PRV-1 viral load in whole blood was higher than PRV-2 loads, indicating more efficient replication of PRV-1 in blood cells, comparing PRV-1 and PRV-2 induced gene expression did not reveal any significant expression differences after two weeks. This is consistent with the PCA plot, where data from PRV-1- and PRV-2- injected individuals clustered together (Supplementary File B, Figure A1). A comparison between PRV-1 and PRV-3 revealed 148 host genes exhibiting higher expression in PRV-3- infected blood, as opposed to only 6 genes expressed higher in PRV-1- infected blood. This, together with the higher numbers of DEGs upregulated for the PRV-3 infected group versus controls, may indicate a stronger and faster response to PRV-3 than PRV-1. Transcriptional differences between PRV-1 and vaccinated controls were only few at week two. By week five, PRV-1 infected blood showed distinct transcriptional differences compared to the PRV-2 injected group and vaccinated controls, with approximately 1000 genes higher expressed and 500 genes lower expressed in PRV-1 infected blood (Figure 2C). It is worth noting that no differentially expressed genes were detected when comparing vaccinated with PRV-2 infected fish at week two, and only a total of 24 genes differed at week five Figure 2B). A comparison between PRV-3 and PRV-2 revealed that at week two, 449 genes had significantly higher expression in PRV-3- infected blood, whereas only 19 genes showed lower expression (higher expression in PRV-2- infected). By week five, 509 genes were identified with higher expression in PRV-3- infected blood, compared to 259 genes with higher expression for PRV-2 (Supplementary File F, Figure A1). A similar relationship was found between PRV-3 and vaccinated controls at both week two and five (Figure 2B), based on gene numbers.

3.4. Categorization of differentially expressed genes (DEGs) into functional groups and heatmaps for targeted differential expression analysis

DEGs in whole blood of infected A. salmon compared to uninfected controls at week two were categorized into functional groups using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology (GO) enrichment analyses for biological process (GO:BP) and molecular function (GO:MF) (Supplementary File C, Table A1-3). Transcriptional analysis of whole blood from vaccinated fish (compared to uninfected control) revealed a total of 13 DEGs (Figure 2A). Thus, KEGG and GO enrichment analyses were not applicable. The categorization of the DEGs between PRV-3 vs PRV-2 and PRV3 vs InPRV-1 are provided in Supplementary File F (Figure A2-3). It is worth noting that several genes identified in A. salmon whole blood were not categorized into any functional group (uncategorized genes).

Transcriptional responses in whole blood of PRV-1- injected fish were compared to PRV-2, -3 and InPRV-1 at weeks two and five. Functional groups and heatmaps of each comparison with PRV-1 are provided separately in Figures 3, 6 and 8.

3.4.1. Comparison of whole blood transcriptional responses to PRV-1 versus PRV-3 over time

Enrichment analysis for DEGs with higher expression in PRV-1-infected blood cells compared to PRV-3, revealed only three functional groups related to innate and adaptive immune responses (Figure 3A) At two weeks, PRV-1 infection resulted in the induction of 31 genes encoding proteins with transcription regulatory activity, while only 9 genes involved in immune system processes (Supplementary File C, Table A1). In comparison, PRV-3 infection led to induction of 147 genes involved in immune system processes and 99 genes associated with responses to intracellular and/or external stimuli (Supplementary File C, Table A2). Both PRV-1 and PRV-3 infection suppressed genes involved in apoptosis, transmembrane transporter and transcription regulator activity. PRV-1 suppressed 12 genes involved in the MAPK signaling pathway (Supplementary File C, Table A3).

PRV-1 and PRV-3 replicated at a similar level in A. salmon blood, but diverged in the ability to cause HSMI pathology. The comparison of whole blood gene expression in response to PRV-1 versus PRV-3 revealed a total of 154 DEGs at week two and only 2 at week five (Figure 2C). Focusing on week two, 6 genes exhibited higher expression level in response to PRV-1 and 148 genes in response to PRV-3 (Figure 2C). Indicatively, genes such as proteasome 26S subunit ATPase 3 (*PSMC3*) interacting protein (*PSMC3IP*) involved in meiotic recombination, and cell surface protein tetraspanin 8-like (*TSPAN8*), showed higher expression levels in PRV-1 only. In contrast, genes with higher expression level in PRV-3 were primarily associated with innate and adaptive immune processes (Figure 3D). The expression pattern of these genes in response to PRV-1 became equivalent to PRV-3 by week five. A few genes, such as IQ motif containing GTPase activating protein 2 (*IQGAP2*), urokinase plasminogen receptor (*uPAR*) and lysosomal protease cathepsin B (*CATB*), were slightly inhibited in response to PRV-1 at week two. However, their expression levels exceeded those induced by PRV-3 by week five (Figure 3B-C, E).

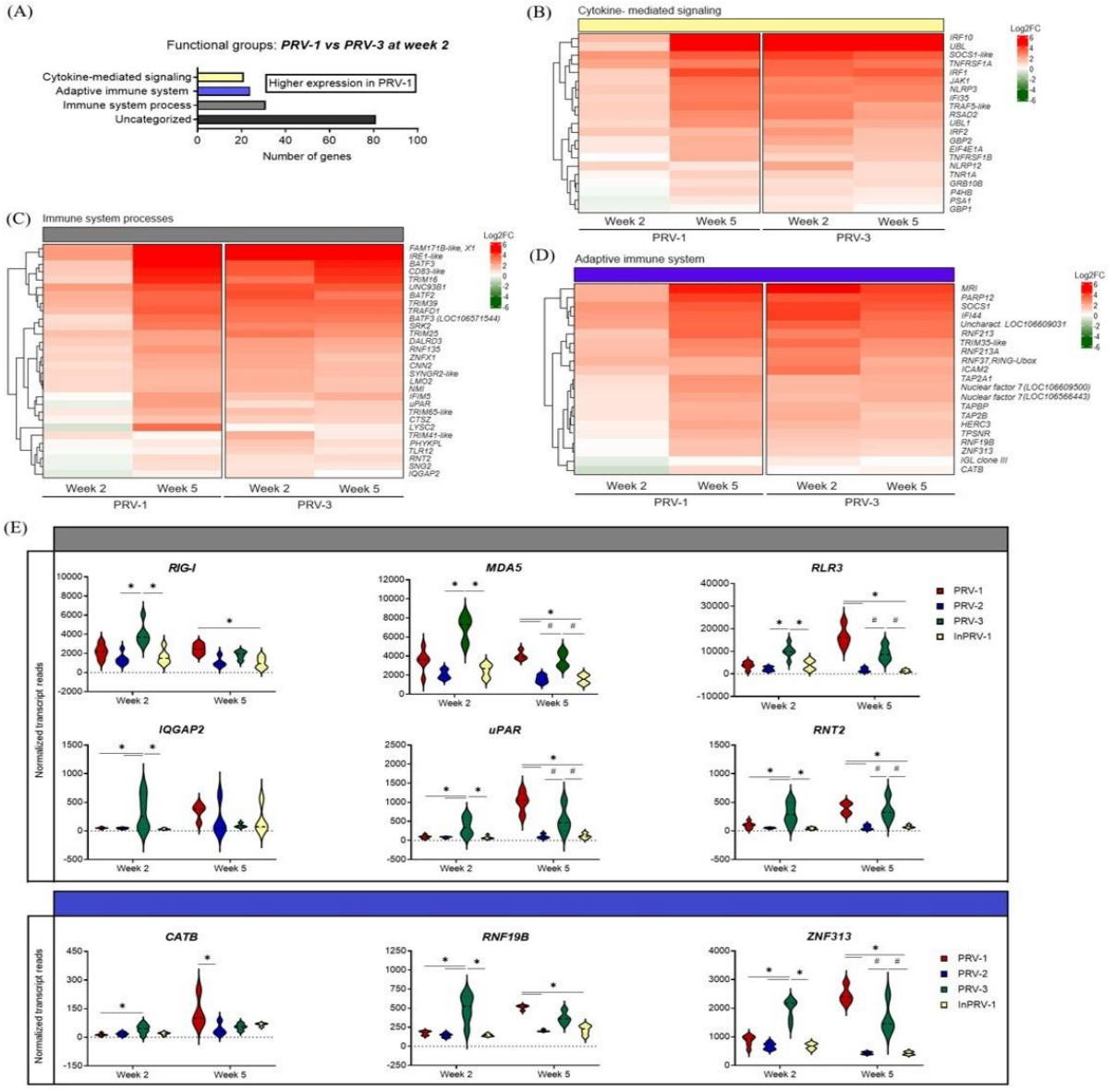


Figure 3. Gene expression profile in whole blood of A. salmon injected with PRV-1 and PRV-3. DEGs with fold- change > 2 (higher expression induced by PRV-1) and < 0.5 (lower expression induced by PRV-1) were included in the analysis. Log2-fold change of the selected DEGs compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five compared to week zero; Green: Lower expression level at week two/five compared to week zero; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs between PRV-1 and PRV3 at week two. (B) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in immune system processes over time, compared to uninfected fish at week zero. (C) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in adaptive immune system, compared to uninfected fish at week zero. (D) Gene expression pattern of DEGs between PRV-1 and PRV-3, involved in cytokine mediated signaling, compared to uninfected fish at week zero. The colored band at the top of each heatmap corresponds to the functional group shown in A. (E) Expression levels of selected genes involved in "Immune system process" and "Adaptive immune system" groups as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes or vaccinated. *: $p \leq 0.01$.

Some uncategorized genes were strongly induced by PRV-3 at week two, including, interferon-induced protein with tetratricopeptide repeats 9 (IFIT9) and galectin 9 (LEG9) (Figure 4). These genes are also

involved in activation of innate immunity and antiviral defense and have previously been found induced in A. salmon erythrocytes in response to PRV-1 *in vivo* at later stages of infection [22]. Genes such as protein phosphatase Mg^{2+}/Mn^{2+} dependent (PPM) 1H (*PPM1H*) and *PPM1F*, as well as RNA binding motif protein 38 (*RBM38*), potentially associated with cell survival and viral genome replication [39, 40], also exhibited high differential expression in PRV-3 infected relative to control blood at week two (Figure 3 and 4, respectively). DEGs of all identified functional groups and uncategorized DEGs in PRV-1 and PRV-3 at week two are provided in Supplementary File E.

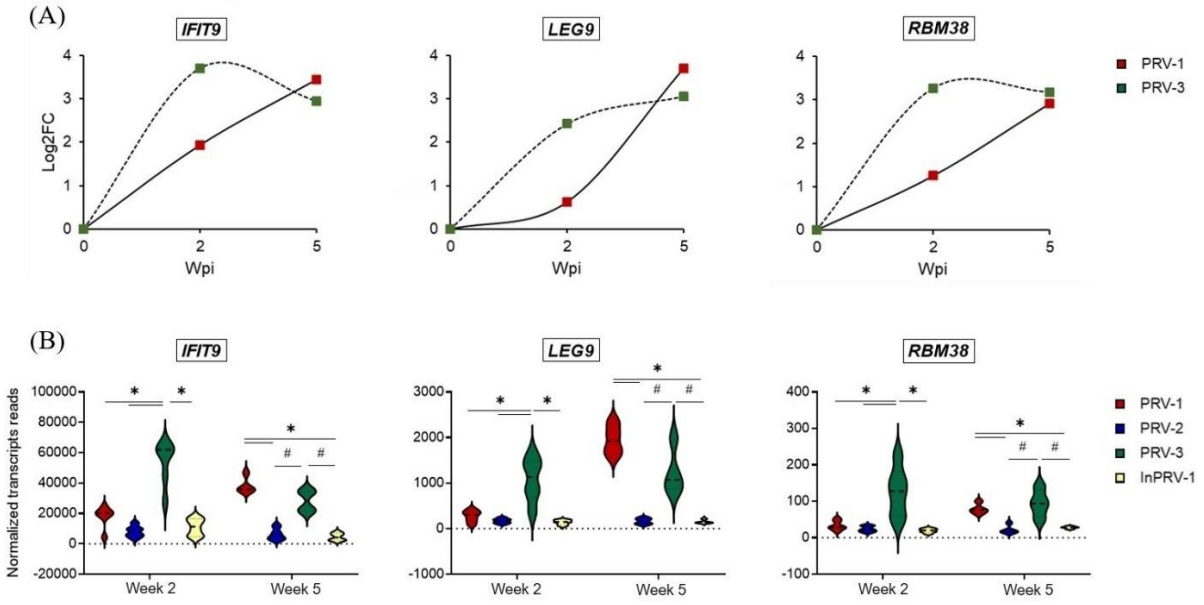


Figure 4. Examples of uncategorized genes with higher relative expression in PRV-3 compared to PRV-1 infected blood at two and five weeks post infection. (A) DEGs with higher relative expression induced by PRV-3 than by PRV-1. Log2FC of DEGs between PRV1 and PRV-3 was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of the same genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes or vaccinated. *: $p \leq 0.01$.

At week five, the two genes with higher expression induced by PRV-1 compared to PRV-3 encode barrier-to-autointegration factor (BANF) b and BANF-like DNA-binding protein (Figure 5A). In mammals, these genes take part in various biological processes, such as transcription regulation, DNA damage response and innate immunity against viruses [41]. Infection with PRV-1 triggered the strongest transcriptional response of BANFB and BANF-like compared to other PRV genotypes and vaccinated controls (Figure 5B).

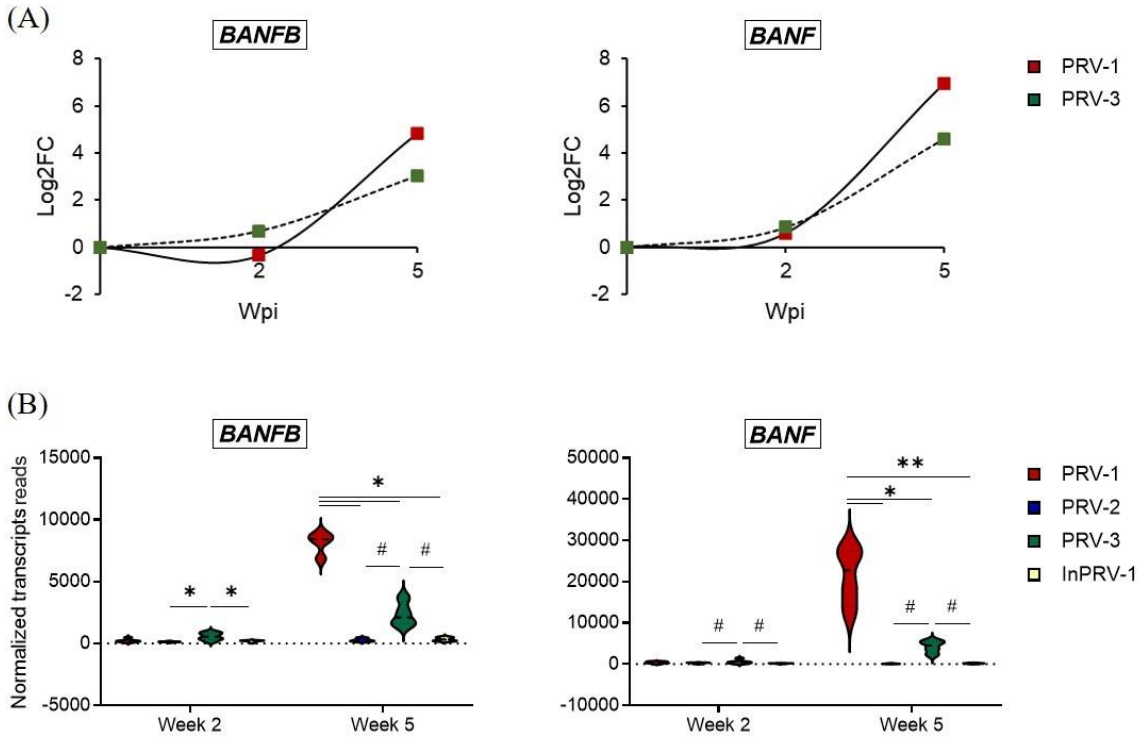


Figure 5. Expression profile of barrier-to-autointegration factor (BANF) b and BANF-like DNA-binding protein genes overtime. BANFB and BANF were the only two genes with significantly higher expression in PRV-1 infected blood relative to PRV-3 at week five. (A) Log2FC of BANFB and BANF between PRV1 and PRV-3 relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of BANFB and BANF genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and inactivated PRV-1 vaccine. *: $p \leq 0.01$.

