UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics
Norwegian College of Fishery Science

Atlantic salmon immune responses after infection with intracellular pathogens

Yorick Andreas van der Wal

A dissertation for the degree of Philosophiae Doctor

November 2022

Acknowledgements

There are so many people that have helped me reach this milestone, and I am so thankful to all of them, that it is hard to express the full extent of my gratitude to everyone.

First, I would like to thank Vaxxinova for enabling and financing my PhD research. Special thanks go to Carla for offering me this opportunity.

I am immensely grateful to both my supervisors, Jaap and Jorunn. Jaap has been a fantastic mentor on scientific, professional, and personal level. It was great to be able to discuss everything openly. Jorunn made me feel very welcome in the field of Fish Immunology and in Tromsø, helped me to grow as a researcher, and did a great job in translating my industry focused project proposal into an academic PhD project.

The support and friendship from my colleagues at Vaxxinova Münster have been invaluable. It was great to know that there was always someone willing to help or have a short talk, whether it was in the lab or in the office. I especially enjoyed sharing my office with Anke and discussing literally anything with Allan.

Everyone in the fish immunology group at the UiT has made me feel very welcome in Tromsø, and I really enjoyed the scientific discussions, dancing lessons, and skiing trip. A special shout out here to Linn and Henriette, who spent a lot of time setting me up in the lab and repeating experiments, to Shiferaw for sharing his expertise on the ELISpot and for proving excellent feedback on my writing, and to Agata and Dhivya for the very nice office talks, even when I was no longer sharing their office.

The first challenge experiments would not have been possible without my colleagues at Vaxxinova Norway, in particular Vicky and Bjørn, who were very helpful and willing to share their extensive experience. For their excellent input and nice discussions, I thank Jacob from Aquagen, Marcos from ADL Chile, and my colleagues at Vaxxinova Nijmegen.

Of course, I would not be here without my parents: Eric, who shared his wisdom and thirst for knowledge and Petra, who thought me the value of determination, which she herself exhibited by reading this entire thesis without background knowledge to find remaining typos. Finally, my thanks go out to my family (my sister Annika) and friends (a.o. Vague Friday and the Bratwurstbende) for their support and for keeping me sane and especially to my loving wife Annieke and my children Roan and Lara for their love, support, and incredible patience.

Many thanks, vielen Dank, tusen takk, heel erg bedankt, muchas gracias!

You will always be in my heart.

Table of Contents

List of papers

Paper I:
Antibody response after intraperitoneal *Piscirickettsia salmonis* infection in Atlantic salmon: duration and protection
Yorick A. van der Wal, Bjørn Krossøy, Marcos Mancilla, Jorunn B. Jørgensen, Jaap Kool
Short communication - unpublished

Paper II:
The importance of the Atlantic salmon peritoneal cavity B cell response: Local IgM secreting cells are predominant upon *Piscirickettsia salmonis* infection.
Yorick A. van der Wal, Shiferaw Jenberie, Henriette Nordli, Linn Greiner-Tollersrud, Jaap Kool, Ingvill Jensen, Jorunn B. Jørgensen
Developmental and Comparative Immunology 123 (2021) 104125

Paper III:
CRISPR-Cas induced IRF3 and MAVS knockouts in a salmonid cell line disrupt PRR signaling and affect viral replication
Yorick A. van der Wal, Henriette Nordli, Allan Akandwanaho, Linn Greiner-Tollersrud, Jaap Kool, Jorunn B. Jørgensen
Manuscript ready for submission

Paper IV:
***Piscirickettsia salmonis* growth in the salmonid cell line CHSE-214 is affected by IRF7, IRF3, and MAVS knockouts**
Yorick A. van der Wal, Allan Akandwanaho, Henriette Nordli, Linn Greiner-Tollersrud, Jaap Kool, Jorunn B. Jørgensen
Short communication - unpublished

Papers published related to the PhD (not part of the thesis):

Microbial Danger Signals Control Transcriptional Induction of Distinct MHC Class I L Lineage Genes in Atlantic Salmon
Steingrim Svenning, Agata T. Gondek-Wyrozemska, Yorick Andreas van der Wal, Børre Robertsen, Ingvill Jensen, Jorunn B. Jørgensen and Eva-Stina Edholm
Front. Immunol. 10:2425.

Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene *ifit5* in a teleost fish
Dennis Berbulla Bela-ong, Linn Greiner-Tollersrud, Yorick Andreas van der Wal, Ingvill Jensen, Ole Morten Seternes, Jorunn B. Jørgensen
Developmental and Comparative Immunology 111 (2020) 103746

Thesis summary

The aquaculture industry has been able to grow extensively during the past decades, in part due to the success of vaccines against extracellular bacteria. Unfortunately, intracellular pathogens have become a bigger threat for farmed fish, and the development of effective vaccines against these has proven to be an issue.

In this thesis, we focused on the responses of two arms of the Atlantic salmon immune system against these pathogens: the antibody (Ab) production by B cells after challenge with the facultative intracellular bacterium *Piscicricketsia salmonis* and the interferon (IFN) responses in a salmonid cell line against salmon alphavirus (SAV), infectious necrotic pancreas virus (IPNV), and *P. salmonis*. Our goal was to develop tools and to generate knowledge on these immune responses that would contribute to the development of effective vaccines against intracellular pathogens, with a special focus on *P. salmonis*.

We optimized a CRISPR-Cas protocol using ribonucleoprotein delivery to induce knock outs (KOs) in the salmonid cell line CHSE-214 and used this to develop IRF3, IRF7, and MAVS KOs. We show that induction of IFN responses is disrupted in the IRF3 and MAVS KOs, while we did not observe effects of similar magnitude in the IRF7 KO. Although replication of SAV was positively affected in the KOs with disrupted IFN induction, IPNV replication and *P. salmonis* growth were negatively affected by these KOs.

In order to investigate Ab production after *P. salmonis* infection of Atlantic salmon, we developed two intraperitoneal (IP) challenge models. In the first of two studies, we observed a significant increase in anti-*P. salmonis* serum Abs at 14 weeks post challenge (wpc), although they were no longer elevated at 18 wpc. The fish were protected against a secondary challenge at 14 wpc, while the protection might have been reduced at the later time point. In the second *in vivo* study, we investigated the origin and specificity of early Ab responses using the ELISpot and ELISA assays we developed. A striking find was that IP challenge resulted in a major increase of leukocytes, total IgM Ab secreting cells (ASC), and anti-*P. salmonis* ASC in the peritoneal cavity (PerC), when compared to the head kidney and spleen. Furthermore, we observed an early increase of non-specific Ab production, while the specific Ab response dominated at the later time point.

At the end, we discuss how our findings fit together in a model of specific and non-specific activation of B cells through B cell receptors and pattern recognition receptors, respectively,

which could explain the early presence of non-specific Abs. Our model addresses the location of early Ab production, the transition to a more specific Ab response, and the possible functions of the PerC and its adipose tissue in this. Finally, we address the duration of Ab responses against *P. salmonis*, how our findings can contribute to the development of vaccine strategies with long term protection, and we suggest possible avenues for future research.

Abbreviations

Ab	Antibody	NC	Negative control(s)
AID	Activation-induced cytidine deaminase	NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
APC	Antigen presenting cell	*P. salmonis*	*Piscirickettsia salmonis*
ASC	Antibody-secreting cell(s)	PAMP	Pathogen associated molecular pattern
AT	Adipose tissue	PD	Pancreatic disease
BCR	B cell receptor	PerC	Peritoneal cavity
CARD	Caspase activation recruitment domain	PRR	Pattern recognition receptor
CPE	Cytopathic effect	RLR	RIG-I-like receptor
dpi	Days post infection	RNP	Ribonucleoprotein
dpv	Days post vaccination	RPS	Relative percent survival
HK	Head kidney	RRV	Simian rhesus rotavirus
IFN	Interferon	SAV	Salmon alphavirus
Ig	Immunoglobulin	Sc	Single cell clone(s)
IP	Intraperitoneal	SRS	Salmonid rickettsial septicemia
IPNV	Infectious pancreatic necrosis virus	TLR	Toll-like receptor
IRF	Interferon regulatory factor	TM	Transmembrane
ISG	Interferon stimulated gene	UK	Bovine UK strain
ISRE	Interferon-sensitive response element	WNV	West Nile virus
KO	Knock out	wpc	Weeks post challenge
MAVS	Mitochondrial antiviral-signaling protein	wt	Wild type
MEF	Mouse embryonic fibroblasts	*Y. ruckeri*	*Yersinia ruckeri*
MHC	Major histocompatibility complex		

1. Introduction

1.1 Aquaculture: vaccines and diseases

The aquaculture industry has become an important part of the global food supply and has grown extensively in the past decades (FAO 2020). The extensive growth of this industry is supported by the development of protective vaccines. The most prominent examples for this are the inactivated bacterial vaccines that allowed the Atlantic salmon industry to grow the last decades (Gudding et al. 2014). Though these vaccines have kept many diseases in check, several new and adapting pathogens require the development of new vaccines. The classical bacterial vaccines contained inactivated pathogens, but emerging viral diseases might warrant other vaccine concepts such as DNA or live vaccines (Adams 2019). Some viral diseases that currently form a big problem for the Norwegian aquaculture industry are the ssRNA virus salmon alphavirus (SAV) that causes pancreatic disease (PD), the dsRNA virus piscine myocarditis virus that causes cardiomyopathy syndrome, and the dsRNA virus piscine reovirus that causes heart and skeletal muscle inflammation (Sommerset et al. 2022). In the Chilean aquaculture industry, intracellular pathogens that pose major threats are the ssRNA virus infectious salmon anemia virus and the facultative intracellular bacterium *Piscirickettsia salmonis*, which causes salmonid rickettsial septicemia (SRS) (Flores-Kossack et al. 2020).

1.2 Salmon alphavirus

SAV is a pathogen of salmonids that causes PD at sea and sleeper disease in fresh water. PD was first reported in Scotland in 1976 (Jansen et al. 2017), and in Norway in 1989 (Poppe et al. 1989). It has been endemic in the south of Norway since 2003 (Aunsmo et al. 2010). PD was named after necrosis in the pancreas, the first described histopathological observation, although the heart is also a major target of infection (McLoughlin and Graham 2007). Affected Atlantic salmon and rainbow trout in sea cages present reduced appetite, lethargy, and increased mortality (McLoughlin and Graham 2007). Despite generally low mortalities, economic losses due to weight loss, reduced filet quality, and preventive measures are significant; Aunsmo et al. (2010) predicted that PD resulted in an 14,4 million NOK increase costs for a 500.000 smolt site.

SAV is an enveloped, ssRNA alphavirus belonging to the family of the Togaviridae (McLoughlin and Graham 2007). The two open reading frames in the SAV genome encode a polyprotein that is cleaved into nsP1, nsP2, nsP3, and nsP4 and a 26S mRNA that produces the

glycoproteins E1, E2, E3, and 6K, where the latter is translated in the 6K protein that has a role in viral release or alternatively in the trans frame protein TF that is important for spreading within the host (McLoughlin and Graham 2007; Ramsey and Mukhopadhyay 2017). SAV can be cultured on several salmonid cell lines, with CHSE-214 and RTG-2 being originally used (McLoughlin and Graham 2007). Due to varying degrees of visible cytopathic effect (CPE) and low titers, other cell lines have been tested for SAV cultivation, with varying results (Herath et al. 2009). Originally, an analysis of genetic variation identified 3 subtypes of SAV: SAV1 contained isolates from PD in Atlantic salmon in Ireland and Scotland, SAV2 contained isolates from sleeper disease in rainbow trout, and SAV3 isolates were from PD affected Atlantic salmon in Norway (Weston et al. 2005). However, these subtypes were later expanded with 3 additional subtypes: SAV4 isolates came from Atlantic salmon farmed in Ireland and Scotland, SAV5 only included Scottish isolates from Atlantic salmon, and one isolate from PD affected Atlantic salmon in Ireland formed a separate clade: SAV6 (Fringuelli et al. 2008). The current subtypes circulating in Norway are maritime SAV2 and SAV3, both of which affect Atlantic salmon and rainbow trout at sea (Jansen et al. 2017). Graham et al. (2014) found strong cross-neutralization of antibodies (Abs) between the 6 subtypes, indicating that they are not subtypes according to the official alphavirus criteria, but they suggest keeping the nomenclature as it was established. This cross-reactivity of anti-SAV Abs suggests that there is a possibility of developing a monovalent SAV vaccine.

Commercial vaccines against PD have been available in Norway since 2002, but the disease has remained an issue, and the development of additional PD vaccines continued (Karlsen et al. 2012). Still, PD vaccination has helped to reduce mortalities and disease severity in the industry and was shown to lead to reduced shedding of SAV and thus reduced transmission (Skjold et al. 2016).

Due to the intracellular nature of the pathogen and the variable vaccine efficacy in the field, the use of DNA vaccines against PD has been evaluated. Robertsen and co-workers have evaluated an experimental DNA vaccine containing either the SAV3 structural polyprotein or the E2 protein alone and observed a markedly increased effect of the polyprotein containing DNA vaccine compared to the E2 alone or a commercial inactivated vaccine (Chang et al. 2017). Later that year, Elanco's PD DNA vaccine 'Clynav' was accepted in the European Union. When this DNA vaccine was compared with an inactivated commercial vaccine in an experimental setting, it resulted in superior Ab titres, reduced disease prevalence, and higher weight gain, but comparable survival after 1041°days (Thorarinsson et al. 2021). In another

investigation that included two controlled field studies, several commercial PD vaccines resulted in variable efficacies (Røsaeg et al. 2021). The only experimental group with significantly higher harvest weight than the other groups had been vaccinated with Clynav (Røsaeg et al. 2021), although full protection against mortality was not achieved.

1.3 Piscirickettsia salmonis

Viruses are not the only intracellular pathogens in salmonids, as some intracellular bacteria are also known to cause diseases. One important pathogenic bacterium is *P. salmonis*, the causative agent of SRS (Fryer et al. 1992). SRS is a systemic infection that has been, and still is, a major threat to the Chilean aquaculture industry (Flores-Kossack et al. 2020).

P. salmonis is a gram-negative Gamma proteobacterium that is related to the *Coxiella* and *Francisella* genera (Rozas and Enríquez 2014). It was first identified as member of the *Rickettsia* but was later reclassified and assigned its own family: *Piscirickettsiaceae* (Fryer and Lannan 2015). *P. salmonis* was initially described as being obligatory intracellular since it apparently only grew on cells, although survival in sea water for extended periods was observed (Lannan and Fryer 1994). In 2008, two different cell free agar media were developed that allowed the extracellular cultivation of *P. salmonis* (Mauel et al. 2008; Mikalsen et al. 2008), while a blood free medium was developed in 2012 (Yañez et al. 2012). The cultivation on cell free media confirmed the facultative intracellular nature of *P. salmonis*.

Routes of host entry for *P. salmonis* infection include the skin, gills, or intestine (Smith et al. 1999), and this leads to a systemic infection. Kidney, liver, spleen, intestine, brain, and ovary are internal organs that have been found to be affected, while external symptoms of SRS include skin lesions, ulcers, pale gills, and haemorrhages at the base of fins (Fryer et al. 1990; Rozas and Enríquez 2014). *P. salmonis* preferentially infects macrophages, where it replicates in cytoplasmic vacuoles (McCarthy et al. 2008; Rozas and Enríquez 2014). Its entry into the macrophage-like SHK-1 cell line is clathrin dependent and leads to rearrangements of the cytoskeleton (Ramírez et al. 2015).

P. salmonis infections have been observed in several fish species and in different countries: Canada, Scotland, Ireland, Norway, and Chile. Still, SRS seems to only be a major problem in the Chilean salmon industry. Chile is the second largest producer of salmonid fish, and the aquaculture industry is thus an important part of its economy. SRS outbreaks in Chile often lead to high mortalities and result in total annual losses of over $700 million USD (Maisey et

al. 2017). Due to these losses, SRS is the main reason for use of antibiotics in Chilean aquaculture, even though it is difficult to get high enough antibiotic concentrations in the intracellular compartment to kill the bacteria (Maisey et al. 2017). Emerging resistance in *P. salmonis* is another drawback of the antibiotic use (Henríquez et al. 2016).

Vaccination would be a great preventive measure against SRS, and several experimental vaccines have been tested (Evensen 2016). Early trials with formalin inactivated bacteria gave variable results (Smith et al. 1997), while heat- or formalin-inactivated vaccine preparations of a Scottish isolate gave 49,6% and 70,7% relative percent survival (RPS), respectively, to a challenge after 194 days (Birkbeck et al. 2004). Experimental subunit vaccines were also developed. Kuzyk et al. (2001) showed a protection of 58,6% RPS 8 weeks after vaccination with an OspA fusion protein, which was increased to up to 83,0% RPS when T cell epitopes were included in the fusion protein. A second study, by Wilhelm et al. (2005), showed variable protection 49 days after vaccination with different compositions of possible protective proteins, with the combination of Hsp60, Hsp70, and FlgG resulting in the highest protection: 95,8% RPS. The sera of surviving fish from this group reacted to the recombinant proteins in Western blot for a period of 8 months after vaccination (Wilhelm et al. 2005). Finally, a DNA vaccine composed of expression vectors with inserts from a *P. salmonis* DNA library was tested in coho salmon. Although serum Abs recognizing *P. salmonis* were increased 60 days post vaccination (dpv), protection was low with only 20% of vaccinated fish surviving the challenge at 60 dpv (Miquel et al. 2003).

The commercially available vaccines in Chile that contain SRS components numbers over 30. Most of them are inactivated, while a few are subunit vaccines, and one live attenuated vaccine is available (Maisey et al. 2017). Intraperitoneal (IP) injection is the common delivery route, although two vaccines are administered orally. Intesal, a Chilean government body, tested the most used vaccines in 2014 for long term protection (1500°days) in a cohabitation challenge of Atlantic salmon and rainbow trout. Of all the tested vaccines, one gave an RPS of 16,4%, while the rest all were below 4% (Intesal 2014), showing that long term protection is a major issue for SRS vaccines. One solution to increase long term protection is to use booster vaccination, but IP boosters are not feasible once the fish are at sea. The oral vaccine from Centrovet enables booster vaccination against SRS, and they have shown that repeated oral boosters after initial IP vaccination keep serum Ab concentration at an elevated level that seems to protect against SRS outbreaks in the tested cages (Tobar et al. 2015).

1.4 Atlantic salmon immune responses after infection with intracellular pathogens

While SAV and *P. salmonis* are good examples of pathogenic viruses and intracellular bacteria in salmon, respectively, there are more intracellular pathogens affecting Atlantic salmon. While the viruses all need to infect host cells to replicate, several of the intracellular bacteria are facultative intracellular; they prefer infection of host cells to evade immune responses and to replicate, but they can also grow extracellularly (Rozas and Enríquez 2014). Generally, these pathogens follow several steps during the infection of the host. First, they need to enter the body and invade the host cells. After successful establishment in the cells, they replicate in the cells, sometimes relocate due to movements of the cells in the body, and finally release themselves from the cell to infect new host cells. Host immune responses mainly focus on prohibiting entry, reducing replication, and clearing the pathogens (Abbas 2020).

Entry in the host is mainly countered through physical barriers combined with mucosal immunity, based on innate antimicrobial peptides, natural and adaptive Abs, and immune cells (Uribe et al. 2011). The skin in the largest physical barrier of the fish, and its protection is augmented by the layer of mucus that covers it (Uribe et al. 2011). Other sites of possible entry also enlist mucosal defenses: the gills, gut, and nose (Gomez et al. 2013).

Interferon (IFN) responses, which we will address in more detail in section 1.5, are innate responses important in inhibiting entry, establishment, and replication in host cells. In short, recognition of pathogens leads to expression of IFNs, which in turn lead to expression of interferon stimulated genes (ISGs) that have several functions to counter viral infection and replication in host cells (Robertsen 2018).

Just like extracellular pathogens, intracellular pathogens can be internalized by antigen presenting cells (APCs) such as macrophages, after which antigens will be presented on major histocompatibility complex (MHC) molecules (Abbas 2020). This process can partially be evaded by intracellular pathogens while they reside inside host cells (Guo et al. 2019). Antigen presentation leads to adaptive immune responses in the form of Ab production after B cell activation, which we will discuss in section 1.6, or/and activation of cytotoxic T lymphocytes (Abbas 2020). Abs bind and neutralize intracellular pathogens while they are extracellular, mainly inhibiting reinfection after release from infected cells. In addition, Abs target natural killer cells to infected cells through Ab-directed cell-mediated cytotoxicity (Abbas 2020). Cytotoxic T lymphocytes are antigen specific, and binding of MHCI complexes that present

antigens on infected cells will lead to clearance of the infected cells (Fischer et al. 2013). These adaptive immune responses are very important in clearing the pathogens from the host, while the innate responses are more focused on prohibiting entry and slowing down replication.

1.5 IFN responses

An important component of innate immune responses against intracellular pathogens, in particular viruses, is the IFN induced antiviral state in host cells. Since teleost fish are more dependent on their innate immune system than mammals (Uribe et al. 2011), it is not surprising that they have an extensive IFN repertoire. This is illustrated by the vast number of IFN genes in salmonid genomes, of which it is unclear whether all have been identified (Sun et al. 2009; Zou et al. 2014; Liu et al. 2020, appendix). Evaluating the interactions of intracellular pathogens with the host IFN responses could lead to new insights that can help designing more effective vaccines, for example through the addition of novel adjuvants that modulate IFN responses.

IFNs are cytokines that play an important role in immune signaling. Type I (IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, and IFNτ) and type III (IFNλ1, IFNλ2, and IFNλ3) IFNs signal in innate immunity, while IFNγ, the type II IFN, bridges innate and adaptive immunity (Robertsen 2018). Type I and II IFNs are present in teleost fish but type III IFNs have not been identified at this time (Robertsen 2018). Teleost fish possess several type I IFNs, although these are not orthologues of the mammalian type I IFNs: both sets of type I IFN subtypes have evolved independently from single progenitors (Robertsen 2018).

Type I IFN transcription is generally induced after host cells recognize pathogen invasion through pattern recognition receptors (PRRs) (Robertsen 2018). IFNs signal paracrine or endocrine through IFN receptors, although autocrine signaling of alternatively spliced IFN1 through an intracellular IFN receptor was suggested for rainbow trout (Chang et al. 2013). IFN binding by IFN receptors on cells nearby or else in the body activates the JAK-STAT pathway, resulting in the expression of ISGs (Robertsen 2018). ISGs are diverse genes with several functions, but they usually antagonize various stages of viral infection. Classical examples of ISGs are Mx genes, IFIT genes, and PKR (Robertsen 2018). Several ISGs have been identified in teleost fish, although only a few have been characterized (Robertsen 2018). In Atlantic salmon, Mx1 (Larsen et al. 2004) and IFIT5 (Bela-Ong et al. 2020) show antiviral activity.

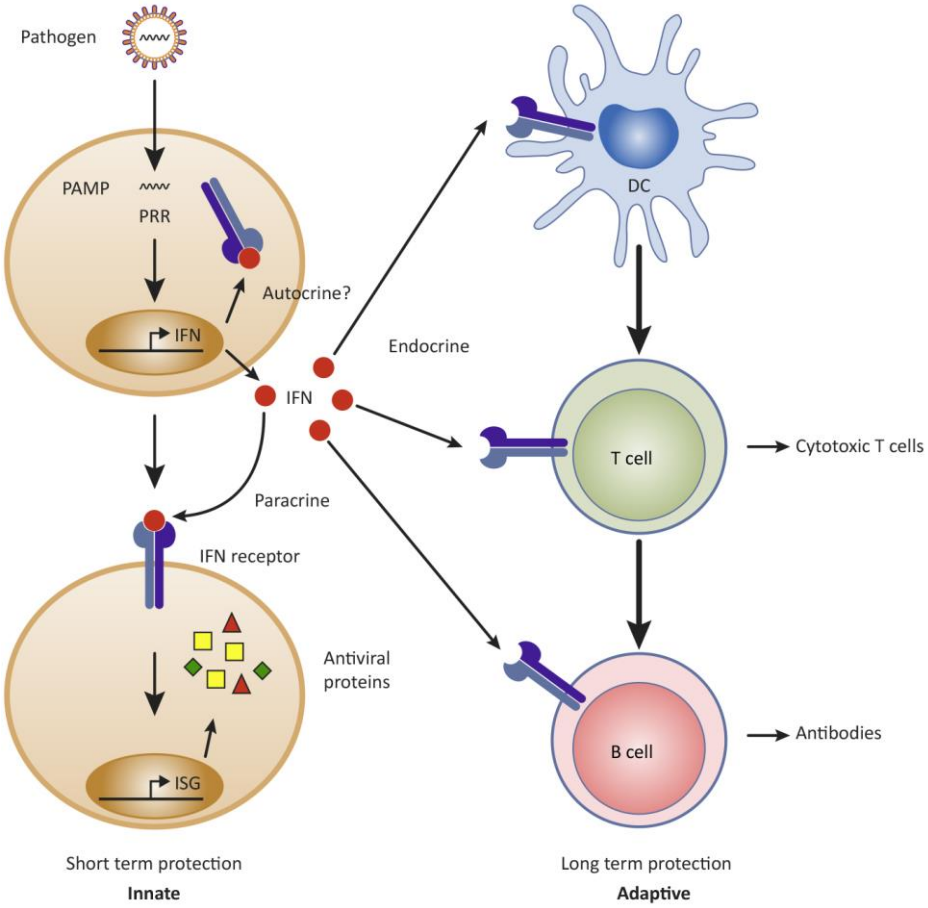


Figure 1: *Activation and functions of IFN responses. Modified from Robertsen (2018).*[1]

In mammals, IFN signaling also bridges innate and adaptive immunity by stimulating T cells, B cells, and dendritic cells (Lazear et al. 2019), and IFNa stimulation of B cells was recently observed in rainbow trout (Benedicenti et al. 2020). As a result, IFN inducing PRR ligands such as poly I:C and CpGs have been observed to increase Ab levels when used as adjuvants in both mammals (Le Bon et al. 2001; Liu et al. 2011) and Atlantic salmon (Strandskog et al. 2008; Thim et al. 2012).

PRRs enable host cells to sense pathogens by recognizing pathogen associated molecular patterns (PAMPs). Over the years, several families of PRRs have been identified, such as toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (Liao and Su 2021). The TLRs were the first to be described and are also the most numerous. TLRs are anchored in the membrane, can be extracellular or endosomal, and different TLR subfamilies bind different PAMPs: TLR1 lipopeptides, TLR3 dsRNA, TLR4 LPS, TLR5 flagellin, TLR7

[1] The original figure was published in Developmental and Comparative Immunology, 80, Børre Robertsen, The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon, 41-52, Copyright Elsevier (2018).

ssRNA (including TLR9 CpG DNA), and TLR11 proteins and nucleic acid (including TLR21 CpG DNA and TLR22 dsRNA) (Liao and Su 2021). Vertebrate species have different numbers of TLR genes, with 10 in human, 13 in mice, and more, including some fish- specific, identified in most teleost fish, such as 20 in Atlantic salmon (Arnemo et al. 2014; Lee et al. 2014) and up to 41 TLR gene copies in Atlantic cod (Khan et al. 2019; Liao and Su 2021). After ligand binding, TLRs initiate a signaling pathway that is dependent on one of two adaptors: MyD88 or TRIF, which leads to the activation of the transcription factors interferon regulatory factor (IRF) 3, IRF7, and/or nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Liao and Su 2021). All these transcription factors initiate IFN expression, although NFκB mainly induces transcription of proinflammatory cytokines (Liao and Su 2021).

The RLR family of PRRs consists of three members: RIG-I, MDA5, and LGP2. These cytosolic receptors sense ssRNA or dsRNA (Jami et al. 2021). The function of LGP2 is probably regulatory due to interactions with the other two RLRs, while RIG-I and MDA5 activate a signaling pathway (Jami et al. 2021). RIG-I and MDA5 contain a caspase activation recruitment domain (CARD) that interacts with the CARD domain on mitochondrial antiviral-signaling protein (MAVS) upon ligand binding (Jami et al. 2021). MAVS is anchored to the mitochondrial membrane through a transmembrane (TM) domain that is essential for its function and can activate TBK1 (Chen et al. 2017). The RLR signaling pathway results in activation of IRF3 and/or IRF7 and the transcription of IFNs (Chen et al. 2017).

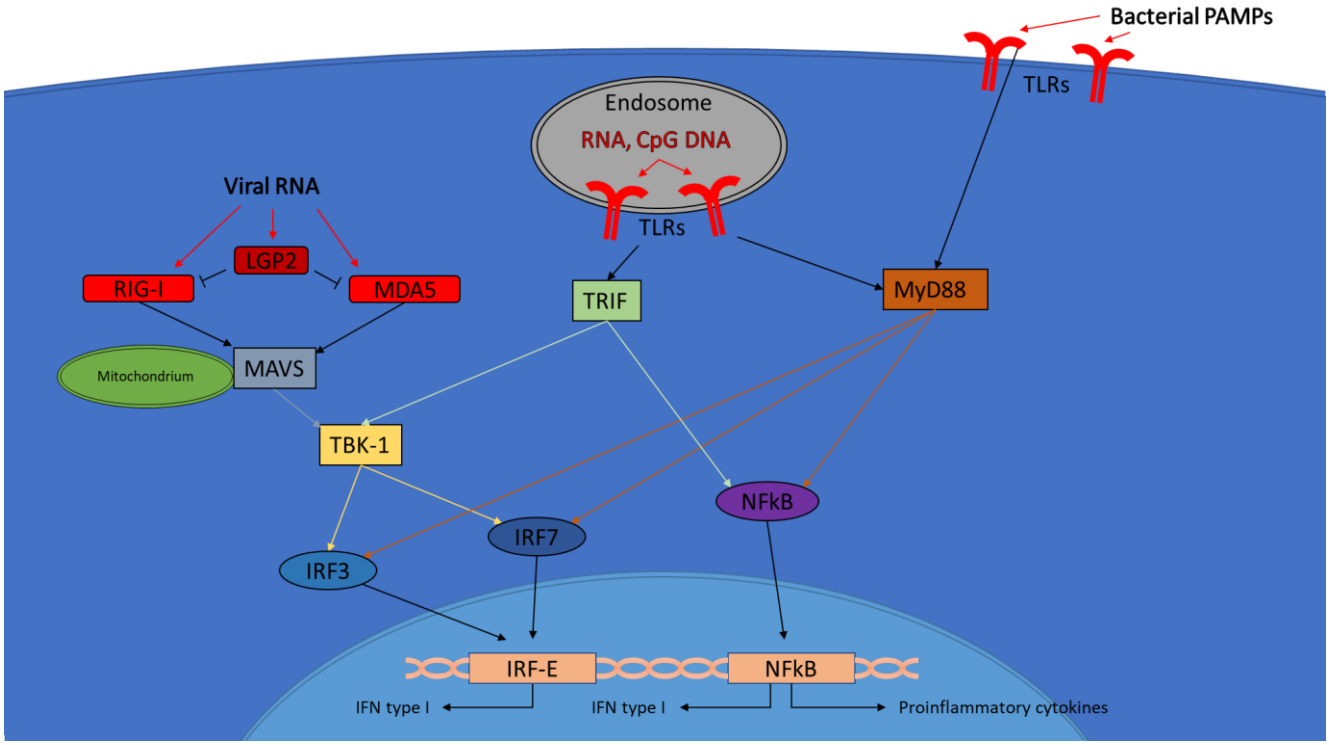


Figure 2: *PRR signaling pathway leading to IFN expression.*

1.5.1 MAVS

MAVS is known under several names (IPS-1, VISA, and cardif) after it was described by different studies at around the same time (Seth et al. 2005; Kawai et al. 2005; Meylan et al. 2005; Xu et al. 2005). It has been identified as a critical component of viral recognition and IFN response initiation (Seth et al. 2005) and is a central part of RLR signaling. Seth et al. (2005) showed that IRF3 and NFκB were not activated by Sendai virus infection of HEK293 cells after silencing of MAVS, while MAVS overexpression resulted in increased IFN expression. In addition, both the conserved CARD and TM domains were found to be necessary for MAVS function.

Biacchesi et al. (2009) cloned MAVS from several teleost fish species and observed strong protection against viral infection in CHSE-214 and EPC cells when salmon MAVS was overexpressed. This overexpression also led to increased IFN and ISG levels in EPC cells. Mutations in the CARD or TM domains abolished induction of protection against viral infection, similar to what was observed in the mammalian system (Biacchesi et al. 2009). At the same time, Lauksund et al. (2009) presented similar results for Atlantic salmon IPS-1 (MAVS). MAVS overexpression in CHSE-214 cells activated the salmon IFNa1 promoter and an NFκB dependent promoter and protected cells against infectious pancreatic necrosis virus (IPNV) infection. Again, these observations were dependent on intact CARD and TM domains. Carp MAVS overexpression also induces the IFN promoter as shown by Zhang et al. (2014) in CAB cells, and this is dependent on TBK1, IRF3, and IRF7. Intracellular poly I:C activation of IFN promoter activity was increased by carp MAVS overexpression but inhibited by dominant negative mutants of MAVS and RIG-I, indicating that these are necessary for the signaling in CAB cells (Zhang et al. 2014).

Several studies investigated the effect of MAVS knock outs in mice, but the results on viral replication differ strikingly. One study showed that IPS-1 (MAVS) knock out (KO) mouse embryonic fibroblasts (MEFs) did not express ISG54 after viral infection, but production of viral antigen differed between viruses (Loo et al. 2008). Production for Sendai virus was comparable to wild type (wt) cells, while a reovirus, dengue virus type 2, and two influenza viruses showed reduced production in MAVS KO MEFs (Loo et al. 2008). Similar results were reported by Sen et al. (2011), who showed that MAVS KO MEFs no longer produced IFN or ISG56 after poly I:C transfection or infection with either of the rotaviruses bovine UK strain (UK) or simian rhesus rotavirus (RRV). However, the replication of the investigated viruses

was again differently affected, with no change in RRV titers and increased titers for UK in MAVS KO MEFs. An interesting difference between these viruses is that RRV inhibits IFN production in wt MEFs, while IFN production is induced by UK infection (Sen et al. 2011).

In vivo viral infections in MAVS KO mice show tissue specific effects on viral loads. The Dengue virus load in infected cardif (MAVS) KO mice, for example, was increased in lymph nodes and bone marrow, but not significantly different in kidney, liver, and intestine compared to wt mice (Perry et al. 2009). Coxsackie B virus infection of MAVS KO mice resulted in reduced survival time and produced less IFNα and IFNβ, but viral titers in sera were not higher when compared to wt mice (Wang et al. 2010).

1.5.2 IRF3

Human IRF3 was first described by Au et al. (1995) and was observed to be phosphorylated during viral infection. This results in relocation of IRF3 from the cytoplasm to the nucleus, where it activates interferon-sensitive response element (ISRE) containing promoters (Hiscott et al. 1999). IRF3 has now been established as one of the main transcription factors responsible for IFN expression after PRR activation in mammals (Dalskov et al. 2020). IRF3 contains several important functional domains: the N-terminal DNA binding domain, the IRF association domain, and the phosphorylation sites or viral activated domain at the C-terminus (Hiscott et al. 1999).

Atlantic salmon and crucian carp IRF3 also contain the conserved DNA binding, IRF association, and virus activated domains (Bergan et al. 2010; Sun et al. 2010). The salmon IRF3 IRF association domain was necessary for induction of the IFNa1 promoter (Bergan et al. 2010), and a similar effect of phosphorylation on relocation and transcriptional activation as in mammals was observed in carp (Sun et al. 2010). MyD88, an adaptor protein important in TLR signaling, interacts with IRF3, and has a synergetic effect on promoter activity in Atlantic salmon (Iliev et al. 2011).

A KO of IRF3 generally seems to increase viral replication or disease severity in mammalian models, but cell type specific differences in IFN responses have been observed. IRF3 KO and IRF3-IRF7 dKO mice show increased mortalities after West Nile virus (WNV) infection, but IRF3 KO in mice only has a minor effect on type I IFN induction (Daffis et al. 2007), whereas IRF3-IRF7 dKO mice had significantly lower type I IFN production (Daffis et al. 2009). Daffis et al. (2007) also showed that macrophages from IRF3 KO mice had increased viral titers and

increased type I IFN production after WNV infection, which is probably IRF7 dependent, whereas primary neurons from IRF3 KO mice were only slightly more susceptible to infection and showed markedly decreased type I IFN production after WNV infection. Similarly, IRF3 KO MEFs lacked the ability to produce IFN after poly I:C stimulation or rotavirus infections and facilitated increased viral replication (Sen et al. 2011). Finally, Intervet has shown in a recent patent application (Langereis et al. 2020) that a KO of IRF3 and/or IRF7 in the MDBK cell line affects IFN signaling and increases titers of bovine respiratory syncytial virus.

1.5.3 IRF7

IRF7 was first identified during an investigation of Epstein-Barr virus latency and has an DNA binding domain that binds to certain ISREs in mammalian or viral promoters (Zhang and Pagano 1997). Human IRF7 is constitutively expressed in lymphoid cells but can also be induced in other cell types (Ning et al. 2011). Although IRF7 can initiate IFN responses in immune cells, it has an especially important role in the amplification of IFN responses (Dalskov et al. 2020). Like IRF3, IRF7 contains several regulatory domains aside from the DNA binding domain (Zhang and Pagano 2002).

Both Atlantic salmon IRF7A and IRF7B have the conserved DNA binding, IRF association, and virus activated domains, similar to Atlantic salmon IRF3 and their mammalian counterpart (Kileng et al. 2009; Bergan et al. 2010). Although MyD88 cotransfection could increase IRF3 mediated ISRE promoter activity in CHSE-214 cells, it failed to do so for both IRF7A and IRF7B (Iliev et al. 2011). Furthermore, cotransfection of MyD88 with the IRF7 expression plasmids into TO cells even led to a reduction of Mx1 or ISRE promoter activity, while the activity of a NFκB promoter was increased (Iliev et al. 2011). It is interesting to note that IRF7 was found to be constitutionally expressed in most investigated organs in both Atlantic salmon and crucian carp (Bergan et al. 2010; Zhang et al. 2003), which seems to be different from the lymphocyte specific expression in mammals.

Stable IRF7 KO in MDCK cells increased viral replication of at least two influenza strains (Hamamoto et al. 2013). Similar, IRF7 KO in the chicken cell line DF-1 increased avian influenza replication after 12 hours (Kim et al. 2020). IRF7 -/- KO mice showed more severe pathogenesis and increased viral replication in several tissues after WNV infection (Daffis et al. 2008). Increased replication was additionally observed in IRF7 -/- KO MEFs, macrophages, dendritic cells, and cortical neurons (Daffis et al. 2008). IRF7 -/- KO mice survival after herpes simplex virus type 1 infection was much lower than in IRF3 -/- KO mice, which showed no

mortalities, like the wt (Honda et al. 2005). In the same investigation, encephalomyocarditis virus infection of mice led to reduced survival in IRF3 -/- KOs, but survival in IRF7 -/- KO mice was even lower (Honda et al. 2005).

1.5.4 IFN responses viruses

Type I IFN responses against several viruses in salmonids have been studied, and the effects of viral infection on type I IFN responses are virus dependent. This is not surprising since viruses would have evolved to counter the effects of protective IFN responses (Guo et al. 2019). IFN responses in Atlantic salmon were first investigated in relation to IPNV infection and were shown to be very effective against this virus, as illustrated by the protection against IPNV in recombinant IFN-treated CHSE-214 (Robertsen et al. 2003; Ooi et al. 2008; Skjesol et al. 2009), ASK, and TO cells (Sun et al. 2011; Svingerud et al. 2012). However, IPNV seems to evade these effects by inhibiting the induction of IFN in certain cell types, since IPNV infection does not activate the Mx-1 promoter in RTG-P1 cells (Collet et al. 2007) and does not increase IFNa expression in CHSE-214 cells (Skotheim 2009), SHK-1 cells (Reyes-Cerpa et al. 2012), and TO cells (Lauksund et al. 2015). Several IPNV proteins seem to contribute to this effect, since preVP2, VP3, VP4, and VP5 all inhibit IPS-1 (MAVS) activation of IFNa, IRF1, and IRF3 promoters in TO cells (Lauksund et al. 2015). Nevertheless, some Atlantic salmon cells do retain the ability to mount a type I IFN response after IPNV infection, as indicated by Mx-1 promoter activation in primary macrophages (Collet et al. 2007) and IFN a1 expression in several tissues of IPNV infected salmon (Skjesol et al. 2011; Reyes-Cerpa et al. 2012).

In contrast to IPNV, SAV infection induces IFN responses in most cell types: CHSE-214 (Skotheim 2009), SHK-1 (Gahlawat et al. 2009), TO (Gahlawat et al. 2009; Xu et al. 2010; Xu et al. 2016), ASK (Munir et al. 2020), and ssp-9 (Bela-Ong et al. 2020). Interestingly, coinfection of SAV and IPNV in CHSE-214 cells did not induce IFN responses, indicating that the inhibitory effects of IPNV affect the ability of SAV to induce IFN as well (Skotheim 2009). Tissue and time dependent upregulation of type I IFN expression was also observed in SAV infected Atlantic salmon (Xu et al. 2012; Herath et al. 2013; Svenning et al. 2019). Similar to IPNV, recombinant type I IFN protects against SAV infection *in vitro* (Xu et al. 2010; Sun et al. 2011; Chang et al. 2016), mainly by reducing viral replication. In a number of *in vivo* experiments, expression plasmids of IFNb and IFNc induced protection against SAV and infectious salmon anemia virus infections in Atlantic salmon (Chang et al. 2014; Chang et al.

2015; Chang et al. 2016). It thus seems that SAV infections are more affected by type I IFN responses than IPNV infections.