3.4.2. Comparison of whole blood transcriptional responses to PRV-1 compared to PRV-2 over time

Whole blood from PRV-2 infected fish showed 88 genes with lower expression level compared to uninfected controls, but only 3 categories were generated from the GO and KEGG analysis. These genes belonged to functions related to MAPK signal transduction, protein folding and apoptosis (Supplementary File C, Table A3).

PRV-2 did not exhibit the same level of replication in whole blood of A. salmon compared to PRV-1 and PRV-3, but showed similar host gene expression profile to PRV-1 at week two. DEGs between PRV-1 and PRV-2 were explored to identify gene expression associated with virus replication. Only the phospholipase *DDHD1*-like (*DDHD1A*) gene exhibited 2-fold higher expression in PRV-2- relative to PRV-1- infected blood (raw data file- BioProject PRJNA1148351).

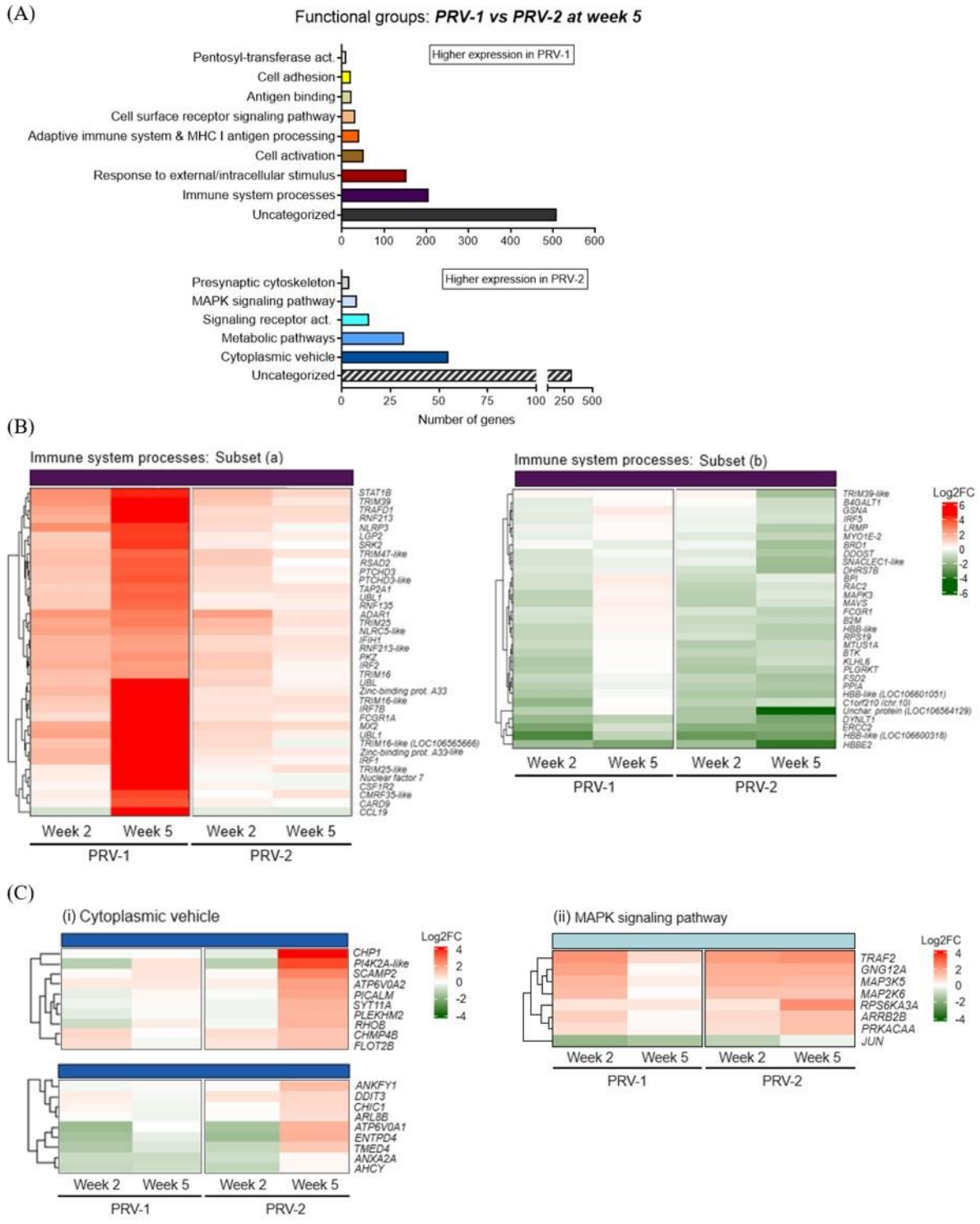


Figure 6. Gene expression profile in whole blood of A. salmon injected with PRV-1 and PRV-2. DEGs with fold- change > 2 (higher expression induced by PRV-1) and < 0.5 (lower expression induced by PRV-1) were included in the analysis. Log2-fold change of the selected DEGs were compared to uninfected controls at week zero. Red: Higher expression level at week two and/or five; Green: Lower expression level at week two/five; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs with higher and lower expression in PRV-1 (top and bottom, respectively) compared to PRV-2 at week five. (B) Transcriptional profile of DEGs between PRV-1 and PRV-2, involved in immune system processes (left) and identified functional groups (right) compared to uninfected fish at week zero. The colored panel to the left of each heatmap corresponds to a functional group from

Fig.6A (top). (C) Gene expression pattern of selected functional groups "(i) Cytoplasmic vehicle" and "(ii) MAPK signaling pathway". The colored panel at the top of each heatmap corresponds to a functional group from A (bottom).

Although gene expression in whole blood of A. salmon in response to PRV-1 and PRV-2 was equivalent at week two, many genes were differentially expressed between the two genotypes by week five (Figure 2C). Setting aside the group of uncategorized genes, the majority of DEGs with higher expression in PRV-1 was involved in immune system processes and signaling pathways activated in response to various stimuli (Figure 6A, top). Transcriptional effectors typically involved in regulation of innate immune gene responses, such as signal transducer and activator of transcription 1B (*STAT1B*) and TRAF-type zinc finger domain-containing protein 1 (*TRAFD1*) genes, together with genes related to antiviral defense, such as interferon regulatory factor (*IRF*) 1, *IRF7* and myxovirus resistance protein 2 (*MX2*), exhibited the highest expression levels in PRV-1- infected blood at week five. In comparison, no alterations in the expression profile of the same genes were observed in response to PRV-2 over time (Figure 6B). Some genes associated with signal transduction and immune defense, including kinases (e.g. mitogen- activated protein kinase 3 (*MAPK3*)), small GTPases (e.g. Ras-related C3 botulinum toxin substrate 2 (*RAC2*)) and intermediary adapters (e.g. mitochondrial antiviral signaling protein (MAVS)), were expressed lower two weeks after both PRV-1 and PRV-2 exposure (Figure 6B, subset b). However, by week five, they were slightly higher expressed in response to PRV-1, while showing even lower expression in response to PRV-2, compared to uninfected controls from week zero (Figure 6B, subset b).

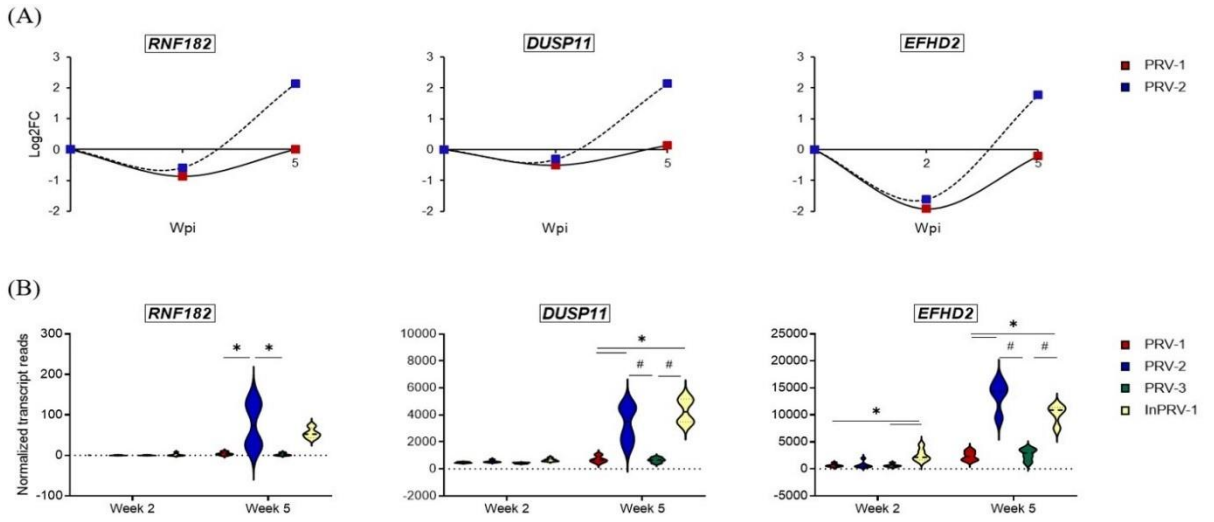


Figure 7. Examples of uncategorized genes with higher relative expression in PRV-2 compared to PRV-1 infected blood at weeks two and five. (A) DEGs with higher relative expression induced by PRV-2. Log2FC of DEGs was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and the inactivated PRV-1 vaccine. *: $p \leq 0.01$.

A total of 428 genes exhibited higher expression levels in response to PRV-2 at week five compared to PRV-1 (Figure 2C). Gene ontology and KEGG pathway enrichment analyses revealed five main

functional groups, as shown in the lower panel of Figure 6A. The genes were implicated in intracellular trafficking, potentially associated with protein folding and degradation through vehicles, as well as in metabolic processes and signal transduction involving the activation of nuclear receptor subfamily members and MAP kinases.

Although many genes within the A. salmon genome were not grouped into specific cellular functions (Figure 6A, bottom, "Uncategorized genes"), their counterparts in mammalian cells have been studied. For instance, genes encoding regulatory proteins, such as ring-finger protein 182 (*RNF182*) and dual specificity phosphate 11 (*DUSP11*), along with regulators of programmed cell death, such as calcium binding adaptor protein EF Hand domain family member D2 (*EFHD2*) were among genes with the highest differential expression in response to PRV-2 relative to control, in contrast to their significantly lower differential expression in response to PRV-1 at week five (Figure 7). DEGs linked to specific functional groups and uncategorized DEGs induced by PRV-1 and PRV-2 at weeks two and five are provided in Supplementary File E.

3.4.3. Comparison of whole blood transcriptional responses to PRV-1 and InPRV-1 vaccine

The gene expression profile in whole blood of A. salmon infected by PRV-1 and vaccinated with InPRV-1 had only 58 DEGs at week two (Figure 2B), despite the total inactivation and adjuvant added to the vaccine, and the high level of replication for PRV-1. By week five, the groups exhibited significant divergence, with 1583 genes differentially regulated (Figure 2B). To elucidate the differences in transcriptional responses to InPRV-1 and PRV-1 over time, we first generated a heatmap for the 58 DEGs at week two (25 genes - higher expression induced by PRV-1; 28 genes - higher expression induced by InPRV-1) (Supplementary file D, Figure A1). Genes involved in regulation of immune functions (e.g. *IRF1*, *TRAFD1*, *BATF3* and *IFI44*) and host genome replication (e.g. MCM2, -3, and -6) showed higher expression levels in the PRV-1 infected group at week two, which further increased by week five. This is consistent with PRV-1 being an actively infecting and replicating virus, distinguishing it from the inactive InPRV-1.

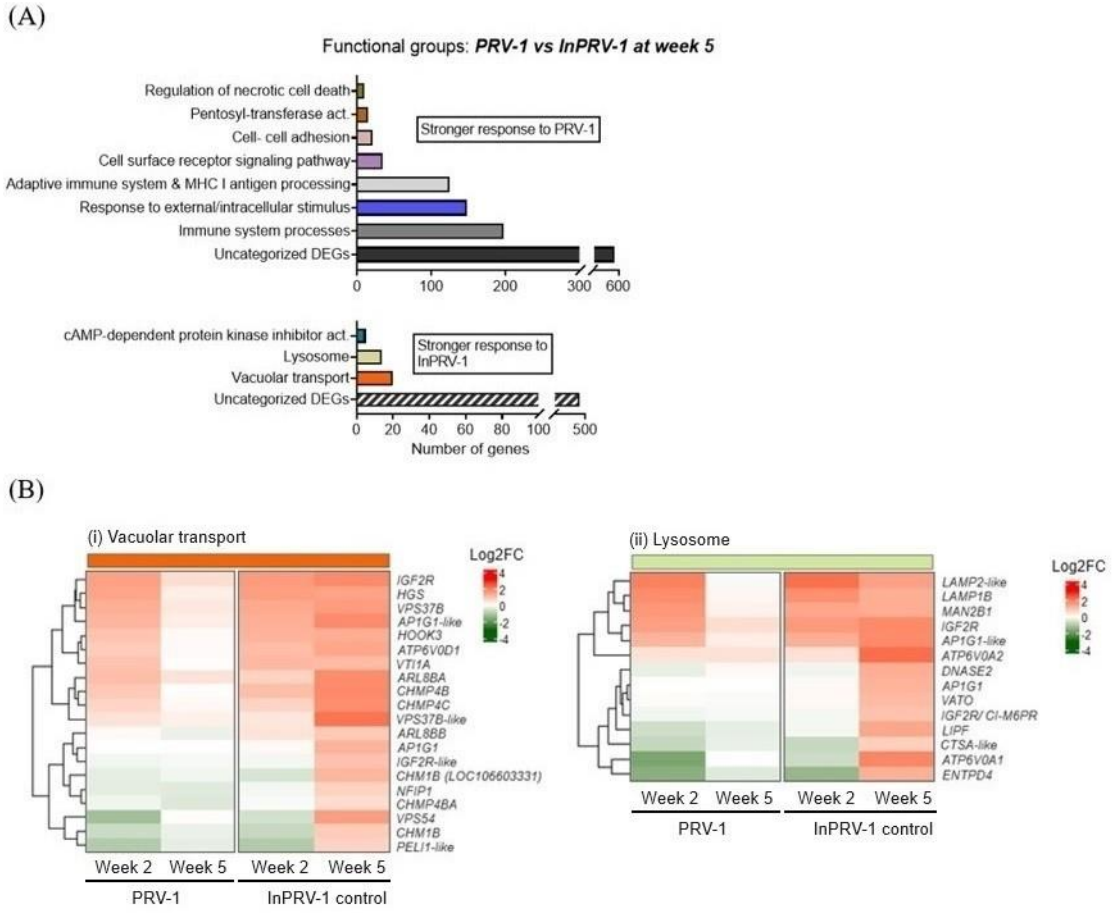


Figure 8. Gene expression profile in whole blood of A. salmon injected with PRV-1 and InPRV-1 vaccine. DEGs with fold-change > 2 (higher expression in PRV-1) and < 0.5 fold- change (lower expression in PRV-1) were included in the analysis. Log2-fold change of the selected DEGs compared to uninfected controls at week zero. Red: Higher expression level at week two/five; Green: Lower expression level at week two/five; White: No expression difference between week two/five and week zero. The darker the color, the stronger the regulation (higher or lower). (A) Functional groups of DEGs with higher and lower expression in PRV-1 (top and bottom, respectively) compared to InPRV-1 at week five. (B) Transcriptional regulation of DEGs induced by PRV-1 compared to InPRV-1 within the identified functional groups (i) Vacuolar transport and (ii) Lysosome.

In general, most genes involved in innate and adaptive immunity exhibited a similar expression pattern in whole blood of A. salmon injected with PRV-1 - and InPRV-1 after two weeks. Only a few regulatory transcription factors involved in immune responses showed higher expression level in response to PRV-1 at the early infection stage. However, by week five, PRV-1 infection strongly induced genes related to antiviral defense, cytokine production and MHC I antigen processing, responses not further induced, and for some genes, even suppressed by InPRV-1. The responses are as expected due to increasing PRV-1 RNA levels in whole blood over time. Genes involved in diverse biological functions, such as responses to stimuli, cell- cell adhesion, pentosyltransferase activity and necrotic cell death, were also induced only in response to replicating PRV-1 (Supplementary File D, Figure A1). Only a few genes with higher expression in InPRV-1 were identified with specific biological functions using GO and KEGG pathway enrichment analyses (Figure 8A). Examples were genes encoding proteins located in

lysosomes, and proteins that regulate intracellular transport through vacuoles (Figure 8B). For instance, vacuolar protein sorting-associated proteins (*VPSs*) and ATPase H+ transporting V0 subunit A1 (*ATP6V0A1*) genes, were highly expressed only in response to InPRV-1 at week five. DEGs linked to specific functional groups and uncategorized DEGs for the PRV-1 and InPRV-1 comparison at weeks two and five is provided in Supplementary File E.

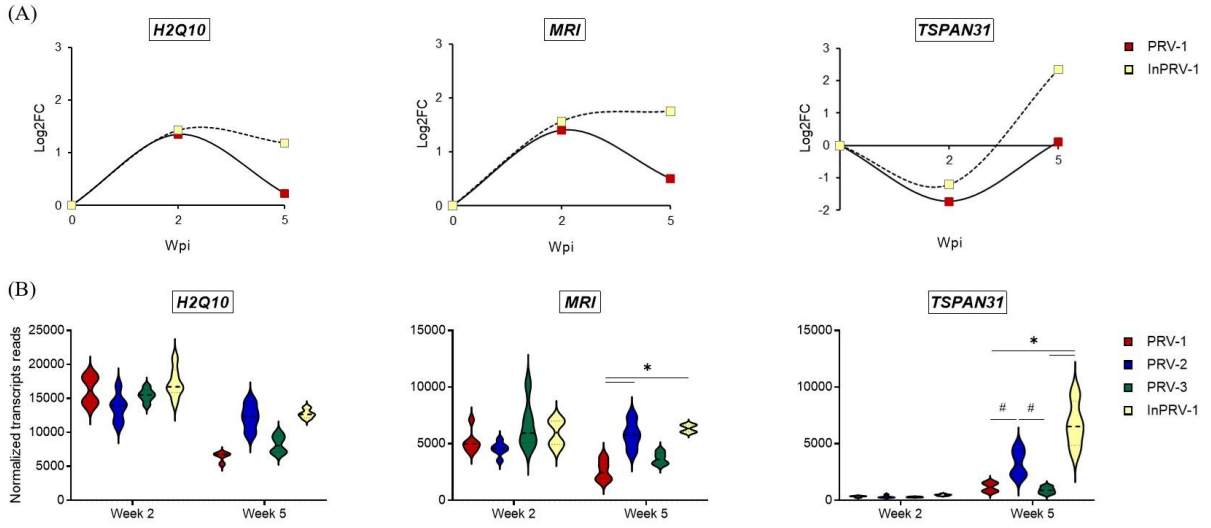


Figure 9. Examples of genes with higher relative expression in vaccinated controls (InPRV-1) compared to PRV-1 at weeks two and five. (A) DEGs with higher relative expression in vaccinated controls compared to PRV-1 infected fish. Log2FC of DEGs for PRV1 and vaccinated controls was calculated relative to controls from week zero. Wpi: Weeks post infection. (B) Expression levels of genes as normalized transcript reads in whole blood of A. salmon infected with PRV genotypes and the inactivated PRV-1 vaccine. *: $p \leq 0.01$.

Interestingly, some genes related to MHC class I antigen processing and adaptive immunity, such as histocompatibility 2 Q region locus 10 (*H2-Q10*), MHC class I-related gene protein-like (*MR1*) and *TSPAN31* showed higher transcription levels in the blood of vaccinated and PRV-2 infected fish compared to replicating PRV-1 and PRV-3 five weeks post infection. In particular, these genes exhibited >2-fold higher expression to InPRV-1 vaccine relative to uninfected controls at week five (Figure 9). A complete overview of DEGs linked to specific functional groups and uncategorized DEGs in response to PRV-1 and vaccinated controls at weeks two and five is provided in Supplementary File E.

4. Discussion

The outcome of the infections with the three known PRV genotypes are different in A. salmon. They differ in the efficacy of replication in blood (PRV-1 > PRV-3 > PRV-2), induction of heart pathology (PRV-1 only), and their potential to cross protect against secondary infection (PRV-3 > InPRV-1 vaccine > PRV-2) [29]. In this study, we explored whether gene expression differences in blood cells in response to the three PRV genotypes and an inactivated vaccine could provide more information on mechanisms of replication, pathogenesis and cross-protection.

Transcriptional responses in blood cells potentially linked to PRV replication

The three PRV genotypes showed relatively similar RNA loads in whole blood of A. salmon two weeks post injection of the virus, with somewhat higher levels of the pathogenic PRV-1 and the non-pathogenic PRV-3, compared to PRV-2 [29]. Three weeks later (week five) PRV-1 and PRV-3 continued to replicate in blood, PRV-1 to somewhat higher levels than PRV-3, whereas PRV-2 RNA levels were reduced in blood, indicating no further replication. Based on this replication pattern one could expect to see a similar cellular response to PRV-1 and PRV-3 and a divergent response to PRV-2. Interestingly, this was not the case after two weeks. While PRV-3 triggered a strong transcriptional response after two weeks relative to uninfected and vaccinated controls, blood cells showed a much lower response to both PRV-1 and PRV-2. After five weeks, PRV-1 had replicated to the highest level in blood, and as expected regulated the highest number of host genes relative to vaccinated controls. PRV-3 had also replicated to higher levels between two and five weeks, and the transcriptional responses to the virus remained relatively similar to responses at week two. Responses to PRV-2 were still low, in line with the lack of further replication.

PRV-3 induced the strongest innate antiviral responses at week two, with the transcriptional levels of most genes remaining relatively stable until week five, while the virus continued to replicate. This indicates that the innate immune gene expression was initiated long before the virus reached the highest replication levels in blood cells, contradictory to responses to PRV-1, that peaked later [9, 22]. Both the PRV-3 response at week two and five, and the strong response to PRV-1 at week five were characterized by effects on a similar set of innate antiviral genes, indicating that the difference in response was mainly associated with timing. Genes associated with a typical dsRNA-induced antiviral response, including PRRs (e.g. *RLR1, RLR3* and *MDA5),* transcription regulators (e.g. *IRF-3/7),* cytokine signaling mediators (e.g. *JAK1, STAT1*, galectin 9 (*LEG9*)) and IFN- inducible effectors (e.g. *Mx2* and *ISG15),* have previously been associated with progression of PRV-1 infection in RBCs *in vivo* [2, 22], studied mainly in the period between the viral peak in blood and the onset of HSMI, and not during early infection [15, 22, 29]. PRV-1 infection is persistent in blood cells, and previous research has indicated that the antiviral response to the virus is long lasting [29]. PRV-3 RNA is also shown to persist in infected A. salmon for at least 10 weeks, but the antiviral responses appear to weaken over time [29].

Given the genetic variation in PRV sequence identity, modest responses to PRV-2 may be attributed to a low rate of amino acid (aa) conservation in segments essential for viral binding, entry and/or replication, compared to host- specific PRV-1 [42]. In this sense, PRV-2 may have not been internalized and the dsRNA genome not sensed by A. salmon RBCs, in line with low replication potential. This is further supported when investigating the expression of genes related to viral dsRNA recognition, such as *TLR3*, *RLR1*, *RLR3* and *MDA5*, and transcription regulation, such as *IRF-1* and IRF-3/7 that did not respond to PRV-2. Interestingly, five weeks post PRV-2 exposure, the genes encoding ring finger

protein 182 (*RNF182*) and dual-specificity phosphatase 11 (*DUSP11*) were significantly induced only in response to PRV-2. In mammals, *RNF182* and *DUSP11* interact with *TLR3* and *RLR1*, respectively, suppressing the IFN- mediated pathway and antiviral defense [43, 44]. This may also explain the limited activation of innate immune antiviral responses to PRV-2 over time. An interesting observation in this context, is that the fish injected with PRV-2, when exposed to a secondary PRV-1 infection, PRV-1 appeared to replicate more efficiently in spleen of some individuals, reaching maximal Ct levels of 10-12, compared to peak Ct levels of 13-14 in PRV-1 control groups [29]. Therefore, the activation of such genes by PRV-2, with inhibitory effect in viral genome recognition and initiation of antiviral responses, may also favor PRV-1 to replicate more efficiently upon consecutive infection.

PRV-3 exhibits higher genetic similarity to PRV-1 [42] in genomic segments which were previously suggested to facilitate virus internalization, propagation and persistence in blood cells and spleen of A. salmon [29]. However, the mechanisms of PRV-3 interaction with A. salmon RBCs may be less evolutionary adapted. Similar to MRV infection mechanism, PRV-1 and PRV-3 likely enters A. salmon RBCs through receptor- dependent endocytosis [45]. The outer part of the double capsid of the internalized virion is partly disassembled in the endosomes and the inner capsid containing the dsRNA genome is transferred to the cell cytoplasm. There, viral factories are formed to serve as production sites for viral amplification, keeping the dsRNA genome protected from exposure to host immune defense mechanisms [25]. While PRV-3 appeared to replicate equally well as PRV-1 in A. salmon RBCs, differences in the infection mechanisms may have led to more exposure of the dsRNA genome in PRV-3 infected cells, a putative explanation on why antiviral responses were triggered earlier.

Are specific transcriptional responses to PRV-1 linked to dissemination and HSMI?

Although PRV-3 may infect and replicate in A. salmon blood cells, and elicit strong antiviral responses when injected IP, transmission of PRV-3 does not occur naturally from infected to naïve cohabitant A. salmon [29, 46]. For PRV-1, transmission to shedders is associated with the early phase of replication when intact virus is released from RBCs to blood plasma. This phase is also associated with virus dissemination to the heart, preceding HSMI [28]. PRV is shown to shed through feces, and also to infect new individuals over the intestinal mucosa [47]. It is unknown whether the lack of transmission of PRV-3 in A. salmon is due to the virus low ability to disseminate into blood plasma and/or be shed through feces, or if PRV-3 is shed but unable to cross mucosal surfaces and enter the blood stream. Previous observations indicate that PRV-3 does not infect the A salmon heart to the same degree as PRV-1, which may explain why PRV-3 does not lead to HSMI [29, 46]. This points towards the dissemination step into blood plasma as the determining factor and indicates that replication and antiviral mechanisms in RBCs may be key. Thus, PRV-1 may escape host antiviral responses and promote its replication/dissemination through inhibition and delay of the same antiviral mechanisms that inhibit PRV-3 dissemination.

For instance, the genes encoding *IQGAP2* and urokinase plasminogen activator receptor (*uPAR*) protein showed lower expression two weeks post PRV-1 infection relative to uninfected controls. In contrast, their expression in response to PRV-3 was significantly higher. The mammalian *IQGAP2* gene serves as an IFN effector, essential for the transcription activation of IFN stimulated genes in response to viral encounter, through interaction with the P65 subunit of nuclear factor-κB (NF- κB) complex [48]. While NF- κB has been characterized as a critical component in reovirus replication and apoptosis induction in host cells in general, so far the involvement in PRV infection of A. salmon RBCs is not evident [2, 22, 23]. Neither NF- κB nor subunits P50 and P65 were induced at the transcriptional level in response to any PRV genotypes here, but may be activated at a post-transcriptional level. Although the *IQGAP2* regulation may have a critical role in host-virus interaction in teleosts, similar to NF- κB in MRV [48], this mechanism is yet to be explored. The protein *uPAR* (also known as CD87) serves a role in immune cell adhesion and migration [49], and is upregulated in response to many viruses [50]. In mammals, it is also implicated in blockage of HIV release from the infected blood cells at the late phase of viral replication [51]. In this sense, inhibition of *uPAR* transcription by PRV-1 at early stages of infection may favor its release into/from RBCs before the initiation of innate immune responses. It is worth noting that a recent proteomic analysis concluded *uPAR* protein as a good candidate biomarker for PRV-1 pathogenesis due to its elevated levels in plasma of PRV-1 positive A. salmon, primarily in association with HSMI onset [52]. The *uPAR* gene activation in blood as reported in our study at week two and five, may further strengthen its biomarker potential.

Another set of genes that showed distinctively high response only to PRV-1 at both two and five weeks post infection were the barrier to autointegration factor proteins *BANFB* and *BANF*. These genes are implicated in host protection by intercepting foreign genomes, a mechanism previously shown to be exploited by mammalian retroviruses to enhance their replication and prevalence in host cells [41]. In addition, *BANF*, in association with serine- threonine vaccinia- related kinases (VRKs) 1 and 3, whose high expression was also induced primarily by PRV-1 infection, is involved in maintaining the integrity of nuclear envelope. Acting as a transcription regulator, *BANF* is also implicated in signaling pathways, potentially moderating gene expression and cell survival [53]. While the functionality of *BANFB* and *BANF* in fish is poorly understood, their overexpression in whole blood of PRV-1 infected fish may suggest their implication in cellular functions associated with both viral replication and persistence mechanism, and the observed survival of infected RBCs [16]. In addition, given the significantly high transcriptional levels of *BANFB* and *BANF* only in response to PRV-1, future investigation of these genes and their proteins as potential biomarkers of PRV-1 infection and pathogenicity may be worth exploring.

MRV infection has been reported to induce the release of lysosomal *CATB* protein, which serves as a "danger" signal activating host innate immune responses [54, 55]. According to this, inhibition of *CATB* expression, observed only in PRV-1 infected blood cells, may also constitute a viral mechanism of

immune evasion, not evolved for PRV-3. Other genes encoding lysosomal proteins, such as ribonuclease T2 (*RNT2* or *RNASET2*), exhibited similar expression profile to *CATB* in PRV-1 and PRV-3 infected blood cells, which may also suggest their implication in virus- host interaction mechanisms.

Transcriptional changes induced by non-replicating PRV genotypes

Vaccination of A. salmon with adjuvanted InPRV-1 has been shown to induce moderate protection against consecutive PRV-1 infection, primarily when fish were challenged through cohabitation [29, 30]. Here, the transcriptional profile of genes implicated in mediation of innate and adaptive immunity was similar in whole blood of vaccinated and PRV-2 infected fish, both at week two and five. However, InPRV-1 vaccine blocked PRV-1 infection and HSMI more efficiently than PRV-2 [29]. This may indicate that physiological and immunological events (e.g. T-cell responses) taken place in other lymphoid tissues primarily contributed to this outcome. It is worth noting that the adjuvant used in the preparation of InPRV-1 vaccine has not been revealed in detail, and the interpretation of the data should consider that the observed responses may both result from the adjuvant, or from the inactivated virus itself. No PRV-1 specific antibodies have been demonstrated after InPRV-1 vaccination [29].

Genes implicated in adaptive immunity and major histocompatibility complex (MHC) class I antigen processing in blood cells were significantly induced primarily by PRV-3, in a manner similar to PRV-1, which may coincide with the efficient protection of A. salmon against PRV-1 and HSMI. Interestingly, some genes involved in MHC class I antigen presentation in mammals, such as histocompatibility 2 Q region locus 10 (*H2-Q10*) and MHC class I-related gene protein-like (*MR1*) [56, 57], exhibited significantly higher expression in blood cells of vaccinated and PRV-2 infected fish relative to PRV-1 and PRV-3. The same was also observed for the genes encoding EF-hand domain-containing protein D2 (*EFHD2*), associated with regulation of T cell- mediated inflammation [58] and *TSPAN31*, linked to apoptosis through a PI3K/Akt pathway [59]. Although the observed transcriptional differences elicited by the PRV genotypes in whole blood may be linked to differential pathogenicity and cross- protection in A. salmon, the functional role of these genes in salmonid blood cells needs to be further explored.

5. Conclusion and future perspectives

This transcriptional study demonstrated that PRV-3, a cross-protective PRV-genotype that does not cause HSMI in A. salmon, triggered potent innate antiviral responses during early replication in blood cells. In comparison, PRV-1 triggered a delayed but similar antiviral response. This difference in the timing of antiviral response may provide an evolutionary advantage for PRV-1, allowing it to disseminate and infect the A salmon heart, a prerequisite for HSMI onset. The early and robust antiviral response to PRV-3 in blood cells likely contributes to the subsequent production of cross-binding anti-PRV antibodies, efficiently blocking PRV-1 infection and the development of HSMI.

Currently, the mechanisms of PRV entry-exit and dissemination within the host cells remain poorly understood. Functional studies on interesting gene candidates with differential expression between PRV-1 and PRV-3, such as *IQGAP2* and *BANF*, may help establish their role in RBCs and viral transmission to cardiomyocytes. Addressing these knowledge gaps would significantly enhance the understanding of the mechanisms involved in PRV pathogenesis and cross-protection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.110088.