1.5.5 IFN responses intracellular bacteria

Type I IFN responses are not only induced after viral infection, but after bacterial infections as well (Perry et al. 2005). This indicates that IFN responses are not solely anti-viral but could also have an anti-bacterial function. Type I IFN responses induced protection against the extracellular bacteria *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, but increased damage by *Staphylococcus aureus* infection in mice (Parker and Prince 2011). Similarly, the effects of type I IFN on intracellular bacterial infections depend on the pathogen (Snyder et al. 2017). Type I IFN has been described to have detrimental effects on the host during infections with *Listeria monocytogenes* (O'Connell et al. 2004), *Mycobacterium tuberculosis* (Mayer-Barber et al. 2014), *Staphylococcus aureus* (Martin et al. 2009), and *Mycobacterium bovis* (Bouchonnet et al. 2002), while only beneficial effects have been observed in *Legionella pneumophila* infections (Coers et al. 2007; Plumlee et al. 2009). The effects of type I IFN responses on infection with *Coxiella burnetti*, which is related to *P. salmonis*, were tissue dependent (Hedges et al. 2016). In salmonids, the effect of type I IFN responses has only been investigated in relation to viral infections, but there are indications that type I IFN responses are affected in *Renibacterium salmoninarum* infected Chinook salmon (Rhodes et al. 2009) and Atlantic salmon (Eslamloo et al. 2020).

Although, like other salmonid intracellular bacteria, the effects of type I IFN response on *P. salmonis* infection have not been investigated, several studies mention some effect of *P. salmonis* on IFN expression. Our group has observed minor upregulation of IFN and ISG expression after *in vivo P. salmonis* infections (Svenning et al. 2019; Bela-Ong et al. 2020), while IFN expression in the muscle of Atlantic salmon in another infection study was downregulated (Tacchi et al. 2011). No significant changes in IFN expression were observed in other organs in the same study (Tacchi et al. 2011), nor in other transcriptome investigations (Pulgar et al. 2015; Rozas-Serri et al. 2018). After vaccination with a live attenuated SRS vaccine, expression of IFN was upregulated in the head kidney (HK) only at 5 days post infection (dpi), which corresponded to increassed expression of the other investigated immune genes (Vargas et al. 2021).

1.6 Antibody responses

Although innate immune responses are important for protection against infection, the adaptive immune responses are often needed to clear pathogens. Abs constitute a main component of the adaptive immunity (Abbas 2020). Ab responses are the most common correlations of protection and one of the main responses evaluated in vaccine development (Plotkin 2010). These responses are most effective against extracellular pathogens, but also play important roles in protection against intracellular pathogens.

According to the mammalian dogma, B cells produce Abs after activation by helper T cells (T cell dependent) or without T cell help (T-cell independent), while natural B1 B cells spontaneously produce Abs (Zubler 2001; Baumgarth 2013). Follicular B cells have a very high diversity of B cell receptors (BCRs) due to somatic gene rearrangement (Bengtén and Wilson 2015). After exclusion of B cells with self-reactive BCRs, B cells generally remain in a resting state, especially in the lymphatic tissues and peripheral blood (Zubler 2001). During an infection, presentation of antigens along with costimulatory signals from helper T cells activate those B cells whose BCRs bind the pathogen antigens best (Abbas 2020). Only these B cells will start proliferation and differentiation, resulting in clonal selection and expansion of B cells with highly specific BCRs (Abbas 2020). These B cells differentiate into plasmablasts and plasma cells which secrete Abs that have the same affinity as the original BCRs, and finally memory B cells (Abbas 2020). Somatic hypermutation during the humoral response to infection leads to even more specific Abs (affinity maturation), while isotype switching increases immunoglobulin (Ig) diversity leading to different effector functions (Zubler 2001). Memory B cells remain in the circulation and different tissues after clearance of the infection and will mount a fast and strong humoral response when the same pathogen is encountered and recognized again: the secondary response or booster effect (Abbas 2020).

Although teleost fish humoral responses generally function similar to mammalian responses, there are some major differences. The teleost humoral responses have been described as being slower and having only a minor secondary response after repeated antigen encounter (Ye et al. 2013). While affinity maturation in teleosts has originally been debated, we now know that it is present in fish (Ye et al. 2013; Wu et al. 2019), although its localization is still not definitively characterized (Magor 2015). Ab responses in teleosts also lack isotype switching, which is confirmed by the lack of switch regions in the Ig genes (Yu et al. 2020). Even though the

humoral responses in teleosts seem to be less pronounced than in mammals, they still form a very important part of the protection against pathogens.

The HK and spleen are the main systemic immune organs in fish. The HK has comparable functions to the mammalian bone marrow: it is the major location of hematopoiesis and houses most of the long-living plasma cells (Uribe et al. 2011; Ma et al. 2013). Lymph nodes are not present in fish, but the spleen covers most of its functions and contains memory B cells (Uribe et al. 2011; Ma et al. 2013). In addition, fish have several mucosa-associated lymphoid tissues, originally described at the gut, gills, skin, and nose, but more are being characterized (Yu et al. 2020).

Aside from producing Abs, teleost B cells have been found to actively phagocytize fluorescent beads and bacteria (Li et al. 2006), constitutively express several TLRs (Abós et al. 2013; Jenberie et al. 2018), and respond to inflammatory cytokines (Castro et al. 2014). In this, they show remarkable similarities to mammalian B1 cells, which is a mammalian B cell population with more innate functions, such as phagocytosis and antigen presentation (Prieto and Felippe 2017; Parra et al. 2012). In addition, teleost B cell expression of CD genes is clearly similar to that of mammalian innate B cells and not B2 cells (Peñaranda et al. 2019). Mammalian B1 cells are primarily located in the peritoneal cavity (PerC) but are also found in other tissues and are the main producers of natural Abs (Baumgarth et al. 2015). These natural Abs are polyreactive and secreted before any antigen encounter; they form a first innate humoral protection (Baumgarth et al. 2015).

1.7 Aims of study

The general problem of lacking effective vaccines against many intracellular pathogens of Atlantic salmon motivated our investigations into immune responses in Atlantic salmon after infections with these intracellular pathogens. More specifically, our goal was to increase knowledge on interactions and immune responses of Atlantic salmon and the intracellular pathogens *P. salmonis* and SAV3. To this end, we investigated the characteristics (kinetics and duration, protective potential, production, and specificity) of Ab responses in Atlantic salmon after IP *P. salmonis* injection and the characteristics of IFN induction and IFN mediated innate immune responses against SAV and *P. salmonis*.

1.7.1 Specific objectives

- Develop *P. salmonis* challenge models in Atlantic salmon
- Develop assays to quantify Abs and Ab secreting cells (ASC) from Atlantic salmon
- Investigate kinetics, duration, and protective effect of Ab responses after *P. salmonis* infection in Atlantic salmon
- Investigate location of ASC and Ab specificity in *P. salmonis* infected Atlantic salmon
- Develop a workflow for efficient generation of CRISPR-Cas edited single cell clones (Sc) from salmonid cell lines
- Investigate the effects of IRF3, IRF7, and MAVS KO on type I IFN induction and pathogen replication in salmonid cell lines

2 Methods: description and discussion

2.1 *P. salmonis* challenge

We developed two challenge models for *P. salmonis* at two different locations.

The first challenge, used in paper I, was based on the LF89 strain, and the inoculum was prepared by harvesting infected CHSE-214 cell cultures, followed by centrifugation of the bacteria to remove cell debris and resuspension in phosphate-buffered saline. Very small cell debris made direct quantification of bacteria through optical density measurement difficult, so the number of bacteria in the harvest used for the inoculum had to be estimated based on earlier trials and titrated on CHSE-214 cells that were read after two weeks. This inability to quantify the inoculum before use in the challenge was the main downside of this method and led to large deviations from the estimated inoculum in the rechallenge experiments, even though the inoculum titers in pilots were much more stable.

In paper II, the challenge model was adapted from the company ADL, who also generously shared their EM90-like strain. This strain was grown on agar plates, which resulted in yields with less variation and the possibility to quantify the number of bacteria based on the optical density. This led to improved estimations of the inoculum at time of challenge, and the titer was confirmed through colony-forming unit determination after growth on agar plates.

The route of infection, IP, used in both challenge models is useful to investigate the effect of vaccination on the Atlantic salmon, since IP is still the most common route of vaccination for salmon. A cohabitation challenge, however, is more suitable to mimic a *P. salmonis* infection on the field. Our research focused mainly on immune responses connected to vaccine development, so the IP route was the preferred option. For the second challenges in paper I, a cohabitation challenge would have been better since we ideally had simulated a 'natural' challenge. Time, cost, and infrastructure unfortunately limited our options.

2.2 ELISpot & ELISA for anti-*P. salmonis* and anti-*Yersinia ruckeri* Abs

We developed ELISpot assays to quantify anti-*P. salmonis* and anti-*Y.ruckeri* ASC, and used the same coating for corresponding ELISA assays. The bacteria were grown in medium (agar for *P. salmonis* and liquid for *Y. ruckeri*), and the harvest was heat-inactivated and sonicated to obtain antigens for coating. By using whole inactivated bacteria to coat, we measure Abs that recognize any epitope on the bacteria. This includes all specific Abs but may also include Abs

that recognize epitopes that are shared between bacteria. A commonly used alternative is to coat with a recombinant protein. This would be bound by a much more selective, and more specific, subgroup of Abs. For the ELISpot assays we decided on whole inactivated bacterial coating to get a general overview of the ASC, but some of the 'specific' ASC might actually recognize epitopes present on both tested bacteria. The ELISA used to quantify the anti-*P. salmonis* Ab level in the serum during the rechallenge experiment used recombinant OspA as coating, meaning that the Abs we measured there were much more specific.

Although ELISpot assays are very useful to quantify ASC, some questions on Ab production were not fully addressed by this method. ASC can secrete different amounts of Ab, mainly depending on their development. Just the quantity of the ASC in an organ does not provide the data required to conclude that it houses the main Ab production. The intensity of the ELISpot spots can give an indication of the amount of secreted Ab, although this could be less straightforward to elucidate from the data than the number of spots. Similarly, the size of the spots is dependent on the amount of Ab produced and the affinity of the Abs to the antigen and could be useful in estimating the relative production of Abs per ASC. Together, the spot count, intensity, and size could give an indication of Ab production per organ, although quantification is not possible due to missing knowledge on how spot size and intensity relate exactly to amount of produced Ab.

2.3 CRISPR-Cas protocol for gene editing in salmonid cell lines

Setting up an efficient protocol for CRISPR-Cas editing in salmonid cell lines was not straightforward due to hard to transfect cell lines, reagents and methods optimized for use in mammals, and a lack of comparable investigations, although this last part has been rectified in recent years. Nucleofection has been proposed as a solution for hard to transfect cell lines and gave us the best transfection results, especially after optimization, compared to lipofection.

When using standard CRISPR-Cas plasmids in the CHSE-214 cells, the efficiency of expression was very low based on the presence of fluorescent cells. Only one cell with fluorescence was observed after transfection of a CRISPC-Cas plasmid with orange fluorescent protein coupled to Cas9. This issue could be connected to plasmid components (promoters, linkers) designed for mammalian systems having less or no function in fish systems as well as to toxicity of the introduced DNA. Both these issues were solved by using the ribonucleoprotein (RNP) approach where a recombinant Cas9 protein is precomplexed with the sgRNA molecule

before introducing the complex into the cell, thus eliminating the need for plasmid elements or introduction of DNA. An additional perk of this method is that Cas9 has a much more limited duration of effect in the cell compared to Cas9 expressed by plasmids, which should reduce the off-target effects (Elke Lorbach May 2018). In addition, we were interested in generating edited cell lines without introducing foreign DNA since this could facilitate registration in the event that one of the KO cells could be used as substrate for vaccine production.

In order to evaluate the editing efficiency during the development of the CRISPR-Cas protocol, we tested several different read-outs. The T7 endonuclease assay and polyacrylamide gel electrophoresis assay often did not yield clear enough results to evaluate editing. Some gel bands were barely visible, especially if the editing efficiency was low. This led us to use sequencing of the targeted location as a read out for editing efficiency. The online TIDE tool from the Netherlands Cancer Institute analyses the shifts in sanger sequencing peaks in a pool of mutated cells and reports the frequencies of the different indels (Brinkman et al. 2014). This allowed quantification of the efficiency, while it simultaneously gave information on whether the present mutations would induce a frameshift and a possible KO.

We decided to test the introduced mutations in Sc, since wt cells still accounted for a substantial population of the edited pools and since not all mutations would lead to a KO. One surprising find that resulted from this was that different wildtype clones of the CHSE-214 cell line already showed a significant difference in IPNV yield (paper III). To reduce the effect of these differences as much as possible in our investigation, we decided to perform the CRISPR-Cas editing on one Sc and to use this clone as negative control (NC) in all experiments. These findings and our solution have been confirmed in mammalian cell lines by Westermann et al. (2022). Other groups planning investigations involving Sc should take this into account.

2.4 Ethical considerations: animal experiments

*Adapted from the student paper 'Challenge of Atlantic salmon with *Piscirickettsia salmonis*' for the course Animal Aquatic Welfare (van der Wal 25-April-2019).

Two of the included investigations were based on challenge experiments that, unfortunately, required the use of experimental animals. We reduced animal suffering as much as possible in these experiments by including the 3 Rs in the design. It was not possible to *replace* the challenge model since other models, such as computer models are not available. In addition, the investigated immune responses are part of an integrated system which prohibits testing in reduced biological systems such as cell culture.

We *reduced* the fish in each group to the lowest numbers that should still give valid results based on previous findings on variation. This reduction, combined with higher moralities than planned, led to too small group sizes for sufficient statistical weight in the challenge experiment in paper I. This indicates that reduction of group sizes should not be overdone, since it can hugely reduce the value of an experiment and thus make it not worth the cost at the end. The number of experimental groups and sampling time points were also reduced to numbers necessary for a well-supported answer to the research questions. Finally, the duration of suffering was reduced by limiting the duration of the experiments to the minimum necessary for the research questions.

The experimental designs were *refined* by ensuring a general welfare of the fish through optimized environmental conditions, suitable anesthetics during handling, and daily observation by well-trained personnel. In addition, humane endpoints were defined to minimize suffering of moribund fish. By using several assays to measure different variables, investigating multiple organ samples per sampled fish, and sharing samples with other researchers for other studies, we also successfully maximized the number of endpoints.

A *cost/benefit evaluation* of the animal welfare in these experiments indicates a high cost, since the challenge experiments led to severe suffering of a few hundred fish, caused by SRS. To be ethically feasible, this cost should be offset by the direct and indirect benefits. The direct benefits of new challenge models, assays, and knowledge on salmon immune responses are moderate and by themselves not sufficient to offset the high costs. The assays and increased knowledge can be used to evaluate the induction of protection through Ab production and ASC numbers. Using these correlates of protection instead of a challenge can reduce the number of fish exposed to severe suffering from SRS in future experiments, thus countering the costs of the current experiments. Finally, the knowledge should further our understanding on the development of an effective SRS vaccine, thus protecting many times the number of fish used here from disease.

3 Results

3.1 Summary of papers

Paper I:

Antibody response after intraperitoneal *Piscirickettsia salmonis* infection in Atlantic salmon: duration and protection

Yorick A. van der Wal, Bjørn Krossøy, Marcos Mancilla, Jorunn B. Jørgensen, Jaap Kool

In this small-scale IP challenge study, we compared the duration of protection in *P. salmonis* infected Atlantic salmon, where an initial challenge of the bacterium was followed by a second homologous challenge after 14 or 18 weeks. The 14 weeks (1200°days) challenge revealed a full protection in survivors (0% mortality), which was significantly different from naïve salmon, which suffered 80% cumulative mortalities. Anti-*P. salmonis* Ab levels in the serum of these survivors were significantly increased compared to naïve fish at the time of rechallenge (14 weeks post challenge (wpc)). For the group of survivors that was rechallenged at 18 wpc (1500°days), we observed 4% cumulative mortality, which was not significantly different from the naïve fish. The latter group suffered 15% cumulative mortality. This suggests that protection was reduced at this time point and corresponded with a reduction in serum anti-*P. salmonis* Abs in the surviving group that was no longer significantly different from the naïve fish. We did not observe increased serum anti-*P. salmonis* levels 6 weeks after the rechallenge in either (14 or 18 wpc) twice challenged group. We conclude that a *P. salmonis* infection confers protection against homologous challenge up to 1200°days, that this protection seems to be reduced at 1500°days, and that serum anti-*P. salmonis* Abs can serve as correlate of protection. A booster effect was not observed in twice challenged salmon after 6 weeks.

Paper II:

The importance of the Atlantic salmon peritoneal cavity B cell response: Local IgM secreting cells are predominant upon *Piscirickettsia salmonis* infection.

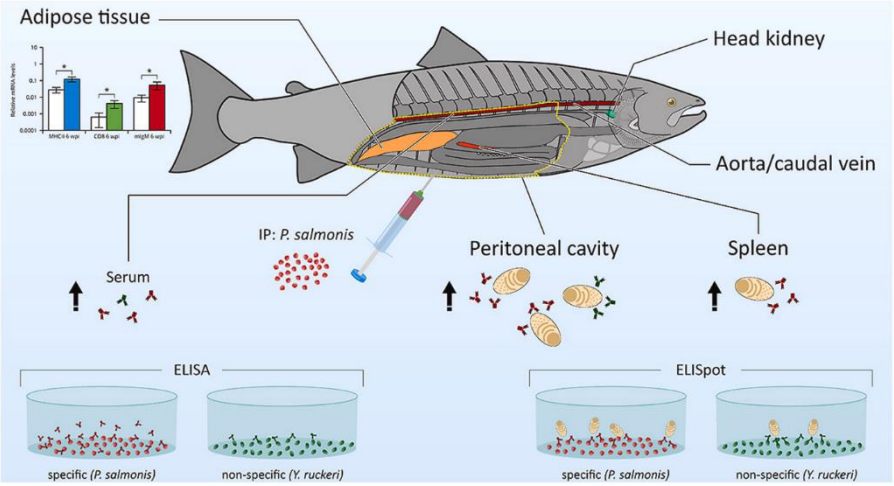
Yorick A. van der Wal, Shiferaw Jenberie, Henriette Nordli, Linn Greiner-Tollersrud, Jaap Kool, Ingvill Jensen, Jorunn B. Jørgensen

Developmental and Comparative Immunology 123 (2021) 104125

We investigated Ab responses and production in Atlantic salmon early after IP *P. salmonis* infection (from 3 days post challenge to 6 wpc). The IP infection with *P. salmonis* resulted in a substantial local increase in leukocytes, total IgM ASC, and specific anti-*P. salmonis* ASC in the PerC up to 6 wpc. During the same time frame, total IgM ASC were increased to a lesser extend in HK and, only at 3 wpc, in spleen. Specific anti-*P. salmonis* ASC were moderately increased in the spleen, and only slightly increased in the HK at 6 wpc. Non-specific *Y. ruckeri* recognizing ASC were only significantly increased in the PerC at 6 wpc. From this, we conclude that local ASC are mainly responsible for early Ab production in Atlantic salmon and suggest that these activated B cells later migrate to the systemic immune organs.

Serum Abs recognizing *P. salmonis*, *Y. ruckeri*, or the model antigen TNP-KLH were significantly increased at 3 and 6 wpc, with the specific Ab levels increasing substantially more at the latest time point compared to the non-specific Abs. This suggests that poly reactive or natural Abs have a clear role in early Ab responses, while specific Abs take over and dominate later in the immune reaction.

Finally, the increased expression of genes connected to several immune relevant cell types (IgM, IFNγ, CD40L, CD4, CD8, MHCI, CD40, MARCO, CD83, MHCII, and TNFα) in the PerC adipose tissue (AT) suggests that the AT has an immunological, possible regulating, role.



Graphical abstract paper II: *The importance of the Atlantic salmon PerC B cell response: Local IgM secreting cells are predominant upon P. salmonis infection.*

Paper III:

CRISPR-Cas induced IRF3 and MAVS knockouts in a salmonid cell line disrupt PRR signaling and affect viral replication

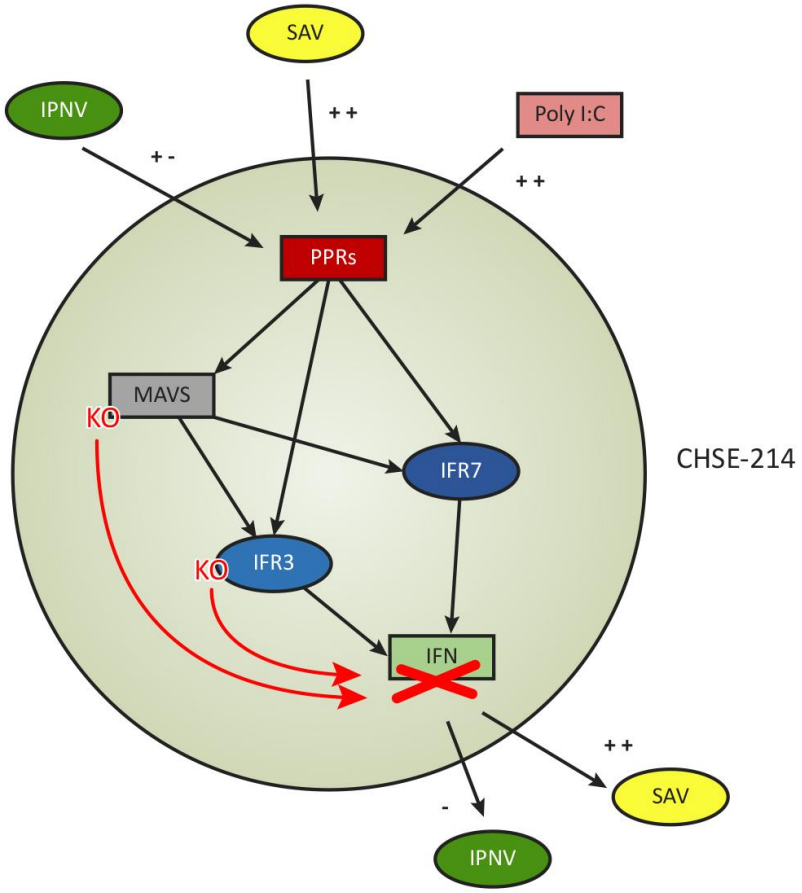
Yorick A. van der Wal, Henriette Nordli, Allan Akandwanaho, Linn Greiner-Tollersrud, Jaap Kool, Jorunn B. Jørgensen

We successfully generated KO cell lines based on a Sc from CHSE-214 cells using nucleofection of CRISPR/Cas RNPs. We obtained IRF3, IRF7, and MAVS single KOs and an IRF7/IRF3 double KO. These genes are important in the signaling system that induces IFN responses after recognition of pathogens. Intracellular poly I:C stimulation induced expression of *ifna*, *ifit5*, and *mx* genes, but not *ifnc* in the wildtype clone. The KO of IRF7 only showed minor changes in gene expression, while the IRF3 and IRF7/IRF3 KOs lost their ability to induce IFN and ISG gene expression. The MAVS KO had reduced induction of IFN and ISG transcripts compared to the wildtype, but some induction was still observed. As expected, intracellular poly I:C stimulation also activated the promoters for IFIT5, Mx2, and IFNa1 in a luciferase promoter reporter assay in the wildtype clone. Activation in the IRF7 KO clone was mostly comparable to the wildtype, while activation in the IRF3 and IRF7/IRF3 clones was completely inhibited, similar to what we observed for gene expression. The MAVS KO clone showed reduced activation of the promoters, but not complete inhibition. We thus conclude that the IRF3, IRF7/IRF3, and MAVS KO clones have an impaired PRR signaling, while the IRF7 KO does not affect PRR signaling after intracellular poly I:C stimulation.

Although SAV infection of CHSE-214 cells usually results in minor CPE as we observed for the wildtype clone and the IRF7 KO clone, SAV infection of IRF3, IRF7/3 and MAVS KO clones resulted in extensive CPE at 6 dpi. SAV RNA copies and infectious SAV titers in the supernatant were increased in the KO clones with impaired PRR signaling, while they were slightly decreased in the IRF7 KO clone. SAV infection of the wildtype and IRF7 KO clones induced expression of *ifna*, *ifit5*, and *mx* genes, similar to the intracellular poly I:C stimulation, but also slightly induced expression of *ifnc* at 6 dpi. Again, we observed complete inhibition of gene induction in the IRF3 and IRF7/IRF3 KO clones, while a partial reduction was visible for the MAVS KO clone. The impaired PRR signaling in the IRF3, IRF7/IRF3, and MAVS KO clones results in increased SAV replication.

Interestingly, and contrary to what we observed after SAV infection, IPNV infection of IRF3, IRF7/IRF3, and MAVS KO clones resulted in lower titers compared to the wildtype or IRF7

KO clones. Although induction of expression of the tested ISGs in IPNV infected clones was low, we could still observe a reduction in induction in the IRF3, IRF7/3, and MAVS KOs.



Graphical abstract paper III: *CRISPR-Cas induced IRF and MAVS KOs in CHSE-214 disrupt PRR signaling and affect viral replication.*

Paper IV:

***Piscirickettsia salmonis* growth in the salmonid cell line CHSE-214 is affected by IRF7, IRF3, and MAVS knockouts**

Yorick A. van der Wal, Allan Akandwanaho, Henriette Nordli, Linn Greiner-Tollersrud, Jaap Kool, Jorunn B. Jørgensen

P. salmonis infection of a wildtype Sc of CHSE-214 cells did not induce expression of *ifna*, *ifnc*, *ifit5*, or *mx*. A minor, though not significant induction in *ifna*, *ifnc*, and at 48 hours, *ifit5* was observed for the IRF3 KO, while no changes were evident in the IRF7 or MAVS KOs.

P. salmonis titers in the supernatant of infected IRF7/IRF3 and MAVS KO clones were significantly reduced compared to the wildtype clones, while the IRF7 KO clone had similar titers. Surprisingly, the IRF3 KO clone had increased, though not significant, *P. salmonis* titers, which is the first time this clone showed a clearly different phenotype than the IRF7/IRF3 KO clone.

3.2 Interconnection results

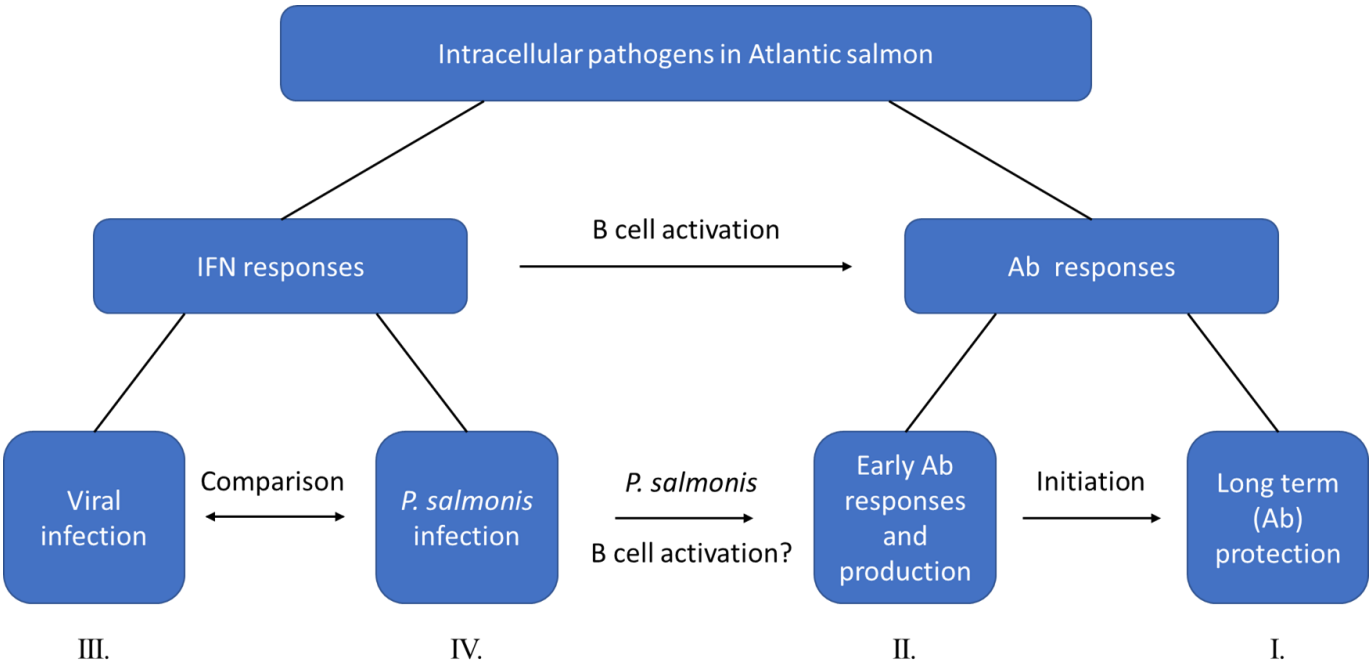


Figure 3: *Interconnection of the results from papers included in the thesis*

We investigated two different immune responses against intracellular pathogens: the IFN and the Ab responses. IFN responses in host cells do not only play a major role in determining the outcome of infections but can also activate B cells in a non-specific manner (Jenberie et al. 2018).

The results from papers III and IV clearly show that the effect of IFN responses on intracellular pathogens is specific for the pathogen. Inactivation of a part of the PRR signaling in CHSE-214 increased SAV replication but was slightly detrimental for IPNV and *P. salmonis* replication. Although *P. salmonis* infection does not induce IFN or ISG expression in CHSE-214 (paper IV), observations in tissues of infected salmon (Svenning et al. 2019; Bela-Ong et al. 2020) indicate that IFN responses can be activated by *P. salmonis*. Non-specific activation of B cells through IFN responses could lead to the increase in non-specific Ab observed in the early immune response in Atlantic salmon (paper II). The overwhelming local increase of ASC in the PerC (paper II) points out that we should focus more on B cell activation in the PerC when investigating possibilities to induce protection that lasts longer than after a *P. salmonis* infection (paper I).

4 Discussion

The main findings in this thesis fit together in a model on B cell activation and Ab production in Atlantic salmon after *P. salmonis* infection. First, we will discuss the KO cell line models we developed to investigate IFN signaling in salmonids and their effect on different intracellular pathogens. Then we will present the general model and discuss the findings that correspond to each of the steps in the model in more detail.

4.1 CRISPR KO of PRR signaling molecules

Overexpression studies have been extensively used to investigate the functions and importance of multiple proteins in immune responses, but CRISPR-Cas induced KOs can give us additional information. Overexpression of proteins can affect non-related pathways due to the increased protein production, while a KO without off-target effects should only affect related pathways and feedback loops. In addition, when a functional CRISPR-Cas protocol is available, it is generally easier and faster to investigate multiple proteins in comparison to overexpression. Investigations into the functions of salmonid immune proteins using CRISPR-Cas are just starting (Dehler et al. 2019, paper III), since effective protocols had to be developed first (Dehler et al. 2019; Gratacap et al. 2020a; Gratacap et al. 2020b, paper III). Our work on optimizing CRISPR-Cas protocols in salmonid cell lines, which was performed during the same time frame, collaborates the findings by Gratacap et al. (2020a) that electroporation of RNPs of precomplexed CRISPR-Cas protein with the sgRNA is an effective method of inducing CRISPR-Cas gene editing in salmonid cell lines (paper III).

This optimized protocol allowed us to generate Sc of CHSE-214 cells with IRF3, IRF7, IRF7/3, and MAVS KOs. The results of the intracellular poly I:C stimulation experiments from paper III confirm conservation of the essential roles of IRF3 and MAVS in PRR signaling in teleost fish, as we discussed for mammals in sections 1.5.1 and 1.5.2. We observed full inhibition of gene induction as measured by expression or promoter activation in IRF3 KOs after intracellular poly I:C stimulation or SAV infection, while MAVS KOs resulted in a partial to full inhibition of this induction (paper III).

In contrast to the clear effect of IRF3 and MAVS KOs, the IRF7 KO did not seem to have an effect, since its phenotype remained very similar to the NC in most experiments (paper III). This is surprising since IRF7 also has a clear role in inducing IFN responses in mammals (Ning et al. 2011) and is being expressed in homeostatic CHSE-214 cells (paper III). Since, in

mammals, IRF7 expression is mainly confined to lymphocytes (Ning et al. 2011), it is interesting that we observed a steady state expression of IRF7 in CHSE-214 cells, which are not lymphocyte like. Still, we observed a much more pronounced increase in IRF3 expression than IRF7 induction after poly I:C stimulation of wt CHSE-214 cells. Together with our other findings, this indicates that IRF3 is indispensable for PRR signaling after poly I:C stimulation. In contrast, IRF7 does not seem to have an essential function within the parameters we tested in CHSE-214 cells. It is interesting to compare these results with the induction of the AsIFNa1 promoter by expression constructs of AsIRF3 and AsIRF7B in salmonids (Bergan et al. 2010). In the study by Bergan et al. (2010), AsIRF3 resulted in high induction at all tested concentrations, while the induction by AsIRF7B was dose dependent. This, together with the synergetic effect of both AsIRF7A and AsIRF7B with AsIRF3 on IFNa1 promoter induction (Bergan et al. 2010), could indicate a directly stimulating effect of IRF3 that is enhanced in a controlled way by IRF7, the latter of which might not be essential for IFN activation.

Since the IRF7 KO did not result in significant changes, it was not surprising that the double IRF7/3 KO yielded similar results as the IRF3 KO. Still, if there was a minor difference between the IRF3 and IRF7/3 values, the IRF7/3 value was always skewed towards the value of the IRF7 KO. This, together with the steady-state expression and induction after stimulation (paper III), indicates that IRF7 has a function in CHSE-214, although it is not essential for IFN induction.

Salmonids have a very large number of IFN genes, many of which have not been investigated or even identified until recently (Liu et al. 2020, appendix). In our recent bioinformatics study (appendix), several copies of each IFN class were found that seem to be functional and will probably have slight differences in function or induction of expression as shown by Chang et al. (2014) and Svingerud et al. (2012) for IFNa, b, and c and by Sun et al. (2009) for IFNa1 and IFNa3. In addition, we observed no induction of IFNc after poly I:C stimulation, but a small induction after SAV3 or IPNV (paper III). It thus seems clear that different IFNs have distinct expression and induction in salmonids.

4.2 IFN responses after *in vitro* infection with intracellular pathogens

KOs of the targeted PRR signaling molecules IRF3, IRF7, and MAVS are known to disrupt PRR signaling and increase viral replication in several mammalian systems as discussed in sections 1.5.1 through 1.5.3. The effect of these KOs on replication was, however, dependent

on the specific pathogen and system since multiple KOs resulted in no change or even reduced replication of pathogens (Loo et al. 2008). Our findings mirror this, since replication of SAV was increased, while replication of IPNV and the intracellular bacterium *P. salmonis* was slightly decreased in Sc with disrupted PRR signaling (paper III, paper IV). An interesting similarity between IPNV and *P. salmonis* is that they did not induce/mildly induced IFN responses, while SAV infection results in strong induction of IFN expression. A similar distinction was observed by Sen et al. (2011) between the rotaviruses UK and RRV, where UK induced IFNβ in wt MEFs and had increased replication in MAVS KO MEFs, while RRV, which did not induce IFNβ, replication did not change in MAVS KO MEFs. In contrast, Loo et al. (2008) found that sendai virus, reovirus T3D, and dengue virus type 2 all induced ISG54 protein levels, while their viral protein levels were similar or lower in MAVS KO MEFs. Interestingly, the induction of ISG54 after RRV infection without IFNβ induction (Sen et al. 2011) suggests that alternative pathways can lead to ISG induction. We thus hypothesize that replication of viruses (and intracellular bacteria) that successfully interfere in PRR signaling and do not induce type I IFN responses will not be positively impacted by a KO of these pathways. Of course, we should consider that intracellular pathogens have evolved many different mechanisms to deal with innate immune responses and that these responses can differ wildly between cell types (Daffis et al. 2007), so exceptions can be expected to be found.

The lack of strong IFN induction for both IPNV and *P. salmonis* in CHSE-214 cells does not mean that these pathogens will not induce IFN responses in other cell types. In fact, the differences in PRR expression between cell types makes this a probable scenario. IPNV, for example, was described not to induce type I IFN responses in CHSE-214, TO, and SHK-1 cells (Skotheim 2009; Reyes-Cerpa et al. 2012; Lauksund et al. 2015; Robertsen 2018), while induction of these responses was observed in RTG-2 cells (Sena and Rio 1975), primary macrophages from Atlantic salmon (Collet et al. 2007), and in several tissues of infected Atlantic salmon (Skjesol et al. 2011). Similarly, several transcriptome analyses of *P. salmonis* infected Atlantic salmon did not mention induction of type I IFN responses in several tissues (Tacchi et al. 2011; Pulgar et al. 2015; Rozas-Serri et al. 2018) while one observed a downregulation of *type I IFN* in the muscle only (Tacchi et al. 2011). Our group described moderate induction of IFN and ISG genes in several tissues, mainly from immune organs, of infected Atlantic salmon (Svenning et al. 2019; Bela-Ong et al. 2020), and Vargas et al. (2021) observed induction of *ifna* in the HK of vaccinated Atlantic salmon. It thus seems that *P. salmonis* induces type I IFN responses in certain cell types, and observations indicate that these

might be mainly immune cells. Investigating the effect of IRF3, IRF7, and MAVS KOs on *P. salmonis* growth and IFN response induction in salmonid macrophages and B cells would be a logical next step.

IPNV proteins can interact with PRR signaling molecules and thus inhibit IFN response induction (Lauksund et al. 2015), and it seems probable that some *P. salmonis* proteins have similar effects based on our findings that *P. salmonis* infection does not induce IFN responses after infection of CHSE-214 cells. Since we observed a small but significant reduction in IPNV and *P. salmonis* replication in Sc with disrupted PRR signaling, it is possible that IPNV and *P. salmonis* have become partially dependent on these interactions during pathogen-host co-evolution. Additional investigations into the interaction of *P. salmonis* with immune system components would be very interesting. These could shed light on the immune suppressive effects of *P. salmonis*, helping with vaccine design, and on dependence and co-evolution of *P. salmonis* and its host.

The minor induction of IFN expression in immune organs and the fact that teleost B cells express several PRRs suggest that *P. salmonis* infection can induce IFN responses in salmon B cells. This possible induction and activation of B cells by *P. salmonis* leads us to our proposed model.

4.3 Model on the initiation, production, and duration of Ab responses

We propose a model on the initiation and subsequent development of Ab responses after *P. salmonis* infection of Atlantic salmon. Teleost B cells are not only equipped with specific BCRs, but also express several PRRs (Abós et al. 2013; Jenberie et al. 2018) and are therefore capable of detecting a wide range of PAMPs such as lipopeptides, flagellin, RNA, and CpG DNA. This non-specific stimulation of B cells can lead to activation, since CpG and/or poly I:C stimulated B cells had upregulated IgM expression (Jenberie et al. 2018), IgM production (Simón et al. 2019), and showed signs of differentiation to plasmablasts (Simón et al. 2019). Although paper IV shows that *P. salmonis* infection line does not induce IFN expression in CHSE-214 cells, it is conceivable that it will stimulate immune cells such as B cells based on *in vivo* expression of IFNs and ISGs (Svenning et al. 2019; Bela-Ong et al. 2020). A *P. salmonis* infection in the PerC would thus activate both specific B cells through BCRs recognizing *P. salmonis* antigen, as well as ‘natural’ B cells producing polyreactive Abs through PRRs. This could account for the increase of non-specific Abs in the serum early after *P. salmonis* challenge

(paper II). A combination of clonal selection and affinity maturation would gradually lead to a more *P. salmonis* specific Ab response, as we observed in paper II. We (paper I) and Tobar et al. (2015) found that the duration of the elevation of specific serum Abs directed against *P. salmonis* or its outer surface protein A, respectively, is about 1200°days. The decrease of serum Abs seems to coincide with a loss of protection against a new *P. salmonis* challenge, indicating that any immunological memory induced by *P. salmonis* infection (paper I) or an IP inactivated vaccine (Tobar et al. 2015) is insufficient. Since a specific booster at the time of reduced Ab levels seems to maintain protection (Tobar et al. 2015), it would be interesting to evaluate the effect of a non-specific booster consisting of a TLR or RLR ligand such as CpGs or poly I:C, for example.