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Paper III

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Effects of glucocorticoid receptor activation on gene expression and antiviral response in Atlantic salmon red blood cells

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Effects of glucocorticoid receptor activation on gene expression and antiviral responses in Atlantic salmon red blood cells

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Abstract

Farmed Atlantic salmon are subjected to different stressors during management routines, leading to prolonged elevated cortisol levels in the blood, a hallmark of chronic stress responses.(1,2) In this study, we investigate how dexamethasone, an agonist for glucocorticoid receptors (GRs), and cortisol, an endogenous glucocorticoid, regulate gene expression in isolated A. salmon red blood cells (RBCs). Salmonid RBCs express GR genes at high levels. Transcriptional analysis revealed that dexamethasone significantly upregulated 156 genes, and led to > 100-fold induction of the FKBP prolyl isomerase 5 (*FKBP5*), the Krueppel-like factor 9 (*KLF9*), and the DNA damage-inducible transcript 4 (*DDIT4*) genes. The significant upregulation of *FKBP5* persisted for up to two weeks post RBC stimulation, indicating its potential as a biomarker candidate for chronic stress exposure. We compared *ex vivo* transcriptional responses in RBCs with responses in blood cells from A. salmon injected intraperitoneally with hydrocortisone, and found that *DDIT4* may be a promising biomarker candidate for stress response *in vivo*. Dexamethasone and cortisol also downregulated antiviral and proteasome genes triggered by double stranded RNA in RBCs. The results indicate that glucocorticoids have a profound effect on gene expression and putative functions of A. salmon RBCs, and particularly block the antiviral response.

1. Introduction

Modern salmonid farming is characterized by high- density rearing, short production cycles and intensive handling routines such as mechanical delousing. These practices increase the likelihood of stress responses and pose significant risk to fish welfare. Farmed Atlantic (A.) salmon (*Salmo salar*) are prone to viral diseases, with stress often identified as the key factor that triggers disease and mortality (3). Stress initiates the release of catecholamines and glucocorticoids (GCs) into the bloodstream (4,5). Cortisol is the major glucocorticoid in fish and produced by steroidogenic cells located in the head kidney interrenal tissue. Although cortisol is widely used as an indicator of elevated stress in salmonid farming (4–6), various factors can influence stress intensity and hormonal profile, potentially leading to inaccurate estimation of physiological stress. These factors, often overlooked, include fish manipulation during sampling (e.g. fish crowding in the net-pen), anesthetics, or physiological adaptations to prolonged stress exposure (7).

The stress response is regulated by the hypothalamus- pituary- interrenal axis (HPI axis). Upon stress, corticotropin- releasing factor (CRF) prompts the secretion of adrenocorticotropic hormone (ACTH), which in turn stimulates cortisol production and release into circulation (6). The physiological effects of cortisol on target tissues are mediated by its binding to glucocorticoid and/or mineralocorticoid receptors (GR and MR, respectively) (8).

Fish red blood cells (RBCs) are nucleated, multifunctional cells with diverse physiological and immunological properties (9–12). Salmonid RBCs play a crucial role in mediating innate immunity (10,11,13), and have been identified as primary targets of *Piscine orthoreovirus* (PRV) (14). PRV is a double- stranded (ds) RNA virus with three distinct genotypes (PRV-1, -2 and -3), all etiologically linked to circulatory diseases in different salmonid species (15–17). PRV-1 is the etiological agent of heart and skeletal muscle inflammation (HSMI), a significant disease in A. salmon aquaculture in Norway (15,18). Fish infected with PRV-1 often exhibit sensitivity to stress (19,20), suggesting that HSMI- related mortality may result from a synergistic effect of stress and viral infection (20).

Chronic stress has been linked to immunosuppression in fish (4,5,21). Experimental studies have shown that other viruses with significant impact on Norwegian aquaculture, such as infectious pancreatic necrosis virus (IPNV) and salmon gill poxvirus (SGPV) (3), can persist in asymptomatically infected individuals, while the onset of clinical disease in such populations is often preceded by elevated cortisol levels (22–24). Although mortality of fish with viral diseases has been associated with stress, the molecular mechanisms underlying this interaction remain relatively uncharacterized. One suggestion is that to restore homeostasis, the cells may prioritize functions essential for stress adaptation, often at the expense of other energy demanding processes, including immune protection (25).

In this study, we hypothesize that A. salmon RBCs respond to activation of the GR pathway, which is stimulated by dexamethasone, a synthetic agonist (26), and hydrocortisone. Our aim is to elucidate the

effects of stress hormones on RBCs, and to identify novel biomarker candidates indicating the secondary effects of chronic stress beyond hormonal screening in plasma.

Following *ex vivo* stimulations of A. salmon RBCs with dexamethasone and hydrocortisone, we aimed to determine whether the induction of the same putative biomarker candidates could also be observed in blood cells from A. salmon injected with cortisol *in vivo*.

In addition, we aim to provide insights into secondary physiological and immunological stress- mediated effects in fish blood. Specifically, we investigate whether dexamethasone and cortisol attenuate the dsRNA mediated antiviral responses in A. salmon RBCs. Polyinosinic: polycytidylic acid (poly (I:C)) is a synthetic dsRNA analogue shown to induce innate immune responses in the RBCs of rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*Oncorhynchus tshawytscha*) *ex vivo* and *in vivo* (13,27). Poly (I:C) binds to toll like receptor (TLR) 3 and retinoid acid- inducible gene- I (RIG- I)- like receptors (RLRs), activating the type I interferon (IFN)- mediated signaling pathway (13,28,29). Based on this, poly (I:C) has been used as a promising adjuvant for commercial vaccines against viral infections in salmonid aquaculture (30,31). To the best of our knowledge, this is the first study on glucocorticoid effects on gene expression and antiviral responses in A. salmon RBCs.

2. Material and methods

2.1. Blood sampling and isolation of red blood cells

Blood (approx. 1mL) was collected from the caudal vein of A. salmon in BD vacutainer lithium heparin tubes (VWR International, LLC). The fish (*Salmo salar* L., AquaGen genetic line), were provided by the aquatic research facility at the University of Life Sciences, Ås, Norway, where they were kept in fresh water at 13-15°C at a density of 6-10kg/m^3. The average weight of the fish was approximately 170 g. The fish were anesthetized prior to sampling by bath immersion in Finquel vet (0.5 g/L water) and euthanized using Finquel vet overdose (1g/L water) for 2 min. The blood was used for isolation of red blood cells (RBCs).

The heparinized blood was diluted 1:10 in sterile phosphate buffered saline (dPBS). RBCs were isolated by layering on a dPBS buffered Percoll (GE healthcare, Uppsala Sweden) gradient with a bottom layer of 49% Percoll and a top layer of 34%. The diluted blood cells were centrifuged (500 x G, 4 °C, 20 min), and the purified RBCs pellet was harvested and washed with dPBS, as previously described (14). Cell quantity and viability (%) were measured using Countess (Invitrogen, Eugene, Oregon, USA). The isolated RBCs were resuspended to a concentration of 2×10^7 cells/mL in Leibovitz's L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with fetal calf serum (2%) (Sigma- Aldrich) and gentamicin (50 mg/mL- Lonza Biowhittaker, Walkersville, USA). Culture purity was assessed by examining three areas under a light microscope (approximately 100 cells/area, ≥300 cells in total). The

culture was considered pure if no more than 2 cells lacked typical RBCs morphology (99% culture purity) (10). The cultures were incubated overnight at 15°C under constant agitation (225 rpm).

2.2. *Ex vivo* stimulation of red blood cells

To investigate the effects of glucocorticoid receptor activation on the expression profile of RBCs and their antiviral innate immune responses induced by poly I (:C), three experimental conditions were established alongside unstimulated controls: RBCs stimulated with (i) glucocorticoids, (ii) poly (I:C) and (iii) glucocorticoids followed by poly (I:C) stimulation. Both stimulated RBCs and unstimulated controls were incubated at 15°C under constant agitation (225 rpm) until sampling.

Dexamethasone and hydrocortisone (both from Sigma- Aldrich Solutions, Darmstadt, Germany) were prepared at stock concentrations of 50 μM and 100 μM, respectively. RBCs were treated with either dexamethasone (test concentrations: 1, 10 and 100 μM; n =6 per conc.) or hydrocortisone (test concentrations: 20, 50, 100, 150 μM; n =6 per conc.) and incubated for 1-14 days before harvest.

Four concentrations of poly (I:C) (Sigma-Aldrich, USA)(25, 50, 100 and 200 μg/mL) and three different harvest points (1, 3 and 7 days) were initially investigated, identifying 50 μg/mL with a three-day-stimulation period as optimal for inducing significantly stable dsRNA antiviral responses in A. salmon RBCs.

To investigate the effects of GCs on innate immune responses to poly (I:C), RBCs were pretreated with either dexamethasone or hydrocortisone for 24 h, followed by exposure to 50 μg/mL poly (I:C) and incubated for an additional 72 h prior to harvest (i.e. four days of incubation in total). RBCs treated with either dexamethasone or hydrocortisone for 4 days, as well as RBCs exposed only to 50 μg/mL poly (I:C) for 3 days, were also included in the analysis. Untreated RBCs incubated for 4 days served as controls.

RBCs were harvested by centrifugation (500 x G, 14 °C, 10 min). After media removal, the cell pellets were washed with dPBS and lysed in 400 μL MagNA Pure LC Isolation Tissue kit (Roche Diagnostics, Germany). Lysed cells were stored at -20 °C.

2.3. *In vivo* experimental trial and blood sampling

Detailed information on the previously published trial can be found in Thoen et al., 2020 (22). In the original trial, the aim was to study the effects of cortisol on fish infected with salmon gill pox virus (SGPV). Briefly, 220 naïve fish with an average body weight of 50 g were divided into four experimental groups. Two groups received IP injection of a depot matrix with hydrocortisone (HC), and two with depot matrix only (Sigma Aldrich, St. Louis, MO, USA). In the original trial, groups were later exposed to salmon gill pox virus (SGPV), but no virus- infected samples were used in the current analyses.

To reduce effects of handling on plasma cortisol prior to blood sampling, fish were first sedated with a low dose of iso-eugenol (2mg/ml), and finally euthanized with an iso-eugenol overdose (30 mg/ml).

Here, blood samples from 5 HC- injected fish and 5 control fish (depot matrix injected only) were studied at two time points: 2 and 4 days post-injection (n= 20). Blood cell pellets (20 µL) were resuspended in 400 µL lysis buffer from the MagNA Pure Tissue RNA Isolation kit (Roche) and stored at -20 °C for further analysis.

2.4. RNA isolation

Lysed RBCs (*ex vivo* experiment) and blood cell pellets (*in vivo* trial) were homogenized using 5 mm steel beads and a TissueLyser II (Qiagen, Germany) for 3 min at 25 Hz. Total RNA was extracted using MagNA Pure 96 Cellular RNA Large Volume Kit, compatible with the automated MagNA Pure 96 system (Roche Diagnostics, Germany), following the manufacturer's instructions. RNA was quantified using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, Norway).

2.5. qPCR analysis

Reverse transcription was performed on RBC samples from the *ex vivo* stimulation experiments, using 200 ng total RNA per sample. The RNA was incubated with gDNA Wipeout Buffer (Qiagen) at 42 °C for 2 min to remove genomic DNA, followed by cDNA synthesis using the QuantiTect Reverse Transcriptase Kit (Qiagen). Quantitative PCR (qPCR) was conducted in duplicate on cDNA corresponding to 5 ng RNA input. Gene expression analysis was performed using SsoAdvanced Universal SYBR Green Supermix (BIO-RAD Laboratories, USA) and 400 nM specific A. salmon primers (listed in Table 1). The amplification run for 40 cycles of 95 °C/ 15 s, 60 °C/ 30 s in an CFX384 Touch Real- Time PCR Detection System (BIO-RAD Laboratories, USA). The analysis of RT- qPCR data was performed in GraphPad Prism 9, and significant differences were estimated using one- or two-way ANOVA with Dunnett's or Tukey's multiple comparison test, respectively.

Table 1. Primers used for RT- qPCR analysis.

Primer	Primer sequence (5' → 3')	Accession #
EF1ab	Fwd- TGCCCCTCCAGGATGTCTAC	Q9DDK2
	Rev- CACGGCCCACAGGTACTG	
MX1	Fwd- GATGCTGCACCTCAAGTCCTATTA	BT043721.1
	Rev- CACCAGGTAGCGGATCACCAT	
ISG15	Fwd- ATATCTACTGAACATATATCTATCATGGAAACTC	BT048733
	Rev- CCTCTGCTTTGTTGTGGCCACTT	
FKBP5	Fwd- TGCTGAGCTTCAAAGGGGAG	BT048177.1
	Rev- AGAGAAGGTAGGTCTGCCTCA	

2.6 Illumina RNA sequencing

A total of 40 samples- 20 from the *ex vivo* RBC stimulation and 20 from the *in vivo* trial- were sent to the Norwegian Sequencing Centre (NSC, Norway). The RNA quality (RIN >8) was ensured using Agilent 2100 Bioanalyser (Agilent, USA) before samples were sent for sequencing. Library preparation was performed using strand- specific TruSeq mRNA-seq Library prep kit (Illumina, CA, USA). The experimental groups of the *ex vivo* experiment included untreated controls (Ctrl, n= 5), and RBCs stimulated with poly(I:C) 50 μg/mL (P(I:C), n= 5), dexamethasone 100 μM (Dex, n= 5) or a combination of dexamethasone 100 μM and poly(I:C) 50 μg/mL (DexP, n= 5). For the *in vivo* trial, the experimental groups included mock controls (n= 5 at day two; n= 5 at day four) and HC- injected fish (n= 5 at day two; n= 5 at day four). The libraries from *ex vivo* and *in vivo* experiments were pooled separately, and sequenced on one lane of Illumina NovaSeq S4 flow cell to obtain 150bp paired-end reads. The raw sequencing data are available in BioProjects PRJNA1042786 and PRJNA1042788, respectively.

2.7 Bioinformatic processing and statistical analysis

Raw sequence data (Fastq files) were processed to trim/remove adapter and low quality sequences using BBDuk tool in BBMap v.38.18 suite (parameters: ktrim=r, k=23, mink=11, hdist=1, tbo, tpe, qtrim=r, trimq=15, maq=15, minlen=36, forcetrimright=149) (32). The mapping of cleaned reads to *Salmo salar* genome (NCBI GCF Ssal_v3.1) was performed using the HISAT2 v.2.2.1 (parameters: –rna-strandness RF) (33). To estimate the number of reads and aligning against the reference genes in NCBI GCF_905237065.1 annotation, FeatureCounts v.1.4.6-p1 (parameters: -p -s 2) was used (34). Initial raw data analysis was performed using SARTools v.1.7.4 and R v.4.1.1 (35,36). Normalization and differential expression between groups and against the control were performed using DESeq2 v.1.34.0, separately for the *ex vivo* and *in vivo* experiments (37). The annotation tables were cleaned using median count reads > 10 as a cut off, to omit genes with zero or low counts. Adjusted p-value (padj) was calculated using Benjamin- Hochberg (BH) correction and gene with padj below 0.05 were considered as differentially expressed genes (DEGs). To assess the variability of the *ex vivo* and *in vivo* samples within each experimental condition, we performed principal component analysis (PCA) (Supplementary File A, Figure 1 and 2). RBCs from the *ex vivo* stimulations showed wide dispersion along the first principle component, but they formed two major clusters, with RBCs replicates from the same condition pairing together. When plotted against the second principle component, the RBCs samples exhibited lower variability, clustering by condition. One outlier that deviated in both PCA plots was omitted from further analysis.

For gene regulation, upregulated features with less than 2-fold change and downregulated features with higher than 0.5- fold change in expression ($0.5 < \text{fold change} < 2$) were filtered out. STRING Database v.12.0 was used for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with 0.05 as p-value cutoff, BH adjusted (38).