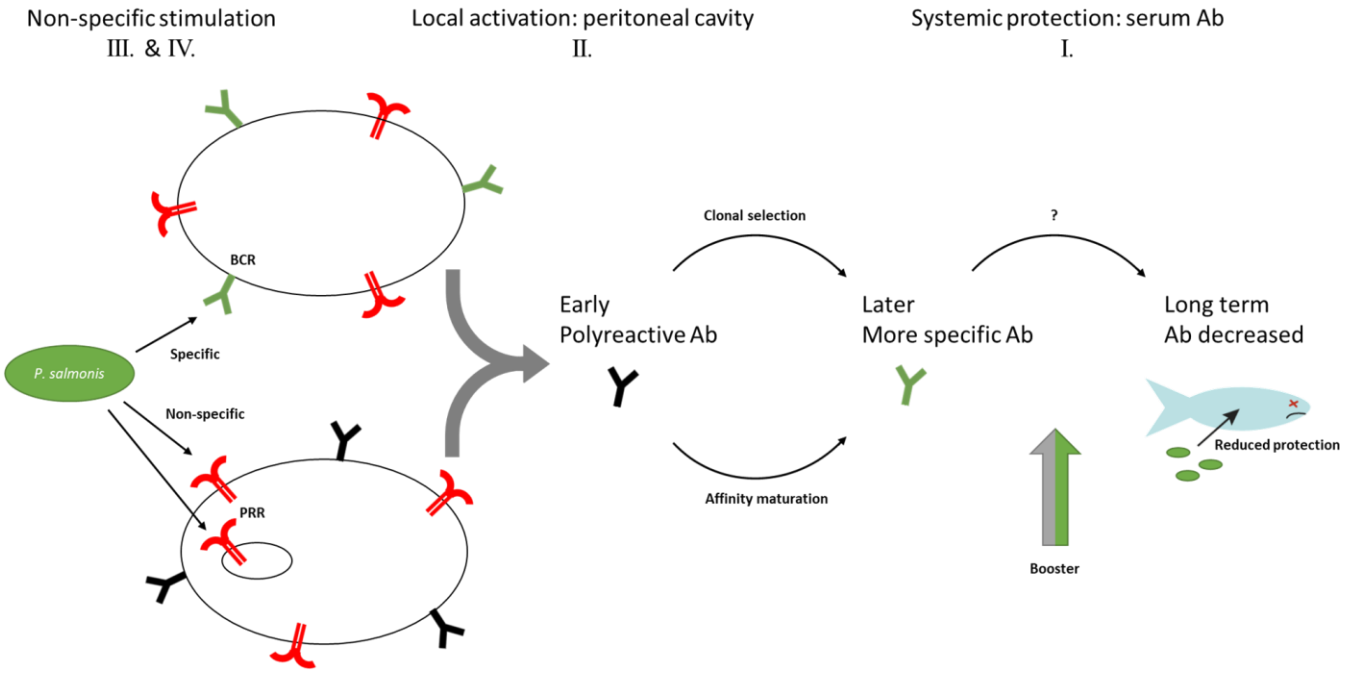


Figure 4: model on Ab response initiation, production, and duration in Atlantic salmon after P. salmonis encounter. *B cells can recognize P. salmonis antigens through BCRs that recognize pathogen specific epitopes or through PRRs that recognize PAMPs that are shared by several pathogens (straight arrows, left side). Non-specific activation of B cells leads to production of low-affinity, poly-reactive Abs (black) in a large number of B cells, which leads to many poly-reactive Abs during the early phase of the humoral immune response (cell, bottom left). B cells that specifically recognize P. salmonis will start to produce specific Abs (green) with higher affinity (cell, top left). Clonal selection of B cells with high-affinity BCRs and/or affinity maturation lead to more specific Abs (curved arrows, middle). Over time, the level of P. salmonis recognizing Abs decreases (curved arrow, right). When this level drops below a certain threshold, protection against a new P. salmonis infection is lost (fish, right). A booster before this event can keep the salmon protected (block arrow, bottom right).*

4.4 Specificity of early Ab responses and non-specific B cell activation

Teleost B cells share several characteristics with mammalian B1 cells, which are generally referred to as innate B cells (Abós et al. 2018). Both are phagocytic (Li et al. 2006), respond to cytokines (Castro et al. 2014), and express several TLRs (Abós et al. 2013; Jenberie et al. 2018). CpG stimulation of Atlantic salmon B cells induces IFNa and IFNb expression, as well as IgM expression and secretion (Jenberie et al. 2018). The stimulation of rainbow trout B cells with CpG, poly I:C, and/or rIFNa can lead to activation, Ab production, and differentiation to plasma blasts (Abós et al. 2015; Simón et al. 2019; Benedicenti et al. 2020), raising question as to the role of this non-specific activation of teleost B cells. We hypothesize that non-specific activation of B cells could lead to production of poly-reactive (non-specific) Abs as shown in mammals by Gunti et al. (2015), which would explain the larger portion of non-specific binding Abs at earlier time points after *P. salmonis* infection (paper II) or after vaccination (Lund et al. 2019). Interestingly, vaccination of coho salmon with expression plasmids containing a *P. salmonis* DNA library only induced *P. salmonis* specific Abs and not Abs recognizing *Y. ruckeri* or *R. salmoninarum* (Miquel et al. 2003), suggesting that this method mainly resulted in specific stimulation of B cells. In addition, this specific stimulation apparently did not activate non-specific B cells, which could mean that induction of non-specific Ab production might be limited to non-specific PRR stimulation. It would be interesting to further investigate the role of non-specific B cell activation and its interplay with BCR activation in the mounting of Ab responses. This could improve future vaccine design: broader protection, increased Ab levels and duration, and practical booster options.

Over time, the observed Ab responses became more specific: a larger portion of the serum Abs recognized *P. salmonis* specifically (paper II). In mammals, this transition is a result of clonal expansion of high affinity B cells and somatic hypermutation driven affinity maturation (Abbas 2020). Clonal selection of B cells in teleost fish has been suggested based on observations in rainbow trout: increasing affinity of Abs after immunization (Ye et al. 2011) or clonal expansion within Ig repertoires after viral infection (Castro et al. 2013). Whether the expanded clones were already present in the repertoire before insult or were the result of somatic hypermutation is not easily determined. A transition to more specific Abs was also described by Lund et al. (2019), who observed an increase of non-specific serum Abs in vaccinated Atlantic salmon that started slightly earlier than the increase of specific Abs. The increase of specific Abs relative to the NC was higher than the non-specific increase at the later time points,

comparable to our results (paper II). Interestingly, the increase of specific Abs at 21 dpv coincided with a significant increase in the number of unique clonotypes of the IgM variable region (Lund et al. 2019). They suggest that this, coupled with a reduction in complementarity determining region 3, indicates expansion of recent B cell clones. These emerging B cell clones could be the result of somatic hypermutation driven affinity maturation.

The existence of affinity maturation in teleosts has long been debated, since only low levels have been observed when compared to mammals (Kaattari et al. 2002; Ye et al. 2011). Still, some degree of affinity maturation can be observed, and activation-induced cytidine deaminase (AID), the enzyme necessary for somatic hypermutation, has functional homologues in teleost fish (Magor 2015). The location of affinity maturation in teleosts is also unclear, since fish lack germinal centers or other follicular structures, where affinity maturation occurs in mammals. Work in channel catfish has identified AID expressing cells near melanomacrophage clusters, where antigen presentation and somatic hypermutation could occur (Magor 2015; Muthupandian et al. 2021). These melanomacrophage clusters might be early versions of germinal centers and thus one possible location of affinity maturation in fish. Interestingly, Magor (2015) even suggests a reason for the slow and low-level affinity maturation in fish based on the ratio of APCs to AID expressing cells in these melanomacrophage clusters. In mammalian germinal centers, the numbers of APCs such as follicular dendritic cells are very limited which would lead to a limited amount of antigen presentation. In turn, this would only allow the best binding B cells to expand through antigen-driven selection of B cells. In contrast, the melanomacrophage clusters in channel catfish contained many more APCs in relation to AID expressing cells, which would mean that there will be sufficient antigen presented to allow less specific B cells to expand as well (Magor 2015). This would result in the slower affinity maturation observed in fish. This less restrictive selection of B cells with a broader range of affinities could, at least in part, lead to the initial expansion of low affinity ASC and hence the slow transition to more specific Abs that we observed in paper II. Thus, clonal selection and affinity maturation both seem to be functional in teleosts, although their respective contributions to mounting a specific Ab response remain to be elucidated.

4.5 PerC as location of early Ab production

Our findings of major increases of leukocytes, total IgM ASC, and anti-*P. salmonis* ASC in the PerC of *P. salmonis* infected Atlantic salmon (paper II), together with similar findings in the PerC of SAV3 infected salmon (Jenberie et al. 2020), suggest that this strong local B cell

expansion could be a general feature in Atlantic salmon after IP infection. The large number of IgM producing B cells at the infection site indicates a local activation and expansion, and/or migration of activated B cells to the site of infection, as shown for the heart of SAV3 infected Atlantic salmon (Bakke et al. 2020).

Local activation of B1 cells in mammals has been observed after different stimulations or infections and is usually connected with a migration towards the spleen (Baumgarth et al. 2015). Our results on anti-*P. salmonis* ASC at 3 wpc in the different organs seem to agree with this. The major increase in specific ASC in the PerC, the minor increase in the spleen, and no changes in the HK (paper II) suggest activation in the PerC and could indicate a migration to the spleen. On the other hand, the increase in total ASC in the PerC at 3 wpc probably has not been solely due to activation, since the total IgM ASC numbers almost reached the total leukocyte counts in the PerC of NC fish (paper II). This indicates that there should be an expansion in - and/or migration of IgM ASC to the PerC. Since there is an increase in total IgM ASC numbers in the spleen and HK as well, expansion seems to be reasonable, although another reservoir such as the blood or the AT, would also be possible. We suggest that a combination of local activation and expansion, together with a migration to and from the systemic immune organs (observed in Atlantic salmon by respectively Iliev et al. (2013) and Bakke et al. (2020)) results in the observed increases in ASC in the PerC of *P. salmonis* infected salmon, as proposed in the model of Jenberie (2019). The location and migration of ASC in Atlantic salmon after different vaccinations or infections would be interesting to investigate at early and late time points to get more insight in the development of protective humoral responses.

While the PerC showed a large increase in both total and specific ASC in the weeks after *P. salmonis* challenge, the ASC increase in the spleen was dominated by specific ASC (paper II). Although this might be the results of migration of specific ASC, it is interesting to note that Perdiguero et al. (2021) observed 10 clusters of B cells in the blood of rainbow trout after Sc sequencing. This diversity is probably the result on B cells in different stages of differentiation and activation but could also indicate the existence of more subpopulations of B cells in teleost fish than currently assumed (Perdiguero et al. 2021). If teleost fish harbor several subtypes of B cells, our results suggest that the spleen contains mainly B cells that will produce specific Ab after activation (B2-like), while the PerC contains more 'natural' B cells (B1- like).

Recently, the PerC AT has been recognized as having an important immunological role, but its role in teleost fish has only been investigated in the last few years (Simón et al. 2022). Pignatelli

et al. (2014) have identified several B cell subsets in the rainbow trout AT and showed changes in immune gene expression after viral hemorrhagic septicemia virus infection. Correspondingly, our expression data of the AT in Atlantic salmon suggested the presence of all immune cells necessary for antigen presentation and showed a very clear response after *P. salmonis* infection (paper II). Interestingly, Simón et al. (2022) characterized B cells in the rainbow trout AT and found increased IgM secretion in the AT during the first 4 weeks after immunization with TNP-LPS, comparable to the spleen and HK, suggesting that this might be an important immune organ. Related to this, it would be interesting to evaluate the AT as location of possible affinity maturation in future research, since this has been described for the omentum in mice (Rangel-Moreno et al. 2009). A possible way to perform this is to look for AID expression in macrophage clusters in the AT, since that could be indication of germinal center like structures where affinity maturation might be occurring (Magor 2015).

An important implication of the strong local responses in the PerC after IP infection is that certain challenge experiments could overestimate the systemic protection of tested vaccines. IP delivered vaccines are probable to induce similar strong local responses as we observed during infection, which means that an IP challenge would be strongly affected by these local responses. The results of an IP challenge administered early after IP vaccination could thus indicate strong protection, while this might not be indicative of the induced systemic protection and only reflect the local protection at the injection site. We thus suggest designing future vaccine efficacy studies with this in mind. The use of cohabitation or immersion challenges circumvents these issues and will mirror a more natural route of infection. Alternatively, if IP challenge is the only available option, a challenge at later time points might be possible, although we have not determined the duration of the strong local responses. An additional perk of using challenges at later time points is that this will give a better view on the duration of protection.

4.6 Duration of Ab response after *P. salmonis* infection

The specific Ab response after IP *P. salmonis* infection was found to be significantly increased until (at least) 1200°days but was no longer increased at 1500°days (paper I). This fits well with the composed field results from Tobar et al. (2015), where Ab levels in vaccinated Atlantic salmon were elevated until 1200°days as well. They observed starting SRS mortalities after this time point and hypothesized that when the serum Ab level falls below a protective threshold, the salmon are susceptible to SRS again (Tobar et al. 2015). Our results support this hypothesis, since we observed a clear protection when Ab levels were increased, but not after Ab levels

were no longer elevated (paper I). An implication of this waning protection is that long term protection against *P. salmonis* should be correctly assessed: a test should be performed at least after 1200°days.

Although our and Tobar et al. (2015) results suggest that serum Ab levels could be a correlate of protection against SRS, cellular immune responses might be more important for successful protection. Expression analyses after SRS vaccination of Atlantic salmon show upregulation of genes connected to cellular immune responses during the time of protection (Vargas et al. 2021; Rozas-Serri 2022), which suggest that these are also a possible correlate of protection. Considering the intracellular nature of *P. salmonis* and the limited duration of protection from oil adjuvanted vaccines, the observed correlation between protection and Ab levels might be a coincidence, or at least connected through cellular immune responses. Future research should focus on identifying correlates of protection connected to cellular immunity. A possible functional assay for cellular immune responses could be the recently developed IFNγ ELISpot assay that works well for Atlantic salmon leukocytes (unpublished data).

4.7 Extending duration of protection

The duration of protection from vaccination can be extended by improving the initial vaccination or by applying boosters when protection wanes. Most vaccines for Atlantic salmon that give a long duration of protection after the initial vaccination are oil-adjuvanted and probably result in long term protection due to depot forming (Evensen 2009). These vaccines, however, do not work as effectively against intracellular pathogens, suggesting that cellular immunity is not sufficiently triggered through these formulations. DNA vaccines have been giving promising results for some viral diseases and two have been registered for use in Atlantic salmon (Adams 2019; Thorarinsson et al. 2021). Miquel et al. (2003) investigated the protection of DNA vaccination with a library of *P. salmonis* in Coho salmon and observed increased Ab titers specific to *P. salmonis*. However, a challenge after 60 days resulted in 80% mortality in vaccinated fish compared to 100% in controls, indicating a minor protective effect that is unfortunately insufficient. Another alternative vaccination method to induce cellular responses is a live attenuated vaccine, and the registered Pharmaq SRS live vaccine was found to induce cellular immune responses (Vargas et al. 2021). However, long term performance in the field combined with our findings of reduced protection after a *P. salmonis* infection, suggest that the protection from a live attenuated vaccine also could have insufficient duration.

Since the duration of protection from oil-adjuvanted vaccines is established for extracellular bacteria, we suggest investigating ways to modulate the induced immune responses to include cellular responses. This could be achieved through addition of immune modulating adjuvants, for example PRR ligands, although more work on the signaling pathways and effects in Atlantic salmon will be necessary to identify interesting targets. Another possibility is to deliver the antigens in nanoparticles, since certain sizes of nanoparticles result in a more Th1-type response in rats (Kanchan and Panda 2007). In combination with oil adjuvant, this could result in a depot of antigen that continuously stimulates cellular responses and thus gives long term protection against *P. salmonis* and other intracellular pathogens.

A generally common practice to extend duration of protection from vaccines is to apply boosters. In mammalian species, a clear secondary response (booster effect) can be observed where the immune response to the secondary insult is many orders of magnitude higher than the initial response (Abbas 2020). A classical secondary response of this magnitude had initially not been observed in fish, leading to the question whether fish have adaptive immunity, but later work has shown that teleost do have secondary responses (Ye et al. 2013; Kaattari et al. 2002). The effect of booster vaccinations against SRS is similarly unclear. We did not observe a clear effect on serum Ab levels from a second *P. salmonis* challenge, since the serum Ab levels were not elevated in comparison to either once challenged or not challenged salmon (paper I). In contrast, Tobar et al. (2015) observed serum Ab levels that were significantly higher compared to controls in salmon populations from farms where an oral booster was applied, which seemed to coincide with a prolonged protection against SRS outbreaks. Similar effects have been observed in prime-boost vaccination trials for other diseases in salmon (Evensen 2016). Although the observations of Tobar et al. (2015) suggest a protective effect of a prime-boost regimen against SRS, statistical modeling on field data did not find a significant positive effect of boosters on mortalities or time to onset of outbreaks in Atlantic salmon on Chilean farms (Happold et al. 2020). An earlier statistical investigation found that the group of salmon with oral booster had significantly lower odds to get high SRS mortalities compared to two other groups that received one IP prime vaccination, while it was not significantly better when compared to the group that got the same IP prime vaccination as the boostered group (Jakob et al. 2014). Based on these findings, we suggest that booster vaccinations should be able to extend the duration of protection against SRS, but that there is a lot of room for improvement.

Applying boosters to Atlantic salmon in sea cages is not straightforward since IP injection and immersion are basically impossible at that stage of the production cycle. Oral boosters are more practical, but still have issues connected to dosage per individual, efficient uptake of antigens, and run-off into the environment (Gudding et al. 2014). If boosters prove to be necessary to protect against several intracellular pathogens in the future, it would be practical to have these combined in just one application for multiple vaccines. For this, it would be interesting to evaluate non-specific stimulation as booster. If this would be possible, it could result in a booster that functions for multiple diseases at once. TLR signaling has several effects in mammalian B-cell development and activation (Hua and Hou 2013), and *in vitro* CpG stimulation of several populations of B cells from Atlantic salmon enhances Ab secretion (Jenberie et al. 2018). The CRISPR protocol and the sgRNAs we developed (paper III) could be used for an investigation into the connections between PRR signaling and teleost B-cell activation by knocking out IRF3, IRF7, and MAVS in teleost B cells.

5 Main conclusions

- Anti-*P. salmonis* serum Ab levels in challenged Atlantic salmon were elevated at 14 wpc, while they are no longer elevated at 18 wpc.
- Significant increase of anti-*P. salmonis* serum Ab levels seemed to coincide with protection against homologous secondary challenge.
- IP challenge of Atlantic salmon with *P. salmonis* resulted in a major increase of leukocytes, IgM ASC, and anti-*P. salmonis* ASC in the PerC for at least 6 wpc.
- Immune cell marker expression after *P. salmonis* IP challenge suggests an immune role for the PerC AT.
- IRF3 and MAVS KO in CHSE-214 disrupted PRR signaling, as evaluated by IFN and ISG expression and promoter activation, while IRF7 KO did not.
- SAV replication was increased in IRF3 and MAVS KO CHSE-214 cells, while IPNV replication was reduced in these cells.
- *P. salmonis* growth on IRF7/3 and MAVS KO CHSE-214 cells was reduced.

References

Abbas, Abul K. (2020): Basic Immunology E-Book: Functions and Disorders of the Immune System: Elsevier.

Abós, Beatriz; Bird, Steve; Granja, Aitor G.; Morel, Esther; More Bayona, Juan A.; Barreda, Daniel R.; Tafalla, Carolina (2018): Identification of the First Teleost CD5 Molecule: Additional Evidence on Phenotypical and Functional Similarities between Fish IgM+ B Cells and Mammalian B1 Cells. In *Journal of immunology (Baltimore, Md. : 1950)* 201 (2), pp. 465–480. DOI: 10.4049/jimmunol.1701546.

Abós, Beatriz; Castro, Rosario; González Granja, Aitor; Havixbeck, Jeffrey J.; Barreda, Daniel R.; Tafalla, Carolina (2015): Early activation of teleost B cells in response to rhabdovirus infection. In *Journal of virology* 89 (3), pp. 1768–1780. DOI: 10.1128/JVI.03080-14.

Abós, Beatriz; Castro, Rosario; Pignatelli, Jaime; Luque, Alfonso; González, Lucia; Tafalla, Carolina (2013): Transcriptional heterogeneity of IgM+ cells in rainbow trout (Oncorhynchus mykiss) tissues. In *PloS one* 8 (12), e82737. DOI: 10.1371/journal.pone.0082737.

Adams, Alexandra (2019): Progress, challenges and opportunities in fish vaccine development. In *Fish & shellfish immunology* 90, pp. 210–214. DOI: 10.1016/j.fsi.2019.04.066.

Arnemo, Marianne; Kavaliauskis, Arturas; Gjøen, Tor (2014): Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (Salmo salar). In *Developmental and comparative immunology* 46 (2), pp. 139–145. DOI: 10.1016/j.dci.2014.03.023.

Au, W. C.; Moore, P. A.; Lowther, W.; Juang, Y. T.; Pitha, P. M. (1995): Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. In *Proceedings of the National Academy of Sciences of the United States of America* 92 (25), pp. 11657–11661. DOI: 10.1073/pnas.92.25.11657.

Aunsmo, Arnfinn; Valle, Paul Steinar; Sandberg, Marianne; Midtlyng, Paul Johan; Bruheim, Torkjel (2010): Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (Salmo salar L.). In *Preventive veterinary medicine* 93 (2-3), pp. 233–241. DOI: 10.1016/j.prevetmed.2009.10.001.

Bakke, Anne Flore; Bjørgen, Håvard; Koppang, Erling Olaf; Frost, Petter; Afanasyev, Sergey; Boysen, Preben et al. (2020): IgM+ and IgT+ B Cell Traffic to the Heart during SAV Infection in Atlantic Salmon. In *Vaccines* 8 (3). DOI: 10.3390/vaccines8030493.

Baumgarth, Nicole (2013): Innate-like B cells and their rules of engagement. In *Advances in experimental medicine and biology* 785, pp. 57–66. DOI: 10.1007/978-1-4614-6217-0_7.

Baumgarth, Nicole; Waffarn, Elizabeth E.; Nguyen, Trang T. T. (2015): Natural and induced B-1 cell immunity to infections raises questions of nature versus nurture. In *Annals of the New York Academy of Sciences* 1362, pp. 188–199. DOI: 10.1111/nyas.12804.

Bela-Ong, Dennis Berbulla; Greiner-Tollersrud, Linn; van der Andreas Wal, Yorick; Jensen, Ingvill; Seternes, Ole Morten; Jørgensen, Jorunn B. (2020): Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene ifit5 in a teleost fish. In *Developmental and comparative immunology* 111, p. 103746. DOI: 10.1016/j.dci.2020.103746.

Benedicenti, Ottavia; Wang, Tiehui; Morel, Esther; Secombes, Christopher J.; Soleto, Irene; Díaz-Rosales, Patricia; Tafalla, Carolina (2020): Type I Interferon Regulates the Survival and

Functionality of B Cells in Rainbow Trout. In *Frontiers in immunology* 11, p. 1494. DOI: 10.3389/fimmu.2020.01494.

Bengtén, Eva; Wilson, Melanie (2015): Antibody Repertoires in Fish. In *Results and problems in cell differentiation* 57, pp. 193–234. DOI: 10.1007/978-3-319-20819-0_9.

Bergan, Veronica; Kileng, Øyvind; Sun, Baojian; Robertsen, Børre (2010): Regulation and function of interferon regulatory factors of Atlantic salmon. In *Molecular immunology* 47 (11-12), pp. 2005–2014. DOI: 10.1016/j.molimm.2010.04.015.

Biacchesi, Stéphane; LeBerre, Monique; Lamoureux, Annie; Louise, Yoann; Lauret, Emilie; Boudinot, Pierre; Brémont, Michel (2009): Mitochondrial antiviral signaling protein plays a major role in induction of the fish innate immune response against RNA and DNA viruses. In *Journal of virology* 83 (16), pp. 7815–7827. DOI: 10.1128/JVI.00404-09.

Birkbeck, T. Harry; Rennie, Shona; Hunter, Douglas; Laidler, L. Anthony; Wadsworth, Simon (2004): Infectivity of a Scottish isolate of Piscirickettsia salmonis for Atlantic salmon Salmo salar and immune response of salmon to this agent. In *Diseases of aquatic organisms* 60 (2), pp. 97–103. DOI: 10.3354/dao060097.

Bouchonnet, Francine; Boechat, Neio; Bonay, Marcel; Hance, Allan J. (2002): Alpha/beta interferon impairs the ability of human macrophages to control growth of Mycobacterium bovis BCG. In *Infection and immunity* 70 (6), pp. 3020–3025. DOI: 10.1128/IAI.70.6.3020-3025.2002.

Brinkman, Eva K.; Chen, Tao; Amendola, Mario; van Steensel, Bas (2014): Easy quantitative assessment of genome editing by sequence trace decomposition. In *Nucleic acids research* 42 (22), e168. DOI: 10.1093/nar/gku936.

Castro, Rosario; Jouneau, Luc; Pham, Hang-Phuong; Bouchez, Olivier; Giudicelli, Véronique; Lefranc, Marie-Paule et al. (2013): Teleost fish mount complex clonal IgM and IgT responses in spleen upon systemic viral infection. In *PLoS pathogens* 9 (1), e1003098. DOI: 10.1371/journal.ppat.1003098.

Castro, Rosario; Martínez-Alonso, Susana; Fischer, Uwe; Haro, Neila Álvarez de; Soto-Lampe, Verónica; Wang, Tiehui et al. (2014): DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle. In *Vaccine* 32 (10), pp. 1160–1168. DOI: 10.1016/j.vaccine.2013.11.062.

Chang, C. J.; Gu, J.; Robertsen, B. (2017): Protective effect and antibody response of DNA vaccine against salmonid alphavirus 3 (SAV3) in Atlantic salmon. In *Journal of fish diseases* 40 (12), pp. 1775–1781. DOI: 10.1111/jfd.12644.

Chang, Chia-Jung; Jenssen, Iris; Robertsen, Børre (2016): Protection of Atlantic salmon against salmonid alphavirus infection by type I interferons IFNa, IFNb and IFNc. In *Fish & shellfish immunology* 57, pp. 35–40. DOI: 10.1016/j.fsi.2016.08.020.

Chang, Chia-Jung; Robertsen, Camilla; Sun, Baojian; Robertsen, Børre (2014): Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. In *Vaccine* 32 (36), pp. 4695–4702. DOI: 10.1016/j.vaccine.2014.05.059.

Chang, Chia-Jung; Sun, Baojian; Robertsen, Børre (2015): Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model. In *Vaccine* 33 (21), pp. 2442–2448. DOI: 10.1016/j.vaccine.2015.03.093.

Chang, Ming-Xian; Zou, Jun; Nie, Pin; Huang, Bei; Yu, Zhanglong; Collet, Bertrand; Secombes, Chris J. (2013): Intracellular interferons in fish: a unique means to combat viral infection. In *PLoS pathogens* 9 (11), e1003736. DOI: 10.1371/journal.ppat.1003736.

Chen, Shan Nan; Zou, Peng Fei; Nie, Pin (2017): Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in fish: current knowledge and future perspectives. In *Immunology* 151 (1), pp. 16–25. DOI: 10.1111/imm.12714.

Coers, Jörn; Vance, Russell E.; Fontana, Mary F.; Dietrich, William F. (2007): Restriction of Legionella pneumophila growth in macrophages requires the concerted action of cytokine and Naip5/Ipaf signalling pathways. In *Cellular microbiology* 9 (10), pp. 2344–2357. DOI: 10.1111/j.1462-5822.2007.00963.x.

Collet, Bertrand; Munro, Eann S.; Gahlawat, Suresh; Acosta, Felix; Garcia, Jose; Roemelt, Christina et al. (2007): Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. In *Fish & shellfish immunology* 22 (1-2), pp. 44–56. DOI: 10.1016/j.fsi.2006.03.011.

Daffis, Stephane; Samuel, Melanie A.; Keller, Brian C.; Gale, Michael; Diamond, Michael S. (2007): Cell-specific IRF-3 responses protect against West Nile virus infection by interferon-dependent and -independent mechanisms. In *PLoS pathogens* 3 (7), e106. DOI: 10.1371/journal.ppat.0030106.

Daffis, Stephane; Samuel, Melanie A.; Suthar, Mehul S.; Keller, Brian C.; Gale, Michael; Diamond, Michael S. (2008): Interferon regulatory factor IRF-7 induces the antiviral alpha interferon response and protects against lethal West Nile virus infection. In *Journal of virology* 82 (17), pp. 8465–8475. DOI: 10.1128/JVI.00918-08.

Daffis, Stephane; Suthar, Mehul S.; Szretter, Kristy J.; Gale, Michael; Diamond, Michael S. (2009): Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7. In *PLoS pathogens* 5 (10), e1000607. DOI: 10.1371/journal.ppat.1000607.

Dalskov, Louise; Narita, Ryo; Andersen, Line L.; Jensen, Nanna; Assil, Sonia; Kristensen, Kennith H. et al. (2020): Characterization of distinct molecular interactions responsible for IRF3 and IRF7 phosphorylation and subsequent dimerization. In *Nucleic acids research* 48 (20), pp. 11421–11433. DOI: 10.1093/nar/gkaa873.

Dehler, Carola E.; Lester, Katherine; Della Pelle, Giulia; Jouneau, Luc; Houel, Armel; Collins, Catherine et al. (2019): Viral Resistance and IFN Signaling in STAT2 Knockout Fish Cells. In *Journal of immunology (Baltimore, Md. : 1950)* 203 (2), pp. 465–475. DOI: 10.4049/jimmunol.1801376.

Elke Lorbach (2018): CRISPR. Tips and Tricks for Efficient Delivery into Primary Cells and Cell Lines. Lonza webinar. Lonza. Online, May 2018, checked on May 2018.

Eslamloo, Khalil; Caballero-Solares, Albert; Inkpen, Sabrina M.; Emam, Mohamed; Kumar, Surendra; Bouniot, Camila et al. (2020): Transcriptomic Profiling of the Adaptive and Innate Immune Responses of Atlantic Salmon to Renibacterium salmoninarum Infection. In *Frontiers in immunology* 11, p. 567838. DOI: 10.3389/fimmu.2020.567838.

Evensen, Ø. (2009): Development in fish vaccinology with focus on delivery methodologies, adjuvants and formulations. In C. Rogers, B. Basurco (Eds.): Options Méditerranéennes, vol. 86. Montpellier, pp. 177–186.

Evensen, Øystein (2016): Immunization Strategies against Piscirickettsia salmonis Infections: Review of Vaccination Approaches and Modalities and Their Associated Immune Response Profiles. In *Frontiers in immunology* 7, p. 482. DOI: 10.3389/fimmu.2016.00482.

FAO (2020): The State of World Fisheries and Aquaculture 2020: FAO.

Fischer, Uwe; Koppang, Erling Olaf; Nakanishi, Teruyuki (2013): Teleost T and NK cell immunity. In *Fish & shellfish immunology* 35 (2), pp. 197–206. DOI: 10.1016/j.fsi.2013.04.018.

Flores-Kossack, C.; Montero, R.; Köllner, B.; Maisey, K. (2020): Chilean aquaculture and the new challenges: Pathogens, immune response, vaccination and fish diversification. In *Fish & shellfish immunology* 98, pp. 52–67. DOI: 10.1016/j.fsi.2019.12.093.

Fringuelli, E.; Rowley, H. M.; Wilson, J. C.; Hunter, R.; Rodger, H.; Graham, D. A. (2008): Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. In *Journal of fish diseases* 31 (11), pp. 811–823. DOI: 10.1111/j.1365-2761.2008.00944.x.

Fryer, J. L.; Lannan, C. N.; GARCES, L. H.; LARENAS, J. J.; Smith, P. A. (1990): Isolation of a rickettsiales-like organism from diseased coho salmon (Oncorhynchus kisutch) in Chile. In *Fish Pathol.* 25 (2), pp. 107–114. DOI: 10.3147/jsfp.25.107.

Fryer, J. L.; Lannan, C. N.; Giovannoni, S. J.; Wood, N. D. (1992): Piscirickettsia salmonis gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. In *International journal of systematic bacteriology* 42 (1), pp. 120–126. DOI: 10.1099/00207713-42-1-120.

Fryer, John L.; Lannan, Catharine N. (2015): Piscirickettsia. In William B. Whitman, Fred Rainey, Peter Kämpfer, Martha Trujillo, Jonsik Chun, Paul DeVos et al. (Eds.): Bergey's Manual of Systematics of Archaea and Bacteria: Wiley, pp. 1–8.

Gahlawat, Suresh K.; Ellis, Anthony E.; Collet, Bertrand (2009): Expression of interferon and interferon--induced genes in Atlantic salmon Salmo salar cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV. In *Fish & shellfish immunology* 26 (4), pp. 672–675. DOI: 10.1016/j.fsi.2009.02.021.

Gomez, Daniela; Sunyer, J. Oriol; Salinas, Irene (2013): The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. In *Fish & shellfish immunology* 35 (6), pp. 1729–1739. DOI: 10.1016/j.fsi.2013.09.032.

Graham, D. A.; Rowley, H. R.; Frost, P. (2014): Cross-neutralization studies with salmonid alphavirus subtype 1-6 strains: results with sera from experimental studies and natural infections. In *J Fish Diseases* 37 (8), pp. 683–691. DOI: 10.1111/jfd.12167.

Gratacap, Remi L.; Jin, Ye Hwa; Mantsopoulou, Marina; Houston, Ross D. (2020a): Efficient Genome Editing in Multiple Salmonid Cell Lines Using Ribonucleoprotein Complexes. In *Marine biotechnology (New York, N.Y.)* 22 (5), pp. 717–724. DOI: 10.1007/s10126-020-09995-y.

Gratacap, Remi L.; Regan, Tim; Dehler, Carola E.; Martin, Samuel A. M.; Boudinot, Pierre; Collet, Bertrand; Houston, Ross D. (2020b): Efficient CRISPR/Cas9 genome editing in a salmonid fish cell line using a lentivirus delivery system. In *BMC biotechnology* 20 (1), p. 35. DOI: 10.1186/s12896-020-00626-x.

Gudding, Roar; Lillehaug, Atle; Evensen, Øystein (2014): Fish Vaccination: John Wiley & Sons, Inc.

Gunti, Sreenivasulu; Messer, Ronald J.; Xu, Chengfu; Yan, Ming; Coleman, William G.; Peterson, Karin E. et al. (2015): Stimulation of Toll-Like Receptors profoundly influences the titer of polyreactive antibodies in the circulation. In *Scientific reports* 5, p. 15066. DOI: 10.1038/srep15066.

Guo, C. J.; He, J.; He, J. G. (2019): The immune evasion strategies of fish viruses. In *Fish & shellfish immunology* 86, pp. 772–784. DOI: 10.1016/j.fsi.2018.12.013.

Hamamoto, Itsuki; Takaku, Hiroshi; Tashiro, Masato; Yamamoto, Norio (2013): High yield production of influenza virus in Madin Darby canine kidney (MDCK) cells with stable knockdown of IRF7. In *PloS one* 8 (3), e59892. DOI: 10.1371/journal.pone.0059892.

Happold, Jonathan; Sadler, Rohan; Meyer, Anne; Hillman, Alison; Cowled, Brendan; Mackenzie, Catriona et al. (2020): Effectiveness of vaccination for the control of salmonid rickettsial septicaemia in commercial salmon and trout farms in Chile. In *Aquaculture* 520, p. 734968. DOI: 10.1016/j.aquaculture.2020.734968.

Hedges, Jodi F.; Robison, Amanda; Kimmel, Emily; Christensen, Kelly; Lucas, Erin; Ramstead, Andrew; Jutila, Mark A. (2016): Type I Interferon Counters or Promotes Coxiella burnetii Replication Dependent on Tissue. In *Infection and immunity* 84 (6), pp. 1815–1825. DOI: 10.1128/IAI.01540-15.

Henríquez, P.; Kaiser, M.; Bohle, H.; Bustos, P.; Mancilla, M. (2016): Comprehensive antibiotic susceptibility profiling of Chilean Piscirickettsia salmonis field isolates. In *J Fish Diseases* 39 (4), pp. 441–448. DOI: 10.1111/jfd.12427.

Herath, T.; Costa, J. Z.; Thompson, K.; Adams, A.; Richards, R. (2009): Alternative cell line for the isolation of salmonid alphavirus-1. In *Icelandic Agricultural Sciences* 22, pp. 19–27.

Herath, Tharangani K.; Thompson, Kim D.; Adams, Alexandra; Richards, Randolph H. (2013): Interferon-mediated host response in experimentally induced salmonid alphavirus 1 infection in Atlantic salmon (Salmo salar L.). In *Veterinary immunology and immunopathology* 155 (1-2), pp. 9–20. DOI: 10.1016/j.vetimm.2013.05.011.

Hiscott, J.; Pitha, P.; Genin, P.; Nguyen, H.; Heylbroeck, C.; Mamane, Y. et al. (1999): Triggering the interferon response: the role of IRF-3 transcription factor. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 19 (1), pp. 1–13. DOI: 10.1089/107999099314360.

Honda, Kenya; Yanai, Hideyuki; Negishi, Hideo; Asagiri, Masataka; Sato, Mitsuharu; Mizutani, Tatsuaki et al. (2005): IRF-7 is the master regulator of type-I interferon-dependent immune responses. In *Nature* 434 (7034), pp. 772–777. DOI: 10.1038/nature03464.

Hua, Zhaolin; Hou, Baidong (2013): TLR signaling in B-cell development and activation. In *Cellular & molecular immunology* 10 (2), pp. 103–106. DOI: 10.1038/cmi.2012.61.

Iliev, Dimitar B.; Sobhkhez, Mehrdad; Fremmerlid, Kjersti; Jørgensen, Jorunn B. (2011): MyD88 interacts with interferon regulatory factor (IRF) 3 and IRF7 in Atlantic salmon (Salmo salar): transgenic SsMyD88 modulates the IRF-induced type I interferon response and accumulates in aggresomes. In *The Journal of biological chemistry* 286 (49), pp. 42715–42724. DOI: 10.1074/jbc.M111.293969.

Iliev, Dimitar B.; Thim, Hanna; Lagos, Leidy; Olsen, Randi; Jørgensen, Jorunn B. (2013): Homing of Antigen-Presenting Cells in Head Kidney and Spleen - Salmon Head Kidney Hosts Diverse APC Types. In *Frontiers in immunology* 4, p. 137. DOI: 10.3389/fimmu.2013.00137.

Intesal (2014): EVALUACIÓN DE LA DURACIÓN DE LA EFICACIA DE VACUNAS PARA EL CONTROL DE PISCIRICKETTSIOSIS EN TRUCHA ARCOÍRIS Y SALMÓN DEL ATLÁNTICO MEDIANTE DESAFÍO POR COHABITACIÓN. Eficacia de vacunas para el control de SRS en trucha arcoíris y salmón del atlántico 1500 UTA. With assistance of SalmonChile. Intesal.

Jakob, Eva; Stryhn, Henrik; Yu, Jenny; Medina, Matias H.; Rees, Erin E.; Sanchez, Javier; St-Hilaire, Sophie (2014): Epidemiology of Piscirickettsiosis on selected Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) salt water aquaculture farms in Chile. In *Aquaculture* 433, pp. 288–294. DOI: 10.1016/j.aquaculture.2014.06.018.

Jami, Raphaël; Mérour, Emilie; Lamoureux, Annie; Bernard, Julie; Millet, Jean K.; Biacchesi, Stéphane (2021): Deciphering the Fine-Tuning of the Retinoic Acid-Inducible Gene-I Pathway in Teleost Fish and Beyond. In *Frontiers in immunology* 12, p. 679242. DOI: 10.3389/fimmu.2021.679242.

Jansen, M. D.; Bang Jensen, B.; McLoughlin, M. F.; Rodger, H. D.; Taksdal, T.; Sindre, H. et al. (2017): The epidemiology of pancreas disease in salmonid aquaculture: a summary of the current state of knowledge. In *Journal of fish diseases* 40 (1), pp. 141–155. DOI: 10.1111/jfd.12478.

Jenberie, Shiferaw; Peñaranda, Ma Michelle D.; Thim, Hanna L.; Styrvold, Morten Bay; Strandskog, Guro; Jørgensen, Jorunn B.; Jensen, Ingvill (2020): Salmonid Alphavirus Subtype 3 Induces Prolonged Local B Cell Responses in Atlantic Salmon (Salmo salar) After Intraperitoneal Infection. In *Frontiers in immunology* 11, p. 1682. DOI: 10.3389/fimmu.2020.01682.

Jenberie, Shiferaw; Thim, Hanna L.; Sunyer, J. Oriol; Skjødt, Karsten; Jensen, Ingvill; Jørgensen, Jorunn B. (2018): Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. In *Scientific reports* 8 (1), p. 3565. DOI: 10.1038/s41598-018-21895-9.

Jenberie, Shiferaw T. (2019): Atlantic salmon B cells- local and systemic responses to intraperitoneally administered salmonid alphavirus. PhD. UiT - The Arctic University of Norway, Tromsø.

Kaattari, S.; Zhang, H.; Khor, I.; Kaattari, I.; Shapiro, D. (2002): Affinity maturation in trout: clonal dominance of high affinity antibodies late in the immune response. In *Developmental & Comparative Immunology* 26 (2), pp. 191–200. DOI: 10.1016/S0145-305X(01)00064-7.

Kanchan, Vibhu; Panda, Amulya K. (2007): Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. In *Biomaterials* 28 (35), pp. 5344–5357. DOI: 10.1016/j.biomaterials.2007.08.015.

Karlsen, Marius; Tingbø, Terje; Solbakk, Inge-Tom; Evensen, Øystein; Furevik, Anette; Aas-Eng, Anne (2012): Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family Togaviridae). In *Vaccine* 30 (38), pp. 5688–5694. DOI: 10.1016/j.vaccine.2012.05.069.

Kawai, Taro; Takahashi, Ken; Sato, Shintaro; Coban, Cevayir; Kumar, Himanshu; Kato, Hiroki et al. (2005): IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. In *Nature immunology* 6 (10), pp. 981–988. DOI: 10.1038/ni1243.

Khan, Imran; Maldonado, Emanuel; Silva, Liliana; Almeida, Daniela; Johnson, Warren E.; O'Brien, Stephen J. et al. (2019): The Vertebrate TLR Supergene Family Evolved Dynamically by Gene Gain/Loss and Positive Selection Revealing a Host–Pathogen Arms Race in Birds. In *Diversity* 11 (8), p. 131. DOI: 10.3390/d11080131.

Kileng, Oyvind; Bergan, Veronica; Workenhe, Samuel Tekeste; Robertsen, Børre (2009): Structural and functional studies of an IRF-7-like gene from Atlantic salmon. In *Developmental and comparative immunology* 33 (1), pp. 18–27. DOI: 10.1016/j.dci.2008.07.020.

Kim, Tae Hyun; Kern, Colin; Zhou, Huaijun (2020): Knockout of IRF7 Highlights its Modulator Function of Host Response Against Avian Influenza Virus and the Involvement of MAPK and TOR Signaling Pathways in Chicken. In *Genes* 11 (4). DOI: 10.3390/genes11040385.

Kuzyk, Michael A.; Burian, Ján; Machander, Dave; Dolhaine, Daphne; Cameron, Stephen; Thornton, Julian C.; Kay, William W. (2001): An efficacious recombinant subunit vaccine against the salmonid rickettsial pathogen Piscirickettsia salmonis. In *Vaccine* 19 (17-19), pp. 2337–2344. DOI: 10.1016/S0264-410X(00)00524-7.

Langereis, Martijn A.; Groof, Ad de; Simmilenk, Bartjan W.; Vermeij, Paul (2020): MDBK IRF3/IRF7 Knock out mutant cell and its use for vaccine production. Applied for by Intervet International B.V. Patent no. WO2020245355A1.

Lannan, C. N.; Fryer, J. L. (1994): Extracellular survival of Piscirickettsia salmonis1. In *J Fish Diseases* 17 (5), pp. 545–548. DOI: 10.1111/j.1365-2761.1994.tb00251.x.