3. Results

3.1. Effects of glucocorticoids on the transcriptional profile of A. salmon red blood cells *ex vivo* and blood cells *in vivo*

The transcriptional responses of A. salmon RBCs stimulated with dexamethasone and cortisol *ex vivo* and compared to those of cortisol-injected A. salmon blood cells *in vivo* are provided in the following sections (3.1.1- 3.1.3)

3.1.1. Effects of dexamethasone on the transcriptional profile of A. salmon red blood cells compared to unstimulated controls *ex vivo*

Transcriptional analysis of purified A. salmon RBCs in their resting state revealed the expression of genes encoding proteins involved in stress hormone responses in mammals. These include transmembrane glucocorticoid receptors (GR) and accessory molecules which are implicated in regulation of glucocorticoid- induced signaling pathways (Table 2). The normalized median transcript reads (ntr) of 2 commonly used house-keeping genes, elongation factor 1A and 1B (*EF1A* and *EF1B*), are also included in Table 2 for comparison. Four GR genes were found to be expressed. The same genes were found in a previous characterization of GR variants in the A. salmon genome (39). The GR genes are predicted to correspond to GR1 (located in chromosomes 4 and 13) and GR2 (located in chromosomes 5 and 9) (39).

Table 2. Normalized median transcript reads (ntr) of genes encoding glucocorticoid receptors (GR) and accessory proteins implicated in regulation of glucocorticoid- induced signaling pathways in A. salmon RBCs in resting state. For comparison, transcript levels of the EF1A and EF1B housekeeping genes are also given.

Gene reference	Gene name	Control (ntr)
LOC106604224	*GR (ssa05)*	1511
LOC106567492	*GR (ssa13)*	1082
LOC100380779	*GR (ssa04)*	1556
LOC106612223	*GR (ssa09)*	1612
LOC106584263	*GMEB1*	3260
LOC106572177	*GMEB2*	1138
LOC106590407	*SGK3*	1236
LOC100306837	*GLCI1*	1520
Housekeeping genes		
LOC100136525	*EF1A*	215
LOC100195925	*EF1B*	625

*GR variants identified in chromosomes 4 (ssa04), 5 (ssa05), 9 (ssa09) and 13 (ssa13) of A. salmon genome.

Differential gene expression analysis was performed to examine the effects of dexamethasone on the transcription profile of RBCs, compared to the unstimulated controls. The GR agonist dexamethasone significantly induced 156 genes and inhibited six genes 24 h after addition to RBCs *ex vivo*. Of the upregulated DEGs, 12 genes exhibited a 40-600 fold increase, with krueppel- like factor 9 (*KLF9*) showing the most pronounced upregulation (600- fold change) (Table 3). In addition to *KLF9*, the DNA damage-inducible transcript 4 protein (*DDIT4*) and FKBP prolyl isomerase 5 (*FKBP5*) genes also demonstrated substantial upregulation, with over 200- fold increases. The elongation of very long chain fatty acids protein 4 (*ELOVL4*) gene, which is involved in regulation of polyunsaturated fatty acid (PUFA) biosynthesis in fish (40), was also significantly activated, with an approximately 90-fold increase in response to dexamethasone. Among the downregulated genes, phosphatidylinositol 4-phosphate 5-kinase-like protein 1 (*PIP5KL1*) and sorting nexin 7 (*SNX7*) have been involved in intracellular protein trafficking via phosphatidylinositol signaling system in mammals (41,42). The lists of all upregulated and downregulated DEGs in response to dexamethasone are provided in Supplementary file B.

Table 3. Normalized median transcript reads (ntr) of the twelve most highly induced genes in response to dexamethasone (100μM) in A. salmon RBCs.

Gene reference	Gene name	Control (ntr)	Dexamethasone 100 μM (ntr)	Fold- change
LOC106585360	***KLF9***	**2**	**1157**	**600.62**
LOC106592479	***DDIT4***	**39**	**10961**	**268.21**
LOC100196052	***FKBP5***	**32**	**7557**	**218.95**
LOC106563189	*INPP5K*	1	154	138.50
LOC106564638	*ELOVL4*	86	10370	92.46
LOC106563188	*SMTNA*	3	258	74.12
LOC106577395	*DDIT4-like*	51	3499	66.62
LOC106590608	*MTRR*	455	28134	59.14
LOC123732461	*PRELID3A*	1	83	46.25
LOC106569334	*HIGD1A*	92	3945	41.89
LOC106587747	*MAP1B-like*	5	238	41.45
LOC106575072	*CDN1B*	2	138	41.45

3.1.2. Transcriptional responses of *FKBP5* in A. salmon red blood cells exposed to dexamethasone and hydrocortisone *ex vivo*

FKBP5 was further investigated as a potential biomarker for chronic stress in RBCs. The relative expression of *FKBP5* in RBCs after a four-day exposure to different concentrations of dexamethasone (1- 100 μM) and hydrocortisone (20- 150 μM) was assessed using quantitative PCR (Figure 1A). *FKBP5* was significantly upregulated across all tested concentrations of both dexamethasone and

hydrocortisone, showing a >100 fold- increase relative to the controls. Dexamethasone induced high *FKBP5* expression, with approximately a 200-300- fold increase (Figure 1A, left panel). In contrast, the lowest concentration of hydrocortisone tested (20 µM) was associated with the lowest *FKBP5* expression. Hydrocortisone at concentrations of 50 and 100 µM, levels often indicative of stressed fish *in vivo* (43), resulted in an approximate 250- fold increase in *FKBP5* expression, a similar effect observed with 10 µM dexamethasone (Figure 1A, right panel). The highest concentrations of dexamethasone (100 µM) induced a 200-fold upregulation of *FKBP5*, consistent with our transcriptomic findings (Table 3). A similar 200- fold increase was also observed for the highest concentration of hydrocortisone (150 µM) (Figure 1A).

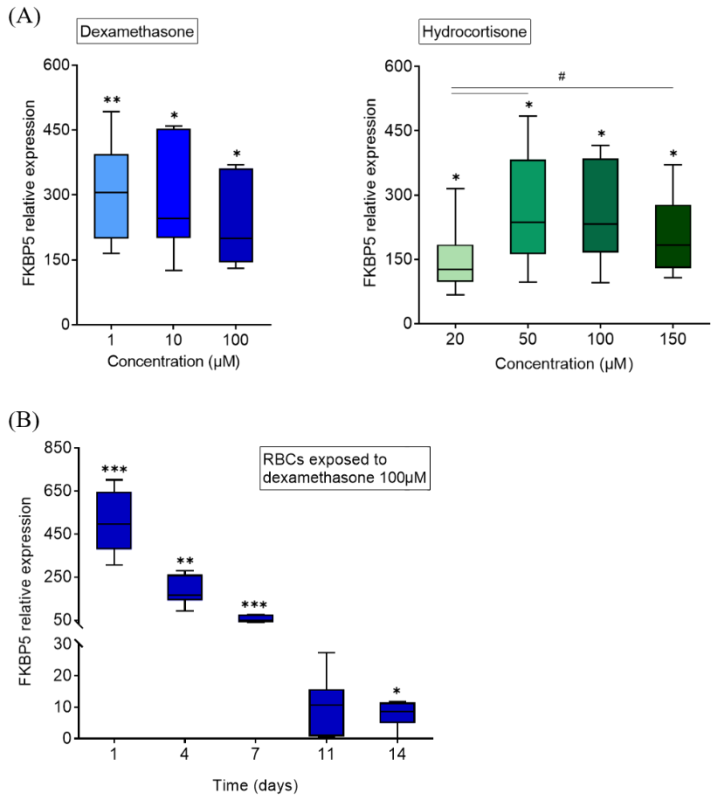

Figure 1. Effects of dexamethasone and cortisol on the transcriptional profile of *FKBP5* in A. salmon red blood cells. Expression of *FKBP5* was measured in purified RBCs by RT-qPCR (n= 6). (A) Relative expression of *FKBP5* in A. salmon RBCs four days post stimulation with dexamethasone at concentrations of 1, 10, 100 µM, and hydrocortisone at concentrations of 20, 50, 100, 150 µM. Data were analyzed using one-way ANOVA Tukey test. *: $p < 0.05$ relative to the control; **: $p < 0.01$ relative to the control; #: $p < 0.05$ between the experimental conditions. (B) Relative expression of *FKBP5* in A. salmon RBCs stimulated with 100 µM dexamethasone over 14 days. Data were analyzed using paired t-test for the treated RBCs of each day, compared to its respective untreated controls (n= 6). *: $p < 0.05$ relative to the control; **: $p < 0.01$ relative to the control; ***: $p < 0.0005$.

The relative expression of *FKBP5* was monitored over 14 days after the activation of the glucocorticoid receptor pathway by 100 µM dexamethasone in RBCs. *FKBP5* showed a > 500- fold upregulation just after 24 h of exposure, suggesting its high responsiveness to glucocorticoids (Figure 1B). Although gene

expression gradually decreased over time, a significant 50-fold upregulation was still observed after seven days of exposure. By days eleven and fourteen, *FKBP5* exhibited approximately a 10-fold upregulation in dexamethasone- stimulated RBCs.

3.1.3. Differential expression analysis of whole blood of Atlantic salmon injected with hydrocortisone compared to sham controls

Transcriptional analysis of A. salmon blood cells was performed at two and four days post IP injection of hydrocortisone, compared to sham controls *in vivo*. These results were used to determine whether elevated cortisol levels induce transcriptional changes similar to those observed in RBCs stimulated with dexamethasone *ex vivo*. As reported by Thoen et al. (2020) and Amundsen et al. (2021) in the original trial, cortisol levels in injected fish were significantly higher on day two, but returned to baseline by day four (Figure 2A) (22,44).

Differential gene expression analysis was performed using a cutoff of > 2- fold change for upregulated genes and < 0.05 for downregulated genes. In response to elevated cortisol levels 2 days post injection, 3 genes were significantly upregulated, while 11 were significantly downregulated in A. salmon blood cells. By day 4, a new set of 5 genes were upregulated and 11 genes were downregulated (Supplementary material C). Only the E3 ubiquitin-protein ligase HERC3 (*HERC3*) gene was significantly suppressed at both day 2 and 4 post injection. The expression level of the *HERC3* gene was not affected in the *ex vivo* stimulation of RBCs with dexamethasone (Figure 2B).

The *DDIT4* gene was significantly induced in both RBCs after *ex vivo* stimulation with dexamethasone, and in blood cells two days post- hydrocortisone injection, when the cortisol levels peaked (Figure 2C). In contrast, the *KLF9* and *FKBP5* genes, which exhibited the greatest transcriptional differences in stimulated RBCs relative to the controls, were not significantly induced in blood cells *in vivo* (Figure 2C). Most of the genes that were significantly upregulated by hydrocortisone in whole blood of A. salmon, were not found expressed in cultured RBCs (zero median transcript count reads), suggesting that their activation may occur in other blood cell types, such as macrophages or leukocytes. In addition, there was no overlap between the downregulated DEGs observed in blood cells and those in dexamethasone- stimulated RBCs.

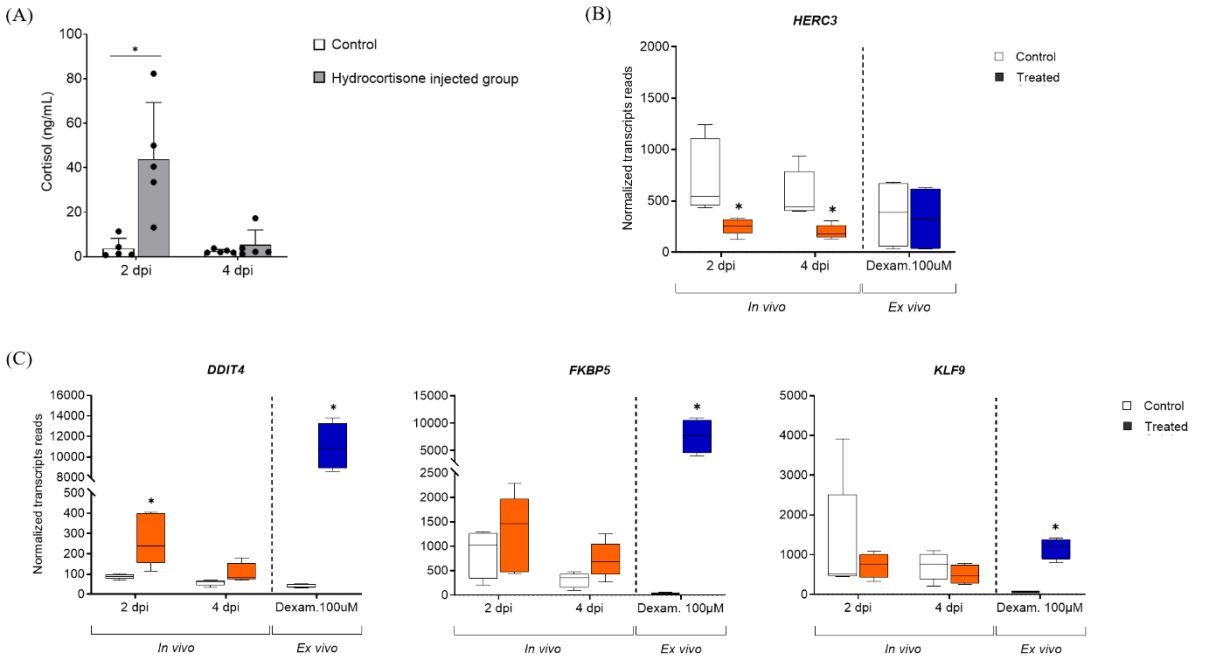

Figure 2. Transcriptional responses in whole blood of A. salmon two and four days post injection (dpi) of hydrocortisone. (A) Cortisol levels in blood plasma of A. salmon detected two and four days post- injection using ELIZA competitive enzyme immunoassay kit, as shown by Thoen et al. (2020) and Amundsen et al. (2021) (22,44). (B) Transcriptional profile of the E3 ubiquitin-protein ligase HERC3 (*HERC3*) gene in blood cells of A. salmon two and four days post- hydrocortisone injection *in vivo* and in purified RBCs four-days post stimulation with dexamethasone *ex vivo*. RBCs *ex vivo*: n= 4. Whole blood *in vivo*: n= 5. *: $p < 0.05$ compared to the controls, estimated during the transcriptional analysis using DESeq2 v.1.34.0. (C) Transcriptional profile of the DNA damage-inducible transcript 4 protein (*DDIT4*), FKBP prolyl isomerase 5 (*FKBP5*) and Krueppel-like factor 9 (*KLF9*) genes in blood cells of A. salmon two and four days post- hydrocortisone injection *in vivo* and in purified RBCs four-days post stimulation with dexamethasone *ex vivo*. RBCs *ex vivo*: n= 4. Whole blood *in vivo*: n= 5. *: $p < 0.05$ compared to the controls, estimated during the transcriptional analysis using DESeq2 v.1.34.0.

3.2. Transcriptional analysis of RBCs stimulated with either dexamethasone, poly (I:C) or dexamethasone and poly (I:C) compared to unstimulated controls *ex vivo*

RBCs from five A. salmon stimulated with either dexamethasone, poly (I:C) or dexamethasone and poly (I:C), and unstimulated controls were analyzed by RNA-seq. Differential gene expression analysis was performed to examine the effects of dexamethasone, poly (I:C) or dexamethasone and poly (I:C) on the transcription profile of RBCs, compared to the unstimulated controls (Figure 3A). In addition, to determine the biological processes and signaling pathways to which DEGs of stimulated RBCs belonged, gene ontology (GO) for Biological Process (GO:BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed (Figure 3B). Given the limited number of downregulated genes, only the upregulated DEGs of RBCs stimulated with poly (I:C) alone or dexamethasone and poly (I:C), were categorized into functional group (Figure 3B).