Larsen, Rannveig; Røkenes, Torunn P.; Robertsen, Børre (2004): Inhibition of infectious pancreatic necrosis virus replication by atlantic salmon Mx1 protein. In *Journal of virology* 78 (15), pp. 7938–7944. DOI: 10.1128/JVI.78.15.7938–7944.2004.

Lauksund, Silje; Greiner-Tollersrud, Linn; Chang, Chia-Jung; Robertsen, Børre (2015): Infectious pancreatic necrosis virus proteins VP2, VP3, VP4 and VP5 antagonize IFNa1 promoter activation while VP1 induces IFNa1. In *Virus research* 196, pp. 113–121. DOI: 10.1016/j.virusres.2014.11.018.

Lauksund, Silje; Svingerud, Tina; Bergan, Veronica; Robertsen, Børre (2009): Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-kappaB and localizes to mitochondria. In *Developmental and comparative immunology* 33 (11), pp. 1196–1204. DOI: 10.1016/j.dci.2009.06.012.

Lazear, Helen M.; Schoggins, John W.; Diamond, Michael S. (2019): Shared and Distinct Functions of Type I and Type III Interferons. In *Immunity* 50 (4), pp. 907–923. DOI: 10.1016/j.immuni.2019.03.025.

Le Bon, Agnes; Schiavoni, Giovanna; D'Agostino, Giuseppina; Gresser, Ion; Belardelli, Filippo; Tough, David F. (2001): Type I Interferons Potently Enhance Humoral Immunity and Can Promote Isotype Switching by Stimulating Dendritic Cells In Vivo. In *Immunity* 14 (4), pp. 461–470. DOI: 10.1016/S1074-7613(01)00126-1.

Lee, P. T.; Zou, J.; Holland, J. W.; Martin, S. A. M.; Collet, B.; Kanellos, T.; Secombes, C. J. (2014): Identification and characterisation of TLR18-21 genes in Atlantic salmon (Salmo salar). In *Fish & shellfish immunology* 41 (2), pp. 549–559. DOI: 10.1016/j.fsi.2014.10.006.

Li, Jun; Barreda, Daniel R.; Zhang, Yong-An; Boshra, Hani; Gelman, Andrew E.; Lapatra, Scott et al. (2006): B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. In *Nature immunology* 7 (10), pp. 1116–1124. DOI: 10.1038/ni1389.

Liao, Zhiwei; Su, Jianguo (2021): Progresses on three pattern recognition receptor families (TLRs, RLRs and NLRs) in teleost. In *Developmental and comparative immunology* 122, p. 104131. DOI: 10.1016/j.dci.2021.104131.

Liu, Fuguo; Wang, Tiehui; Petit, Jules; Forlenza, Maria; Chen, Xinhua; Chen, Liangbiao et al. (2020): Evolution of IFN subgroups in bony fish - 2. analysis of subgroup appearance and expansion in teleost fish with a focus on salmonids. In *Fish & shellfish immunology* 98, pp. 564–573. DOI: 10.1016/j.fsi.2020.01.039.

Liu, Ying; Luo, Xing; Yang, Chunting; Yu, Sanke; Xu, Honglin (2011): Three CpG oligodeoxynucleotide classes differentially enhance antigen-specific humoral and cellular immune responses in mice. In *Vaccine* 29 (34), pp. 5778–5784. DOI: 10.1016/j.vaccine.2011.05.087.

Loo, Yueh-Ming; Fornek, Jamie; Crochet, Nanette; Bajwa, Gagan; Perwitasari, Olivia; Martinez-Sobrido, Luis et al. (2008): Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. In *Journal of virology* 82 (1), pp. 335–345. DOI: 10.1128/JVI.01080-07.

Lund, Hege; Bakke, Anne Flore; Sommerset, Ingunn; Afanasyev, Sergey; Schriwer, Geir; Thorisdottir, Audur et al. (2019): A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon. In *Molecular immunology* 106, pp. 99–107. DOI: 10.1016/j.molimm.2018.12.018.

Ma, Cuiyan; Ye, Jianmin; Kaattari, Stephen L. (2013): Differential compartmentalization of memory B cells versus plasma cells in salmonid fish. In *European journal of immunology* 43 (2), pp. 360–370. DOI: 10.1002/eji.201242570.

Magor, Brad G. (2015): Antibody Affinity Maturation in Fishes-Our Current Understanding. In *Biology* 4 (3), pp. 512–524. DOI: 10.3390/biology4030512.

Maisey, Kevin; Montero, Ruth; Christodoulides, Myron (2017): Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. In *Expert review of vaccines* 16 (3), pp. 215–228. DOI: 10.1080/14760584.2017.1244483.

Martin, Francis J.; Gomez, Marisa I.; Wetzel, Dawn M.; Memmi, Guido; O'Seaghdha, Maghnus; Soong, Grace et al. (2009): Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. In *The Journal of clinical investigation* 119 (7), pp. 1931–1939. DOI: 10.1172/JCI35879.

Mauel, Michael J.; Ware, Cynthia; Smith, Pedro A. (2008): Culture of Piscirickettsia salmonis on enriched blood agar. In *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc* 20 (2), pp. 213–214. DOI: 10.1177/104063870802000211.

Mayer-Barber, Katrin D.; Andrade, Bruno B.; Oland, Sandra D.; Amaral, Eduardo P.; Barber, Daniel L.; Gonzales, Jacqueline et al. (2014): Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. In *Nature* 511 (7507), pp. 99–103. DOI: 10.1038/nature13489.

McCarthy, Una M.; Bron, James E.; Brown, Linton; Pourahmad, Fazel; Bricknell, Ian R.; Thompson, Kim D. et al. (2008): Survival and replication of Piscirickettsia salmonis in rainbow trout head kidney macrophages. In *Fish & shellfish immunology* 25 (5), pp. 477–484. DOI: 10.1016/j.fsi.2008.07.005.

McLoughlin, M. F.; Graham, D. A. (2007): Alphavirus infections in salmonids--a review. In *Journal of fish diseases* 30 (9), pp. 511–531. DOI: 10.1111/j.1365-2761.2007.00848.x.

Meylan, Etienne; Curran, Joseph; Hofmann, Kay; Moradpour, Darius; Binder, Marco; Bartenschlager, Ralf; Tschopp, Jürg (2005): Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. In *Nature* 437 (7062), pp. 1167–1172. DOI: 10.1038/nature04193.

Mikalsen, Jarle; Skjaervik, Olaf; Wiik-Nielsen, Jannicke; Wasmuth, Marit A.; Colquhoun, Duncan J. (2008): Agar culture of Piscirickettsia salmonis, a serious pathogen of farmed salmonid and marine fish. In *FEMS microbiology letters* 278 (1), pp. 43–47. DOI: 10.1111/j.1574-6968.2007.00977.x.

Miquel, Alvaro; Müller, Ilse; Ferrer, Pablo; Valenzuela, Pablo D.; Burzio, Luis O. (2003): Immunoresponse of Coho salmon immunized with a gene expression library from Piscirickettsia salmonis. In *Biological research* 36 (3-4), pp. 313–323. DOI: 10.4067/S0716-97602003000300004.

Munir, Danish; Munro, Eann S.; Secombes, Christopher J.; Dooley, Helen (2020): Atlantic salmon kidney (ASK) cells are an effective model to characterise interferon (IFN) and IFN-induced gene

expression following salmonid alphavirus infection. In *Fish & shellfish immunology* 106, pp. 792–795. DOI: 10.1016/j.fsi.2020.08.043.

Muthupandian, A.; Waly, D.; Magor, B. G. (2021): Do ectothermic vertebrates have a home in which to affinity mature their antibody responses? In *Developmental and comparative immunology* 119, p. 104021. DOI: 10.1016/j.dci.2021.104021.

Ning, S.; Pagano, J. S.; Barber, G. N. (2011): IRF7: activation, regulation, modification and function. In *Genes and immunity* 12 (6), pp. 399–414. DOI: 10.1038/gene.2011.21.

O'Connell, Ryan M.; Saha, Supriya K.; Vaidya, Sagar A.; Bruhn, Kevin W.; Miranda, Gustavo A.; Zarnegar, Brian et al. (2004): Type I interferon production enhances susceptibility to Listeria monocytogenes infection. In *The Journal of experimental medicine* 200 (4), pp. 437–445. DOI: 10.1084/jem.20040712.

Ooi, Ei Lin; Verjan, Noel; Hirono, Ikuo; Nochi, Tomonori; Kondo, Hidehiro; Aoki, Takashi et al. (2008): Biological characterisation of a recombinant Atlantic salmon type I interferon synthesized in Escherichia coli. In *Fish & shellfish immunology* 24 (5), pp. 506–513. DOI: 10.1016/j.fsi.2007.10.004.

Parker, Dane; Prince, Alice (2011): Type I interferon response to extracellular bacteria in the airway epithelium. In *Trends in immunology* 32 (12), pp. 582–588. DOI: 10.1016/j.it.2011.09.003.

Parra, David; Rieger, Aja M.; Li, Jun; Zhang, Yong-An; Randall, Louise M.; Hunter, Christopher A. et al. (2012): Pivotal advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4+ T cells. In *Journal of leukocyte biology* 91 (4), pp. 525–536. DOI: 10.1189/jlb.0711372.

Peñaranda, Ma Michelle D.; Jensen, Ingvill; Tollersrud, Linn G.; Bruun, Jack-Ansgar; Jørgensen, Jorunn B. (2019): Profiling the Atlantic Salmon IgM+ B Cell Surface Proteome: Novel Information on Teleost Fish B Cell Protein Repertoire and Identification of Potential B Cell Markers. In *Frontiers in immunology* 10, p. 37. DOI: 10.3389/fimmu.2019.00037.

Perdiguero, Pedro; Morel, Esther; Tafalla, Carolina (2021): Diversity of Rainbow Trout Blood B Cells Revealed by Single Cell RNA Sequencing. In *Biology* 10 (6). DOI: 10.3390/biology10060511.

Perry, Andrea K.; Chen, Gang; Zheng, Dahai; Tang, Hong; Cheng, Genhong (2005): The host type I interferon response to viral and bacterial infections. In *Cell research* 15 (6), pp. 407–422. DOI: 10.1038/sj.cr.7290309.

Perry, Stuart T.; Prestwood, Tyler R.; Lada, Steven M.; Benedict, Chris A.; Shresta, Sujan (2009): Cardif-mediated signaling controls the initial innate response to dengue virus in vivo. In *Journal of virology* 83 (16), pp. 8276–8281. DOI: 10.1128/JVI.00365-09.

Pignatelli, Jaime; Castro, Rosario; González Granja, Aitor; Abós, Beatriz; González, Lucia; Jensen, Linda B.; Tafalla, Carolina (2014): Immunological characterization of the teleost adipose tissue and its modulation in response to viral infection and fat-content in the diet. In *PloS one* 9 (10), e110920. DOI: 10.1371/journal.pone.0110920.

Plotkin, Stanley A. (2010): Correlates of protection induced by vaccination. In *Clinical and vaccine immunology : CVI* 17 (7), pp. 1055–1065. DOI: 10.1128/CVI.00131-10.

Plumlee, Courtney R.; Lee, Carolyn; Beg, Amer A.; Decker, Thomas; Shuman, Howard A.; Schindler, Christian (2009): Interferons direct an effective innate response to Legionella pneumophila infection. In *The Journal of biological chemistry* 284 (44), pp. 30058–30066. DOI: 10.1074/jbc.M109.018283.

Poppe, T.; Rimstad, E.; Hyllseth, B. (1989): Pancreas disease in Atlantic salmon (Salmo salar) postsmolts infected with infectious pancreatic necrosis virus (IPNV). In *Bulletin of the European Association of Fish Pathologists* 9 (4), pp. 83–85.

Prieto, J. M. B.; Felippe, M. J. B. (2017): Development, phenotype, and function of non-conventional B cells. In *Comparative immunology, microbiology and infectious diseases* 54, pp. 38–44. DOI: 10.1016/j.cimid.2017.08.002.

Pulgar, Rodrigo; Hödar, Christian; Travisany, Dante; Zuñiga, Alejandro; Domínguez, Calixto; Maass, Alejandro et al. (2015): Transcriptional response of Atlantic salmon families to Piscirickettsia salmonis infection highlights the relevance of the iron-deprivation defence system. In *BMC genomics* 16, p. 495. DOI: 10.1186/s12864-015-1716-9.

Ramírez, Ramón; Gómez, Fernando A.; Marshall, Sergio H. (2015): The infection process of Piscirickettsia salmonis in fish macrophages is dependent upon interaction with host-cell clathrin and actin. In *FEMS microbiology letters* 362 (1), pp. 1–8. DOI: 10.1093/femsle/fnu012.

Ramsey, Jolene; Mukhopadhyay, Suchetana (2017): Disentangling the Frames, the State of Research on the Alphavirus 6K and TF Proteins. In *Viruses* 9 (8). DOI: 10.3390/v9080228.

Rangel-Moreno, Javier; Moyron-Quiroz, Juan E.; Carragher, Damian M.; Kusser, Kim; Hartson, Louise; Moquin, Amy; Randall, Troy D. (2009): Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. In *Immunity* 30 (5), pp. 731–743. DOI: 10.1016/j.immuni.2009.03.014.

Reyes-Cerpa, Sebastián; Reyes-López, Felipe E.; Toro-Ascuy, Daniela; Ibañez, Jorge; Maisey, Kevin; Sandino, Ana María; Imarai, Mónica (2012): IPNV modulation of pro and anti-inflammatory cytokine expression in Atlantic salmon might help the establishment of infection and persistence. In *Fish & shellfish immunology* 32 (2), pp. 291–300. DOI: 10.1016/j.fsi.2011.11.018.

Rhodes, Linda D.; Wallis, Steviebrooke; Demlow, S. Ellen (2009): Genes associated with an effective host response by Chinook salmon to Renibacterium salmoninarum. In *Developmental and comparative immunology* 33 (2), pp. 176–186. DOI: 10.1016/j.dci.2008.08.006.

Robertsen, Børre (2018): The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. In *Developmental and comparative immunology* 80, pp. 41–52. DOI: 10.1016/j.dci.2017.02.005.

Robertsen, Børre; Bergan, Veronica; Røkenes, Torunn; Larsen, Rannveig; Albuquerque, Artur (2003): Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 23 (10), pp. 601–612. DOI: 10.1089/107999003322485107.

Røsaeg, Magnus Vikan; Thorarinsson, Ragnar; Aunsmo, Arnfinn (2021): Effect of vaccines against pancreas disease in farmed Atlantic salmon. In *Journal of fish diseases* 44 (12), pp. 1911–1924. DOI: 10.1111/jfd.13505.

Rozas, M.; Enríquez, R. (2014): Piscirickettsiosis and Piscirickettsia salmonis in fish: a review. In *Journal of fish diseases* 37 (3), pp. 163–188. DOI: 10.1111/jfd.12211.

Rozas-Serri, Marco (2022): Why Does Piscirickettsia salmonis Break the Immunological Paradigm in Farmed Salmon? Biological Context to Understand the Relative Control of Piscirickettsiosis. In *Frontiers in immunology* 13, p. 856896. DOI: 10.3389/fimmu.2022.856896.

Rozas-Serri, Marco; Peña, Andrea; Maldonado, Lucerina (2018): Transcriptomic profiles of post-smolt Atlantic salmon challenged with Piscirickettsia salmonis reveal a strategy to evade the adaptive immune response and modify cell-autonomous immunity. In *Developmental and comparative immunology* 81, pp. 348–362. DOI: 10.1016/j.dci.2017.12.023.

Sen, Adrish; Pruijssers, Andrea J.; Dermody, Terence S.; García-Sastre, Adolfo; Greenberg, Harry B. (2011): The early interferon response to rotavirus is regulated by PKR and depends on MAVS/IPS-1, RIG-I, MDA-5, and IRF3. In *Journal of virology* 85 (8), pp. 3717–3732. DOI: 10.1128/JVI.02634-10.

Sena, J. de; Rio, G. J. (1975): Partial purification and characterization of RTG-2 fish cell interferon. In *Infection and immunity* 11 (4), pp. 815–822. DOI: 10.1128/iai.11.4.815-822.1975.

Seth, Rashu B.; Sun, Lijun; Ea, Chee-Kwee; Chen, Zhijian J. (2005): Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. In *Cell* 122 (5), pp. 669–682. DOI: 10.1016/j.cell.2005.08.012.

Simón, Rocío; Díaz-Rosales, Patricia; Morel, Esther; Martín, Diana; Granja, Aitor G.; Tafalla, Carolina (2019): CpG Oligodeoxynucleotides Modulate Innate and Adaptive Functions of IgM+ B Cells in Rainbow Trout. In *Frontiers in immunology* 10, p. 584. DOI: 10.3389/fimmu.2019.00584.

Simón, Rocío; Martín-Martín, Alba; Morel, Esther; Díaz-Rosales, Patricia; Tafalla, Carolina (2022): Functional and Phenotypic Characterization of B Cells in the Teleost Adipose Tissue. In *Frontiers in immunology* 13, p. 868551. DOI: 10.3389/fimmu.2022.868551.

Skjesol, Astrid; Aamo, Toril; Hegseth, Marit Nøst; Robertsen, Børre; Jørgensen, Jorunn B. (2009): The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. In *Virus research* 143 (1), pp. 53–60. DOI: 10.1016/j.virusres.2009.03.004.

Skjesol, Astrid; Skjæveland, Ingrid; Elnæs, Marianne; Timmerhaus, Gerrit; Fredriksen, Børge N.; Jørgensen, Sven Martin et al. (2011): IPNV with high and low virulence: host immune responses and viral mutations during infection. In *Virology journal* 8, p. 396. DOI: 10.1186/1743-422X-8-396.

Skjold, Pål; Sommerset, Ingunn; Frost, Petter; Villoing, Stephane (2016): Vaccination against pancreas disease in Atlantic salmon, Salmo salar L., reduces shedding of salmonid alphavirus. In *Veterinary research* 47 (1), p. 78. DOI: 10.1186/s13567-016-0362-9.

Skotheim, Sven A. (2009): Co-infection with Norwegian Salmonid Alphavirus (NSAV) and Infectious Pancreatic Necrosis Virus (IPNV) in Chinook Salmon Embryo Cells (CHSE-214). Master. University of Bergen, Bergen.

Smith, P. A.; Contreras, J. R.; LARENAS, J. J.; Aguillon, J. C.; GARCES, L. H.; Perez, B.; Fryer, J. L. (1997): Immunization with bacterial antigens: piscirickettsiosis. In *Developments in Biological Standardization* 90, pp. 161–166.

Smith, P. A.; Pizarro, P.; Ojeda, P.; Contreras, J.; Oyanedel, S.; Larenas, J. (1999): Routes of entry of Piscirickettsia salmonis in rainbow trout Oncorhynchus mykiss. In *Diseases of aquatic organisms* 37 (3), pp. 165–172. DOI: 10.3354/dao037165.

Snyder, Deann T.; Hedges, Jodi F.; Jutila, Mark A. (2017): Getting "Inside" Type I IFNs: Type I IFNs in Intracellular Bacterial Infections. In *Journal of immunology research* 2017, p. 9361802. DOI: 10.1155/2017/9361802.

Sommerset, I.; Walde, C. S.; Bang Jensen, B.; Wiik-Nielsen, J.; Bornø, G.; Oliveira, V. H. S. et al. (2022): Fiskehelserapporten 2021. Norwegian Veterinary Institute (2a).

Strandskog, Guro; Skjaeveland, Ingrid; Ellingsen, Terje; Jørgensen, Jorunn B. (2008): Double-stranded RNA- and CpG DNA-induced immune responses in Atlantic salmon: comparison and synergies. In *Vaccine* 26 (36), pp. 4704–4715. DOI: 10.1016/j.vaccine.2008.06.054.

Sun, Baojian; Robertsen, Børre; Wang, Zhiqiang; Liu, Bin (2009): Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. In *Developmental and comparative immunology* 33 (4), pp. 547–558. DOI: 10.1016/j.dci.2008.10.001.

Sun, Baojian; Skjæveland, Ingrid; Svingerud, Tina; Zou, Jun; Jørgensen, Jorunn; Robertsen, Børre (2011): Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. In *Journal of virology* 85 (17), pp. 9188–9198. DOI: 10.1128/JVI.00319-11.

Sun, Fan; Zhang, Yi-Bing; Liu, Ting-Kai; Gan, Li; Yu, Fei-Fei; Liu, Ying; Gui, Jian-Fang (2010): Characterization of fish IRF3 as an IFN-inducible protein reveals evolving regulation of IFN response in vertebrates. In *Journal of immunology (Baltimore, Md. : 1950)* 185 (12), pp. 7573–7582. DOI: 10.4049/jimmunol.1002401.

Svenning, Steingrim; Gondek-Wyrozemska, Agata T.; van der Wal, Yorick Andreas; Robertsen, Børre; Jensen, Ingvill; Jørgensen, Jorunn B.; Edholm, Eva-Stina (2019): Microbial Danger Signals Control Transcriptional Induction of Distinct MHC Class I L Lineage Genes in Atlantic Salmon. In *Frontiers in immunology* 10, p. 2425. DOI: 10.3389/fimmu.2019.02425.

Svingerud, Tina; Solstad, Terese; Sun, Baojian; Nyrud, May Liss J.; Kileng, Øyvind; Greiner-Tollersrud, Linn; Robertsen, Børre (2012): Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues. In *Journal of immunology (Baltimore, Md. : 1950)* 189 (12), pp. 5912–5923. DOI: 10.4049/jimmunol.1201188.

Tacchi, Luca; Bron, James E.; Taggart, John B.; Secombes, Christopher J.; Bickerdike, Ralph; Adler, Michael A. et al. (2011): Multiple tissue transcriptomic responses to Piscirickettsia salmonis in Atlantic salmon (Salmo salar). In *Physiological genomics* 43 (21), pp. 1241–1254. DOI: 10.1152/physiolgenomics.00086.2011.

Thim, Hanna L.; Iliev, Dimitar B.; Christie, Karen E.; Villoing, Stéphane; McLoughlin, Marian F.; Strandskog, Guro; Jørgensen, Jorunn B. (2012): Immunoprotective activity of a Salmonid Alphavirus Vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I:C alone or combined with an oil adjuvant. In *Vaccine* 30 (32), pp. 4828–4834. DOI: 10.1016/j.vaccine.2012.05.010.

Thorarinsson, Ragnar; Wolf, Jeffrey C.; Inami, Makoto; Phillips, Lisa; Jones, Ginny; Macdonald, Alicia M. et al. (2021): Effect of a novel DNA vaccine against pancreas disease caused by salmonid alphavirus subtype 3 in Atlantic salmon (Salmo salar). In *Fish & shellfish immunology* 108, pp. 116–126. DOI: 10.1016/j.fsi.2020.12.002.

Tobar, Iván; Arancibia, Sergio; Torres, Constanza; Vera, Verónica; Soto, Paola; Carrasco, Claudia et al. (2015): Successive Oral Immunizations Against Piscirickettsia Salmonis and Infectious Salmon Anemia Virus are Required to Maintain a Long-Term Protection in Farmed Salmonids. In *Frontiers in immunology* 6, p. 244. DOI: 10.3389/fimmu.2015.00244.

Uribe, C.; Folch, H.; Enriquez, R.; Moran, G. (2011): Innate and adaptive immunity in teleost fish: a review. In *Veterinární Medicína* 56 (10), pp. 486–503.

van der Wal, Yorick A. (25-April-2019): Challenge of Atlantic salmon with Piscirickettsia salmonis. Student paper. UiT - The Arctic University of Norway. Animal Aquatic Welfare.

Vargas, Deborah; Vallejos-Vidal, Eva; Reyes-Cerpa, Sebastián; Oyarzún-Arrau, Aarón; Acuña-Castillo, Claudio; Imarai, Mónica et al. (2021): The Analysis of Live-Attenuated Piscirickettsia salmonis Vaccine Reveals the Short-Term Upregulation of Innate and Adaptive Immune Genes in Atlantic Salmon (Salmo salar): An In Situ Open-Sea Cages Study. In *Microorganisms* 9 (4). DOI: 10.3390/microorganisms9040703.

Wang, Jennifer P.; Cerny, Anna; Asher, Damon R.; Kurt-Jones, Evelyn A.; Bronson, Roderick T.; Finberg, Robert W. (2010): MDA5 and MAVS mediate type I interferon responses to coxsackie B virus. In *Journal of virology* 84 (1), pp. 254–260. DOI: 10.1128/JVI.00631-09.

Westermann, Lukas; Li, Yong; Göcmen, Burulca; Niedermoser, Matthias; Rhein, Kilian; Jahn, Johannes et al. (2022): Wildtype heterogeneity contributes to clonal variability in genome edited cells. In *Scientific reports* 12 (1), p. 18211. DOI: 10.1038/s41598-022-22885-8.

Weston, J. H.; Graham, D. A.; Branson, E.; Rowley, H. M.; Walker, I. W.; Jewhurst, V. A. et al. (2005): Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease. In *Diseases of aquatic organisms* 66 (2), pp. 105–111. DOI: 10.3354/dao066105.

Wilhelm, Vivian; Soza, Cristián; Martínez, Rodrigo; Rosemblatt, Mario; Burzio, Luis O.; Valenzuela, Pablo D. T. (2005): Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen Piscirickettsia salmonis. In *Biological research* 38 (1), pp. 69–82. DOI: 10.4067/S0716-97602005000100009.

Wu, Liting; Fu, Shengli; Yin, Xiaoxue; Guo, Zheng; Wang, Anli; Ye, Jianmin (2019): Long-Lived Plasma Cells Secrete High-Affinity Antibodies Responding to a T-Dependent Immunization in a Teleost Fish. In *Frontiers in immunology* 10, p. 2324. DOI: 10.3389/fimmu.2019.02324.

Xu, Cheng; Evensen, Øystein; Munang'andu, Hetron (2016): De Novo Transcriptome Analysis Shows That SAV-3 Infection Upregulates Pattern Recognition Receptors of the Endosomal Toll-Like and RIG-I-Like Receptor Signaling Pathways in Macrophage/Dendritic Like TO-Cells. In *Viruses* 8 (4), p. 114. DOI: 10.3390/v8040114.

Xu, Cheng; Guo, Tz-Chun; Mutoloki, Stephen; Haugland, Oyvind; Evensen, Oystein (2012): Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. In *Veterinary research* 43, p. 78. DOI: 10.1186/1297-9716-43-78.

Xu, Cheng; Guo, Tz-Chun; Mutoloki, Stephen; Haugland, Øyvind; Marjara, Inderjit S.; Evensen, Øystein (2010): Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro. In *Journal of virology* 84 (17), pp. 8903–8912. DOI: 10.1128/JVI.00851-10.

Xu, Liang-Guo; Wang, Yan-Yi; Han, Ke-Jun; Li, Lian-Yun; Zhai, Zhonghe; Shu, Hong-Bing (2005): VISA is an adapter protein required for virus-triggered IFN-beta signaling. In *Molecular cell* 19 (6), pp. 727–740. DOI: 10.1016/j.molcel.2005.08.014.

Yañez, A. J.; Valenzuela, K.; Silva, H.; Retamales, J.; Romero, A.; Enriquez, R. et al. (2012): Broth medium for the successful culture of the fish pathogen Piscirickettsia salmonis. In *Diseases of aquatic organisms* 97 (3), pp. 197–205. DOI: 10.3354/dao02403.

Ye, Jianmin; Kaattari, Ilsa M.; Kaattari, Stephen L. (2011): The differential dynamics of antibody subpopulation expression during affinity maturation in a teleost. In *Fish & shellfish immunology* 30 (1), pp. 372–377. DOI: 10.1016/j.fsi.2010.11.013.

Ye, Jianmin; Kaattari, Ilsa M.; Ma, Cuiyan; Kaattari, Stephen (2013): The teleost humoral immune response. In *Fish & shellfish immunology* 35 (6), pp. 1719–1728. DOI: 10.1016/j.fsi.2013.10.015.

Yu, Yongyao; Wang, Qingchao; Huang, Zhenyu; Ding, Liguo; Xu, Zhen (2020): Immunoglobulins, Mucosal Immunity and Vaccination in Teleost Fish. In *Frontiers in immunology* 11, p. 567941. DOI: 10.3389/fimmu.2020.567941.

Zhang, Jun; Zhang, Yi-Bing; Wu, Min; Wang, Bing; Chen, Chen; Gui, Jian-Fang (2014): Fish MAVS is involved in RLR pathway-mediated IFN response. In *Fish & shellfish immunology* 41 (2), pp. 222–230. DOI: 10.1016/j.fsi.2014.09.002.

Zhang, L.; Pagano, J. S. (1997): IRF-7, a new interferon regulatory factor associated with Epstein-Barr virus latency. In *Molecular and cellular biology* 17 (10), pp. 5748–5757. DOI: 10.1128/MCB.17.10.5748.

Zhang, Luwen; Pagano, Joseph S. (2002): Structure and function of IRF-7. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 22 (1), pp. 95–101. DOI: 10.1089/107999002753452700.

Zhang, Yi-Bing; Hu, Cheng-yu; Zhang, Jing; Huang, Guo-ping; Wei, Li-hua; Zhang, Qi-ya; Gui, Jian-Fang (2003): Molecular cloning and characterization of crucian carp (Carassius auratus L.) interferon regulatory factor 711The nucleotide sequence data reported in this paper has been submitted to the GenBank under accession number AY177629. In *Fish & shellfish immunology* 15 (5), pp. 453–466. DOI: 10.1016/S1050-4648(03)00025-1.

Zou, Jun; Gorgoglione, Bartolomeo; Taylor, Nicholas G. H.; Summathed, Thitiya; Lee, Po-Tsang; Panigrahi, Akshaya et al. (2014): Salmonids have an extraordinary complex type I IFN system: characterization of the IFN locus in rainbow trout oncorhynchus mykiss reveals two novel IFN subgroups. In *Journal of immunology (Baltimore, Md. : 1950)* 193 (5), pp. 2273–2286. DOI: 10.4049/jimmunol.1301796.

Zubler, R. H. (2001): Naive and memory B cells in T-cell-dependent and T-independent responses. In *Springer seminars in immunopathology* 23 (4), pp. 405–419. DOI: 10.1007/s281-001-8167-7.

Paper I

Antibody response after intraperitoneal *Piscirickettsia salmonis* infection in Atlantic salmon: duration and protection

Authors/contributors

Yorick A. van der Wal[1,2], Bjørn Krossøy[3], Marcos Mancilla[4], Jorunn B. Jørgensen[2], Jaap Kool[1]

1 Vaxxinova Research & Development GmbH, Münster, Germany
2 Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, Tromsø, Norway
3 Vaxxinova Norway AS, Bergen Norway
4 ADL Diagnostic, Puerto Montt, Chile

Author contributions

YvW and JK conceived and designed research. YvW, BK, and MM performed research and analyzed data. BK and JJ helped design experiments. YvW, JK, and JJ wrote the paper. All authors reviewed and approved the manuscript.

Abstract

Since most vaccines against *Piscirickettsia salmonis* still give insufficient protection in the field, we investigated antibody responses and protection after an IP *P. salmonis* infection in Atlantic salmon. Survivors were protected against a second challenge 14 weeks post challenge. Anti-*P. salmonis* Ab levels in the serum were significantly increased at this time point but were reduced to base levels at 18 weeks post challenge. Observations of mortalities at the later time point suggest a reduced protection, but the 18 wpc challenge did not induce sufficient mortalities in the control group to allow a definitive conclusion. A *P. salmonis* infection thus can induce protective immune responses in Atlantic salmon for at least 1,200 degree days, but these seem to be diminished after 1,500 degree days. Finally, we suggest that serum Ab levels could be used as a correlate of protection for SRS.

Introduction

The salmon aquaculture industry is very important for the Chilean economy (Flores-Kossack et al. 2020). Unfortunately, diseases form a major threat to salmonid aquaculture, leading to reduced animal welfare and significant economic losses. One of the major threats to the Chilean salmon aquaculture industry is salmonid rickettsial septicemia (SRS), which is caused by the facultative intracellular bacterium *Piscirickettsia salmonis* (Fryer et al. 1992). SRS outbreaks result in extensive mortalities and lead to annual losses of $700 million USD in Chile (Maisey et al. 2017). Several experimental SRS vaccine candidates have been evaluated, and over 30 vaccines that include an *P. salmonis* component

are available in Chile (Maisey et al. 2017; Servicio Agrícola y Ganadero 2020). Nearly all the commercial SRS vaccines are inactivated vaccines, containing whole inactivated *P. salmonis* or components thereof. Unfortunately, none of these vaccines induce a high level of protection in field conditions during the full production cycle (Intesal Febuary 2014; Happold et al. 2020). Interestingly, multiple outbreaks per cage during the production cycle have been documented (Jakob et al. 2014), which could indicate that the protection acquired after an SRS infection is for a limited period whereafter fish become susceptible to SRS infection again. To gain more insight into the kinetics and duration of the development of protective responses after *P. salmonis* infection in salmon, we evaluated the antibody (Ab) production and long-term protection after infection with this bacterium under controlled conditions. To this end, we intraperitoneally (IP) challenged Atlantic salmon with *P. salmonis*, measured specific Abs in the serum, rechallenged at different time points, and observed mortalities.

Materials & Methods

Bacterial cultivation: Piscirickettsia salmonis (LF-89, ATCC VR-1361) was grown on CHSE-214 cells in L15 with 10% FBS and 1% L-glutamine. Inoculum was prepared by harvesting supernatant at 50% CPE and centrifuging at 300 x g for 10 minutes (4°C) to remove cell debris. Bacteria were pelleted by centrifuging at 4,000 x g for 10 minutes (4°C) and resuspended in PBS. We performed titration on CHSE-214 cells to determine the actual titer of the inoculum.

Experimental challenge. Atlantic salmon (*Salmo salar*) parr of 30 g were obtained from the test facility (Industrilaboratoriet, Bergen) and were certified as pathogen-free before the experiment started. The fish were kept in fresh water at 12°C with 12h:12h light dark cycles, fed according to appetite, and starved for 24h before handling. Fish were be anesthetized by immersion in 100 mg/L Finquel vet. (ScanVacc, Norway) before tagging and challenge.

Table 1: Experimental groups and treatments.

Group	Tank at first challenge	Tank at second challenge	Challenged (wpc)	Sampled (wpc)
1A	Tank 1 (C)	Tank 1	0, 14	14, 20
1B	Tank 5 (NC)	Tank 1	14	
2A	Tank 2 (C)	Tank 2	0, 18	14, 24
2B	Tank 5 (NC)	Tank 2	18	
IC	Tank 3 (C)	NA	0	14, 18, 20
NC	Tank 5 (NC)	NA	NA	0, 14, 18, 20

NA: not applicable, no challenge performed. Wpc: weeks post challenge. NC: negative control.

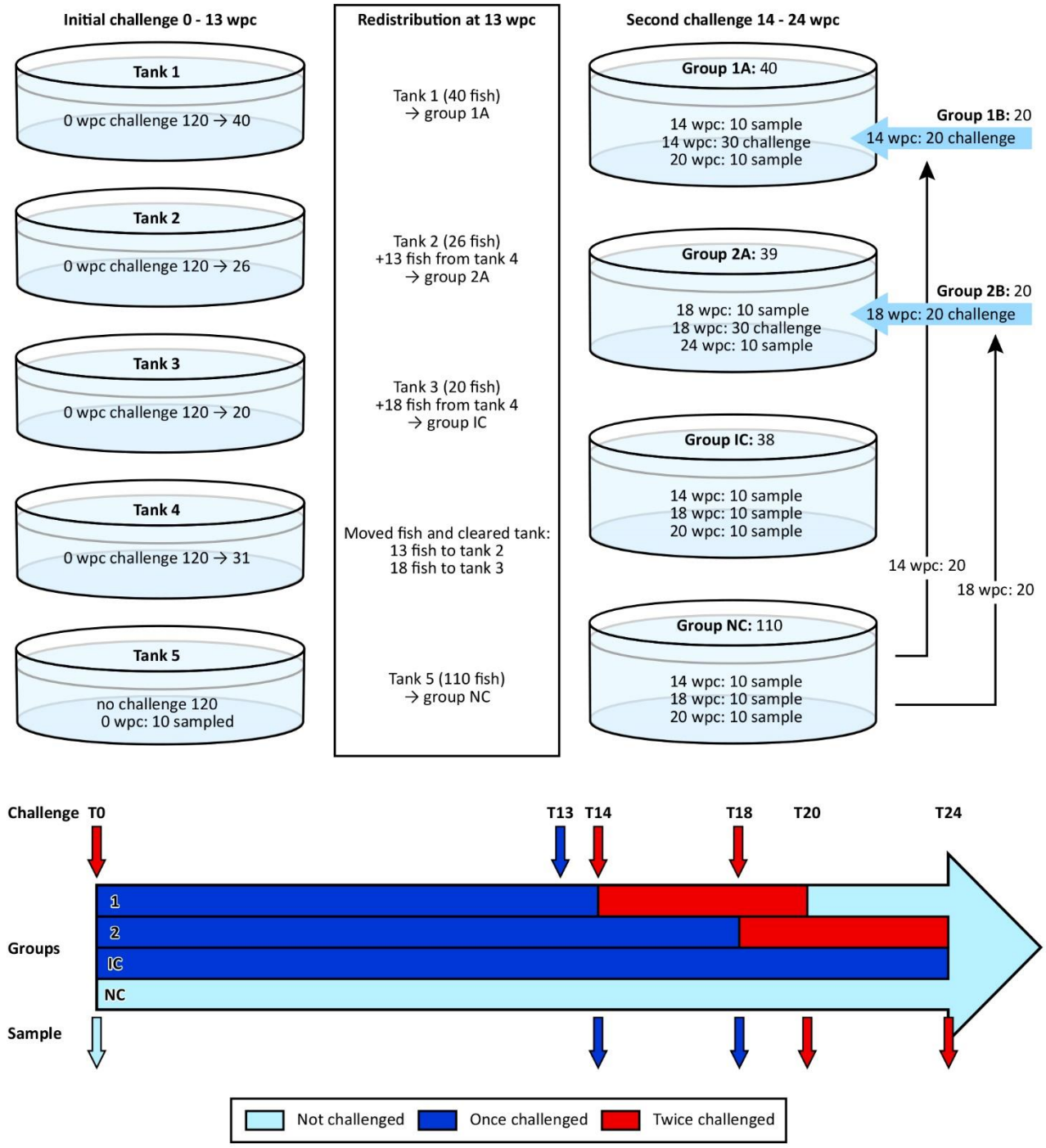


Figure 1: Top: Overview of challenges, transfers, and samplings in the tanks. 0 weeks post challenge (wpc) challenge in tanks 1-4 (left hand side). Transfer of fish from tank 4 at 13 wpc to form final experimental groups (central block). 14 wpc challenge in tank 1 and transferred group from tank 5 (black and blue arrows). 18 wpc challenge in tank 2 and transferred group from tank 5 (black and blue arrows). Sampling time points and number of fish indicated per group. Bottom: Timeline of the experiment with the four experimental groups included in the main arrow. Challenge or transfer events indicated by arrows at the top (red arrows for challenge, blue arrow for transfer). Samplings indicated by arrows at the bottom, with colors following the status of the experimental group with the most challenges per sampling: light blue for not challenged, dark blue for once challenged (NC group also sampled), and red for twice challenged fish (NC and once challenged fish also sampled).

Table 1 and fig. 1 present an overview of the experimental groups and procedures. Fish were split randomly in five tanks with 120 fish each, and fish in tanks 1-4 were IP challenged with 0.1 mL inoculum (5.8E+04 $TCID_{50}$/mL), while the fish in tank 5 (group NC: negative control, no challenge) were mock challenged with 0.1 mL PBS. At 13 wpc, surviving fish from tank 4 were transferred to tank 2 and 3 to obtain comparable group sizes (38-40) in tanks 1, 2, and 3 (respectively group 1A, 2A, and initial challenge (IC)). At 14 wpc, 10 fish from groups 1A, IC, and NC were sampled. In addition, 20 NC fish from tank 5 were tagged, challenged (0.1 mL, IP, 4.2E+04 $TCID_{50}$/mL), and transferred to tank 1 to form group 1B, while all remaining fish in tank 1 (group 1A) were rechallenged with the same dose. At 18 wpc, 10 fish from groups 2A, IC, and NC were sampled. In addition, 20 NC fish from tank 5 were tagged, challenged (0.1 mL, IP, 2.1E+05 $TCID_{50}$/mL), and transferred to tank 2 to form group 2B, while all remaining fish in tank 2 (group 2A) were rechallenged with the same dose. At 20 wpc, 10 fish from group 1A, IC, and NC were sampled. At 24 wpc, 10 fish from group 2A were sampled. Blood samples were stored at 4°C overnight and centrifuged 3000 rpm for 10 minutes at 4°C. Serum was stored with 50% glycerol at -20°C.

Fish were observed daily, and moribund fish were euthanized, checked for clinical signs, and counted as mortality. The animal experiment was evaluated and approved by the Norwegian Food Safety Authority according to the European Union directive 2010/63/EU for animal experiments (FOTS ID13650).

For the quantification of antibody levels present in the serum of fish we used an IgM ELISA described by Tobar et al., 2015. In short, serum from 5 healthy Atlantic salmon was harvested, pooled, and centrifuged after overnight incubation at 4°C. IgM was chromatographically purified using sephacryl S300 (GE Lifesciences) and identified in the obtained fractions using dot plot and SDS-PAGE. Protein concentration (860 µg/mL) of the purified IgM was measured with a Qubit fluorometer (Invitrogen), and a two-fold dilution series was prepared in carbonate buffer. Eight dilutions (860 - 6.72 µg/mL) of this standard were included in duplicate on each ELISA plate to allow quantification of IgM concentrations.

Anti P. salmonis ELISA. ELISA plates were coated with 100 µL of the standard dilution series (1:50 diluted to concentrations of 17,2 – 0,13 µg/mL, earlier tests indicated linearity between 0,13 – 1,08 µg/mL, see suppl. fig. 1) for 2 hours at 37°C or with 5 µg/well of recombinant *P. salmonis* outer surface protein A (rOspA, produced by ADL) overnight at 4°C. Wells were washed with PBS-Tween20 (T-PBS), blocked with skim milk, and washed three more times. Serum samples from experimental groups were diluted 1:50 in PBS with 1% nonfat milk and added to the wells coated with rOspA. After incubation, wells were washed three times with T-PBS and incubated with monoclonal anti-salmon IgM (LS-C63026-100, clone IPA-5F12, HRP conjugated) before final washing. Hundred µL TMB substrate

(T0440-100 ML, Sigma-Aldrich) was added for 20 minutes, after which 50 µL HCl 1M was added and OD_{450} measured (EPOCH2, Biotek).

Statistical analyses were performed in GraphPad Prism version 8.4.1. The Log-rank Mantel-Cox test was used for survival curves. A Mann-Whitney test (two groups) or a Kruskal-Wallis ANOVA test (three groups) was used to test differences between antibody levels in serum of different groups at the same time point after exclusion of outliers.

Results & Discussion

The initial challenge at 0 wpc led to mortalities between 70 and 80% in the tanks 1-4 (fig. 2a). Since this was higher than expected, we reduced the planned number of experimental groups and divided the fish from tank 4 among the other groups to increase and even out the group sizes. The unexpectedly high mortality was a result of difficulties with estimating the challenge dose, even after several pilots. The precise titer of the challenge material was determined with a titration taking two weeks and found to be 8.5 times higher than planned for the 0 wpc challenge. Pilot studies directly using frozen inoculum were not successful. The final established titers of the challenge doses at 14 and 18 wpc were closer to the estimated dose at the time of challenge (0.5 and 2.6 times higher, respectively).

All the sampled deceased individuals showed clinical signs corresponding with SRS, such as discoloration or bleeding in the liver and other organs. Furthermore, the kinetics of the mortality curves followed those of earlier pilot studies, where the presence of bacterial DNA in head kidney and liver was demonstrated (results not shown). Together, these data indicate a successful infection with *P. salmonis*.

The challenge at 14 wpc, corresponding roughly to 1,200 degree days, led to 80% mortalities in control group 1B, while the previously challenged group 1A did not show any mortalities (Fig. 2b). The difference in mortality between group 1A and 1B is statistically significant (Log-rank Mantel-Cox test, $p<0.05$), indicating a full protection 14 weeks after a *P. salmonis* challenge. In contrast, the challenge at 18 wpc (1,500-degree days) only induced 15% mortalities in the control group 2B (Fig. 2c). Apparently, this challenge was less effective, although the bacterial titers were higher than in the inoculum at 14 wpc. The age of the salmon could have an influence on this, since older/larger fish have higher natural antibody titers (Magnadottir et al. 2009) and probably have stronger cellular responses as well. The less effective challenge at 18 wpc induced 4% mortalities in the previously challenged group 2B, although this corresponds to only one dead fish. The difference between the mortalities in group 2A and 2B at 18 wpc was not statistically significant (Log-rank Mantel-Cox test, $p > 0.05$), suggesting a reduced protection when compared to the 14 wpc. Unfortunately, the mortalities in the control group (2B) are not high enough to draw conclusions from this 18 wpc challenge.

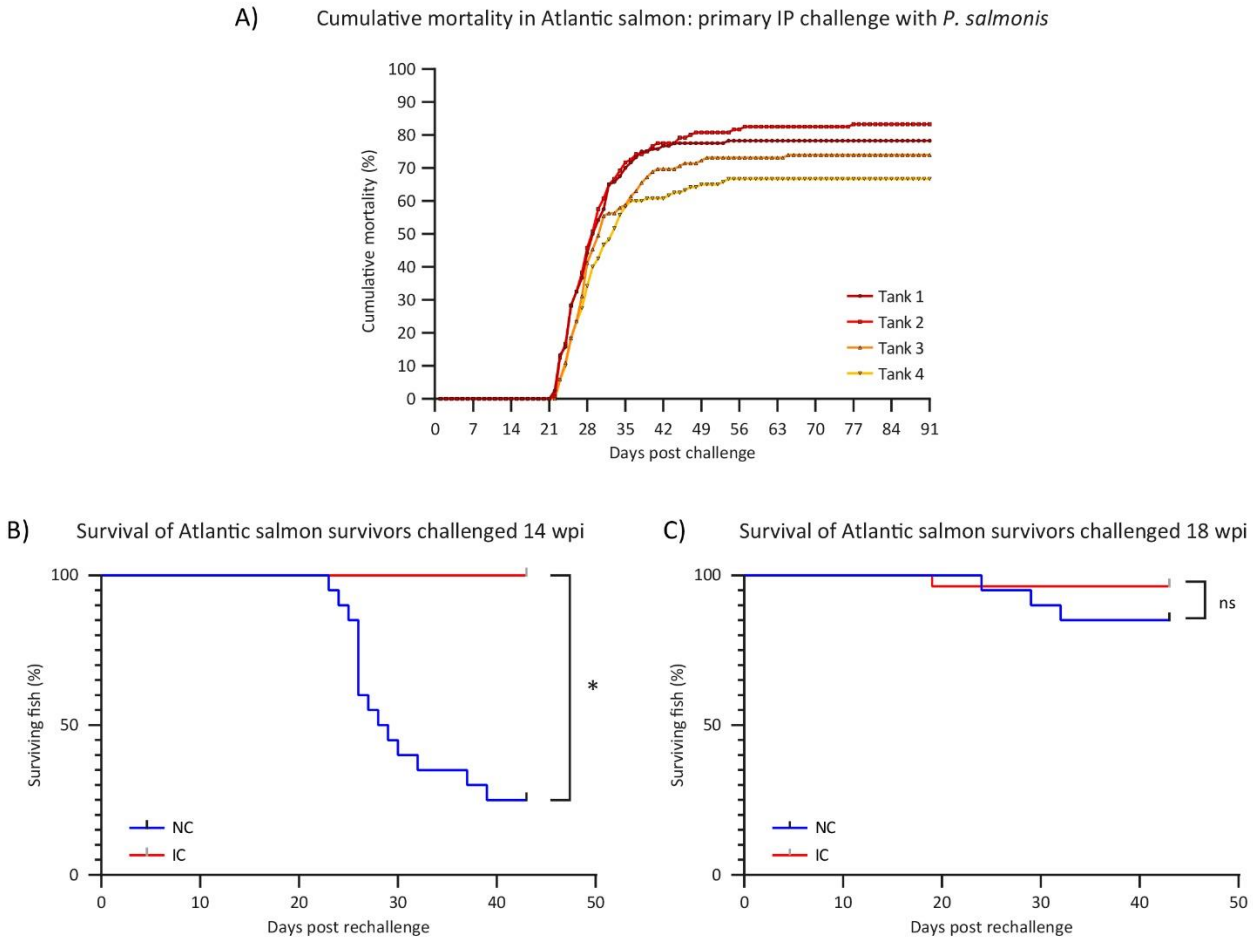


Figure 2: Mortality and survival curves after the three challenges. A: cumulative mortalities in the four replicate tanks after the 0 weeks post challenge (wpc) challenge. B: survival curves in group 1A (IC, initial challenge, red line) and 1B (NC, no challenge, blue line) after the 14 wpc challenge. C: survival curves in group 2A (IC, initial challenge, red line) and 2B (NC, no challenge, blue line) after the 18 wpc challenge. 120 fish per group in panel A, and 20 fish per group in panels B and C. (*) Statistically significant difference in survival: Log-rank Mantel-Cox test, $p < 0.05$.

The anti-*P. salmonis* antibodies in serum remained relatively constant in the initial NC group, though seemed to increase slightly with age (fig. 3). This is in agreement with observations in other teleost fish where natural antibody levels increase with age/size (Magnadottir et al. 2009). Antibody titers in challenged fish were significantly higher at 14 wpc compared to NC (Mann-Whitney test between group NC and groups 1A and IC together (same experimental status, only received the initial challenge up to this point), $p < 0.05$). In contrast, no difference in anti-*P. salmonis* antibodies was observed between challenged and control fish at 18 wpc. (Mann-Whitney test between group NC and groups 2A and IC together (same experimental status, only received the initial challenge up to this point), $p > 0.05$). In addition, we evaluated whether there was a booster effect from the second challenge after 6 weeks (at 20 and 24 wpc for the 14 and 18 wpc challenges, respectively), but we could not observe any significant differences (fig. 3).

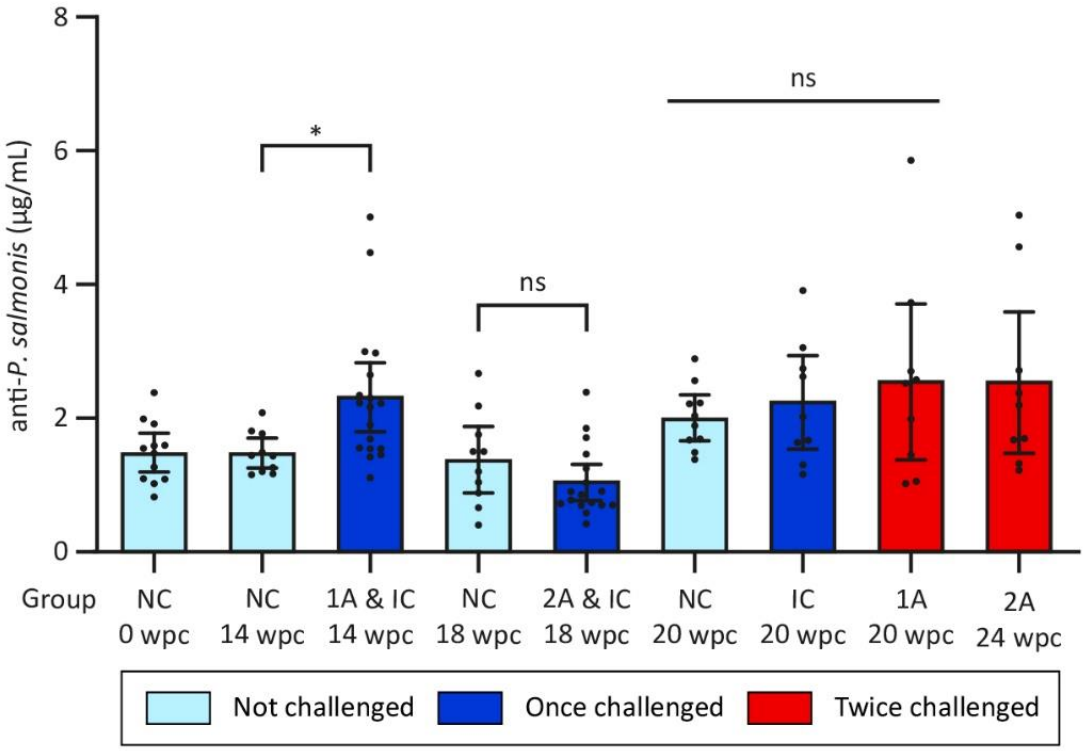


Figure 3: Amount of anti-*P. salmonis* in serum of negative control (NC) and challenged Atlantic salmon presented as µg/mL, calculated using an included standard dilution. Time points presented as weeks post the initial challenge at 0 weeks post challenge (wpc). n=10 for all groups, except 0 wpc (n=12). In addition, data points from experimental groups 1A & IC and 2A & IC were combined at time points 14 and 18 wpc respectively since they had the same experimental status at those time points. Brackets at 14 and 18 wpc: (*) Statistically significant ($p < 0.05$) between the challenged group and the NC (Mann-Whitney test). Line at 20 wpc: no statistically significant difference ($p > 0.05$) between the NC, the challenged group, and the rechallenged group (ANOVA; Kruskal-Wallis test). Error bars indicate 95% confidence interval.

The elevated antibody titers at 14 wpc corresponded with full protection, while the possibly reduced protection at 18 wpc coincided with no observable elevation of antibodies. This seems to agree with observations of Tobar et al. 2015 that waning antibody titers after vaccination in farms are an indication of susceptibility to SRS. It is interesting to note that, although the difference in survival curves was not significant, there could still be some residual protection in group 2A. Since antibody titers were no longer elevated, this might be connected to cellular immune responses. *P. salmonis* is an intracellular pathogen, it is therefore reasonable to assume that cell-mediated immunity is required for long term immunity against this intracellular bacterium.

The lack of visible booster effects at both investigated time points (20 and 24 wpc) puts into question the strength of the salmon immunological memory, if not for all pathogens, then at least for *P. salmonis*. Interestingly, slight booster effects on antibody titers can be observed with oral boosters after IP vaccination against *P. salmonis*, ISAV, and IPNV (Tobar et al. 2015; Evensen 2016). The discrepancy with our missing booster effect could be due to the difference in booster delivery, IP versus oral, or that our booster was a fully intact pathogen instead of a vaccine formulation, as described by Tobar et al. (2015) and Evensen (2016). Local responses at the injection site in the peritoneal cavity, as observed early after IP *P. salmonis* infection (van der Wal et al. 2021), could result in local antigens not arriving

in systemic immune organs such as the spleen and head kidney, where the booster response is probably originating. It is also conceivable that immune-suppressive effects of intact *P. salmonis* on the immune system inhibit a booster effect, although we do not know enough about these effects to confirm this.

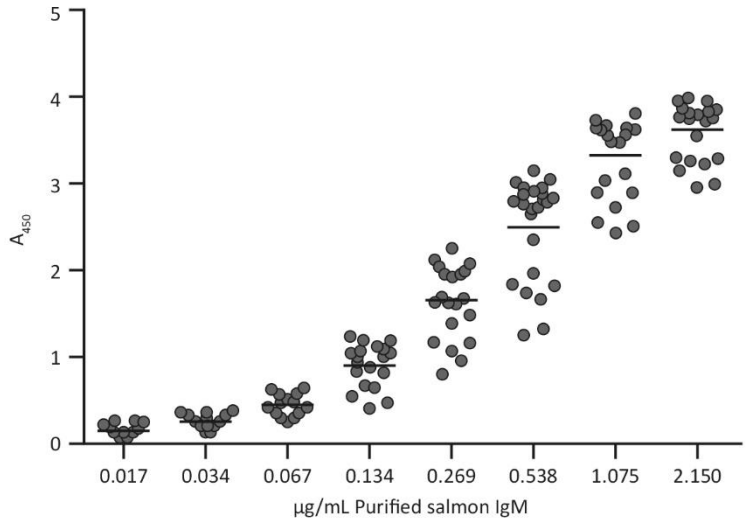
An important note on the experimental setup is that the second challenge was performed on survivors of the initial challenge, which might have induced a bias through selection. The survivors might have been more resistant to *P. salmonis* infection than the fish that died during the initial challenge due to genetic or other biological variation, leading to an overestimation of the protection during the later challenges. This is a reasonable possibility since we did not have the possibility to use clonal salmon and Atlantic salmon families have shown clear differences in susceptibility to SRS (Pulgar et al. 2015). In addition, the fact that we used the IP route for both challenges could result in an effect of the local immune response on the measured protection, as suggested by van der Wal et al. 2021. However, in that study we focused on early time points (up to 6 weeks post challenge). It is presently unclear if and how long this local response would last, but if there still is some effect at 14 and 18 wpc, some of the observed protection might be attributed to local rather than to systemic responses.

For the development of SRS vaccines with long term (> 6 months) protection, it would be useful to identify possible correlates of protection. Identifying good correlates of protection would facilitate experiments on protection induced by various vaccine strategies. In early trials, the correlates of protection can be used as read-out of induced protection without the need for costly challenge trials and thus reduce the need for animal experimentation. Only promising vaccine candidates would then later be used in challenge experiments. Furthermore, the correlates of protection can give an insight in the mechanisms important for protection and how these can be induced by vaccines. Our results, as well as the data from Tobar et al. 2015, indicate that serum antibody levels probably are useful as correlate of protection. Since T cell responses, in general, are important for the protection against intracellular pathogens, it would be interesting to investigate whether correlates of protection connected to T cell responses can be established. For *P. salmonis* specifically, Kuzyk et al. 2001 show a markedly increased RPS after adding T cell epitopes to their prototype vaccine.

Conclusion

We have shown that surviving Atlantic salmon are protected against a second challenge 14 weeks post IP *P. salmonis* challenge. This protection at 14 wpc corresponded with a significantly increased Ab level in the serum, that was reduced to base levels at 18 wpc. Protection against rechallenge at this timepoint could be reduced, but the challenge in the current study was not sufficient to draw a definitive conclusion. We conclude that a *P. salmonis* infection induces protective immune responses in Atlantic salmon for at least 1,200 degree days, but which seem to be diminished after 1,500 degree days. Furthermore, our results indicate that serum Ab levels could be a correlate of protection for SRS.

Supplementary material



Supplementary figure 1: Linearity of ELISA on purified Atlantic salmon IgM dilution series. The OD_{450} of wells coated with different dilutions of purified IgM was plotted against the concentration in the coating dilution. Data from several ELISA assays were combined.

Publication bibliography

Chen, Lihan; Evensen, Øystein; Mutoloki, Stephen (2015): IPNV Antigen Uptake and Distribution in Atlantic Salmon Following Oral Administration. In *Viruses* 7 (5), pp. 2507–2517. DOI: 10.3390/v7052507.

Evensen, Øystein (2016): Immunization Strategies against Piscirickettsia salmonis Infections: Review of Vaccination Approaches and Modalities and Their Associated Immune Response Profiles. In *Frontiers in immunology* 7, p. 482. DOI: 10.3389/fimmu.2016.00482.

Flores-Kossack, C.; Montero, R.; Köllner, B.; Maisey, K. (2020): Chilean aquaculture and the new challenges: Pathogens, immune response, vaccination and fish diversification. In *Fish & shellfish immunology* 98, pp. 52–67. DOI: 10.1016/j.fsi.2019.12.093.

Happold, Jonathan; Sadler, Rohan; Meyer, Anne; Hillman, Alison; Cowled, Brendan; Mackenzie, Catriona et al. (2020): Effectiveness of vaccination for the control of salmonid rickettsial septicaemia in commercial salmon and trout farms in Chile. In *Aquaculture* 520, p. 734968. DOI: 10.1016/j.aquaculture.2020.734968.

Intesal (Febuary 2014): EVALUACIÓN DE LA DURACIÓN DE LA EFICACIA DE VACUNNAS PARA EL CONTROL DE PISCIRRICKETTSIOSIS EN TRUCHA ARCOÍRIS Y SALMÓN DEL ATLÁNTICO MEDIANTE DESAFÍO POR COHABITAACIÓN. SEGUNDO REPORTE. Intesal. Chile.

Jakob, Eva; Stryhn, Henrik; Yu, Jenny; Medina, Matias H.; Rees, Erin E.; Sanchez, Javier; St-Hilaire, Sophie (2014): Epidemiology of Piscirickettsiosis on selected Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) salt water aquaculture farms in Chile. In *Aquaculture* 433, pp. 288–294. DOI: 10.1016/j.aquaculture.2014.06.018.

Kuzyk, Michael A.; Burian, Ján; Machander, Dave; Dolhaine, Daphne; Cameron, Stephen; Thornton, Julian C.; Kay, William W. (2001): An efficacious recombinant subunit vaccine against the salmonid rickettsial pathogen Piscirickettsia salmonis. In *Vaccine* 19 (17-19), pp. 2337–2344. DOI: 10.1016/S0264-410X(00)00524-7.

Magnadottir, Bergljot; Gudmundsdottir, Sigridur; Gudmundsdottir, Bjarnheidur K.; Helgason, Sigurdur (2009): Natural antibodies of cod (Gadus morhua L.): specificity, activity and affinity. In *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology* 154 (3), pp. 309–316. DOI: 10.1016/j.cbpb.2009.07.005.

Maisey, Kevin; Montero, Ruth; Christodoulides, Myron (2017): Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. In *Expert review of vaccines* 16 (3), pp. 215–228. DOI: 10.1080/14760584.2017.1244483.

Pulgar, Rodrigo; Hödar, Christian; Travisany, Dante; Zuñiga, Alejandro; Domínguez, Calixto; Maass, Alejandro et al. (2015): Transcriptional response of Atlantic salmon families to Piscirickettsia salmonis infection highlights the relevance of the iron-deprivation defence system. In *BMC genomics* 16, p. 495. DOI: 10.1186/s12864-015-1716-9.

Servicio Agrícola y Ganadero (2020): PRODUCTOS BIOLÓGICOS INMUNOLÓGICOS CON REGISTRO PROVISIONAL USO EN SALMÓNIDOS. Available online at http://www.sag.cl/sites/default/files/lista_salmonidos_biol-inmunol_registro_provisional_20-2-2020.pdf, updated on 2/20/2020.

Tobar, Iván; Arancibia, Sergio; Torres, Constanza; Vera, Verónica; Soto, Paola; Carrasco, Claudia et al. (2015): Successive Oral Immunizations Against Piscirickettsia Salmonis and Infectious Salmon Anemia Virus are Required to Maintain a Long-Term Protection in Farmed Salmonids. In *Frontiers in immunology* 6, p. 244. DOI: 10.3389/fimmu.2015.00244.

van der Wal, Yorick A.; Jenberie, Shiferaw; Nordli, Henriette; Greiner-Tollersrud, Linn; Kool, Jaap; Jensen, Ingvill; Jørgensen, Jorunn B. (2021): The importance of the Atlantic salmon peritoneal cavity B cell response: Local IgM secreting cells are predominant upon Piscirickettsia salmonis infection. In *Developmental and comparative immunology* 123, p. 104125. DOI: 10.1016/j.dci.2021.104125.

Paper II

Developmental and Comparative Immunology 123 (2021) 104125

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Full Length Article

The importance of the Atlantic salmon peritoneal cavity B cell response: Local IgM secreting cells are predominant upon *Piscirickettsia salmonis* infection

Yorick A. van der Wal [a,b], Shiferaw Jenberie [b], Henriette Nordli [b], Linn Greiner-Tollersrud [b], Jaap Kool [a], Ingvill Jensen [b], Jorunn B. Jørgensen [b,*]

[a] Vaxxinova Research & Development GmbH, Münster, Germany
[b] Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT the Arctic University of Norway, Tromsø, Norway

ARTICLE INFO

Keywords:
Piscirickettia salmonis
Atlantic salmon
Peritoneal cavity
ELISpot
B cell
Adipose tissue

ABSTRACT

The intraperitoneal route is favored for administration of inactivated and attenuated vaccines in Atlantic salmon. Nevertheless, the immune responses in the teleost peritoneal cavity (PerC) are still incompletely defined. In this study, we investigated the B cell responses after intraperitoneal *Piscirickettsia salmonis* (*P. salmonis*) challenge of Atlantic salmon, focusing on the local PerC response versus responses in the lymphatic organs: spleen and head kidney. We observed a major increase of leukocytes, total IgM antibody secreting cells (ASC), and *P. salmonis*-specific ASC in the PerC at 3- and 6-weeks post infection (wpi). The increase in ASC frequency was more prominent in the spleen and PerC compared to the head kidney during the observed 6 wpi. The serum antibody response included *P. salmonis*-specific antibodies and non-specific antibodies recognizing the non-related bacterial pathogen *Yersinia ruckeri* and the model antigen TNP-KLH. Finally, we present evidence that supports a putative role for the adipose tissue in the PerC immune response.

1. Introduction

Although humoral responses are crucial to the protection induced by vaccines, in depth studies on B cell biology, including how the exposure to pathogens initiates B cell responses and leads to subsequent antibody (Ab) production in Atlantic salmon (*Salmo salar* L), are elusive. Teleosts lack germinal centers and follicular structures, which in mammals have roles in the proliferation and differentiation of B cells to plasma cells, and there is no class switching. Furthermore, the mechanisms and limitations of immunological memory in teleosts are still being questioned (Yamaguchi et al., 2019). One reason for this is that the teleost secondary immune response results in only slightly higher Ab responses than the primary response (Cossarini-Dunier, 1986). By contrast, 100–1000 times more antibodies are produced in the mammalian secondary response. Like mammals, teleosts have non-specific or natural Abs that are present without prior specific antigen encounters (Magnadóttir 2006), but their role in immune responses after pathogen encounters remains to be elucidated. Finally, the existence of affinity maturation of Abs in teleosts has been questioned, but several recent studies have identified the occurrence of affinity maturation in bony fish (Wu et al. 2019a, 2019b; Ye et al., 2013). Nevertheless, the location and mechanisms of the teleost affinity maturation are still largely unknown (Magor 2015), and the affinity maturation in fishes is generally considered as much less efficient than in mammals (Kaattari 2002). The absence of true germinal centers is often provided as an explanation, resulting in an inefficient selection of high-affinity clones (Magor 2015; Muthupandian et al., 2021).

Only recently, the role of local B cell responses in the peritoneal cavity (PerC) of teleosts has gained more attention. This is remarkable because intraperitoneal injection (IP) is the most commonly used route of vaccine administration in Atlantic salmon (Plant and Lapatra 2011). Castro et al., (2017) found an increase of B cell numbers in the PerC within 2–3 days after IP stimulation of rainbow trout with *Escherichia coli* or viral haemorrhagic septicemia virus. After 6 days, they also observed a significant increase of antibody secreting cells (ASC) in the PerC. In concordance with these results, Jenberie et al., (2020) showed a significant increase in ASC in the PerC after IP challenge with salmonid alphavirus subtype 3 (SAV3) over a period of nine weeks. In this study, the increase of ASC in the PerC was higher than in the head kidney (HK)

* Corresponding author. Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, N-9037 Tromsø, Norway.
E-mail address: jorunn.jorgensen@uit.no (J.B. Jørgensen).

https://doi.org/10.1016/j.dci.2021.104125
Received 2 March 2021; Received in revised form 1 May 2021; Accepted 1 May 2021
Available online 1 June 2021
0145-305X/© 2021 Elsevier Ltd. All rights reserved.

Abbreviations

Ab (Abs)	antibody (antibodies)
ASC	antibody-secreting cell(s)
AT	adipose tissue
DC	dendritic cells
Dpi	days post infection
HK	head kidney
IP	intraperitoneal
NC	negative control
P. salmonis	*Piscirickettsia salmonis*
PerC	peritoneal cavity
SRS	salmonid rickettsial septicemia
TLRs	Toll-like receptors
Wpi	weeks post infection
Y. ruckeri	*Yersinia ruckeri*

and spleen. This raises questions on the formation, location, and migration of ASC after IP stimulation with different pathogens. Additionally, Pignatelli et al., (2014) suggested an immunological role for the adipose tissue (AT) in the rainbow trout PerC, where it may take part in regulating PerC immune responses. Clarifying the participation and contribution of B cell responses after IP encounter with pathogens or immunogens is crucial to perceive how the PerC and the systemic sites interplay, and how this affects the overall humoral immune response. In the future, an extended understanding of the dynamics between different immune sites in bony fish may give clues on how to improve protective responses against diseases for which effective vaccines are currently lacking.

One such a disease is salmonid rickettsial septicemia (SRS), a severe systemic disease that mainly affects salmonid species and was first described in 1989 (Rozas and Enríquez 2014; Fryer et al., 1992). SRS is a major concern for the Chilean aquaculture industry, where it causes severe mortalities and, in extension, annual economic losses estimated at USD $700 million (Maisey et al., 2017). The facultative intracellular bacterium *Piscirickettsia salmonis* (*P. salmonis*) causes SRS (Fryer et al., 1992). The natural route of infection for *P. salmonis* is through the gills and wounds in the skin, leading to a systemic infection (Smith et al., 1999). The bacteria are targeting hepatocytes and liver associated macrophages, as well as kidney, spleen, and peripheral blood macrophages (reviewed in Almendras and Fuentealba 1997). Over thirty vaccines containing *P. salmonis* are currently available for aquaculture in Chile. Most of these vaccines are inactivated or subunit vaccines, while a recent vaccine contains a live-attenuated strain (Maisey et al., 2017). Even though initial experimental tests for the vaccines show promising protection shortly after vaccination (usually around 600 degree-days), evaluated vaccines failed to protect at 1500 degree-days (Intesal, 2014). The knowledge concerning the host response to this pathogen is still limited and mainly based on *in vivo* transcriptomics or *in vitro* studies (Rozas and Enríquez 2014). This incomplete understanding of the Atlantic salmon immune responses probably contributed to the inability to develop efficacious vaccines against *P. salmonis* for thirty years.

To explore how B cell responses are induced upon infection with a bacterial pathogen, we here examined the characteristics of Atlantic salmon humoral responses in various sites, including the PerC, spleen, and HK, after IP challenge with *P. salmonis*. We observed a major increase of leukocytes, total IgM ASC, and *P. salmonis*-specific ASC in the PerC. Additionally, we found that the early response included an induction of non-specific Abs. To our knowledge, this is the first description in a teleost species that a bacterial infection in the PerC elicits a strong local antigen specific Ab response over the course of several weeks. This raises the question where and how local and systemic responses develop after IP immunization in salmon. Correlations between serum Abs titre and frequency of ASC in the PerC and spleen suggest that this early Ab production is mainly located in these two sites. In accordance with earlier studies (Veenstra et al., 2018; Pignatelli et al., 2014), the infection induced the upregulation of different immune genes, including markers for different B and T cell populations and professional antigen presenting cells, in the PerC AT. This indicates a role as an immune site in salmonids. Finally, we discuss how the strong immune response in the PerC could influence the predicted efficacy of vaccines.

2. Materials and methods

2.1. Bacterial strains and antigen

P. salmonis strain PM15972 (EM-90-like) was obtained from Marcos Mancilla (ADL Diagnostic Chile), cultivated for two passages on PSA agar plates at 17,5 °C (as described in Henríquez et al., 2016), and harvested after 5 days. The harvested bacterial suspension had an OD600 of 0,210, corresponding to around 7×10^7 CFU/mL, and was kept on ice until use as challenge material. *P. salmonis* antigen for ELISpot and ELISA was generated by heat-inactivation (60 °C for 15 min) and sonication (90 cycles for 2 min) of the challenge material. *Yersinia ruckeri* (*Y. ruckeri*) strain CCUG 14190, A4-53 was obtained from Lill-Heidi Johansen (NOFIMA) and cultivated on LB agar and in liquid LB medium at room temperature. *Y. ruckeri* antigen was generated by heat inactivating (65 °C for 30 min) and sonicating (90 cycles for 2 min) a liquid culture at 0,8 OD_{600}.

2.2. Fish and P. salmonis challenge

Atlantic salmon presmolts (Aquagen standard) were produced and housed at the Tromsø Aquaculture Research Station in fresh water at 10 °C and acclimatized to the tank for three weeks. Fish were kept at 24 h light and were fed commercial feed (Skretting) according to appetite. At the start of the experiment, the mean weight of the fish was 56,2 g. Fish were starved one day before challenge or sampling and anesthetized (40 μg/mL) or euthanized (80 μg/mL) using benzocaine (ACD Pharmaceuticals) before handling. The Atlantic salmon were randomly allocated to two tanks: 40 fish for infection and 24 for control. Fish were IP challenged with 0,1 mL *P. salmonis* (0,21 OD_{600}, corresponding to approximately 7×10^6 CFU/fish) or injected IP with 0,1 mL PBS to form the negative control (NC) group. In addition, four fish were sampled at day 0. The animal experiment was evaluated and approved by the Norwegian Food Safety Authority (ID 21507).

2.3. Sampling and leukocyte isolation

Blood, PerC cells, AT (see Fig. 3e), liver, spleen, and HK were sampled from 8 NC and 8 infected fish at 3 days post infection (dpi), 3 weeks post infection (wpi), and 6 wpi, with the exception of blood samples at 6 wpi being taken from 12 individuals. Peritoneal washes with visible blood contamination were removed. At 0 dpi, the same samples were taken from 4 non-injected fish. Blood samples (0,3 to 1 mL, depending on fish size) were stored in regular Eppendorf tubes overnight at 4 °C, centrifuged at 2000×*g* for 10 min, serum was harvested, diluted 1:1 in glycerol, and stored at −20 °C until analysis. AT and liver samples were stored in RNAlater (Invitrogen) at −20 °C after overnight incubation at 4 °C. HK and spleen samples were kept in transport medium (L-15 with 2% FBS, 0,4% heparin, 10 U/mL penicillin, and 10 μg/mL streptomycin) on ice until further processing. PerC cells were harvested as described by Jenberie et al., (2020) by washing and gently scraping the abdominal wall of the PerC three times with 1 mL wash medium (PBS with 2% FBS and 20U/mL heparin). The PerC cell suspension was collected in 2 mL transport medium on ice until further processing.

Leukocytes were isolated from PerC, spleen, and HK as described earlier (Iliev et al., 2010; Jenberie et al., 2020). Spleen and HK tissue were dissociated using a 100 μm cell strainer (Falcon). Cells were collected in transport medium, layered on 25%/54% discontinuous Percoll (GE Healthcare) gradients, and centrifuged 400×*g* for 40 min at 4 °C. Leukocytes were collected from the interface, washed twice in L-15 with 10 U/mL penicillin and 10 μg/mL streptomycin, counted (Countess II FL; Invitrogen), and kept on ice. To compare the total number of leukocytes per organ, we sampled the complete spleen and weighed the sampled HK. Due to a technical issue with only part of the HK being sampled from infected fish at 3 dpi, those numbers were normalized (see suppl. Table 1).

2.4. Total IgM ASC ELISpot assay

An ELISpot assay was performed to enumerate the number of IgM-producing cells as described by Jenberie et al., (2020). In short, MSIPS4510 plates (Merck Millipore) were activated with 35% ethanol, washed with water and PBS, and coated with 1.5 μg/well capture Ab (Anti-trout IgM/F1-18, kindly provided by Dr. Karsten Skjødt) diluted in PBS (100 μL/well) at 4 °C overnight. This mAb was originally produced towards purified IgM from rainbow trout plasma but was later shown to bind both subtypes of Atlantic salmon IgM (IgM A and B) (Hedfors et al., 2012). After washing four times with PBS, the membrane was blocked with 100 μL L-15 with 10 U/mL penicillin, 10 μg/mL streptomycin, and 2% BSA per well for 90 min. Isolated leukocytes were seeded at 12500 cells per well and incubated for 48 h at 15 °C. This was followed by incubating with 100 μL of anti-trout IgM (F1-18), biotinylated using the EZ-Link NHS-PEG solid phase biotinylation kit (Thermo Fisher Scientific), diluted in PBS with 1% BSA and 0,1% Tween 20, per well for 90 min. Plates were then developed using 100 μL streptavidin-HRP conjugate (Mabtech) diluted 1:500 in PBS per well for 1 h, and 100 μL filtrated TMB ultra substrate (Mabtech) per well for 10 min. Washing of the plates with PBS was performed before each of these steps. Finally, the plates were washed under tap water to stop the reaction, air-dried overnight, and spots were counted using the S6 Ultra-V analyzer and ImmunoSpot software (both from ImmunoSpot, CTL). No cell- and no biotinylated Ab-controls were included.

2.5. Specific ELISpot assay for P. salmonis and Y. ruckeri

To enumerate the number of ASC producing *P. salmonis* or *Y. ruckeri*-recognizing IgM, we established two specific ELISpot assays. The ELISpot assays were performed as described above, with the following deviations: ELISpot wells were coated with 8 μg/well *P. salmonis* or *Y. ruckeri* antigen (preparation described in 2.1) in 100 μL PBS at 4 °C overnight. Leukocytes were seeded at 250000 cells per well for both the specific ELISpots. No cell- and no biotinylated Ab-controls were included. Limited leukocyte numbers and reagents led to reduced numbers of wells in the NC group for the *Y. ruckeri* ELISpot (specified in the figure caption).

2.6. ELISA

ELISAs were used to measure *anti-P. salmonis*, *anti-Y. ruckeri*, and *anti*-TNP-KLH Abs in serum. ELISA plates (Microlon® 200, Greiner) were coated with 2 μg/well of *P. salmonis* or *Y. ruckeri* antigen, as used for coating of specific ELISpot plates, or with 0.5 μg/well TNP-KLH (LGC Biosearch Technologies), diluted in 100 μl PBS at 4 °C overnight. After washing three times, the wells were blocked with 200 μL PBS with 0,05% Tween 20 and 5% non-fat milk for 1 h. Serum samples were diluted 1:50 (exceptions: 1:200 for *P. salmonis* 6 wpi and all TNP-KLH

Table 1
List of primers used in this study with references to original publications of the primers.

Target	FW/RV	Sequence	Published in:
CD4	FW	GTTGAAAGGGCGAAAGTGAG	Sobhkhez et al. (2018)
	RV	GTGCCTTCGATGAGGACATT	
CD40	FW	ATGCCATGCCAAGAGGGTGAAT	Lagos et al. (2012)
	RV	ATTTGCATGGGCTGAGGCTTGT	
CD40L	FW	CACCAGGACCGGGCCACAAC	Lagos et al. (2012)
	RV	TGGGCACACCCCCAGTGAGT	
CD83	FW	GTGGCGGCATTGCTGATATT	Iliev et al. (2013)
	RV	CTTGTGGATACTTCTTACTCCTTTGCA	
CD8a	FW	CGTCTACAGCTGTGCATCAATCAA	Strandskog et al. (2011)
	RV	GGCTGTGGTCATTGGTGTAGTC	
elf2aB	FW	TGCCCCTCCAGGATGTCTAC	Iliev et al. (2013)
	RV	CACGGCCCACAGGTACTG	
IFNγ	FW	AAGGACCAGCTGTTCAACGG	Thim et al. (2014)
	RV	CACACCCTCCGCTCACTGT	
MARCO	FW	AGGACCTGCTGGTGTTAATG	Jenberie et al. (2018)
	RV	CTGCTCTTTCACCCTTCTCTC	
MHCI	FW	GAAGAGCACTCTGATGAGGACAG	Sobhkhez et al. (2018)
	RV	CACCATGACTCCACTGGGGT	
MHCI lga	FW	CACAAAAACCAAGGACGATGAA	Svenning et al. (2019)
	RV	CGGTGCTTTAGTTCAAATGATCTG	
MHCII	FW	AGAAGCCTGGAACAAAGGTCCTGA	Jenberie et al. (2018)
	RV	AACTGTCTTGTCCAGTATGGCGCT	
mIgM	FW	CCTACAAGAGGGAGACCGA	Iliev et al. (2013)
	RV	GATGAAGGTGAAGGCTGTTTT	
Myeloperoxidase (MPO)	FW	CGAAACACGACCTTCAACAAC	Jenberie et al. (2020)
	RV	AACTCGCTATCGTTCACTACAC	
Pax5	FW	ACGGAGATCGGATGTTCCTCTG	Zwollo et al. (2008)
	RV	GATGCCGCGCTGTAGTAGTAC	
P. salmonis 16S	FW	AGGGAGACTGCCGGTGATA	Karatas et al. (2008)
	RV	ACTACGAGGCGCTTTCTCA	
sIgM	FW	CTACAAGAGGGAGACCGGA	Iliev et al. (2013)
	RV	AGGGTCACCGTATTATCACTAGTTT	
TNFα	FW	TGCTGGCAATGCAAAAGTAG	Iliev et al. (2010)
	RV	AGCCTGGCTGTAAACGAAGA	

samples, and 1:10 for *Y. ruckeri* samples) in PBS with 0,05% Tween 20 and 1% non-fat milk, and 100 µL was added to each well for 1 h after washing. After washing, 100 µL/well of the secondary Ab (LS-C63026-100, clone IPA-5F12, HRP conjugated – Bio-Rad) diluted 1:2000 in PBS with 0,05% Tween 20 and 1% BSA was added and incubated for 1 h. Wells were developed for 20 min using 100 µL/well 1-step ultra TMB-ELISA substrate (Thermo Fisher Scientific) before stopping the reaction by adding 100 µL/well 2M H_2SO_4. Optical density was read at 450 nm using a Sunrise absorbance reader (Tecan). Ab titers are presented as percentage of a positive standard for the antigens used; pooled sera from infected fish at 6 wpi for *P. salmonis* and TNP-KLH, or sera from salmon vaccinated for *Y. ruckeri* (courtesy of Vaxxinova Norway AS), by using a standard curve. This method is also described by Bailey et al., (2004).

2.7. P. salmonis qPCR

DNA from liver and HK samples on RNAlater or from isolated PerC leukocytes was isolated using the QIAamp cador Pathogen kit (Qiagen) as per manufacturer's instructions with lysis of organ samples using buffer ATL (Qiagen). Five µL DNA template (1:10 diluted for mortality and PerC samples, otherwise undiluted) was mixed with 10 µL 2x KAPA SYBR FAST qPCR master mix (Kapa Biosystems), 4 µL water, and 0,5 µL of both *P. salmonis* 16S primers (Table 1). These reactions were run on the CFX96 system (Bio-Rad) at 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 65 °C for 20 s, along with a dilution series of an amplicon with known copy numbers. Melting curves (increase from 65 °C to 97 °C with standard ramp rate) were used to verify the presence of a single product.