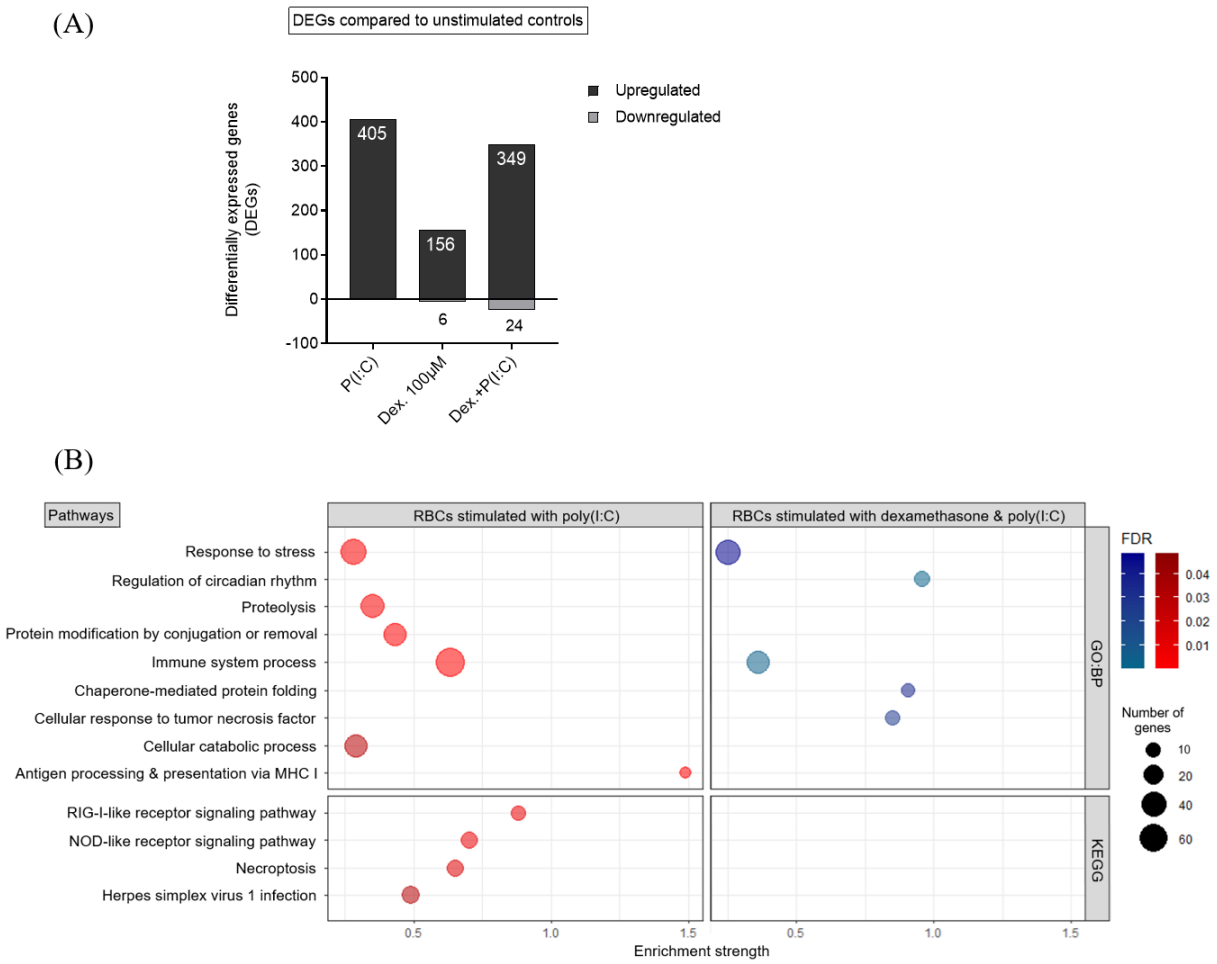

Figure 3. Transcriptional analysis of A. salmon RBCs stimulated either with 100 µM dexamethasone, 50 µg/mL poly (I:C) or 100 µM dexamethasone and 50 µg/mL poly (I:C), compared to unstimulated controls *ex vivo*. Only genes with genes with normalized median count reads ≥ 10 were included in the analysis. A cutoff ≥ 2-fold change was used for upregulated DEGs, and ≤ 0.5-fold change for downregulated DEGs. (A) Differential gene expression analysis of A. salmon RBC exposed to dexamethasone, poly (I:C), and their combination, compared to their unexposed controls. (B) Enriched Gene Ontology (GO) terms within the "Biological Process" (GO: BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway datasets. Only functional groups and pathways with FDR (adjusted p value) ≤0.05 were considered significant. GO: BP (top) and KEGG pathways (bottom) enriched in RBCs exposed to poly (I:C) (red), and dexamethasone and poly (I:C) (blue).

3.3. Effects of dexamethasone and hydrocortisone on poly (I:C)- induced antiviral responses in A. salmon red blood cells *ex vivo* analyzed by RT- qPCR

The mRNA levels of *MX1* and *ISG15* were analyzed in RBCs stimulated with poly (I:C) at concentrations of 25, 50, 100 and 200 µg/mL, following incubation for one, three and seven days. No significant gene induction was observed on day one at any concentration. *MX1* was significantly upregulated by day three post stimulation, exhibiting a similar response pattern at 50, 100 and 200 µg/mL poly (I:C), with an approximate 20- fold increase relative to the controls. Peak expression occurred on day seven, particularly at 50 µg/mL poly (I:C), where *MX1* showed a significant 50- fold upregulation (Supplementary file D, Figure 1A). The expression of *ISG15* was generally higher than

MX1, with approximately 70- fold and 200- fold increases in response to 50 and 200 µg/mL poly (I:C) on days three and seven, respectively (Supplementary file D, Figure 1B). Based on these observations, along with the substantial variation in responses among replicates on day seven, a three day- exposure of RBCs to 50 µg/mL poly (I:C) was selected as the optimal *ex vivo* stimulation for eliciting significant antiviral responses.

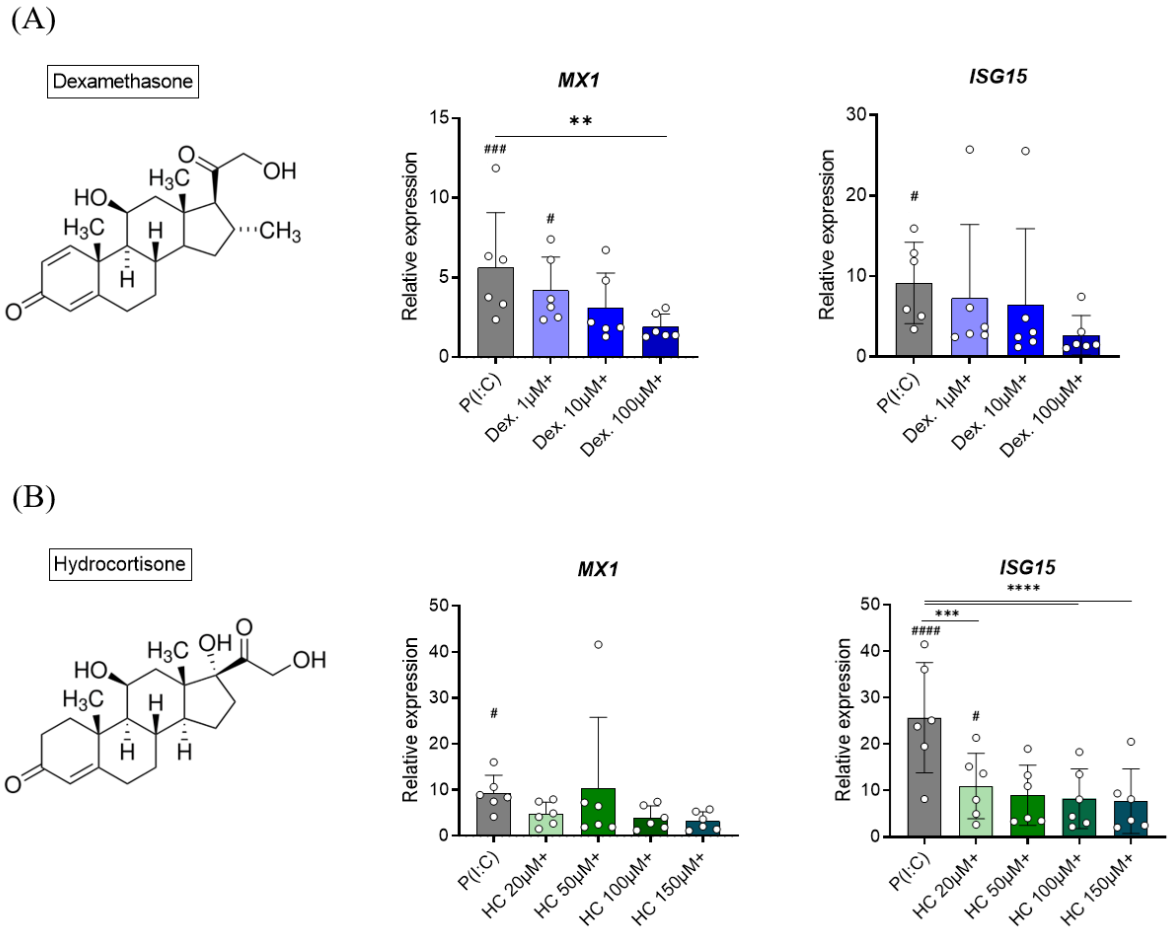

Figure 4. Activation of the glucocorticoid receptor (GR)- mediated pathway by dexamethasone or hydrocortisone suppresses antiviral responses of A. salmon RBCs to poly (I:C). (A) The glucocorticoid receptor agonist dexamethasone (blue), and (B) the chronic stress hormone, hydrocortisone (green). The chemical structures of dexamethasone and hydrocortisone originated from He Y., et al. (2014) (45). Expression levels of *MX1* and *ISG15* were measured by RT-qPCR. RBCs were first stimulated with different doses of dexamethasone or hydrocortisone, incubated for 24 h and thereafter exposed to 50 µg/mL poly(I:C). The expression levels of stimulated RBCs relative to the unstimulated controls were calculated for each sample (n=6). Data were analyzed using two-way ANOVA Tukey's multiple comparison test. #: $p < 0.05$; ####: $p < 0.0001$ compared to the unstimulated controls. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0005$; ****: $p < 0.0001$.

The expression levels of *MX1* and *ISG15* in RBCs were analyzed after *ex vivo* stimulation with dexamethasone or hydrocortisone, followed by 50 µg/mL poly (I:C), to assess whether prior exposure to glucocorticoids suppressed antiviral responses. Post- stress cortisol levels in fish can range from 20 to 500 ng/mL, depending on the species and stress intensity (46). To determine the threshold at which immunosuppression occurs, three different concentrations of dexamethasone (1, 10, 100 µM) and four

of hydrocortisone (20, 50, 100, 150 µM) within the designated physiological range were tested. Both dexamethasone and hydrocortisone similarly attenuated dsRNA- induced antiviral responses in RBCs, with dose-dependent immunosuppressive effects (Figure 4). Specifically, *MX1* expression was significantly suppressed following exposure to 100 µM dexamethasone (Figure 4A), while *ISG15* expression was significantly suppressed by 20, 100 and 150 µM hydrocortisone (Figure 4B).

3.4. Effects of dexamethasone on poly (I:C)- induced transcriptional responses of A. salmon red blood cells *ex vivo* analyzed by RNA-seq

A three- day exposure of RBCs to poly (I:C) induced 405 DEGs, while no genes were significantly downregulated compared to unstimulated controls (Figure 3A). Several upregulated genes were primarily associated with regulation of cellular homeostasis ("Response to stress) and antiviral innate immune protection, including dsRNA- mediated signaling pathways, such as "RIG-I-like receptor" and "Herpes simplex virus 1 infection" (Figure 3B, left panel). Similar gene activation has previously been observed following a 24-h exposure of A. salmon RBCs to PRV-1 *ex vivo* (12), as well as during the early phase of PRV-1 infection (of A. salmon RBCs) *in vivo* (10).

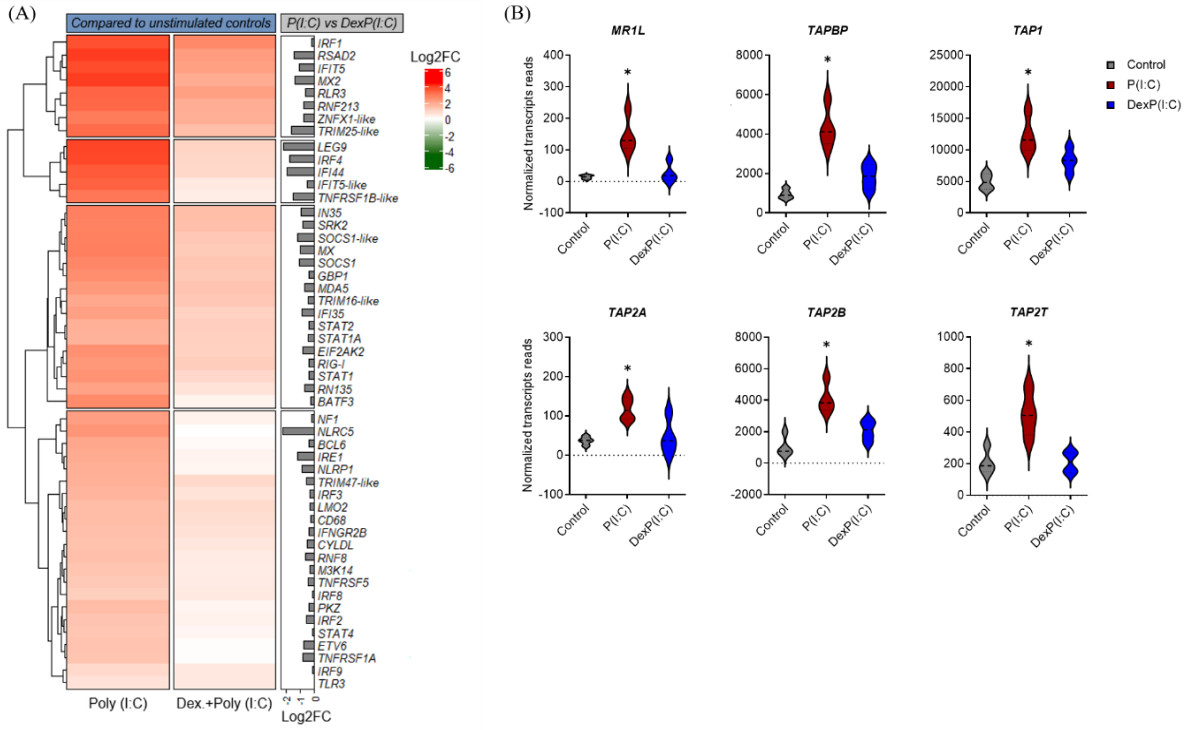

Figure 5. Transcriptional analysis of A. salmon RBCs stimulated with poly (I:C) or dexamethasone and poly (I:C), compared to unstimulated controls. (A) Gene expression profile in RBCs of A. salmon stimulated with either poly (I:C) or dexamethasone and poly (I:C), compared to unstimulated controls. Log2-fold change (Log2FC) of selected DEGs involved in "Immune system process" (GO:BP), as well as "RIG-I-like receptor", "NOD-like receptor" and "Herpes simplex virus 1 infection" signaling pathways (KEGG), compared to unstimulated controls (heatmap). Log2FC of selected DEGs is also provided from the comparison of poly (I:C)- with dexamethasone and poly (I:C)- stimulated RBCs (grey bar plot). Red: Higher expression level in stimulated RBCs compared to unstimulated controls; Green: Lower expression level in stimulated RBCs compared to

unstimulated controls; White: No expression difference between stimulated and unstimulated RBCs. The darker the color, the stronger the regulation (higher or lower). (B) Selected genes involved in MHC class I antigen processing and presentation with significantly different expression patterns between RBCs stimulated with poly (I:C) alone and those treated with dexamethasone and poly (I:C), compared to unstimulated controls. *$p \leq 0.01$ in poly (I:C)- stimulated RBCs compared to unstimulated controls; # $p \leq 0.01$ in dexamethasone and poly (I:C)- stimulated RBCs compared to unstimulated controls.

When combined, dexamethasone and poly (I:C) resulted in 349 upregulated and 24 downregulated DEGs (Figure 3A). This is a decrease in number of significantly upregulated DEGs compared to poly (I:C) alone (56 DEGs less).

The expression patterns of selected poly (I:C)-induced DEGs involved in immune system processes (GO:BP, Figure 3B) and PRR- mediated antiviral pathways (KEGG pathways, Figure 3B), as well as how their responses to poly (I:C) were attenuated by dexamethasone, are shown in a heatmap (Figure 5A). Although the transcriptional levels of several antiviral genes, including RIG-like receptor 3 (*RLR3*), interferon (IFN) induced protein with tetratricopeptide 5 (*IFIT5-like*) and viperin- like (referred to as *RSAD2*) genes, were reduced, their expression was not completely blocked. In addition, key mediators of antiviral response in A. salmon RBCs (10,12), such as *TLR3* and interferon regulatory factors (IRFs) 1, 3 and 9 genes, were significantly induced by poly (I:C) but unaffected by dexamethasone.