2.8. RT-qPCR on PerC AT

Total RNA was isolated from AT using the RNeasy Mini Kit (Qiagen), and 0,5 µg RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), both as per manufacturer's instructions. Six µL 1:10 diluted cDNA was used per 15 µL PCR reaction using 2 × Fast SYBR® Green Master Mix (Applied Biosystems) with primer concentrations of 0,5 µM (see Table 1 for all primers). The reactions were run under the following conditions: 95 °C for 5 min and 45 cycles of 95 °C for 5 s, 60 °C for 15 s, and 72 °C for 15 s (7500 Fast Real-Time PCR System, Applied Biosystems). Melting curves were used to verify the presence of a single product, and the used primers had previously determined efficiencies between 2,04 and 2,22 (see Table 1 for references per primer). Relative expression was calculated using the delta ct method with *Elf2a* as a reference gene (Schmittgen and Livak

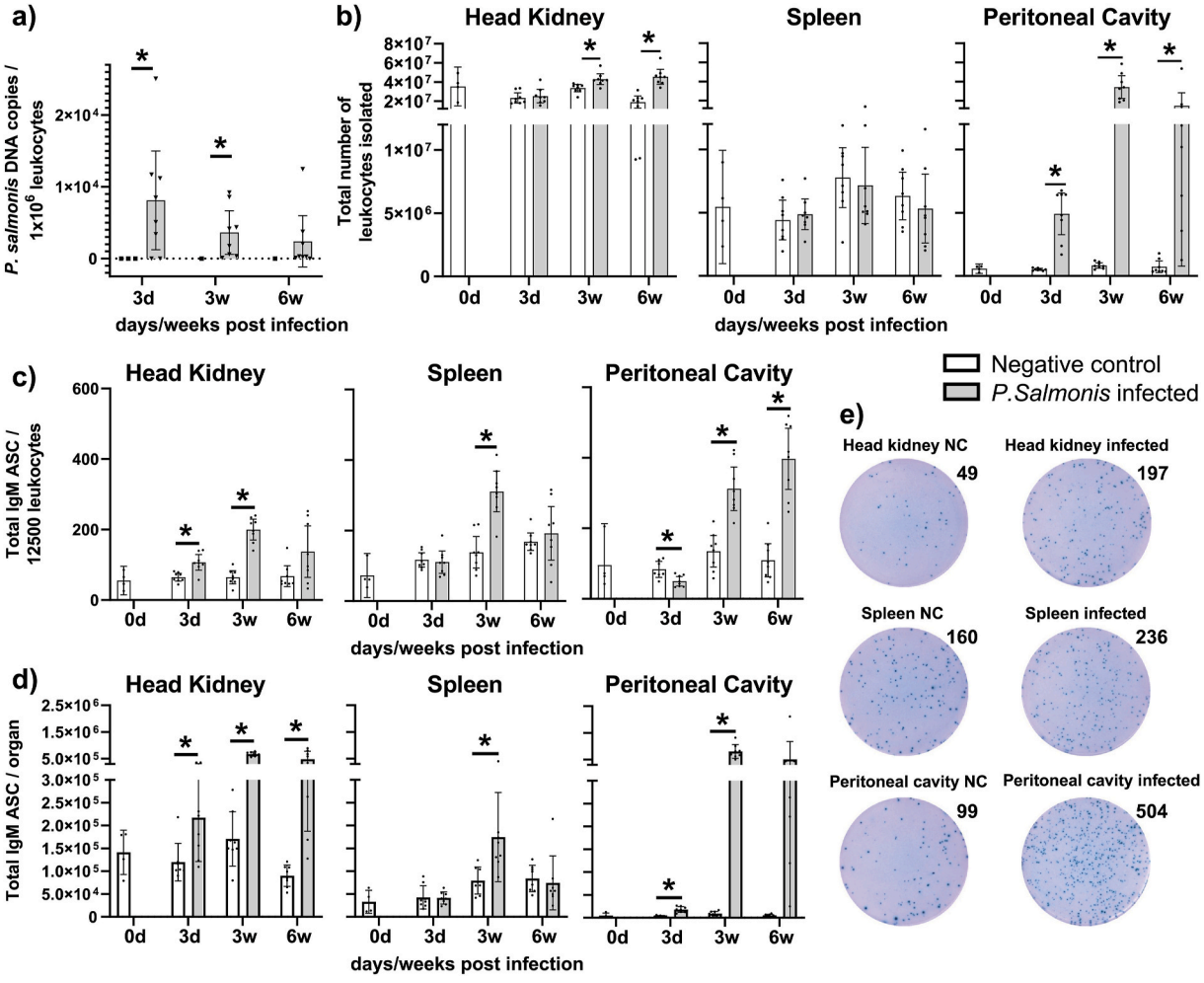


Fig. 1. *P. salmonis* DNA copies, leukocytes, and total IgM ASC in infected Atlantic salmon (a) *P. salmonis 16S* DNA copies per 1 × 10^6 peritoneal cavity leukocytes from negative control (NC) and *P. salmonis* infected Atlantic salmon. n = 8 for the infected salmon at all time points. At 3 days post infection, 8 NC fish were pooled in 3 pools, and at 3- and 6-weeks post infection 8 NC fish were pooled in 1 pool. Individual measurements shown as squares and triangles. **(b)** Total number of leukocytes from NC and *P. salmonis* infected Atlantic salmon per organ. **(c & d)** Number of total IgM ASC per 12500 leukocytes (c) or per organ (d) from NC and *P. salmonis* infected Atlantic salmon. n = 8 for all time points, except for day 0 (n = 4). Individual measurements shown as dots. (*) Statistically significant ($p < 0,05$) between the infected group and the NC, error bars indicate 95% confidence interval. **(e)** Representative total IgM ELISpot wells from NC and *P. salmonis* infected head kidney, spleen, and peritoneal cavity at 6 weeks post infection.

2008).

2.9. Statistical analysis

We performed statistical analyses in GraphPad Prism version 8.4.1. We used unpaired t-tests with Welch's correction to compare the leukocyte numbers, ASC numbers, specific Ab titers, and *P. salmonis* load in the PerC between the *P. salmonis* infected group with the NC group at all time-points. Since we did not deviate from the clearly predefined comparisons, we did not correct for multiple testing in these tests. The fold increase in specific Ab titers was tested using a one-sample *t*-test against the theoretical mean 1 (no change). We used Spearman correlation for correlations between serum IgM and ASC. Welch's ANOVA with Dunnett's T3 post hoc test was used to test gene expression differences between all NC and *P. salmonis* infected groups at time points 3 dpi, 3 wpi, and 6 wpi. The statistical significance level was set to $p < 0,05$ and is indicated with *.

3. Results

3.1. P. salmonis infection in Atlantic salmon

The *P. salmonis* challenge resulted in an infection of the Atlantic salmon based on the following observations. We euthanized four fish according to the humane endpoints (loss of buoyancy, lack of response to stimuli, or severe injury or ulceration) between day 36 and 41 and counted these as mortalities. Furthermore, fish weight was reduced by around 15% in infected fish at 3 wpi, and a similar, though not significant, reduction was observed at 6 wpi. Additionally, we observed discoloration and bleeding in liver and PerC of some infected fish at both 3- and 6-wpi (see Suppl. Table 2), corresponding to symptoms of SRS (Rozas and Enríquez 2014). *P. salmonis* DNA was demonstrated in liver and HK of all four mortalities, as well as in the liver of all infected fish at 3- and 6-wpi (see Suppl. Table 3), indicating that an infection was established. No *P. salmonis* DNA was detected in the liver of infected fish at 3 dpi nor in any NC samples. PerC leukocytes from infected fish were tested positive for *P. salmonis* DNA at the three time points: 3 dpi, 3 wpi, and 6 wpi (Fig. 1a), with the highest signal at 3 dpi. Together, these results demonstrate that *P. salmonis* established an infection in Atlantic salmon.

3.2. Leukocyte and total IgM ASC increase is highest in infected PerC

Previous studies have shown that upon IP injection with bacterial pathogens, IgM + B cells become the most abundant leukocyte population in the PerC (Korytář et al., 2013; Castro et al., 2017). However, these reports have focused on early time points (up to 72 h post infection), whereas studies investigating more prolonged responses are currently lacking. Herein, the total and specific IgM ASC in PerC and systemic tissues of Atlantic salmon were monitored over a period of 6 weeks upon challenge by *P. salmonis*, aiming at understanding more about the interplay between local ASC and systemic responses during a prolonged bacterial infection.

First, the total number of leukocytes isolated per organ was determined at different time-points after infection. In the steady state, at 0 dpi, most leukocytes were present in the HK, followed by the spleen, while in the PerC of non-infected fish the numbers remained very low (>20 times lower than in HK). Upon *P. salmonis* challenge, a very strong increase in leukocyte numbers was evident in the PerC of infected fish at all time-points, with a peak at 3 wpi (Fig. 1b). For the HK, there was also a significant increase in the number of leukocytes at both 3- and 6-wpi (Fig. 1b), while the number of leukocytes in the spleen of infected fish was similar to the controls.

Next, we determined the number of IgM ASC in HK, spleen, and PerC using ELISpot. The relative frequency of total IgM secreting cells per 12500 leukocytes peaked in the HK and spleen of infected fish at 3 wpi (Fig. 1c). In the HK, it was still slightly elevated, though not statistically significant, at 6 wpi. In the spleen, the levels in the infected fish paralleled controls at 6 wpi. In the PerC of infected fish, the frequency of IgM producing cells was slightly reduced at 3 dpi (Fig. 1c). In contrast, a significant IgM ASC increase was seen in the PerC at 3 wpi that increased even further at 6 wpi. By looking at the numbers of IgM secreting cells per organ, we see comparable amounts in the HK and in the PerC of infected fish, and the numbers in both sites were increased compared to negative controls at all time-points (Fig. 1d). The spleen, in contrast, contained lower total numbers and showed an increase only at 3 wpi.

3.3. Specific anti-P. salmonis ASC and non-specific anti-Y. ruckeri ASC are mainly increased in PerC

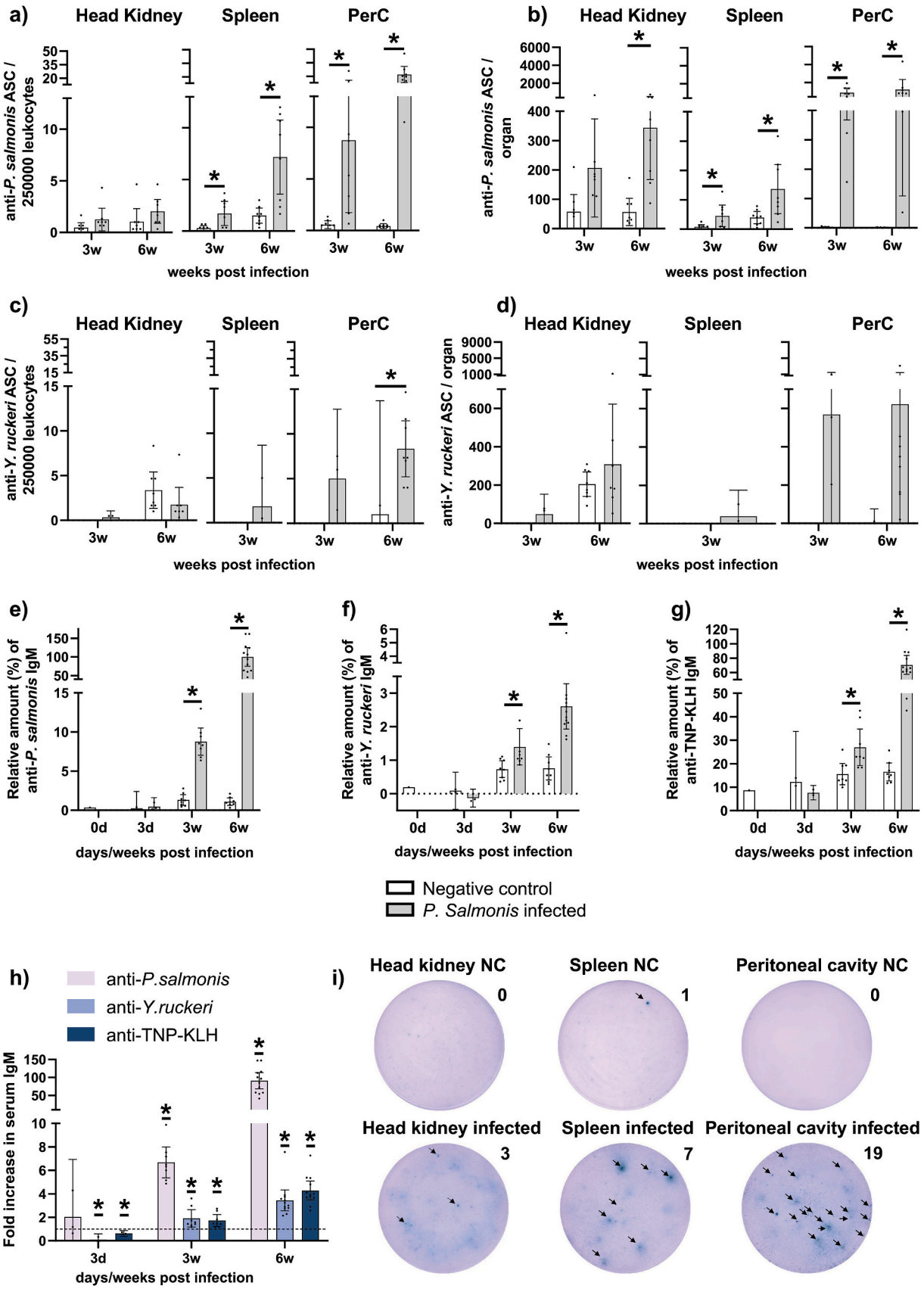
We established a *P. salmonis* ELISpot assay to enumerate the *anti-P. salmonis* ASC in the different organs. We optimized coating and cell seeding densities, while no cell- and no biotinylated antibody-controls showed no to very low background (suppl. Fig. 2c and 2d). In the HK, there was no significant difference in the frequency of *anti-P. salmonis* ASC per 250000 leukocytes between NC and infected fish at both 3- and 6-wpi (Fig. 2a). This contrasted with the frequency of total IgM ASC, which were higher in infected fish at both these time-points, though not significantly at 6 wpi (Fig. 1c). Interestingly, the frequency of *anti-P. salmonis* ASC in the spleen was higher after infection at both time points, while the frequency of total IgM ASC was not increased at 6 wpi (Figs. 1c–2a).

The increase in *anti-P. salmonis* ASC frequency in the PerC was more pronounced compared to the HK and spleen at both time-points, with the highest frequency of specific ASC found at 6 wpi (Fig. 2a). This agrees with earlier findings (suppl. Fig. 1c) and suggests that *anti-P. salmonis* ASC were mainly induced at the site of injection and not in the systemic immune organs at these time points. The total numbers of *anti-P. salmonis* ASC per organ showed similar results (Fig. 2b). For HK, a significant increase in *anti-P. salmonis* ASC per organ appeared at 6 wpi, although the increase compared to the NC was highest in the PerC. The spleen clearly contained the lowest number of *anti-P. salmonis* ASC when compared to HK and PerC. The control group (injected with PBS) did not exhibit any significant change during the study period (Fig. 2).

To investigate the presence of non-specific (cross-reacting) ASC, a second specific ELISpot assay was established using antigen from the unrelated fish pathogen *Y. ruckeri.* Interestingly, we observed a significant increase of non-specific ASC frequency in infected PerC at 6 wpi, which was around 15% of the increase in *anti-P. salmonis* ASC numbers (Fig. 2c). No differences in *anti-Y. ruckeri* ASC frequency were found in HK. At 3 wpi, some *anti-Y. ruckeri* ASC were found in all organs, and, although the number of observations was too low for statistical analysis, the frequencies seemed to be higher in infected Atlantic salmon (Fig. 2c). The total numbers of *anti-Y. ruckeri* ASC per organ showed a similar trend as seen for *anti-P. salmonis* ASC, although they were a bit lower and lacked statistical significance (Fig. 2d).

3.4. Anti-P. salmonis Ab titers, as well as anti-Y. ruckeri and anti-TNP-KLH titers, increase over time

The serum Ab response to *P. salmonis*, as measured by ELISA, was induced at low levels in infected fish after 3 wpi and continued to increase at 6 wpi (Fig. 2e). In addition, non-specific Abs recognizing *Y. ruckeri* and TNP-KLH were detected, and their levels in the infected fish gradually increased until at 6 wpi (Fig. 2f and g). No changes in Ab titers were detectable in the control fish compared to the titers at day 0. The level of *Y. ruckeri* Abs, given as percentage of a reference sample from vaccinated Atlantic salmon with high *Y. ruckeri* Ab titers, was only 2% of this reference. Thus, there was a slight, but clear increase in *Y. ruckeri*-recognizing Abs in the *P. salmonis*-infected fish. For the TNP-KLH Abs, we used pooled sera from the *P. salmonis* infected salmon as a standard, and the levels in infected fish increased at 3- and 6-wpi. The



(caption on next page)

Fig. 2. Specific and non-specific ASC and antibody responses in *P. salmonis* infected Atlantic salmon. (a & b) Number of *anti-P. salmonis* IgM ASC per 250000 leukocytes (a) or per organ (b) from negative control (NC) and *P. salmonis* infected Atlantic salmon. n = 8 for all time points. **(c & d)** Number of *anti-Y. ruckeri* IgM ASC per 250000 leukocytes (c) or per organ (d) from NC and *P. salmonis* infected Atlantic salmon. n = 1–3 for 3 weeks and n = 8 for 6 weeks (NC: PerC n = 2). **(e–g)** Relative amount of *anti-P. salmonis* (e), *anti-Y. ruckeri* (f), and *anti*-TNP-KLH (g) IgM in serum of NC and infected Atlantic salmon presented as a percentage of an included standard (pooled sera from infected fish at 6 weeks post infection for *P. salmonis* and TNP-KLH; serum from salmon vaccinated against *Y. ruckeri* for *Y. ruckeri*). n = 8 for all time points, except 0 days (n = 4, pooled in 1) and infected salmon at 6 weeks post infection (n = 12). At 3 days post infection, the 8 samples were pooled in 3 pools. Individual measurements shown as dots. (*) Statistically significant ($p < 0,05$) between the infected group and the NC, error bars indicate 95% confidence interval. **(h)** Fold increase in serum IgM for *anti-P. salmonis*, *anti-Y. ruckeri*, and *anti*-TNP-KLH relative to the average of the NC group per time point, n = 8–12. At 3 days post infection the 8 samples were pooled in 3 pools. Error bars indicate 95% confidence interval. Statistically significant difference (*) from mean 1 (no change, dotted line) based one-sample *t*-test. **(i)** Representative *anti-P. salmonis* ELISpot wells (96-well) from NC and *P. salmonis* infected head kidney, spleen, and peritoneal cavity at 6 weeks post infection.

use of different reference samples in the *Y. ruckeri* and TNP-KLH ELISAs does not allow direct comparison. Still, if we compare the increases in the infected groups to the NC groups, we can observe similar trends.

To enable a better comparison of the increase of serum Abs with different specificities, we calculated the relative increase of Abs per fish after infection. This increase was calculated by dividing the Ab titer for each infected fish (as presented by the individual dots in Fig. 2e–g) by the average of the NC group for the same time point (as presented by the white bars in Fig. 2e–g). An increase of 7- and 90-fold was visible for *P. salmonis* Abs on 3- and 6-wpi respectively (Fig. 2h). For *Y. ruckeri*, a 2- and 3-fold increase were detected at 3- and 6-wpi, respectively, similar to TNP-KLH, which showed a 2- and 4-fold increase at those time points. A comparison of the non-specific Ab increase with the increase in specific *P. salmonis* Abs shows relatively more non-specific Abs at 3 wpi than at 6 wpi (Fig. 2h). This indicates that although the numbers of non-specific Abs increased in time, the overall response became more specific at 6 wpi, as relatively more specific Abs are present at that time point.

We investigated the correlations between the serum Ab titers for *P. salmonis* and *Y. ruckeri* and the ASC frequencies using Spearman correlation. The analysis was performed for the HK, spleen, and PerC at 3- and 6-wpi. Significant correlations were found between *Y. ruckeri*-recognizing Abs in serum and total ASC in PerC at 3 wpi (rs: 0,76), and between the *Y. ruckeri*-recognizing Abs in the serum and total ASC in spleen (rs: 0,83), total ASC in PerC (rs: 0,86), and *Y. ruckeri*-recognizing ASC in PerC (rs: 0,78, see Suppl. Table 4) at 6 wpi. Surprisingly, correlations between *P. salmonis* Ab titers and ASC numbers in organs were weaker than those for *Y. ruckeri* Ab titers and below the significance limit. The highest correlation coefficients found for *P. salmonis* Ab titers came up with *anti-P. salmonis* ASC and total IgM ASC in the spleen and PerC at 6 wpi (rs: 0,62 to 0,71). All correlation coefficients between serum Ab and ASC in the HK were lower compared to the other two organs at 6 wpi (Suppl. Table 4).

3.5. Immune cell marker expression in PerC at suggests immunological role

A previous study has stated that rainbow trout AT contains various immune cell populations including macrophages, T-, and B cells (Pignatelli et al., 2014). To investigate the possible role of the AT in the immune responses against an IP challenge of *P. salmonis*, we evaluated the expression of several immune cell marker genes and related immune relevant genes. The expression of investigated genes in the NC group was not significantly altered between any of the time points (Fig. 3), which suggests that the PBS injection induced no change in expression and that we measured the steady state for these genes. In this steady state, B cells were clearly present based on the basal expression of *membrane bound IgM* (*mIgM*), *IgT*, and *IgD*, of which *IgM* was most highly expressed, while *IgD* expression was barely detectable (Fig. 3a). Basal levels of *Pax5* in the AT were low as well. *Secreted IgM* (*sIgM*) transcripts were also expressed, indicating the presence of ASC. T cell markers, both *CD4* and *CD8*, were observed, but the low expression of *CD40L* suggests that the majority of T cells was not activated. Professional antigen presenting cells (such as B cells, tissue resident macrophages, and dendritic cells (DCs)) are characterized by their expression of *MHCII*, *CD40*, and *CD83* and were also present based on detected gene expression. Together, this indicates a presence of all cell types necessary for antigen presentation in the steady state AT.

The expression of most of the measured genes changed upon *P. salmonis* challenge. Early in the infection, at 3 dpi, an increase of *IFNγ* expression may have initiated a Th1 response since it coincided with an increase in *CD8* and *MCHI* expression (Fig. 3b). Later in the infection, at 3 and 6 wpi, B cell markers (*Ig*'s, *CD40*, and *MHCII*) were increased, with *sIgM* levels being higher at 6 wpi than at 3 wpi (Fig. 3a & c). The latter indicates a higher proportion of more differentiated B cells (plasmablasts or plasma cells) compared to the earlier time points, although the expression of *Pax5* (immature B cells) did not change significantly. The increase in *IgT* seemed to follow the general increase in *mIgM* and *sIgM*, but the *IgD* expression remained extremely low. Macrophages also seemed to be present in higher numbers at the later time points based on the *MARCO* expression increase. Nevertheless, macrophages might have been less abundant than DCs, since the increase in *CD83* expression was higher than that of *MARCO*.

The Th1 response that started at 3 dpi was more pronounced at later time points based on the increase in *IFNγ*, *CD8*, *MHCI*, and *CD40L* expression (Fig. 3b). T-helper cell and cytotoxic T cell markers (*CD4* and *CD8*) were increased at 3 and 6 wpi, in addition to *CD40L*, a marker for T cell activation. *P. salmonis 16S* RNA was present in the AT of infected fish at 3- and 6-wpi, although the detected increase was not statistically significant due to large variation (Fig. 3d). Interestingly, no inflammation seemed to be present in the AT based on the lack of *myeloperoxidase* expression that would signal neutrophil activity. The bacterium was possibly mainly present in infected or presenting cells, which would explain the Th1 response. Finally, the peak in expression of the pro-inflammatory cytokine *TNFα* at 3 weeks, without a clear inflammatory response in the AT, might indicate a regulation of the immune response in the PerC. Together, the basal expression levels and the induced expression of immune cell markers after *P. salmonis* infection point to an immunological role for the AT.

4. Discussion

4.1. PerC response and leukocyte migration

The main aim of our study has been to define the characteristics of the local (peritoneal) and the systemic B cell Ab responses in Atlantic salmon upon IP challenge with *P. salmonis*. Our results revealed a strong local B cell response in the PerC of the infected fish. This local response was characterized by increased numbers of leukocytes, as well as elevated frequencies of total-, specific-, and non-specific ASC at 3- and 6-wpi. At 3 dpi, an increase of leukocytes in the PerC coincided with a decrease in total IgM ACS, indicating that leukocytes other than plasmablasts or plasma cells were mainly present at that time point. This is in line with the observations by Korytář et al., (2013), where the ratio of myeloid cells in the PerC is increased after *Aeromonas salmonicida* infection at 12 h and was still elevated at 72 h.

In a recent study by our group (Jenberie et al., 2020), the viral pathogen SAV3 was IP administered to Atlantic salmon. They found

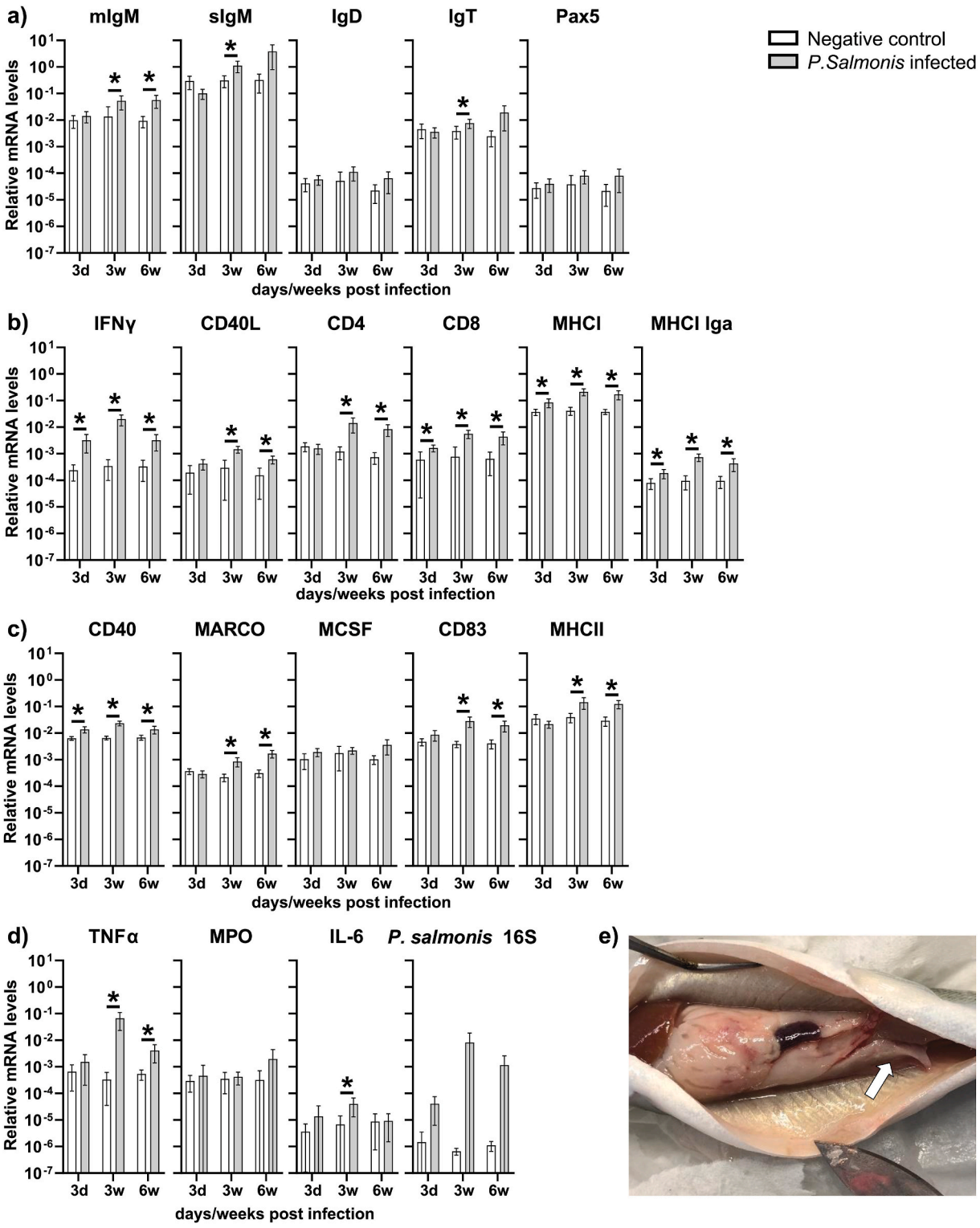


Fig. 3. Relative immune gene expression levels in peritoneal adipose tissue from negative control (NC) and *P. salmonis* infected Atlantic salmon for B cell (a), T cell (b), professional antigen presenting cell (c), and inflammation (d) related genes at 3 days, 3-, and 6-weeks post infection. Graph shows the mean expression of genes, including the bacterial *16S* gene, presented relative to a control gene (*Elf-2a*). n = 7 for NC and n = 8 for infected fish at all time points. (*) Statistically significant between the infected group and the NC, error bars indicate 95% confidence interval. **(e)** Peritoneal cavity of *P. salmonis* infected Atlantic salmon. The sampled adipose tissue is indicated with an arrow.

prolonged ASC responses in the PerC, similar to what we report here. Collectively, the two studies demonstrate the presence and maintenance of ASC in the PerC, highlighting an active role of the PerC to IP challenge. However, when comparing these studies, the bacterial infection resulted in a stronger response (increase in leukocyte- and total IgM ASC numbers) compared to the viral infection. Notably, the effects of the SAV3 infection were milder (no mortalities and less observed pathology) compared to our bacterial challenge. Jenberie et al., (2020) observed a decrease of leukocytes in the HK at 1- and 2-wpi, coinciding with an increase in the PerC. They suggested that the leukocytes might have migrated from the HK to the PerC. In contrast, we did not see a decrease in leukocyte numbers in the HK or spleen at any time point. This suggests either a fast local expansion of leukocytes in the PerC or an influx from other organs than HK or spleen. The AT might be a source of origin of the leukocytes, as well as the peripheral blood. One explanation as to why no decrease in leukocyte numbers in the HK was observed might be the observed presence of *P. salmonis* in the HK of Atlantic salmon as early as 3 dpi (Svenning et al., 2019). This is possible through transport by infected macrophages that return to the HK for antigen presentation. As a result, the presence of *P. salmonis* infected cells could attract more leukocytes to the HK. This trafficking of leukocytes between HK and PerC is in accordance with a previous study in Atlantic salmon, where IP injected fluorescent labeled ovalbumin was rapidly endocytosed by MHCII + cells in the PerC, and subsequently transferred to the HK, where they remained present for at least two weeks (Iliev et al., 2013). At 3 dpi, the leukocyte counts for the infected HK had to be normalized due to a technical issue. Since this normalization could mask a reduction in leukocyte numbers, it cannot be completely ruled out that HK leukocyte numbers were reduced.

This strong local response in the PerC could result in an overestimation of vaccine protection in vaccination/challenge studies, especially when vaccination and challenge are both administered IP. Several *P. salmonis* vaccine efficacy trials performed the challenge not long after vaccination, e.g. after 600° days (around 2 months at 10 °C) (Evensen 2016; Tobar et al., 2011; Wilhelm et al., 2006), probably since longer experiments are costly. The response to an early IP challenge in vaccine trials could be significantly influenced by the observed strong local response in the PerC and lead to lower mortalities and thus higher predicted protection. This could explain, at least partly, why the *P. salmonis* vaccines show protection in experimental settings but fail to do so in the field. However, since our study was performed with a live pathogen, the outcome of vaccine trial using inactivated antigens may elicit responses that are different from reported here. This would be an interesting objective for a future study.

4.2. Non-specific versus specific B cell activation

Another possible reason for overestimation of vaccine efficiency, in this instance against heterologous strains, could be the cross-protection from non-specific Abs. Such Abs, which are present early in the immune response, can bind to a secondary pathogen and lead to cross-protection. The non-specific Abs and ASC that we found at 3- and 6- wpi show that this is probable, especially since they recognize *Y. ruckeri*, a non-related pathogen. At 6 wpi, we found less non-specific Abs relative to specific Abs compared to 3 wpi. This could indicate a reduction in cross-reactivity at later time points, as can be observed after vaccination of rainbow trout against viral haemorrhagic septicemia (Yamaguchi et al., 2019; Lorenzen 2002). A non-specific Ab response after IP vaccination of Atlantic salmon has also been found by Lund et al., (2019). An early challenge with a heterologous strain after vaccination could thus encounter more cross-protection than a challenge after a longer period. Our data indicate that careful design of vaccination experiments is of outmost importance to obtain a readout of specific protection.

This non-specific response could originate from non-specifically activated B cells. In addition to their clonally rearranged B cell receptor that responds to specific antigens, teleost B cells express a range of Toll-like receptors (TLRs) that allows them to react directly to microbial products (Abós et al., 2013; Jenberie et al., 2018; Peñaranda et al., 2019). In mice, the recognition of pathogen associated molecular patterns may lead to the production of non-specific or natural Abs that are poly-reactive and are able to react with foreign antigens that the host never met, as shown by Gunti et al., (2015). The non-specific activation of natural ASC might also explain why correlations between *Y. ruckeri* recognizing Abs and ASC were stronger than between *anti-P. salmonis* Abs and ASC. Several of the found correlations for Y. ruckeri serum Abs were significant at 6 wpi, while the best correlations for *P. salmonis* Abs were just below the significance level. While one would intuitively expect the specific reaction to the pathogen to have a better correlation, a higher variation in the specific responses between individual fish could explain the lower correlations found. An activation through TLRs by pathogen associated molecular patterns would be much less varied than an activation through a B cell receptor that has undergone somatic hypermutations. Additionally, the repertoire of natural Abs is less varied (Bilal et al., 2021), restricting the variation of the response. Finally, we correlated serum Abs with ASC numbers. This could result in lower correlations if some ASC would produce more Ab than others, which could be the case for specific ASC.

The mechanisms of the transition to a more specific Ab response in teleosts still warrants more research. The affinity maturation that leads to specific responses in mammals is driven by somatic hypermutation and clonal selection. This is realized through mutations and antigen-dependent survival of activated B cells. In mammals, this phenomenon is linked to follicular B cells (also named B2 cells), follicular DCs, and T cell help. Although less efficient, activated teleost B cells share the ability to undergo somatic hypermutation and clonal selection with their mammalian counterparts, but germinal centers, the sites of clonal selection, together with follicular DCs, have not been found in teleosts (Magor 2015; Steinel and Bolnick 2017; Stosik et al., 2019). Melano-macrophage centers might function as maturation sites in fish, where macrophages and follicular DC-related reticular cells could take part in retention and presentation of antigen (Magor 2015). According to their observations, melanomacrophage centers appear to contain more antigen-trapping cells than mammalian germinal centers, thus low affinity B cells have a possibility of receiving antigen signals as well. This, in turn, may result in a weaker selection of high-affinity clones. Magor (Magor 2015; Muthupandian et al., 2021) suggests that this could explain the slow and weak affinity maturation found in fish. The main increase of specific ASC in the PerC observed in our study suggests that clonal selection might be occuring locally. A possible location would be the PerC AT, where melanomacrophage centers have been observed after vaccination (Villumsen et al., 2017).

4.3. ASC localization

The fact that the PerC showed the highest induction of all ASC, compared to HK and spleen, raises the question on the main location of systemic Ab production early after IP *P. salmonis* infection. Based on the ASC frequencies, this would be the PerC, followed by the spleen. The correlations between serum Abs and different ASC in the investigated organs support this (Suppl. Table 4). Unexpectedly, the HK showed no significant increase of specific ASC frequency at any of the included time points. This is interesting because the HK is the main hematopoietic organ and the organ considered to be the main location for ASC at later (>8 wpi) time points (Bromage et al., 2004). Bromage et al. describe antigen-specific ASC in blood, spleen, and HK of rainbow trout immunized with TNP-KLH in Freund's Complete Adjuvant. They observed an initial peak of specific ASC numbers in all three organs at 8 wpi, after which only ASC numbers in the HK remained elevated. These HK ASC were hydroxyurea-resistant, meaning that they did not proliferate any more, and their presence coincided with elevated serum titers. This led the authors to propose a model where long-lived (hydroxyurea-resistant) ASC in the HK are mainly responsible for the increased serum

Ab titers late in the response. In comparison, we found significant serum Ab titers and an increased number of specific ASC in the spleen and PerC, not the HK, as early as 3- and 6-wpi. This seems to indicate that the HK is less important for Ab production during the early response, which is in accordance with the kinetics found by Bromage et al., (2004). The observed faster and more pronounced Ab response, compared with Bromage et al., (2004), could be due to a difference in host/pathogen interactions, fish species, and/or environment. A notable difference lies in the antigens used in the two studies; a living pathogen usually leads to a more complete response than a model antigen. Based on a combination of the findings one could propose a model in which Ab secretion is initiated locally in the PerC with contribution from the spleen, while the HK facilitates long-term Ab responses.

Higher ASC frequencies or numbers do not necessarily reflect that more Abs are produced as the ASC in some organs might produce more Abs per ASC. In the present study, the spots in the ELISpot wells differed in size and intensity between the three organs (Fig. 2i). This indicates a difference in the quantity of secreted Abs per specific ASC. The most pronounced difference was present in the spleen samples, where some spots were clearly bigger and more intense than in the other organs, while the HK spots were the faintest. Additionally, the (counted) average spot sizes were larger in both the spleen and PerC compared to the HK at 6 wpi (see suppl. Fig. 3). The total well intensity was also lowest in the HK (data not shown). These results suggest that the spleen and PerC not only had a larger increase in specific ASC numbers, but also produced more Abs per ASC when compared to the HK.

4.4. Immunological role of the PerC AT

A logical explanation for the presence of ASC in the PerC is a local B cell activation. Teleost B cells most closely resemble mammalian innate, or B1, B cells in that they express several pattern recognition receptors, phagocytize actively, and respond to inflammatory signals (Díaz-Rosales et al., 2019; Peñaranda et al., 2019). Additionally, their expression profile of cluster of differentiation molecules corresponds best with mammalian B1 cells: CD5, CD9, CD11a, CD11c, and CD22 (Peñaranda et al., 2019). In mammals, the B1 B cells reside in the PerC and pleural cavity and can be activated in the PerC before migrating to the spleen (Baumgarth 2013). Interestingly, our gene expression analysis of immune cell markers indicates that the PerC AT may harbor the immune cells necessary for antigen presentation and B cell activation. Still, while we expect the expression of *Pax5*, a marker for immature B cells, to go down in a population of maturing B cells, it did not change significantly. A possible explanation is the recruitment of more immature B cells, possibly coupled with an efflux of mature B cells.

In addition to the presence and increase of *IgM* transcripts during the *P. salmonis* infection, an increase in *IgT* transcripts was also evident, indicating a role of IgT^+ B cells in the AT. Pignatelli et al., (2014) also observed increased *IgT* expression after viral challenge of rainbow trout, as well as the presence of IgT^+ cells by flow cytometry of the AT.

The expression of TNFα without an inflammatory response (no increase in *myeloperoxidase* expression) in the AT could furthermore indicate a regulating role for the AT in the PerC immune response. Together with the possible antigen presentation, the early cellular-, and the later humoral response, this indicates an immunological response in the AT to an infection of the PerC. This is in agreement with the hypothesis that the AT is a relevant immune organ in teleosts (Pignatelli et al., 2014; Veenstra et al., 2018). Functional studies are needed to confirm the presence of all components of efficient antigen presentation in the naïve AT and thus its role as a secondary immune organ. Nevertheless, our gene expression results suggest an immune regulatory role for the AT in the PerC.

Our findings indicate that the PerC is an essential site of B cell Ab production upon IP challenge with *P. salmonis* (and probably vaccination) and suggest a more extended role of the PerC than previously assumed. Although our findings only span the first 6 wpi, it is possible that ASC in the PerC continue to produce Abs after 6 wpi. Jenberie et al. (unpublished, personal communication S. Jenberie, April 03, 2020) found this for SAV by showing the presence of SAV specific ASC in the PerC of infected fish at 13 wpi, their latest time point, supporting this view. For IP oil-based vaccines, a long-term presence of ASC in the PerC might additionally be maintained due to depot forming and gradual antigen release. Whether Abs produced in the PerC also contribute to systemic immunity, or whether ASC in spleen and HK are mainly responsible, is a pending question. If the role of the PerC in short- and long-term immunity is elucidated, new vaccines can be evaluated more accurately. Further research into the numbers of ASC in the PerC and their levels of Ab secretion during longer periods after infection or vaccination will help us to expand the current model of B cell biology and ASC localization.

5. Conclusion

The successful development of specific ELISpot assays for *P. salmonis* and *Y. ruckeri* allowed us to observe a large increase in specific ASC in the Atlantic salmon PerC after IP *P. salmonis* challenge. This strong local response, combined with a non-specific response, could possibly lead to overestimation of vaccine efficacy if fish are IP-challenged a few weeks after vaccination. Another intriguing finding is that the *anti-P. salmonis* ASC frequency in PerC, and to a lesser extent in the spleen, is higher than in the HK. This sheds a new light on the main location of Ab production in infected salmon during the early response to *P. salmonis* infection and is a highly relevant issue related to different vaccination regimes. Finally, the expression of several immune cell markers indicates an immunological role for the AT in the PerC. Together, these findings suggest a more important role of the PerC after IP challenge and vaccination than previously held. Taking this into account could have important consequences for future vaccine development.

Author contributions

YvW and JJ conceived and designed research. YvW, SJ, HN, and LGT performed research and analyzed data. SJ, IJ and JK helped design experiments. YvW and JJ wrote the paper. All authors reviewed and approved the manuscript.

Role of funding source

Most of the research was funded by Vaxxinova R&D GmbH. Vaxxinova had an advisory role in the study design and final report.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to acknowledge Dr. Marcos Mancilla and ADL Diagnostic Chile for the *P. salmonis* challenge strain and their support. Furthermore, we like to thank Lill-Heidi Johansen, NOFIMA, for the *Y. ruckeri* strain, dr. Karsten Skjødt for the anti-trout IgM Ab, and the company Cellular Technology Limited for support with ELISpot counting. We thank Guro Strandskog for her help with sampling, and Annemarie van der Zeeuw for her invaluable feedback during the writing process. Finally, we appreciate the Tromsø Aquaculture station for technical assistance with the challenge experiment and Rudi Caeyers and Rod Wolstenholme from the UiT graphical services section for graphical support. The study has been partly financed by UiT- The Artic University of Norway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104125.

References

Abós, Beatriz, Castro, Rosario, Pignatelli, Jaime, Luque, Alfonso, González, Lucia, Tafalla, Carolina, 2013. Transcriptional heterogeneity of IgM+ cells in rainbow trout (Oncorhynchus mykiss) tissues. PloS One 8 (12), e82737. https://doi.org/10.1371/journal.pone.0082737.

Almendras, F.E., Fuentealba, I.C., 1997. Salmonid rickettsial septicemia caused by Piscirickettsia salmonis:a review. Dis. Aquat. Org. 29 (2), 137–144. https://doi.org/10.3354/dao029137.

Bailey, Michael, Haverson, Karin, Miller, Bevis, Jones, Philip, Sola, Isabel, Enjuanes, Luis, Stokes, Christopher R., 2004. Effects of infection with transmissible gastroenteritis virus on concomitant immune responses to dietary and injected antigens. Clin. Diagn. Lab. Immunol. 11 (2), 337–343. https://doi.org/10.1128/CDLI.11.2.337-343.2004.

Baumgarth, Nicole, 2013. Innate-like B cells and their rules of engagement. In: Advances in Experimental Medicine and Biology, vol. 785, pp. 57–66. https://doi.org/10.1007/978-1-4614-6217-0_7.

Bilal, Sumaira, Etayo, Angela, Hordvik, Ivar, 2021. Immunoglobulins in teleosts. Immunogenetics 73 (1), 65–77. https://doi.org/10.1007/s00251-020-01195-1.

Bromage, Erin S., Kaattari, Ilsa M., Zwollo, Patty, Kaattari, Stephen L., 2004. Plasmablast and plasma cell production and distribution in trout immune tissues. Baltimore, Md J. Immunol. 173, 7317–7323. https://doi.org/10.4049/jimmunol.173.12.7317, 12.

Castro, Rosario, Abós, Beatriz, González, Lucia, Granja, Aitor G., Tafalla, Carolina, 2017. Expansion and differentiation of IgM+ B cells in the rainbow trout peritoneal cavity in response to different antigens. Dev. Comp. Immunol. 70, 119–127. https://doi.org/10.1016/j.dci.2017.01.012.

Cossarini-Dunier, Muriel, 1986. Secondary response of rainbow trout (Salmo gairdneri Richardson) to DNP-haemocyanin and Yersinia ruckeri. Aquaculture 52 (2), 81–86. https://doi.org/10.1016/0044-8486(86)90027-X.

Díaz-Rosales, Patricia, Muñoz-Atienza, Estefanía, Tafalla, Carolina, 2019. Role of teleost B cells in viral immunity. Fish Shellfish Immunol. 86, 135–142. https://doi.org/10.1016/j.fsi.2018.11.039.

Evensen, Øystein, 2016. Immunization strategies against Piscirickettsia salmonis infections: review of vaccination approaches and modalities and their associated immune response profiles. Front. Immunol. 7, 482. https://doi.org/10.3389/fimmu.2016.00482.

Fryer, J.L., Lannan, C.N., Giovannoni, S.J., Wood, N.D., 1992. Piscirickettsia salmonis gen. Nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Int. J. Syst. Bacteriol. 42 (1), 120–126. https://doi.org/10.1099/00207713-42-1-120.

Gunti, Sreenivasulu, Messer, Ronald J., Xu, Chengfu, Yan, Ming, Coleman, William G., Peterson, Karin E., et al., 2015. Stimulation of Toll-Like Receptors profoundly influences the titer of polyreactive antibodies in the circulation. Sci. Rep. 5, 15066. https://doi.org/10.1038/srep15066.

Hedfors, Aagård, Ida, Bakke, Hege, Skjødt, Karsten, Grimholt, Unni, 2012. Antibodies recognizing both IgM isotypes in Atlantic salmon. Fish Shellfish Immunol. 33 (5), 1199–1206. https://doi.org/10.1016/j.fsi.2012.09.009.

Henríquez, P., Kaiser, M., Bohle, H., Bustos, P., Mancilla, M., 2016. Comprehensive antibiotic susceptibility profiling of Chilean Piscirickettsia salmonis field isolates. J. Fish. Dis. 39 (4), 441–448. https://doi.org/10.1111/jfd.12427.

Jenberie, Shiferaw, Peñaranda, Ma, Michelle, D., Thim, Hanna L., Styrvold, Morten Bay, Strandskog, Guro, Jørgensen, Jorunn B., Jensen, Ingvill, 2020. Salmonid alphavirus subtype 3 induces prolonged local B cell responses in atlantic salmon (Salmo salar) after intraperitoneal infection. Front. Immunol. 11, 1682. https://doi.org/10.3389/fimmu.2020.01682.

Jenberie, Shiferaw, Thim, Hanna L., Sunyer, J. Oriol, Skjødt, Karsten, Jensen, Ingvill, Jørgensen, Jorunn B., 2018. Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. Sci. Rep. 8 (1), 3565. https://doi.org/10.1038/s41598-018-21895-9.

Iliev, Dimitar B., Jørgensen, Sven Martin, Rode, Marit, Krasnov, Aleksei, Harneshaug, Ingvill, Jørgensen, Jorunn B., 2010. CpG-induced secretion of MHCIIbeta and exosomes from salmon (Salmo salar) APCs. Dev. Comp. Immunol. 34 (1), 29–41. https://doi.org/10.1016/j.dci.2009.07.009.

Iliev, Dimitar B., Thim, Hanna, Lagos, Leidy, Olsen, Randi, Jørgensen, Jorunn B., 2013. Homing of antigen-presenting cells in head kidney and spleen - salmon head kidney hosts diverse APC types. Front. Immunol. 4, 137. https://doi.org/10.3389/fimmu.2013.00137.

Intesal, 2014. Evaluación de la duración de la EFICACIA de vacunnas para el control de PISCIRRICKETTSIOSIS en trucha arcoíris Y salmón del atlántico MEDIANTE desafío por cohabitaación. Intesal, Chile. SEGUNDO REPORTE.

Kaattari, S., 2002. Affinity maturation in trout: clonal dominance of high affinity antibodies late in the immune response. Dev. Comp. Immunol. 26 (2), 191–200. https://doi.org/10.1016/S0145-305X(01)00064-7.

Karatas, S., Mikalsen, J., Steinum, T.M., Taksdal, T., Bordevik, M., Colquhoun, D.J., 2008. Real time PCR detection of Piscirickettsia salmonis from formalin-fixed paraffin-embedded tissues. J. Fish. Dis. 31 (10), 747–753. https://doi.org/10.1111/j.1365-2761.2008.00948.x.

Korytář, Tomáš, Jaros, Joanna, Verleih, Marieke, Rebl, Alexander, Kotterba, Günter, Kühn, Carsten, et al., 2013. Novel insights into the peritoneal inflammation of rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol. 35 (4), 1192–1199. https://doi.org/10.1016/j.fsi.2013.07.032.

Lagos, Leidy X., Iliev, Dimitar B., Helland, Ronny, Rosemblatt, Mario, Jørgensen, Jorunn B., 2012. CD40L–a costimulatory molecule involved in the maturation of antigen presenting cells in Atlantic salmon (Salmo salar). Dev. Comp. Immunol. 38 (3), 416–430. https://doi.org/10.1016/j.dci.2012.07.011.

Lorenzen, N., 2002. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Dev. Comp. Immunol. 26 (2), 173–179. https://doi.org/10.1016/S0145-305X(01)00059-3.

Lund, Hege, Bakke, Anne Flore, Sommerset, Ingunn, Afanasyev, Sergey, Schriwer, Geir, Thorisdottir, Audur, et al., 2019. A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon. Mol. Immunol. 106, 99–107. https://doi.org/10.1016/j.molimm.2018.12.018.

Magnadóttir, Bergljót, 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20 (2), 137–151. https://doi.org/10.1016/j.fsi.2004.09.006.

Magor, Brad G., 2015. Antibody affinity maturation in fishes-our current understanding. Biology 4 (3), 512–524. https://doi.org/10.3390/biology4030512.

Maisey, Kevin, Montero, Ruth, Christodoulides, Myron, 2017. Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. Expet Rev. Vaccine 16 (3), 215–228. https://doi.org/10.1080/14760584.2017.1244483.

Muthupandian, A., Waly, D., Magor, B.G., 2021. Do ectothermic vertebrates have a home in which to affinity mature their antibody responses? Dev. Comp. Immunol. 119, 104021. https://doi.org/10.1016/j.dci.2021.104021.

Peñaranda, M.M.D., Jensen, I., Tollersrud, L.G., Bruun, J.A., Jørgensen, J.B., 2019. Profiling the atlantic salmon IgM+ B cell surface proteome: novel information on teleost fish B cell protein repertoire and identification of potential B cell markers. Front. Immunol. 10, 37. https://doi.org/10.3389/fimmu.2019.00037.

Pignatelli, Jaime, Castro, Rosario, Granja, González, Aitor, Abós, Beatriz, González, Lucia, Jensen, Linda B., Tafalla, Carolina, 2014. Immunological characterization of the teleost adipose tissue and its modulation in response to viral infection and fat-content in the diet. PloS One 9 (10), e110920. https://doi.org/10.1371/journal.pone.0110920.

Plant, Karen P., Lapatra, Scott E., 2011. Advances in fish vaccine delivery. Dev. Comp. Immunol. 35 (12), 1256–1262. https://doi.org/10.1016/j.dci.2011.03.007.

Rozas, M., Enríquez, R., 2014. Piscirickettsiosis and Piscirickettsia salmonis in fish: a review. J. Fish. Dis. 37 (3), 163–188. https://doi.org/10.1111/jfd.12211.

Schmittgen, Thomas D., Livak, Kenneth J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3 (6), 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Smith, P.A., Pizarro, P., Ojeda, P., Contreras, J., Oyanedel, S., Larenas, J., 1999. Routes of entry of Piscirickettsia salmonis in rainbow trout Oncorhynchus mykiss. Dis. Aquat. Org. 37 (3), 165–172. https://doi.org/10.3354/dao037165.

Sobhkhez, Mehrdad, Krasnov, Aleksei, Robertsen, Børre, 2018. Transcriptome analyses of Atlantic salmon muscle genes induced by a DNA vaccine against salmonid alphavirus, the causative agent of salmon pancreas disease (PD). PloS One 13 (10), e0204924. https://doi.org/10.1371/journal.pone.0204924.

Steinel, Natalie C., Bolnick, Daniel I., 2017. Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. Front. Immunol. 8, 827. https://doi.org/10.3389/fimmu.2017.00827.

Stosik, Michał P., Tokarz-Deptuła, Beata, Deptuła, Wiesław, 2019. Melanomacrophages and melanomacrophage centres in Osteichthyes. Cent. Eur. J. Immunol. 44 (2), 201–205. https://doi.org/10.5114/ceji.2019.87072.

Strandskog, Guro, Villoing, Stéphane, Iliev, Dimitar B., Thim, Hanna L., Christie, Karen Elina, Jørgensen, Jorunn B., 2011. Formulations combining CpG containing oligonucleotides and poly I:C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon. Dev. Comp. Immunol. 35 (11), 1116–1127. https://doi.org/10.1016/j.dci.2011.03.016.

Svenning, Steingrim, Gondek-Wyrozemska, Agata T., van der Wal, Yorick Andreas, Robertsen, Børre, Jensen, Ingvill, Jørgensen, Jorunn B., Edholm, Eva-Stina, 2019. Microbial danger signals control transcriptional induction of distinct MHC class I L lineage genes in atlantic salmon. Front. Immunol. 10, 2425. https://doi.org/10.3389/fimmu.2019.02425.

Thim, Hanna L., Villoing, Stéphane, McLoughlin, Marian, Christie, Karen Elina, Grove, Søren, Frost, Petter, Jørgensen, Jorunn B., 2014. Vaccine adjuvants in fish vaccines make a difference: comparing three adjuvants (montanide ISA763A oil, CpG/poly I: C combo and VHSV glycoprotein) alone or in combination formulated with an inactivated whole salmonid alphavirus antigen. Vaccines 2 (2), 228–251. https://doi.org/10.3390/vaccines2020228.

Tobar, Jaime A., Jerez, Sofía, Caruffo, Mario, Bravo, Catalina, Contreras, Francisco, Bucarey, Sergio A., Harel, Moti, 2011. Oral vaccination of Atlantic salmon (Salmo salar) against salmonid rickettsial septicaemia. Vaccine 29 (12), 2336–2340. https://doi.org/10.1016/j.vaccine.2010.12.107.

Veenstra, Kimberly A., Wangkahart, Eakapol, Wang, Tiehui, Tubbs, Lincoln, Arous, Ben, Juliette, Secombes, Christopher, J., 2018. Rainbow trout (Oncorhynchus mykiss) adipose tissue undergoes major changes in immune gene expression following

bacterial infection or stimulation with pro-inflammatory molecules. Dev. Comp. Immunol. 81, 83–94. https://doi.org/10.1016/j.dci.2017.11.001.

Villumsen, Kasper Rømer, Koppang, Erling Olaf, Christensen, Dennis, Bojesen, Anders Miki, 2017. Alternatives to mineral oil adjuvants in vaccines against Aeromonas salmonicida subsp. salmonicida in rainbow trout offer reductions in adverse effects. Sci. Rep. 7 (1), 5930. https://doi.org/10.1038/s41598-017-06324-7.

Wilhelm, Vivian, Miquel, Alvaro, Burzio, Luis O., Rosemblatt, Mario, Engel, Esteban, Valenzuela, Sebastián, et al., 2006. A vaccine against the salmonid pathogen Piscirickettsia salmonis based on recombinant proteins. Vaccine 24 (23), 5083–5091. https://doi.org/10.1016/j.vaccine.2006.03.027.

Wu, Liting, Fu, Shengli, Yin, Xiaoxue, Guo, Zheng, Wang, Anli, Ye, Jianmin, 2019a. Long-lived plasma cells secrete high-affinity antibodies responding to a T-dependent immunization in a teleost fish. Front. Immunol. 10, 2324. https://doi.org/10.3389/fimmu.2019.02324.

Wu, Liting, Fu, Shengli, Yin, Xiaoxue, Leng, Wenna, Guo, Zheng, Wang, Anli, Ye, Jianmin, 2019b. Affinity maturation occurs in channel catfish (Ictalurus punctaus) following immunization with a T-cell dependent antigen. Fish Shellfish Immunol. 84, 781–786. https://doi.org/10.1016/j.fsi.2018.10.057.

Yamaguchi, Takuya, Quillet, Edwige, Boudinot, Pierre, Fischer, Uwe, 2019. What could be the mechanisms of immunological memory in fish? Fish Shellfish Immunol. 85, 3–8. https://doi.org/10.1016/j.fsi.2018.01.035.

Ye, Jianmin, Kaattari, Ilsa M., Ma, Cuiyan, Kaattari, Stephen, 2013. The teleost humoral immune response. Fish Shellfish Immunol. 35 (6), 1719–1728. https://doi.org/10.1016/j.fsi.2013.10.015.

Zwollo, Patty, Haines, Ashley, Rosato, Pam, Gumulak-Smith, Juliann, 2008. Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. Dev. Comp. Immunol. 32 (12), 1482–1496. https://doi.org/10.1016/j.dci.2008.06.008.

Paper III

CRISPR-Cas induced IRF3 and MAVS knockouts in a salmonid cell line disrupt PRR signaling and affect viral replication

Authors

Yorick A. van der Wal[1,2], Henriette Nordli[2], Allan Akandwanaho[1], Linn Greiner-Tollersrud[2], Jaap Kool[1], Jorunn B. Jørgensen[2]

1 Vaxxinova Research & Development GmbH, Münster, Germany

2 Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, Tromsø, Norway

Author contributions

YvW, JK, and JJ conceived and designed research. YvW, AA, HN, and LGT performed research and analyzed data. AA helped design experiments. YvW and JJ wrote the paper. All authors reviewed and approved the manuscript.

Corresponding author:

Jorunn B. Jørgensen, Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, N-9037 Tromsø, Norway. Phone: +47 776 46 716, Email: jorunn.jorgensen@uit.no

Declarations of interest:

YvW, AA, and JK are employees of the company Vaxxinova Research & Development GmbH which amongst others develops and commercializes vaccines for aquaculture. Most of the research was funded by Vaxxinova Research & Development GmbH. Vaxxinova had an advisory role in the study design and final report.

Abstract

Interferon (IFN) responses are very important in the resolution of viral infections and are actively targeted by many viruses. They also play a role in inducing protective responses after vaccination and have been successfully tested as vaccine adjuvants. IFN responses are well conserved and function very similar in teleosts and mammals. Teleost fish have several IFNs, although these are not orthologues of the mammalian IFNs. Since IFN responses are initiated through pattern recognition receptor signaling and this signaling is only not thoroughly investigated in salmonids, we studied the effect of knockouts (KOs) on central IFN signaling components in these pathways in the salmonid cell line CHSE-214. We successfully generated KO clones for IRF3, IRF7, and MAVS, as well as a double KO for IRF7/3 using

an optimized protocol for delivery of CRISPR-Cas ribonucleoproteins through nucleofection. As far as we know, this is the first investigation of the effect of knocking out these genes in teleosts. We found that IRF3 and MAVS KOs inhibited IFN and IFN-stimulated gene induction after intracellular poly I:C stimulation as determined through gene expression and promoter activation assays. In contrast, the IRF7 KO did not have a clear effect. This shows that IRF3 and MAVS are essential for initiation of intracellular RNA-induced IFN responses in CHSE-214 cells. Salmon alphavirus 3 infection in control and IRF7 KO cells yielded similar titers and no cytopathic effect, while IRF3 and MAVS KOs presented with severe cytopathic effect and increased titers 6 days after salmon alphavirus 3 infection. In contrast, infectious pancreatic necrosis virus yields were reduced in IRF3 and MAVS KOs, suggesting a dependency on interactions between viral proteins and pattern recognition receptor signaling components during viral replication. Aside from more insight in this signaling in salmonids, our results indicate a possible method to increase viral titers in salmonid cells.

Key words

Salmon alphavirus, CHSE-214, CRISPR-Cas, IFN responses, PRR signaling, MAVS, IRF

Abbreviations

CARD	Caspase activation recruitment domain	CPE	Cytopathic effect
Dpi	Days post infection	FBS	Fetal bovine serum
HMW	High molecular weight	IFN	Interferon
IPNV	Infectious necrotic pancreas virus	IRF	Interferon regulatory factor
ISG	Interferon stimulated gene	KO	Knock out
LMW	Low molecular weight	MAVS	Mitochondrial antiviral-signaling protein
MEM	Minimum essential media	MOI	Mode of infection
NC	Negative control cells: single cell clone 11	NCBI	National Center for Biotechnology Information
NLR	NOD-like receptors	PRR	Pattern recognition receptor
RLR	RIG-I-like receptors	RLU	Relative light units
RNP	Ribonucleoprotein	SAV	Salmon alphavirus
Sc	Single cell clone	TCID	Tissue culture infectious dose
TLR	Toll-like receptor	Wt	Wild type

1 Introduction

The Atlantic salmon aquaculture industry in Norway has grown extensively over the last decades, but emerging and recurring diseases are still a problem. Many of these diseases are caused by viruses and lack effective vaccines (Adams 2019). The interferon (IFN) induced anti-viral state of host cells is a crucial component of successful protection against viral infection. For salmonid cells, antiviral responses in cell lines have shown a clear influence on viral replication (Dehler et al. 2019; Berg et al. 2009; Ooi et al. 2008; Sun et al. 2011; Robertsen et al. 2003; Xu et al. 2010). These cellular antiviral responses can be induced rapidly after activation of pathogen pattern receptors (PRR). Different PRRs recognize different pathogens or danger associated molecular patterns, in case of viral infection, these are often RNA molecules. Binding of their ligand leads to the activation of the PRR, and the activation of a signaling pathway cumulates mainly in the production of IFNs (Abbas et al. 2020). IFNs are cytokines that bind and activate extracellular IFN-receptors on other cells or, as observed in rainbow trout, intracellular IFN-receptors in the same cell (Chang et al. 2013). Finally, the IFN signaling pathway leads to the expression of interferon stimulated genes (ISGs), most of which have anti-viral functions (Robertsen 2018).

The PRRs consist of different families, such as toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) while additional members have been described in recent years (Chen et al. 2017). The first and most extensively investigated PRRs are the TLRs. The number of TLRs identified in species varies quite a lot within vertebrates, with 13 TLRs described in mammals and 28 functional TLRs in teleosts (Liao and Su 2021; Khan et al. 2019). TLRs can be located on the cell membrane or in endosomal compartments and can recognize a wide range of molecules, such as: LPS, flagellin, single stranded (ss)RNA, double stranded (ds)RNA, and CpG DNA (Liao and Su 2021). TLR signaling can occur through interrelated pathways, that usually include the adaptors MyD88 and/or TRIF, and leads to activation of one or more transcription factors, most notably interferon regulatory factor (IRF) 3, IRF7, and NFκB and finally IFN expression (Liao and Su 2021).

The RLR family consists of three cytosolic receptors: RIG-I, MDA5, and LGP2. These receptors recognize ssRNA or dsRNA. RIG-I and MDA5 ligand binding leads to activation of mitochondrial antiviral-signaling protein (MAVS) after interactions through caspase activation recruitment domains (CARD) on the RLRs and MAVS (Liao and Su 2021), while LGP2 is suggested to have a function regulating the other RLRs. MAVS (also named CARDIF, IPS1, or VISA) contains a transmembrane domain that anchors it to the mitochondrial membrane, which is necessary for its function (Seth et al. 2005; Lauksund et al. 2009). The signaling pathway downstream of MAVS activates similar transcriptions factors as the TLR pathway, followed by IFN transcription and induction of an anti-viral state through ISG expression (Robertsen 2018).

Since these innate anti-viral responses are crucial for protection, it is no surprise that viruses have evolved ways to evade these responses (Dahle and Jørgensen 2019). The naked dsRNA infectious pancreatic necrosis virus (IPNV) is a salmonid virus that very potently inhibits the IFN response. IPNV infection in vitro does not induce IFN expression in certain cell types (Robertsen 2018; Collet et al. 2007), and several IPNV proteins have been shown to inhibit IFNa1 expression (Lauksund et al. 2015). Salmonid alphavirus (SAV), an enveloped ssRNA virus, is another highly pathogenic salmonid virus but it strongly induces IFN responses in infected cell lines, in contrast to IPNV (Skotheim; Gahlawat et al. 2009; Munir et al. 2020; Bela-Ong et al. 2020). An investigation into the role of key components of the PRR signaling that leads to IFN expression can help to gain more insight in these host-pathogen interactions. A knockout of these key components in cell lines through gene editing can shed light on their roles.

In recent years, gene editing has been hugely facilitated through revolutionary advances surrounding CRISPR-Cas. Originally discovered as an innate immune system in bacteria, CRISPR-Cas was soon developed into a cost-effective and fast way to introduce specific and targeted gene edits (Le Cong et al. 2013; Hsu et al. 2014). Although most protocols and reagents have been developed for use in mammalian systems, CRISPR-Cas gene edits have been performed in salmonids after injection in embryos (Edvardsen et al. 2014), transfection of plasmids or ribonuclear proteins (RNPs) in cell lines (Dehler et al. 2016; Gratacap et al. 2020a), or lentiviral delivery (Gratacap et al. 2020b).

We investigated the effect of PRR signaling on ISG expression and pathogen growth in CHSE-214 cells by knocking out the transcription factors IRF3 and IRF7 and the RLR signaling molecule MAVS. To this end, we developed a protocol for efficient CRISPR-Cas editing in CHSE-214 cells using RNP nucleofection and generated four knock out (KO) cell lines: IRF3, IRF7, MAVS, and a IRF7/3 double KO. Since the CHSE-214 cell line seems to have limited TLR activity (Monjo et al. 2017), these KOs would mainly affect RLR signaling. We evaluated the effect of the KOs on PRR signaling and viral growth through titration of virus, expression analysis of ISGs and IFNs, and promoter reporter assays to investigate activation of ISG promoters. Our results demonstrated that IRF3 and MAVS are essential for induction of IFN type I production in CHSE-214 cells, while the IRF7 KO did not affect IFN induction. The inhibition of IFN type I responses resulted in increased SAV3 titers, while IPNV titers were reduced. Those KO cell lines that showed an increased SAV3 replication could be useful for virus production in the industry or for research.

2 Materials and methods

2.1 Culture of cells and pathogens

Chinook salmon embryo cells (CHSE-214) (kindly provided by Bjørn Krossøy, Vaxxinova Norway AS) were grown in growth medium (L15 (PanBiotech) with 1% L-glutamine and 8% FBS (fetal bovine

serum, HyClone)) at 20°C and passaged weekly at 2.5 x 10^6 cells per 75 cm^2 flask. CHSE-214 cells were single cell cloned through limited dilution by plating 4 cells per well in 96-well plates. Single colonies were transferred and expanded. Single cell clone (Sc) 11 was used for transfections and included as negative control (NC) in later experiments.

IPNV (supplied by Vaxxinova Norway AS) was propagated on CHSE-214 cell culture at 18°. The cells were grown to about 80% confluence prior to infection, and IPNV was harvested at extensive cytopathic effect (CPE) after 2 days. The infected cell layer was freeze-thawed once before centrifugation at 5000xg for 10 minutes to remove debris. The remaining supernatant was titrated by end-point titration, calculated by the 50% tissue culture infective dose ($TCID_{50}$) method (REED and MUENCH 1938), and frozen in 1 mL aliquots at -80°C until use in infection experiments.

SAV3 (provided by Øystein Evensen, Norwegian University of Life Sciences) was propagated on CHH-1 cell culture as described by (Jenberie et al. 2020). The supernatant was titrated on CHH-1 cells and frozen in 1 mL aliquots at -80°C until use in infection experiments.

2.2 CRISPR-Cas editing

Bioinformatics

We used genomic data from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) to design sequencing primers on highly conserved regions between both species to sequence parts of the *irf3*, *irf7*, and *mavs* genes in CHSE-214 cells. These sequences were blasted (National Center for Biotechnology Information (NCBI)) against the Chinook salmon (*Oncorhynchus tshawytscha*) genome to verify the genes and obtain the gene IDs: *irf3* (112235560), *irf7* (112252506), and *mavs* (112236223).

We designed sgRNAs using either the Benching Guide RNA design tool or the Geneious CRISPR gRNA Design Software. sgRNAs were designed in batches of three with high predicted efficiency, low off-target effects, and homology with Atlantic salmon as criteria. Synthego produced the modified sgRNA (2'-O-Methyl at first 3 and last 3 bases and phosphorothioate bonds between the first 3 and last 2 bases). We investigated possible duplicate genes in chinook salmon by blasting (megaBLAST) the coding sequences of the targeted genes against the NCBI nucleotide collection for chinook salmon (assembly: Otsh_v2.0). Alignment and generation of phylogenetic trees was performed using Clustal Omega. The NCBI conserved domain search and THTMM tools predicted conserved and transmembrane domains respectively.

Nucleofection of RNPs

The CHSE-214 wildtype single cell clone 11 (NC) was nucleofected with CRISPR RNPs for genome editing using the 4D Nucleofector (Lonza). NC cells were passaged one day before nucleofection and

seeded at 4 x 10^6 cells per 75 cm^2 flask. RNP solution was prepared by mixing 1 µg sgRNA and 2 µg recombinant Cas9 (EnGen® Spy Cas9 NLS, New England Biolabs) with nucleofector solution SE (Lonza) to a final volume of 10 µL, followed by 10 minutes incubation at room temperature for complexing. 4 x 10^5 NC cells were trypsinized, centrifuged at 300xg for 10 minutes, resuspended in 10 µL nucleofector solution SE, mixed with the RNP solution, and added to a well in a 16-well Nucleocuvette strip. After nucleofection with program DS-137, the sample was incubated with 80 µL OptiMEM (Gibco) for 10 minutes at room temperature and seeded in a 12-well plate well in growth medium. Transfection controls with pmaxGFP (Lonza) were evaluated after 2 days incubation at 20°C.

Editing efficiency and KO determination

Samples from transfected cells were lysed in QuickExtract DNA extraction solution (LGC Biosearch) according to manufacturer's instructions, and purified PCR products of the target region were sequenced (Microsynth Seqlab). Sequencing chromatograms with superimposed peaks were analyzed using the online tool TIDE (Brinkman et al. 2014) for editing efficiency and indels present. Transfected pools with highest editing efficiency per target gene were used for single cell cloning and the single cell clones were evaluated by sequencing as described above. Single cell clones with frameshift mutations in both targeted alleles were sampled and re-sequenced twice to verify the mutations. We used Geneious prime to check whether the mutations would result in premature stop codons in the ORF and evaluated whether this disruption would lead to a knockout of the targeted gene.

2.3 Poly I:C transfection and qPCR

NC and KO cells were seeded in 24 well plates with 250.000 cells/well in 1 mL growth medium with crosswise movement to spread the cells equally in the wells. One day later, cells were transfected with high molecular weight (HMW) poly I:C by adding 100 µL minimum essential media (MEM, Gibco), 1,2 µL poly I:C (1 mg/mL stock, Invivogen), and 3 µL TransIT (Mirus) per well. RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer's instructions one and two days after poly I:C transfection. Subsequently, cDNA was synthesized using the QuantiTect RT kit (Qiagen) according to manufacturer's instructions with 500 ng RNA per 20 µL reaction. cDNA was diluted 1:5 for use in qPCR reactions containing 6 µL cDNA, 7,5 µL 2× Fast SYBR® Green Master Mix (Applied Biosystems), and 0,8 µL of each primer (5 µM stock). Taqman PCR reactions consisted of 5 µL cDNA, 7,5 µL 2x TaqMan universal master mix (Applied Biosystems), 0,18 µL of each primer (100 µM), 0,05 µL probe (100 µM, 6FAM-BHQ1), and 2,09 µL water. Table 1 lists all primers used. The qPCR reactions were performed in 384-well plates under the following conditions: 95 °C for 5 minutes and 45 cycles of 95 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds (QuantStudio 6, Applied Biosystems). A melt curve stage was included to confirm the absence of nonspecific products in SYBR Green PCR reactions, primers and their references are presented in table 1, and the efficiencies of tested

primer pairs were between 90 and 110%. Relative expression was calculated using the delta Ct method with Elf2a as a reference gene (Schmittgen and Livak 2008).

Table 1. List of primers used in this study with references to original publications of the primers.

Target	FW/RV	Sequence	Published in: *
IRF3	sgRNA	TTCTAGGAAGGATTGCTCCG	
IRF7	sgRNA	GCGAACAGATAAATAGTGGC	
MAVS (1)	sgRNA	TGTCAGAAGGTGTAAGGCAA	
MAVS (2)	sgRNA	CTGATGCTCCAACAGCTCCA	
MAVS (3)	sgRNA	TTCCTTCTACCAGCTCTGAG	
IRF3	FW	ACTGGCTGATAGAACAAGTG	
	RV	ATGGGGGTCGTTTGAGTCCTTG	
IRF7	FW	TCCCAGTTTACACAGGCTGTCA	
	RV	GGTGCTTTACCTCCTGTGGGT	
MAVS	FW	ACTGGACACCTAGGATCTCTGT	
	RV	CAGCAACAGGAGAAGGTGCT	
qPCR Target	FW/RV	Sequence	Published in: *
Elf2a	FW	TGCCCCTCCAGGATGTCTAC	(Iliev et al. 2013)
	RV	CACGGCCCACAGGTACTG	
IFNa	FW	AAAACTGTTTGATGGGAATATGAAA	(Monjo et al. 2017)
	RV	CGTTTCAGTCTCCTCTCAGGTT	
IFNc	FW	ATGTATGATGGGCAGTGTGG	(Jenberie et al. 2018)
	RV	CCAGGCGCAGTAACTGAAAT	
AllMx	FW	TGCAACCACAGAGGCTTTGAA	(Robertsen et al. 2019)
	RV	GGCTTGGTCAGGATGCCTAAT	
IFIT5	FW	GCTGGGAAGAAGCTTAAGCAGAT	(Bela-Ong et al. 2020)
	RV	TCAGAGGCCTCGCCAACT	
SAV3 nsP1	FW	CCGGCCCTGAACCAGTT	(Sobhkhez et al. 2017)
	RV	GTAGCCAAGTGGGAGAAAGCT	
Elf2a	FW	TGCCCCTCCAGGATGTCTAC	(Iliev et al. 2013)
	RV	CACGGCCCACAGGTACTG	
	Probe	AAATAGGCGGTATTGG	
IFNa (IFNa1-2)	FW	TGACTGGATCCGACACCACT	
	RV	ATCTCCTCCCATCTGGTCCA	
	Probe	AGCGCAGAATACCTTTCCCT	
IFNc (IFNc1-4)	FW	ATACCGCCAGATTGAAGAGAG	
	RV	CAGTCCTTCTGTCCTGATGAGATA	
	Probe	GGGCAGTGTGGATACCAGTG	
IRF3	FW	CAGGATTCCTGCAGCGATGA	
	RV	GTCGCCTTGAACCCTACCAT	
	Probe	ATTTTCAAGGCGTGGGCTGA	
IRF7	FW	CTCCGAGGACGACCGTAAAA	
	RV	CCTTGTCAGTGGGATGCTCA	
	Probe	TATTCAGGGCATGGGCAGTG	
MAVS	FW	GCTGATGAACTGAGGGCAGA	
	RV	GGTAGCAGCAGGTGAAGGAG	
	Probe	AGCACAACCAGAACAATCCCT	

* If no reference is given, the primers were designed specifically for this investigation.

2.4 Luciferase assay

NC and KO cells were seeded in 96 well plates with 16.000 cells/well in 100 µL growth medium with 8% FBS and incubated for 1 day. Then, the cells were transiently transfected by replacing medium with neat L15 and adding 10 µL transfection mix containing 100 ng promoter reporter (firefly luciferase) construct, 10 ng Renilla luciferase vector (Promega- Madison WI), and 0,3 µL TransIT in MEM per well. Atlantic salmon Mx2, IFIT5, and IFNa1 promoter constructs (Li et al. 2016) were investigated, while pGL3-basic was included as empty vector control. The promoters for Mx2 (Robertsen et al. 2019) and IFIT5 (Bela-Ong et al. 2020) were synthesized as GeneArt String fragments by ThermoFischer and cloned into HindIII-linearized pGL3 Basic using the Infusion HD cloning kit (Takara). One day after transfection, medium was replaced with 100 µL neat L15 again, and transfection medium (10 µL MEM with 0,3 µL TransIT and 200 ng stimulant) for HMW poly I:C or low molecular weight (LMW) poly I:C was added. The medium was replaced with growth medium (8% FBS) 5 hours post transfection. All samples for the luciferase assays were set up in quadruplicates and the constitutively expressing Renilla luciferase construct provided an internal control value to which the expression of the experimental firefly luciferase was normalized. Two days after transfection with stimulants, luciferase production was measured using the Dual-Luciferase Reporter Assay System (Promega, Madsion, WI) according to manufacturer's instructions. The results are presented as fold-change in relative light units (RLU) by dividing the RLU of the stimulated samples by the average RLU of the corresponding non-stimulated samples.

2.5 Infections and CPE

SAV3

NC and KO cells were seeded in 24 well plates with 200.000 cells/well in 1 mL growth medium one day before infection. For each cell line, the number of cells per well was counted to calculate the amount of virus to be added to achieve the planned multiplicity of infection (MOI), and growth medium was replaced with 1 mL infection medium with an MOI of 1 before incubation at 15°C. Supernatant for viral RNA qPCR and titration was sampled at 2- and 6-days post infection. At 6 days post infection (dpi), pictures were taken from selected wells to compare CPE. Cells were lysed for RNA extraction and expression analysis as described in 2.3 at 2 and 6 dpi.

IPNV

KO cells were seeded in 24 well plates with 125.000 cells/well in 1 mL infection medium (L15 with 1% L-glutamine and 2% FBS) one day before infection. For each cell line, the number of cells per well was counted to calculate the amount of virus to be added to achieve an MOI of 0.01, and IPNV was added to the wells before incubation at 18°C. At 2 dpi, supernatant for titration was sampled, pictures were

taken from selected wells, and the cell layer was sampled. Cell layers were either fixed with 4% formaldehyde for crystal violet staining or lysed for RNA extraction and qPCR as described in 2.3. Expression analysis was performed at one and two days after infection on infected cells that were originally seeded at 250.000 cells/well. Formaldehyde-fixed cells were washed with phosphate buffered saline (PBS) and stained with 1% crystal violet in PBS to quantify CPE. After 10 minutes incubation at room temperature, the wells were carefully washed three times with H_2O and dried. Non-specific staining was removed from well walls, and the remaining crystal violet eluted by shaking for 5 minutes with 200 µL elution buffer (50% ethanol with 0.05 M sodium citrate and 0.05 M citric acid). The OD_{590} was determined using a Sunrise absorbance reader (Tecan).

2.6 Titrations

IPNV supernatant samples were titrated by end-point titration on CHSE-214 cells with 8 wells per dilution and CPE was scored after 14 days. SAV3 supernatant samples were titrated on MAVS KO CHSE-214 cells (described in section 3.1) with 8 wells per dilution. These cells showed clear CPE after SAV3 infection, and CPE was used to score the titration 14 dpi after we determined that this scoring method was as reliable as staining with anti-SAV antibodies according to Strandskog et al. 2011 (unpublished results). $TCID_{50}$/mL was calculated following the method of Reed and Muench (REED and MUENCH 1938).

2.7 Viral RNA – cDNA and qPCR

The viral RNA from SAV3 infected NC and KO cell supernatants was isolated using the QIAamp Viral RNA mini kit (Qiagen) according to manufacturer's instructions, with the exception that no carrier RNA was used. Subsequently, the QuantiTect RT kit (Qiagen) was used for cDNA synthesis according to manufacturer's instructions with 12 µL isolated RNA per reaction. qPCR reactions contained 6 µL cDNA (1:5 diluted), 7,5 µL 2× Fast SYBR® Green Master Mix (Applied Biosystems), and 0,8 µL of each primer (5 µM stock). The following conditions were used for the amplification: 95 °C for 5 minutes and 45 cycles of 95 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds (7500 Fast Real-Time PCR System, Applied Biosystems). Melting curves were used to confirm the absence of nonspecific products, and the used primers have previously been tested (see table 1 for references). A dilution series of an amplicon with known concentration was included to generate a standard curve for calculation of DNA copies per sample.

2.8 Statistics

We performed statistical tests in GraphPad Prism version 8.4.1. Outliers were removed using the ROUT test with Q=1%. Welch's ANOVA with Dunnett's T3 multiple comparisons test (α=0.05) was performed to find significant differences between the NC and the KOs. The data for IPNV titrations were not normally distributed, and an ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons test

was used instead. One-sample *t*-tests against the theoretical mean 1 (no change) were used to evaluate gene induction in poly I:C stimulated NC.

3 Results

3.1 Efficient CRISPR-Cas editing in CHSE-214 cells through RNP nucleofection

Since our gene editing protocol involved isolating and infecting single cell clones from an edited pool, we investigated whether wildtype single cell clones from the CHSE-214 cell line yielded different IPNV titers after infection. Suppl. fig. 1 shows that there is a significant difference in IPNV titers between individual clones and the CHSE-214 pool. To eliminate the risk of observing differences between gene-edited cell lines resulting from variation between the original cells in which the gene-edits are introduced, single cell clone 11 (NC), which had a significantly lower IPNV replication, was used for all subsequent gene editing experiments and included as a NC in later experiments.

Gene editing efficiencies were quite variable between the sgRNAs tested. The final sgRNAs resulted in editing efficiencies of 73%, 23%, and 76% for IRF7, IRF3, and MAVS, respectively, based on decomposition of sequencing chromatograms by the TIDE webtool. We isolated single cell clones from these edited NC pools and picked one clone per gene edit for further analysis. The indels in all selected single cell clones for IRF3 and IRF7 KOs led to premature stop codons within the first 50 amino acids, which is within the DNA binding domain, visualized in suppl. fig. 2. The mutations in the alleles of the MAVS KO led to premature stop codons after 124 and 125 amino acids respectively (suppl. table 1). The CARD domain would be mostly intact in these truncated proteins, but the C terminal transmembrane domain that is also essential for MAVS function in both human (Seth et al. 2005) and Atlantic salmon (Lauksund et al. 2009) is missing. In conclusion, the verified mutations in the presented single cell clones lead to KO of the genes of interest.

A megaBLAST search of the targeted coding sequence revealed no duplicate genes for IRF3 and IRF7 in the chinook salmon genome (assembly Otsh_v2.0) but identified a possible duplicate MAVS gene. This gene (Gene ID: 112237596) has a 67% homology on the RNA level with our targeted gene (47% on protein level) and is not targeted by the used sgRNAs. The putative duplicate MAVS gene contains a conserved death domain, which could indicate a CARD domain, and a N-terminal transmembrane domain and could therefore have a MAVS-like function (see supplementary material for more details).

Off-target analysis with CCTop did not yield any possible off-target effects in other genes with less than 3 mismatches. Furthermore, a BLAST search of the sgRNA sequences on NCBI only returned high identity results in other species or in the targeted genes in chinook salmon. Thus, the chance of off-

target effects seemed to be quite low, also considering the temporary activity of the Cas9 protein due to delivery in RNP format.

3.2 IRF3 and MAVS KOs inhibit induction of ISG expression

To investigate the effect of the KOs on PRR signaling, we evaluated the ability of KO cells to express IFNs and ISGs upon intracellular poly I:C stimulation after 24 and 48 hours (fig. 1). The expression of mx genes and IFIT5, both ISGs with antiviral activity, was successfully induced at both time points after poly I:C transfection in the wildtype (Wt) NC (fig. 1). The IRF7 KO showed a similar induction as the Wt cells at 24 hours, that was reduced at 48 hours for the ISGs, while the IFNa expression was increased. In stark contrast, for both the IRF3 and IRF7/3 KOs this induction was completely abolished at both time points. In the MAVS KO, the induction was reduced, although not as extreme as the IRF3 and IRF7/3 KOs at 48 hours. A similar trend was observed for IFNa, where the expression was induced in NC and the IRF7 KO, (but) abolished in the IRF3 and IRF7/3 KOs, while the MAVS KO showed reduced IFNA transcript levels. In contrast to mx and IFIT5, IFNa induction in IRF7 KO at 48 hours was higher than the NC. We did not observe a significant-induction of IFNc at these time points, although the IRF3, IRF7/3, and MAVS KOs had a slightly lower induction at 48 hours (fig. 1). These results indicated a disruption of PRR signaling in IRF3 and MAVS KO cells.