Some genes implicated in the homeostatic control of innate immunity and antigen processing and presentation by major histocompatibility complex (MHC) class I, such as NLR family CARD domain containing 5 (*NLRC5*), transporters TAP 1 and 2, and MHC class I- related gene protein (*MR1*), were inhibited by dexamethasone (Figure 5B). Genes involved in regulation of cellular catabolic processes, proteolysis and protein modification were also significantly induced in response to poly (I:C) (Figure 3B, left panel). Among these DEGs were those encoding regulatory and core particles of 26S and 20S proteasomes, which mediate ubiquitin- dependent proteolysis and are involved in antigen processing and presentation, respectively (Figure 6A). Dexamethasone significantly suppressed the expression of most of these genes, including proteasome 20S (PSM) subunit alpha 1 (*PSMA1*) and *PSMB7*, *PSMB9* and *PSMB12* (Figure 6B). This suggests that hormonal stress responses may influence the proteolytic activity and MHC class I antigen processing and presentation following subsequent encounters with pathogens.

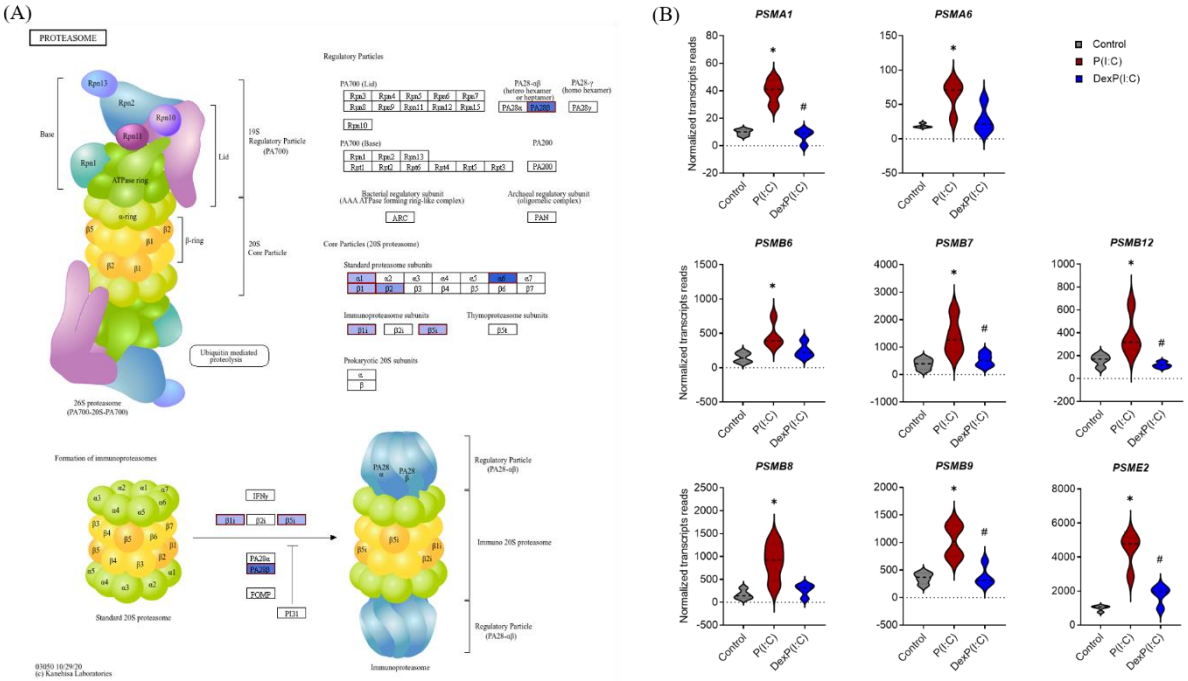

Figure 6. Differential expression analysis of selected genes associated with the formation of the proteasome apparatus in A. salmon RBCs, stimulated either with 50 µg/mL poly (I:C) or 100 µM dexamethasone and 50 µg/mL poly (I:C), compared to unstimulated controls *ex vivo.* (A) Genes encoding regulatory and core particles of the proteasome. Blue: Genes significantly induced by poly (I:C), but suppressed following pre-treatment with dexamethasone. This figure was modified from "Proteasome" pathway- sasa03050, to include only subunits of interest. The darker the color the stronger the effect of dexamethasone (B) Selected genes with significantly different expression patterns between RBCs stimulated with poly (I:C) alone and those treated with dexamethasone and poly (I:C), compared to unstimulated controls. *$p \leq 0.01$ in poly (I:C)-stimulated RBCs compared to unstimulated controls; # $p \leq 0.01$ in dexamethasone and poly (I:C)- stimulated RBCs compared to unstimulated controls.

4. Discussion

Environmental and physical stressors in aquaculture trigger cortisol release and subsequent GR signaling, facilitating physiological adaptations essential for maintaining internal homeostasis (47). While cortisol measurement is an established method for assessing stress in fish (48), this approach may be limited in capturing the secondary effects of cumulative stressors, particularly due to impaired HPI sensitivity under allostatic overload (49,50). In this study, we demonstrated that A. salmon RBCs, which express GRs at high levels (10), responded to GCs both *ex vivo* and *in vivo*, and changes in the expression pattern of some genes, including *DDIT4* and *FKBP5*, could serve as indicators of stress response in fish health assessment. However, it should be noted that the transcriptional responses of RBCs to dexamethasone *ex vivo* showed little resemblance to those induced in blood cells by cortisol injection *in vivo*. This discrepancy, which is discussed further below, may have arisen from the absence of complex systemic factors that regulate gene expression dynamics in a living organism, as opposed to the controlled conditions of cell studies (51).

Cortisol and dexamethasone are structurally similar compounds (45). Dexamethasone is more stable than cortisol, with approximately twice the plasma half life (52). It also exhibits higher receptor binding affinity, and thereby higher potency than cortisol at equivalent doses (45). In A. salmon RBCs, both dexamethasone and cortisol induced comparable transcriptional changes in genes involved in antiviral defense (e.g. *MX1* and *ISG15*) and physiological stress responses (e.g. *FKBP5*) in a dose- dependent manner, as expected. Unlike mammals, most fish possess two GR genes (GR1 and GR2), the activation of which may vary across tissues depending on circulating GC levels during a stress response (39,53–55). Previous genetic analysis revealed four copies of GRs (two GR1 and two GR2) in the A. salmon genome (39). This is consistent with our transcript findings, where all four GR variant genes were found to be highly expressed in RBCs. While GR gene activation has been demonstrated following a 24 h glucocorticoid exposure in several tissues, little is known about their expression kinetics overtime (39). In this sense, the absence of significant differences in GRs transcriptional profile between dexamethasone- stimulated RBCs and unstimulated controls may be attributed to the duration of glucocorticoid exposure and the timing of the analysis. Notably, GR expression levels in blood of A. salmon injected with cortisol *in vivo* remained at baseline at both time points, despite a pronounced cortisol peak observed at day two.

It has previously been reported that gilthead seabream (*Sparus aurata*) treated with cortisol showed higher accumulation of triglycerides in liver, contrasting with increased glycogen storage in dexamethasone- treated fish (56). Here, dexamethasone induced the overexpression of the *ELOVL4* gene, suggesting that GR- mediated signaling may influence lipid metabolism in RBCs. However, whether the activation of genes involved in LC-PUFA biosynthetic pathways (40) serves as a compensatory mechanism for the cells to meet the energy demands of stress responses, and/or whether it affects the regulation of other biological processes in RBCs, such as the innate immune response, remains to be explored.

Both dexamethasone and cortisol acted as potent modulators of the *FKBP5* gene in A. salmon RBCs, reinforcing its potential role in the stress response in fish (57). Previous research of GR signaling in fish and mammalian models demonstrated that when cortisol levels are within the normal physiological range (resting state), the FKBP5 protein interacts with the GR complex hindering its translocation into the nucleus by reducing its affinity for GCs. Once the concentration of circulating GCs increase, FKBP5 is degraded, allowing GR/GC complex to enter the nucleus and initiate the expression of target genes by binding to GR elements (GREs) (57,58). *KLF9* has been characterized as a key regulator of *FKBP5* activity and, consequently, GR- signaling during stress responses in zebrafish (57). Here, the significantly high expression levels of *FKBP5* and *KLF9* following dexamethasone stimulation, compared to their very low expression (close to zero transcripts detected) at resting state, suggests that RBCs may actively contribute to their own regulation of stress responses.

The concentration of cortisol in the bloodstream of fish has been shown to increase within minutes to hours in response to stress (59), typically returning to baseline levels in 24 to 48 h post exposure to the stressor (43,44). Interestingly, the relative expression of *FKBP5* gene in dexamethasone- treated RBCs remained significantly high up to 14 days post stimulation, compared to unstimulated controls. Thus, although plasma cortisol may be depleted within a few days after a stressful event, secondary stress responses may still be detectable. Notably, *FKBP5* activation was triggered even at very low GC doses, without a corresponding increase in gene mRNA levels at higher GC concentrations. Based on these findings, in scenarios where fish were exposed to consecutive stressors of varying intensities, *FKBP5* expression levels in blood would likely remain persistently high. Therefore, targeted transcriptional analysis of *FKBP5* alone may not be a sufficient indicator of the specific effects or intensity of the different stressors. This was evident when investigating transcriptional responses of *FKBP5* in whole blood of A. salmon two and four days post cortisol injection (dpi), compared to untreated controls; despite variability in individual responses, *FKBP5* mean expression levels were not significantly different between control and cortisol injected groups. Although differences in the experimental procedures (e.g. use of anesthesia) or the type of biological material used in each analysis (i.e. whole blood vs isolated RBCs) may contribute to transcriptional inconsistencies between the *ex vivo* and *in vivo* findings, the precise underlying factors are difficult to anticipate.

An intriguing observation was the upregulation of DNA damage inducible transcript 4 (*DDIT4*) gene in both the whole blood of cortisol- injected fish and *ex vivo* dexamethasone- stimulated RBCs. In mammals, *DDIT4* regulation, like that of *FKBP5* and *KLF9*, is directly modulated by GR- signaling (60). Previous transcriptional analyses of mammalian skeletal muscle and blood cells have shown significant upregulation of *DDIT4* expression under hypoxic conditions and/or GC administration, supporting its characterization as a promising biomarker of the stress response (61,62). In A. salmon whole blood, *DDIT4* overexpression was observed at two days post cortisol injection, coinciding with peak cortisol levels. By day four, as cortisol levels declined, *DDIT4* expression returned to basal levels. Similarly, *DDIT4* upregulation was reported in RBCs four days post stimulation with dexamethasone, compared to unstimulated controls. This dose-response relation between GC dynamics and *DDIT4* regulation could serve as a reliable indicator for assessing both the onset and attenuation of stress response in fish.

Fish RBCs are immunologically active, engaging in diverse innate and adaptive immune processes in response to external stimuli (10,63–66). Their role as mediators of innate immunity was first demonstrated in rainbow trout RBCs following a 24 h-exposure to poly (I:C), which triggered the expression of *TLR3, IFNa* and *MX* immune genes (13). Similar antiviral responses, indicative of dsRNA-mediated immunostimulation, were later described in A. salmon RBCs infected by the dsRNA virus PRV-1 in both *ex vivo* and *in vivo* studies (10,12,67). Here, poly (I:C) served as a model to mimic the immune responses typically occur during the acute phase of RBC infection by PRV-1 (10). In particular,

genes implicated in dsRNA recognition, such as MDA5 and RLR-1/-3 (68), IFN-mediated transcriptional regulation (i.e. IRFs genes) and immune protection, were significantly induced following a three-day stimulation with poly (I:C). Genes involved in MHC class I antigen processing and presentation, like *PSMB7* and *MR1*, were also upregulated, the expression of which has not previously been documented in A. salmon RBCs. Despite the consistent effects of poly (I:C) on the gene expression profile of treated cells, the responses among individuals varied substantially. This may be due to differences in RBC maturation stage, with senescent cells displaying reduced reactivity compared to "younger" cells (10,70).

Chronic stress exerts negative effects on innate immune function by promoting prolonged cortisol release through the HPI-axis and stimulating GR/ GC- signaling pathways (21,71). In fish, stress-induced immunosuppression has been linked to the overexpression of anti-inflammatory mediators, such as NF-KB inhibitor alpha protein (*NFKBIAA* or *IKBA*), which inhibits IFNs production by disrupting *NF-KB* transcription regulator activity (21,72). Activation of GR/ CG- signaling pathway in A. salmon RBCs by dexamethasone significantly upregulated *IKBA*, which may underlie the attenuated responsiveness of some IFN- stimulated antiviral effectors (e.g. *RSAD2* and *MX2*) to poly (I:C) through repression of NF-KB and IFN signaling. The decreased expression levels of some cytokine receptor genes, such as tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*) which interacts with secreted TNF-a cytokine inducing innate immune response (73), may further support this hypothesis. In mammals, *NF-KB* has also been shown to interact with *FKBP5*, modulating the antiviral immune response (58,74,75). Specifically, *FKBP5* was found to promote IKBA degradation, thereby enhancing downstream cytokine production through NF-KB signaling (75). However, in the present study, a direct correlation of *FKBP5* role in the regulation of innate immunity in fish RBCs was difficult to establish.

Among the PRRs responding to poly (I:C), only the expression of *NLRC5* gene was entirely blocked by GCs, returning to basal levels. A previous study investigated the *NLRC5* transcriptional profile in several tissues of A. salmon at parr and smolt stages, and linked its downregulation to cortisol elevation, during smoltification (76). While GCs clearly affected the transcriptional regulation of innate immune genes in RBCs, specific molecular interactions between the endocrine and immune systems in this context remain poorly understood and warrants further investigation.

Poly (I:C) induced the expression of several MHC class I antigen and proteasome subunit genes was entirely blocked (down to baseline levels) by dexamethasone. The impairment of antigen processing and presentation by glucocorticoids has previously been demonstrated in mammalian dendritic cells through transcriptional and functional analyses (77). In particular, GR pathway activation prior to infection has been linked to reduced generation of antigenic peptides by disrupting the activity of proteolytic molecules essential for degradation of viral proteins (77). In salmonids, cortisol injection prior to IPNV infection resulted in significant downregulation of MHC class I gene in mucosal and lymphoid tissues

(78). While the exact mechanism in fish is not fully elucidated, suppression of genes encoding proteasome subunits, such as *PSMB-6 -9*, and MHC-I regulatory proteins, such as TAP-binding protein (*TAPBP*) which mediates antigen peptide transport across endoplasmic reticulum (ER), (79) may reduce the capacity of RBCs to present viral peptides on their membrane. This complex interrelation between hormonal stress responses, compromised antiviral immunity, and reduced antigen presentation may create conditions that favor viral replication and dissemination across tissues. This is in line with observation in trials where fish infected by PRV-1 and SGPV exhibited reduced robustness and increased mortality when exposed to stress (19,22).

In conclusion, A. salmon RBCs may play a critical role in physiological adaptations to stress, as they respond to cortisol and dexamethasone, modulating their immune and metabolic responses. *FKBP5* and *KLF9* genes may be key regulators of stress response in RBCs based on their transcriptional upregulation by GR agonists *ex vivo*. In cortisol- injected fish, the *DDIT4* gene, regulation of which appeared to be GC- dependent both *ex vivo* and *in vivo*, may emerge as a promising biomarker candidate of cortisol-mediated responses. Exposure of RBCs to GCs prior to stimulation with poly (I:C), resulted in the attenuation of several genes involved in IFN-mediated immunity and significant suppression of genes involved in proteolytic degradation and MHC class I antigen processing and presentation. Stress-mediated dysregulation of antiviral immune function and immunological memory may reduce both short-term and long term immune protection against viruses, and increase the susceptibility of previously stressed fish to subsequent infections.

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Supplementary File A

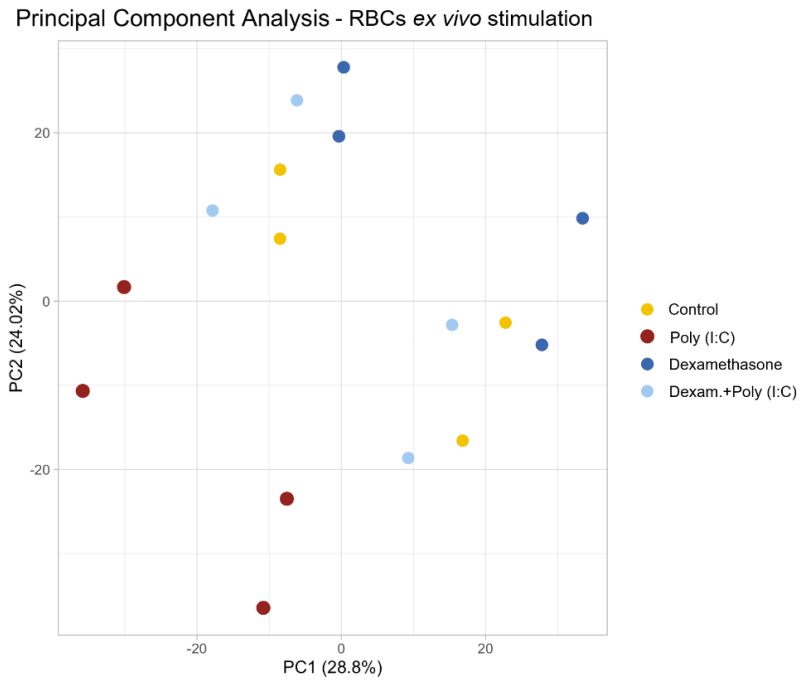


Figure 1. Principal component analysis for Atlantis salmon red blood cells (RBCs) treated with 100 µM dexamethasone (4 days), 50 µg/mL poly (I:C) (3 days), dexamethasone and poly (I:C) together, and untreated controls.

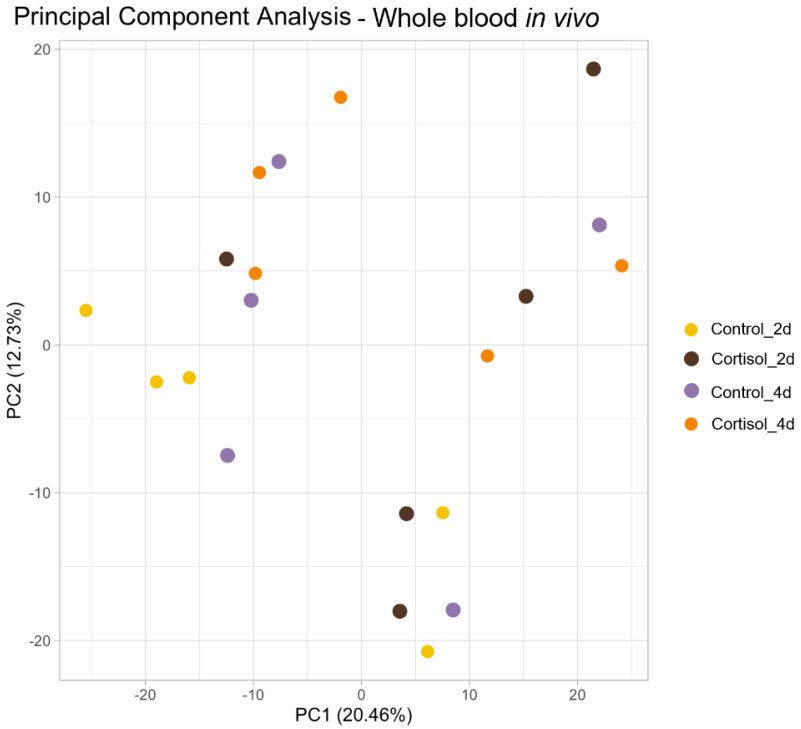


Figure 2. Principal component analysis for whole blood of Atlantic salmon two and four days post injection with cortisol, and non-cortisol injected controls.

Supplementary File B

UPREGULATED

Gene ID	Gene name	Control	Dexamethasone	Dex vs Ctrl_FC
klf9	KLF9	2	1157	600.623
LOC106592479	DDIT4	39	10961	268.208
fkbp5	FKBP5	32	7557	218.954
LOC106563189	INPP5K	1	154	138.504
LOC106565346	FKBP5	5	643	120.473
LOC106564638	ELOVL4	86	10370	92.459
LOC106597646	ELOVL4	76	8321	76.364
smtna	SMTNA	3	258	74.12
LOC106577395	DDIT4-like	51	3499	66.617
LOC106590608	MTRR	455	28134	59.139
LOC123732461	PRELID3A	1	83	46.252
LOC106593866	PRELID3A	2	95	43.909
higd1a	HIGD1A	92	3945	41.898
LOC106587747	MAP1B-like	5	238	41.454
LOC106575072	CDN1B	2	138	41.449
LOC106569333	ACKR2	4	102	26.304
LOC106575012	ZAN-like	156	4384	26.095
LOC123723897	PEX3	314	6762	21.298
LOC106610080	ZNF135-like	13	293	21.107
LOC106583048	TAS1R3	6	131	20.519
LOC106567009	DTX3	9	201	19.959
LOC106609100	CDKN1B-like	0	24	19.761
LOC106590610	CCT5-like	107	1890	17.143
phop1	PHOP1	67	1138	15.211
LOC106611779	FSCB	2	38	13.153
LOC106572176	STK35	37	508	12.881
LOC106588001	KLF13	1293	16126	12.213
LOC106591234	ATP6V0E2	9	126	12.211
LOC106572179	STMN3	4	72	11.42
wu:fb97g03	Prisilkin-39	14	144	9.403
LOC106563187	SLC35E4	628	6167	9.391
LOC106566795	OSBPL2	1807	17270	9.202
LOC106572529	COQ10A, mt-like	67	620	8.966
LOC106600990	SNRP116	6	64	8.371
LOC106592879	ASCC1	132	1067	7.879
myom1b	MYOM1B	1418	12261	7.851
LOC123732469	NSUN5, mt-like	22	185	7.753
LOC106563139	TNFRSF10A-like	0	10	7.577
LOC106583303	NR1D1	199	1394	6.634
LOC106594499	ANAPC16	546	3618	6.582
LOC106568381	ZBTB26-like	5	40	6.526
LOC106575013	ARL13B	85	593	6.389
LOC106565112	CRY1	1648	10204	6.047
LOC106588809	MYLIP-A-like	608	4011	5.957
LOC106568134	DMTN	323	2138	5.955
atpv0e2	ATPV0E2	30	197	5.874
LOC106573641	LYPA3	822	4932	5.59
LOC106582110	NR1D2	317	1767	5.094
errfi	#REF!	5245	26813	4.961
LOC106567511	STK10	2147	10768	4.913
tnfrsf21	TNFRSF21	3164	15386	4.775
LOC106583545	SRSF3-like	966	4776	4.758
mat2aa	MAT2AA	85	506	4.72
djb12	DJB12	303	1450	4.628

nfe2	NFE2	2076	9326	4.407
LOC106607014	EVPL	30	184	4.403
LOC106606241	rogdi atypical leuc	57	282	4.359
LOC106587663	FAM214A	136	624	4.201
LOC106572584	NFE2-like	819	3880	4.186
LOC106562488	FAM214A-like	657	2765	4.158
LOC106603812	CDKL3	5	35	4.133
LOC106587368	EVI5-like	26	122	4.128
lpin2	LPIN2	13732	58296	4.079
LOC106568378	RC3H2-like	28	128	4.052
nfkbiab	NFKBIAB	1240	5201	4.034
LOC106601178	IKBA	3334	12476	3.649
orai2	ORAI2	452	1719	3.64
LOC106569521	SEMA4E	17	70	3.631
bckdk	BCKDK	2045	7568	3.62
LOC106584368	MKNK2-like	3288	12106	3.596
LOC100136378	NR1D2	3815	13606	3.523
LOC106572181	TNFRSF6B-like	6	33	3.513
LOC106570576	MYLIP-A-like	408	1549	3.512
LOC106563185	OSBP2	661	2326	3.437
kel	KEL	7784	27311	3.428
LOC106561157	RASGRF2	748	2900	3.365
LOC106574587	ANKRD34B	22	100	3.332
LOC106560840	ANAPC16	695	2341	3.314
LOC106602713	TSPAN5-like	144	489	3.261
ccng2	CCNG2	346	1158	3.26
LOC106581405	ARAP1	1018	3449	3.236
LOC106608090	CIPC-like (?)	2164	7172	3.223
LOC106574963	CCDC80	2	18	3.204
LOC106607874	CD2AP-like	739	2651	3.148
ccdc113	CCDC113	9	38	3.146
LOC106605267	UB2E1	149	508	3.113
LOC106609880	ABCA1	484	2267	3.101
LOC106574322	Prkab1-like	15	57	3.056
LOC106605200	tmem106b	2158	6634	3.025
LOC106607496	NR1D1	278	902	3.013
acsl4	ACSL4	534	1676	3.004
LOC106563280	XPO6-like	4630	14199	3
LOC106603521	RPS6KB1-like	293	900	2.998
LOC106560826	ASCC1-like	243	730	2.939
LOC123731566	SNORA49	3	46	2.926
ivns1abpa	IVNS1ABPA	418	1291	2.914
LOC106602106	testis-specific gene	37	256	2.906
LOC106567574	UCH2	682	2055	2.881
LOC106605773	HLF	2252	6774	2.877
LOC106603377	FAA4	78	270	2.85
LOC106598261	LRATD2-like	7	56	2.795
LOC106568133	XPO7	8178	24334	2.792
LOC100380819	WEE1	401	1128	2.768
LOC106583166	PL8L1	425	1289	2.768
LOC106563389	PER1	1086	3047	2.758
LOC106563496	FKBP2	16	49	2.752
aurka	AURKA	189	704	2.729
LOC106609509	ARNT-like 1	428	1272	2.696
LOC106593434	FOXK1-like	1128	3070	2.652
LOC106564905	POLG2	157	441	2.627
LOC106607162	TEF	744	1994	2.597
LOC106573643	CK2A1	90	241	2.531
LOC106585919	LMBRD2B-like	395	1029	2.526

LOC106601241	TEF-like	2300	5990	2.517
LOC106569912	CDC42SE1-2	191	567	2.514
LOC106582745	TI17A	506	1291	2.505
LOC106567067	CBX5	587	1480	2.459
LOC106574508	SSBP3	4908	12091	2.371
LOC106607064	DDX5	1286	3247	2.363
LOC106578047	PICALM-like	4482	10775	2.356
akr1a1a	AKR1A1A	24	83	2.301
LOC106583488	CRY1	821	2027	2.298
LOC123742377	A8 protein	73	340	2.273
LOC106609006	EEF2K	294	750	2.272
LOC106590971	MTR	1657	3956	2.264
dbpb	DBPB	1462	3578	2.26
LOC106603519	VMP1-like	1404	3455	2.239
LOC106600803	FBXL19-like	234	551	2.199
LOC106564369	mitoferrin-2-like	769	1737	2.19
egln2	EGLN2	293	670	2.177
LOC106561642	PNPLA8	330	805	2.128
ckmt2	CKMT2	4	25	2.11
LOC106581989	BRD2	706	1544	2.104
LOC106563456	URP2	156	570	2.098
LOC106580771	PPP1R14A	209	508	2.098
LOC106584714	CHRNB4	26	78	2.091
LOC106594327	SPRED2-LIKE	369	898	2.088
LOC106572525	ANKRD52	2262	4994	2.08
LOC106574321	CNPPD1	3774	8583	2.08
LOC106591654	PLPPR3	5	20	2.08
LOC106575382	SSBP3-LIKE	4485	9830	2.068
LOC106579276	PDZD7	9070	23228	2.06
LOC106567068	COPZ1	1191	2636	2.054
LOC106564529	KLF1	349	811	2.034
LOC123732460	*Unchar.*	*1*	*184*	*85.425*
LOC106591240	*Unchar.*	*13*	*346*	*24.523*
LOC106575009	*Unchar.*	*394*	*8945*	*22.457*
LOC106583546	*Unchar.*	*296*	*5610*	*18.568*
LOC106588723	*Unchar.*	*44*	*362*	*7.065*
LOC106587354	*Unchar.*	*4*	*31*	*6.924*
LOC106602409	*Unchar.*	*87*	*381*	*4.216*
LOC106592047	*Unchar.*	*1814*	*7270*	*3.696*
LOC106594392	*Unchar.*	*809*	*2843*	*3.226*
LOC123733435	*Unchar.*	*1141*	*4333*	*3.068*
LOC123743169	*Unchar.*	*2*	*14*	*2.701*
LOC106605611	*Unchar.*	*13*	*48*	*2.147*

DOWNREGULATED

Gene ID	Gene name	Control	Dexamethasone	Dex vs Ctrl_FC
LOC106579975	PIP5KL1	1991	718	0.43
LOC106601396	SMURF2	305	116	0.434
LOC106573960	VLDLR	54	21	0.487
LOC106599289	ST17A	207	86	0.531
snx7	SNX7	345	155	0.538
LOC123729646	TC1A	794	331	0.443

Supplementary File C

UPREGULATED/ **Cortisol 2dpi**

Gene ID	Gene name	Control_2d	Cortisol_2d	Cortisol vs Ctrl_FC
LOC106575926	LIMCH1	4	167	29.28
LOC106592479	DDIT4	88	270	2.853
LOC106601992	TTC30A	12	101	5.703

DOWNREGULATED/ **Cortisol 2dpi**

Gene ID	Gene name	Control_2d	Cortisol_2d	Cortisol vs Ctrl_FC
ier2	IER2	323	79	0.276
LOC106565406	NR4A1-like	3464	978	0.308
LOC106569186	LAMA3-like	148	56	0.422
LOC106590650	IER2	381	85	0.252
LOC106592739	UBAC2	28	9	0.487
LOC106597100	HERC3	735	254	0.404
LOC106611802	PSBP1	186	52	0.324
LOC106613676	NRK2	242	44	0.293
LOC123724386	NRK2-like	212	45	0.333
LOC123732760	BTNL1	126	29	0.356
LOC106611644	Unchar.	63	21	0.404

UPREGULATED/ **Cortisol 4dpi**

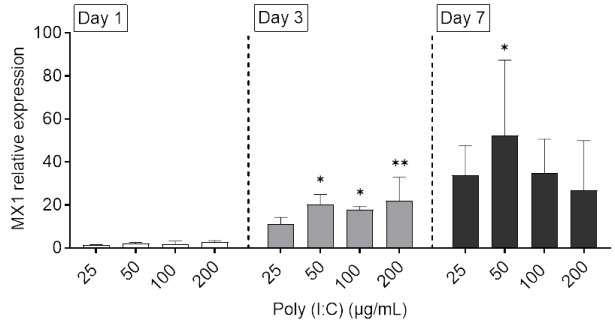
Gene ID	Gene name	Control_4d	Cortisol_4d	Cortisol vs Ctrl_FC
LOC106573520	ZP4	0	521	125
LOC106567858	PRPS1-like	0	88	9.523809524
LOC106567727	ZBTB16A	5	44	6.060606061
LOC123726086	SLAIN-like	0	32	2.202643172

DOWNREGULATED/ **Cortisol 2dpi**

Gene ID	Gene name	Control_4d	Cortisol_4d	Cortisol vs Ctrl_FC
cd37	CD37	53	3	0.087115602
LOC106590043	ZEB1	34	3	0.131682908
LOC123724045	IGIC1S1-like	103	10	0.165700083
LOC106608989	SLAMF9	69	7	0.251572327
LOC106589833	SOCS3	281	66	0.272553829
LOC106605673	CD79A-like	63	19	0.360750361
LOC106597100	HERC3	564	200	0.455788514
LOC100286404	KLF2	103	30	0.527704485
LOC106601675	IgD; MUCM	137	21	0.440334654
LOC123742469	Unchar.	62	2	0.060006001
si:ch211-119e14.1	Unchar.	52	5	0.194061712
si:dkey-24p1.1	Unchar.	66	9	0.243131534

Supplementary File D

(A)



(B)

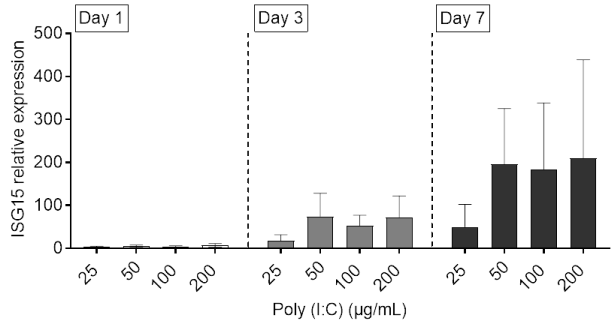


Figure 1. Antiviral responses in Atlantis salmon red blood cells (RBCs) treated with 50 µg/mL poly (I:C). (A) MX1 and (B) ISG15 was measured by RT-qPCR at threes samplings points, one-, three- and seven- days post exposure to poly(I:C). The expression levels in stimulated RBCs relative to the unstimulated controls were calculated for each sample (n=3) and the standard deviation (SD) is shown as error bars. Data were analyzed using One-way ANOVA Dunnett's multiple comparison test. *: $p < 0.05$; **: $p < 0.01$.