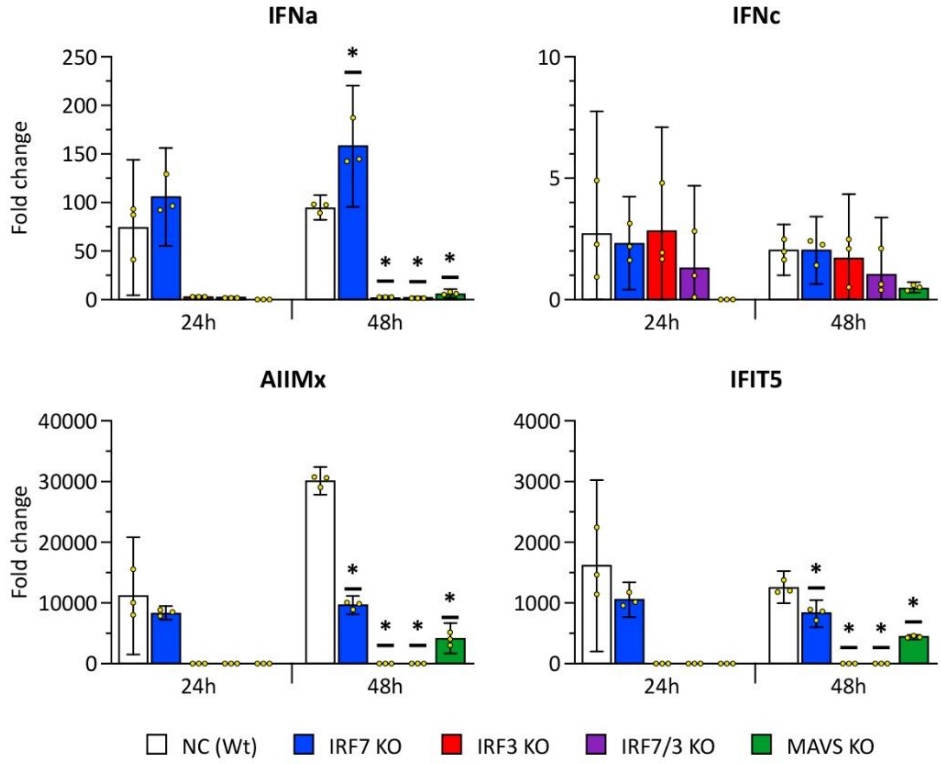


Figure 1: Expression of IFNs and ISGs in IRF7, IRF3, and MAVS KO CHSE-214 cells 24 and 48 hours after HMW poly I:C transfection measured by quantitative PCR. The graphs show the fold change of expression compared to non poly I:C transfected controls and normalized against *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC.

3.3 Reduced ISG promoter activation in IRF3 and MAVS KO cells after PRR stimulation

To confirm the disruption of PRR signaling by IRF3 and MAVS KOs as evidenced by the expression results, we investigated ISG promoter activation upon intracellular poly I:C stimulation of the KO cells (fig. 2). All investigated promoters (IFIT5, Mx2, and IFNa1) showed clear activation in NC and the IRF7 KO 48 hours after both HMW and LMW poly I:C transfection compared to non-stimulated controls (fig. 2). In the IRF3 and IRF7/3 KOs, this activation was almost completely absent, while the MAVS KO results in a reduced activation. Both the empty vector (pGL3-basic) and non-stimulated controls showed very low background activation (suppl. fig. 3A), and a second experiment confirmed the inhibition of activation in the IRF3, IRF7/3, and MAVS KOs (suppl. fig. 3B). Together, these data confirm that the IRF3 and MAVS KOs inhibit IFN responses after intracellular poly I:C stimulation of the cells, while the IRF7 KO does not have a strong inhibitory effect on the IFN response.

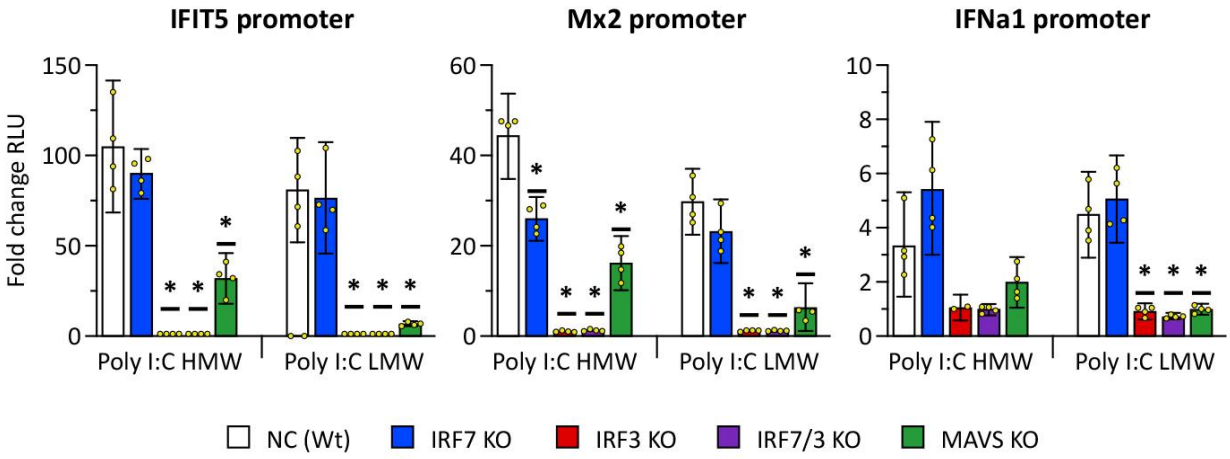


Figure 2: IFIT5, Mx2, and IFNa1 promoter activation in IRF7, IRF3, and MAVS KO CHSE-214 cells 48 hours after HMW or LWM poly I:C transfection. The graphs show the fold change of RLU (normalized against co-transfected Renilla plasmid) compared to non poly I:C transfected controls. Values of the quadruplicates visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC. These data represent one of three repeated experiments which gave reproducible results.

3.4 Increased virus titers and CPE after SAV3 infection of IRF3 and MAVS KO cells

After having investigated the impact on the different KOs on antiviral response assays, we aimed to understand their effects on virus replication. While the IFN response is generally considered as a very broad antiviral system, different classes of viruses have been described to be affected distinctively by various subsets of ISGs (Dahle and Jørgensen 2019). Moreover, viruses have evolved intricate strategies to counteract or repress the effects of the type I IFN system (Dahle and Jørgensen 2019). We therefore tested viral growth for two different salmonid RNA viruses to assess the impact of the different KOs on their growth. The viruses were the enveloped ssRNA virus, SAV3, and the naked dsRNA IPNV. Both

viruses are sensitive to the antiviral effects of type I IFNs, but also possess strategies to counteract/modulate IFN activity (Dahle and Jørgensen 2019)). We first infected the IRF3, IRF7, and MAVS KOs with SAV3 to investigate whether and how the disruption in the PRR signaling pathway would affect virus growth. Some CPE was present in NC at 6 dpi (fig. 3A), which was in line with previous observations that SAV3 infection usually results in minor CPE in CHSE-214 cells. The IRF7 KO cell layer similarly exhibited some CPE (fig. 3B). In contrast, SAV3 infection resulted in massive CPE in the IRF3, IRF7/3, and MAVS KOs, which suggests an increased SAV3 replication (fig. 3C-E). The appearance of clear CPE on IRF3 and MAVS KOs allowed for titration on these cells without staining with antibodies as described in (Strandskog et al. 2011). Visual CPE scoring of titration on MAVS KO cells compared very well with scoring based on antibody staining (data not shown). As a result, we used the MAVS KO cells to determine virus titers.

SAV3 titers in supernatant of the infected IRF7 KO cells were slightly (non-significantly) reduced compared to NC (fig. 3F), but this could be due to the apparent difference in confluence leading to less cells being available for production of viral particles. From three separate experiments, we found a clear increase in viral titers in the IRF3 and MAVS KOs in (fig. 3F). An additional experiment with just these KOs further confirmed the significant increase in SAV3 titers (fig. 3G). Viral RNA in the supernatant as determined by qPCR mimics the trend seen for the titration results: a slight reduction in the IRF7 KO, an increase in the IRF3 and MAVS KOs, and IRF7/3 between IRF3 and IRF7 results (fig. 3H).

Since the IRF3 and MAVS KOs had a clear effect on the expression of ISGs after intracellular poly I:C stimulation, we investigated whether a similar effect could be observed after SAV3 infection. To this end, we measured the expression of the same genes at 2 dpi and 6 dpi (fig. 4). Mx, IFIT5, and IFNa genes were induced in NC and the IRF7 KO (fig. 4). In contrast, IRF3 and IRF7/3 KOs showed no elevated levels of these genes after infection, and for the MAVS KO induction was reduced compared to wild type (fig. 4). These observations were comparable to the poly I:C stimulation results (fig. 1). The later time point (6 dpi) showed a general slight increase in induction of Mx, IFIT5, and both measured IFNs in NC, the IRF7 KO, and MAVS KO compared to 2 dpi. IFNc was not induced at the early time point, as was seen for poly I:C, but showed upregulation (albeit not significant) in NC, the IRF7 KO, and the MAVS KO at the later time point (6 dpi). This was in contrast to the poly I:C stimulated cells where we did not observe a clear induction (fig. 1). This increase of IFNc after SAV infection was absent in the IRF3 and IRF7/3 KOs, similar to the other investigated genes.

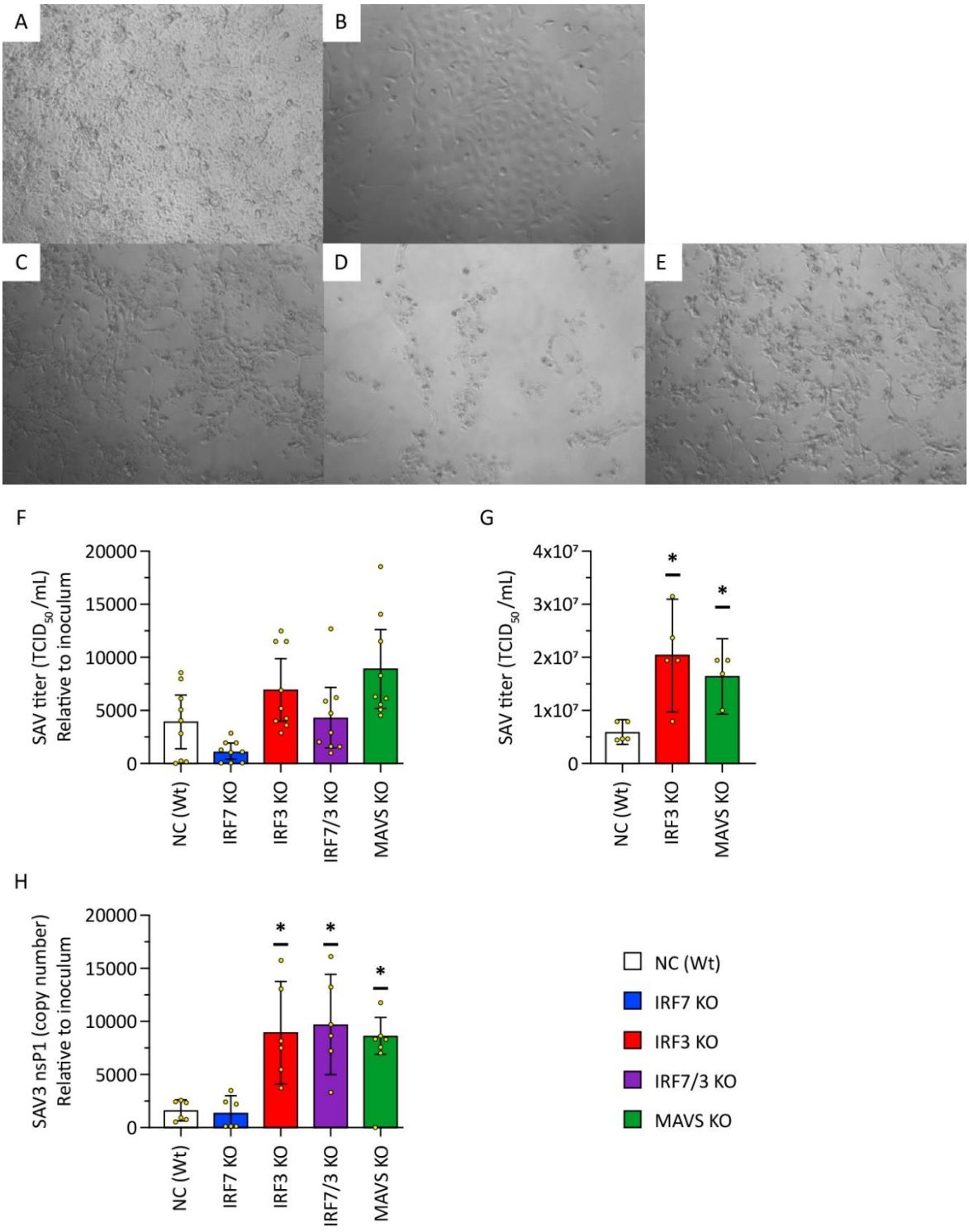


Figure 3: Salmonid alphavirus 3 (SAV3) growth on KO CHSE-214 cells. A-E: representative pictures of CPE on SAV3 infected cells 6 dpi: NC (A), IRF7 KO (B), IRF3 KO (C), IRF7/3 KO (D), and MAVS KO (E). F: SAV3 titers in supernatants of infected KO cells relative to the used inoculum at 6 dpi. G: SAV3 titers in supernatants of infected KO cells with the same inoculum at 6 dpi. H: SAV3 nsp1 transcript levels in supernatant of infected KO cells relative to the used inoculum at 6 dpi. Presented as # DNA copies (SAV3 *snp1*) in 6 µL cDNA from 12 µL RNA isolate divided by the viral titer of the inoculum. Values of triplicates from three (viral titer F), one (viral titer G), or two (viral RNA H) experiments visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC.

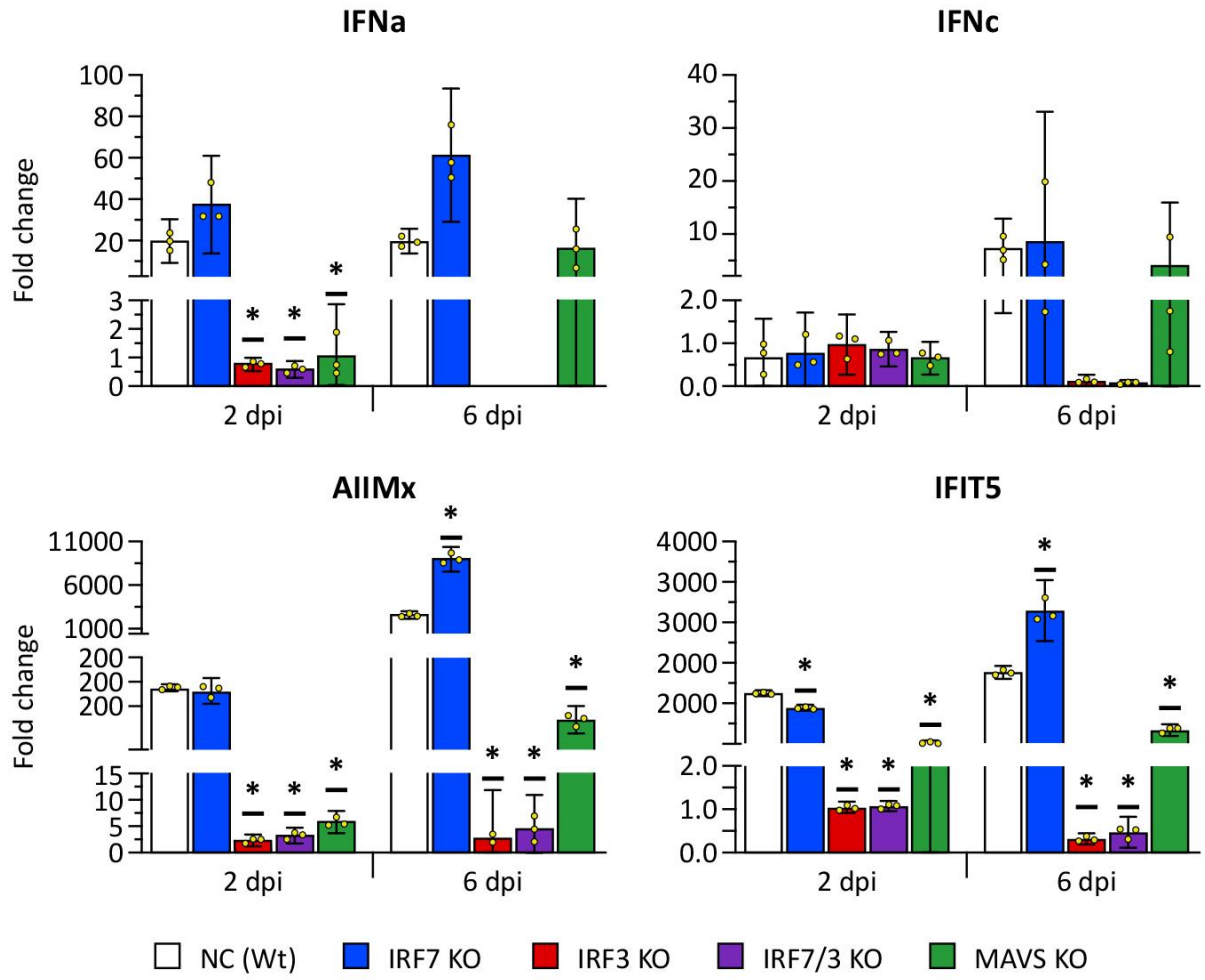


Figure 4: Expression of type I IFN and selected ISGs in IRF7, IRF3, and MAVS KO CHSE-214 cells determined by quantitative PCR, 2 or 6 dpi after Salmonid alphavirus 3 (SAV3) infection. The graphs show the fold change of expression compared to non-infected controls and normalized against *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC.

3.5 Reduced IPNV titers in IRF3 and MAVS KO cells

To investigate whether the increased viral titers was a more common feature on viral replication for IRF3 and MAVS KO cells, we infected the cells with another virus, IPNV. We evaluated IPNV replication on the KO clones by titrating the supernatant harvested at 2 dpi from infected KO cells. Inactivation of IRF7 did not lead to a significant difference, although viral titers were slightly lower (fig. 5). Interestingly, and in contrast to the increase in titers seen for SAV3, IPNV titers were significantly reduced in IRF3, IRF7/3, and MAVS KO cells compared to wild type controls (fig. 5). Determination of CPE through crystal-violet staining showed a significantly reduced CPE for the IRF3 KO, but not for the other clones (suppl. fig. 4). No loss of cells due to CPE was detectable in the MAVS KO cells or the corresponding NC at the time of harvest, so these results were not presented. In summary, disrupting PRR signaling by inactivation of IRF3 and MAVS reduced IPNV replication.

We evaluated ISG and IFN transcript levels in IPNV infected NC and KO cells to investigate the observed differences in effect on viral growth with SAV3. Induction of IFIT5 and Mx was very low after IPNV infection (fig.6) when compared to the induction after SAV3 infection (fig. 4), but seems to be present, nonetheless. Especially after 48 hours, we observed an induction in the wt NC, while this was reduced in the IRF3 and MAVS KOs. The induction of IFIT5 and Mx in IRF7 KO was closer to the NC, as generally observed in our other experiments. IFNa was slightly, though not significantly, induced after 48 hours, which was mainly noticeable due to the apparent reduced induction in the IRF3 and MAVS KOs (fig. 6). Interestingly, IFNc was slightly induced in the NC and IRF7 KO with a smaller induction in the MAVS KO, as seen after SAV3 infection.

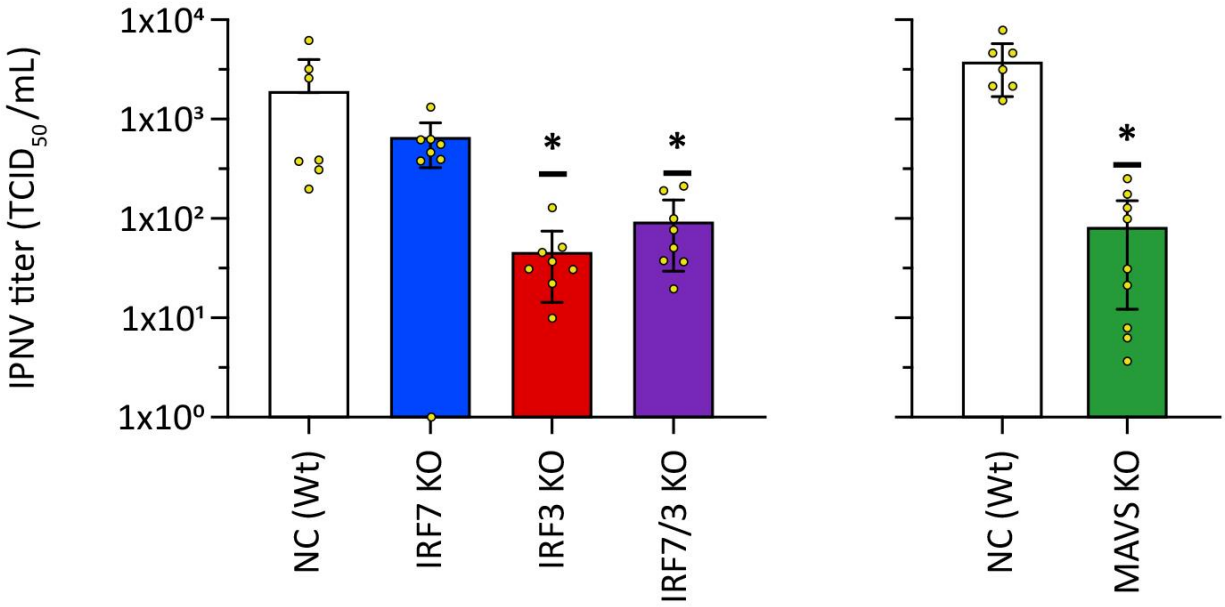


Figure 5: Infectious pancreatic necrosis virus (IPNV) replication in IRF7, IRF3, and MAVS KO CHSE-214 cells. IPNV titers in supernatants of infected KO cells 2 dpi. Values of triplicates from three experiments visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC.

3.6 Expression of IRF3, IRF7, and MAVS in wt cells

To address the observed differences between IRF3 and IRF7 KOs, we examined their transcript levels in non- and poly I:C- stimulated NC. The basal levels of IRF3 and IRF7 mRNAs/transcripts (suppl. fig. 5) were comparable. Both IRF3 and IRF7 were induced after poly I:C stimulation, although IRF3 induction is much higher (fig. 7). MAVS was hardly induced in stimulated NC (fig. 7) but had a higher basal expression than IRF3 and IRF7 (suppl. Fig. 5).

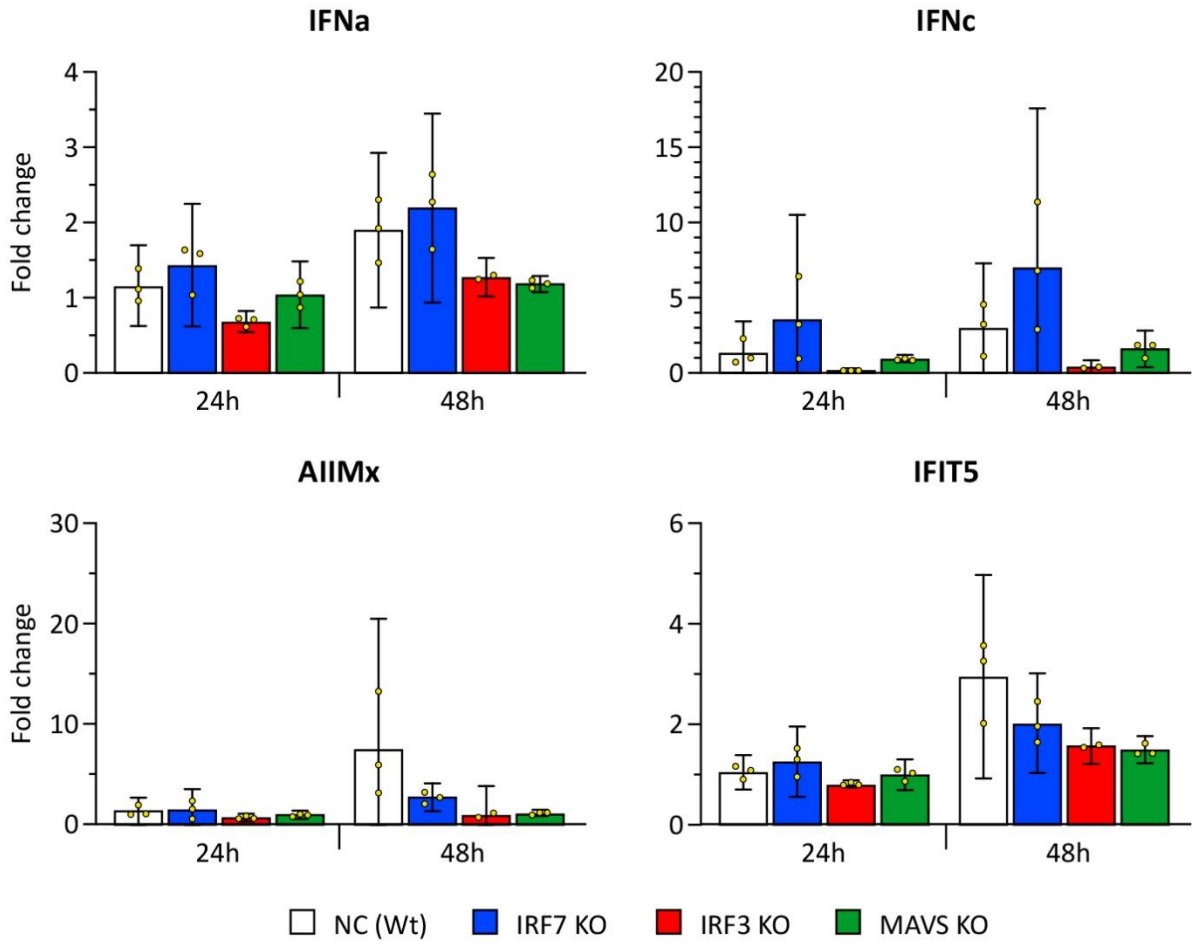


Figure 6: Expression in IRF7, IRF3, and MAVS KO CHSE-214 cells 1 or 2 dpi after *Infectious pancreatic necrosis virus* (IPNV) infection. The graphs show the fold change of expression compared to non-infected controls and normalized against *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval. These data represent one of 2 repeated experiments which gave reproducible results.

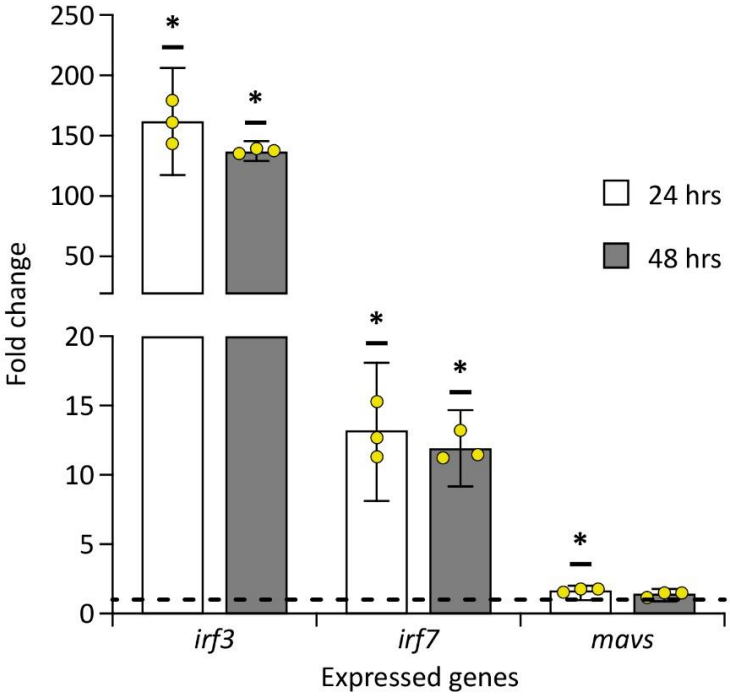


Figure 7: Expression of *irf3*, *irf7*, and *mavs* in NC 24 and 48 hours after HMW poly I:C transfection. The graphs show the fold change of expression compared to non poly I:C transfected controls and normalized against *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant difference (*) from mean 1 (no change, dotted line) based one-sample t-test.

4 Discussion

4.1 Efficient CRISPR-Cas editing in CHSE-214 cells using nucleofection of RNPs

The type I IFN response is the immune system's preferred early weapon against viral infections. It can be triggered in many cell types by detection of viral infections through the activation of different PRRs (Liao and Su 2021). RLRs detect dsRNA in the cytosol and the adapter protein MAVS is essential for their signaling. IRF3 and IRF7 are master transcription factors for the type I IFN response in mammalian species and are also known to be important regulators of IFN-responses in teleosts (Bergan et al. 2010). In this study, we employed a functional genomics approach to elucidate the roles of salmon MAVS, IRF3, and IRF7 in the antiviral responses against viruses in the CHSE-214 cell line. We successfully generated KO clones for IRF3, IRF7, and MAVS, as well as a double KO for IRF7/3. This was possible due to the use of nucleofection to deliver RNPs for CRISPR-Cas editing which enabled us to obtain high editing efficiency. Our results were comparable with the editing efficiencies that (Gratacap et al. 2020a) obtained in salmonid cells using another optimized RNP protocol (slightly over 70%).

Initial testing on IPNV infection of IRF7 edited single cell clones generated from the original CHSE-214 cell line showed significant differences in viral titer between several obtained IRF7 KO clones and between a wt clone and the original CHSE-214 cell line (results not shown). This could have been a result of off-target edits or differences between the single parent cells. However, the chance of all tested clones having off-target edits influencing IPNV replication is probably not that high. We confirmed that different wild-type single cell clones can lead to differences in IPNV replication and used one of these clones to develop KO clones from the same parental clone to reduce any possible effect from different parental cells. This heterogenicity of wildtype cell lines has been confirmed in a mammalian setting, and the use of monoclonal cells for gene editing was found to lead to less variability (Westermann et al. 2022).

4.2 IRF3 and MAVS KO inhibit PRR signaling

The IRF7, IRF3, and MAVS KOs led to different effects on IFN and ISG induction after intracellular poly I:C stimulation: full inhibition in the IRF3 KO clones, reduced inhibition in the MAVS KO clone, and induction that is most comparable to the wt in the IRF7 KO. The results from both the expression and promoter activation experiments indicate that IRF3, and not IRF7, is an essential transcription factor for IFN type I induction in CHSE-214 cells. The clear difference of KO effects between IRF3 and IRF7 is striking, especially since both these transcription factors contribute to PRR signaling and show some synergetic activity in Atlantic salmon (Bergan et al. 2010). Nonetheless, IRF3 was found to be a stronger activator of the IFNa1 promoter in Atlantic salmon cells than IRF7 (Bergan et al. 2010). The most fitting explanation for the fact that the IRF7 KO showed no effect on the signaling pathway, would be that high

basal expression of IRF7 in mammals is largely restricted to immune cells, such as B cells and plasmacytoid dendritic cells (Au et al. 1998). Since CHSE-214 cells are not lymphocyte lineage cells, they would not express IRF7, and a KO of IRF7 thus would have no effect on these cells. Our results on the basal expression of IRF3 and IRF7 (suppl. fig. 5), however, show a comparable expression, which would not be expected according to the mammalian paradigm since CHSE-214 cells are not lymphocyte lineage cells. We did observe higher IRF3 mRNA levels compared to IRF7 after stimulation (fig. 7), which suggests that IRF3 has a more prominent role, in accordance with the KO results. Our results fit in a model where IRF3 is essential in initiating IFN expression, while IRF7 enhances these responses and is more tightly regulated (Sharma et al. 2003; Dalskov et al. 2020).

A less pronounced difference in KO effect was found between IRF3 and MAVS. The results of expression induction and promoter activation for multiple genes after intracellular poly I:C stimulation showed a complete inhibition of induction in the IRF3 KO clones, while the MAVS KO clone at times only led to a partial inhibition. This difference could be due to the PRR pathways these genes have a function in. MAVS is a major component of the RLR pathway but has no major function in other PRR pathways (Chen et al. 2017) . IRF3, in contrast, is involved in signaling of several PRRs (Servant et al. 2002; Liao and Su 2021). Intracellular poly I:C stimulates RLRs, but also certain NLRs and TLRs (Liao and Su 2021). The IRF3 KO would affect all these pathways, while the MAVS KO only affects the RLR pathway. A second explanation for the difference in effect between the IRF3 and MAVS KOs would be the presence of a duplicate MAVS gene in salmonids. We identified a putative duplicate MAVS gene with 67% homology on mRNA level (see suppl. material). Domain predictions and synteny in Atlantic salmon, rainbow trout, and chinook salmon indicate that this gene probably arose from MAVS after a duplication event and could possess MAVS like function. The low level of homology on protein level (47%), however, generates doubts on how much of the original function is kept. If some of the original function is retained in the duplicate gene, this could account for the observed incomplete inhibition of PRR signaling, since the used sgRNAs did not target the duplicate gene. Further investigation could elucidate whether the duplicate MAVS gene has a function and how similar this function is to the original MAVS gene.

Activation of the IFNa1 promoter led to much lower RLU values (suppl. fig. 3) compared to the other used promoter constructs. These values are comparable to the values of an earlier publication that used the same construct (Li et al. 2016). This suggests that the IFNa1 promoter is activated at much lower levels than the ISG promoters from Mx2 and IFIT5 after poly I:C stimulation. IFNs are signaling cytokines whose signal is amplified in receiving cells and that need to be carefully regulated to avoid extreme immune responses (Ivashkiv and Donlin 2014). In contrast, Mx2 and IFIT5 have a direct anti-viral activity for which they need to be expressed at sufficient levels. It is thus logical that the strongly

regulated IFN promoter is less activated as the Mx2 and IFIT5 promoters, which is also in line with our expression results.

The early induction of IFNa, IFIT5, and Mx genes and the missing induction of IFNc after stimulation suggests that IFNa, and not IFNc, is responsible for initial ISG transcription in CHSE-214 cells in response to poly I:C stimulation. Since chinook salmon, like other salmonids, possess multiple IFN genes and our primers amplify mRNA of several genes based on our bioinformatic analyses (unpublished results), additional IFN genes could be involved in the IFN responses initiated by poly I:C transfection. A complete IFN gene expression analysis would be an entire investigation on its own. Still, the late induction of IFNc by SAV3 at 6 dpi and the minor induction after IPNV infection indicates a differential expression pattern of these IFN genes.

4.3 PRR signaling disruption increases SAV3 replication in CHSE-214 cells, while decreasing IPNV replication

Our results clearly show that disrupting PRR signaling positively affects SAV3 replication in CHSE-214 cells, as illustrated by the increased CPE, viral titers, and viral RNA. In addition, inactivation of IRF3 and MAVS abolished the activation of antiviral genes such as IFN, Mx and IFIT5 that we observed in wt cells after SAV3 infection. Still, the effect of IRF3 and MAVS KOs on viral replication seems to be dependent on the combination of virus and cell type. Our results show a different effect of IRF3 and MAVS KOs on the replication of two different viruses, SAV3 and IPNV, on the same cell line. The mammalian literature contains more examples of diverging effects of MAVS KO on viral replication where different viruses or different cell types/tissues have been investigated (Loo et al. 2008; Perry et al. 2009).

One surprising find is that SAV3 replicated equally well in the IRF3 and MAVS KO clones, while the disruptive effect of the IRF3 KO on PRR signaling was more pronounced. It is possible that after IFN responses have been reduced below a critical level, the viral replication is not affected by any further reduction. This would mean that SAV3 already replicates at peak efficiency after partial inhibition of IFN responses and that complete inhibition is not necessary for elevated SAV3 replication. Finally, a difference in replication kinetics between the KOs could result in an over- or underestimation of the titers during a comparison at one time point. We showed a difference in dynamics, but there might still be a comparable final titer if later time points would be analyzed.

The fact that inactivation of PRR signaling did not increase IPNV titers fits well with a model wherein IPNV can inhibit PRR signaling in vitro. This model is based on several observations. Although IFN responses were found to be induced by IPNV in tissues of infected Atlantic salmon (Skjesol et al. 2011), primary macrophages (Collet et al. 2007), and RTG-2 cells (Sena and Rio 1975), it is usually not induced after IPNV infection in the cell lines CHSE-214, TO, and SHK-1 (Robertsen 2018; Lauksund et al.

2015; Reyes-Cerpa et al. 2012; Skotheim). It is interesting that we observed a very minor induction of IFNa, Mx, and IFIT5 expression after in vitro IPNV infection, in contrast to these earlier findings. This induction was mainly visible due to comparison with the non-induced IRF3 and MAVS KO clones, which could be why it was not registered in earlier investigations. In addition, it has been demonstrated that several IPNV proteins interact with and inhibit multiple components taking part in IFN and ISG induction with a profound effect on MAVS-mediated activation of the IFNa1 promoter (Lauksund et al. 2015; Dahle and Jørgensen 2019). Overall, the rapid and extensive CPE that IPNV causes on CHSE-214 cells, suggests that antiviral responses do not strongly inhibit the viral infection in these cells or that the viral replication is fast enough to overwhelm the responses. Our findings that KO in PRR signaling does not increase IPNV replication in CHSE-214 cells further strengthen this model. The interaction of IPNV proteins with PRR signaling components could offer an explanation why IPNV replication decreased in the IRF3 and MAVS KOs. During the evolution of IPNV to combat the antiviral responses, the virus could have become partially dependent on these interactions, besides just inhibiting the antiviral responses. This dependency on interactions with host components would not be surprising considering virus-host co-evolution and can explain the reduced viral replication after our KOs of host PRR signaling.

Our results clearly indicate that IRF3 and MAVS are interesting targets to improve SAV3 growth on the CHSE-214 cells. Using the wt NC for gene editing, we managed to increase the production some 2-3 times. Although this is a modest increase, the optimal timing of harvest could be different for the differently edited clones. Finding the ideal timepoint of harvest could increase the obtained SAV3 titers, but higher titers should also be achievable by selecting other Sc from CHSE-214 for gene editing, possibly leading to a new efficient production substrate for SAV3. The clear CPE on IRF3 and MAVS KO CHSE-214 clones also made it possible to use visual scoring to read-out titrations on these cells. Previously, titrations of SAV3 on CHSE-214 cells would be read-out by ELISA after staining with anti-SAV antibodies (Strandskog et al. 2011) which takes more time and requires expensive antibodies. The use of IRF3 or MAVS KO clones for titration of SAV3 samples thus reduces costs for experiments that would otherwise titrate on the CHSE-214 cell line.

5. Conclusion

We have successfully shown that viral replication in CHSE-214 cells is affected by disrupting PRR signaling with CRISPR-Cas induced IRF3 or MAVS KOs. As far as we know, this is the first investigation of the effect of knocking out these genes in teleosts. KO of IRF7 showed no or minor effects on PRR signaling after internal poly I:C stimulation or viral infections. In contrast, KOs of IRF3 completely blocked the induction of type I IFNs and IFN-induced ISGs, demonstrating the vital importance of IRF3 for IFN induction in non-lymphoid salmonid cells. These responses were also reduced in MAVS KO clones, suggesting that RIG-I signaling is essential in CHSE-214 cells. However,

since IFN-induction was not totally abolished in the MAVS KO, another PRR signaling pathway is likely involved in dsRNA mediated signaling in these cells. The effect of PRR signaling disruption was pathogen dependent, with SAV3 replicating better in IRF3 and MAVS KO clones, but IPNV titers being reduced. Future research could focus on the effect of KO of IRF3 and MAVS on the replication of additional viruses, infectious salmon anemia virus for example, and in other cell lines. This research could lead to enhanced substrates to produce salmonid viruses and thus lower costs for research and vaccine production. In addition, the edited cell lines might even support replication of viruses that cannot be cultivated on currently available cell lines. Finally, deeper insight in the PRR pathways affecting different viruses could be used to generate leads for new adjuvants in the form of PRR ligands for viral vaccines.

Acknowledgements

We would like to acknowledge Guro Strandskog for preparing the initial SAV inoculum and prof. Ole Morten Seternes & dr. Maryam Imam for their contribution to establishing the Atlantic salmon Mx2 and IFIT5 promoter constructs. Furthermore, we would like to thank the Vaxxinova Münster team for their support and assistance with cell culture during absence. Finally, we greatly appreciate the graphical support of Rod Wolstenholme from the UiT graphical services.

Publication bibliography

Abbas, Abul K.; Lichtman, Andrew H.; Pillai, Shiv (2020): Basic immunology. Functions and disorders of the immune system. With assistance of David L. Baker, Alexandra Baker. 6th edition. Philadelphia: Elsevier (Medical Textbooks Immunology).

Adams, Alexandra (2019): Progress, challenges and opportunities in fish vaccine development. In *Fish & shellfish immunology* 90, pp. 210–214. DOI: 10.1016/j.fsi.2019.04.066.

Au, W. C.; Moore, P. A.; LaFleur, D. W.; Tombal, B.; Pitha, P. M. (1998): Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. In *The Journal of biological chemistry* 273 (44), pp. 29210–29217. DOI: 10.1074/jbc.273.44.29210.

Bela-Ong, Dennis Berbulla; Greiner-Tollersrud, Linn; van der Andreas Wal, Yorick; Jensen, Ingvill; Seternes, Ole Morten; Jørgensen, Jorunn B. (2020): Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene ifit5 in a teleost fish. In *Developmental and comparative immunology* 111, p. 103746. DOI: 10.1016/j.dci.2020.103746.

Berg, Kristel; Svingerud, Tina; Sun, Baojian; Robertsen, Børre (2009): An antiserum against Atlantic salmon IFNa1 detects IFN and neutralizes antiviral activity produced by poly I:C stimulated cells. In *Developmental and comparative immunology* 33 (4), pp. 638–645. DOI: 10.1016/j.dci.2008.11.005.

Bergan, Veronica; Kileng, Øyvind; Sun, Baojian; Robertsen, Børre (2010): Regulation and function of interferon regulatory factors of Atlantic salmon. In *Molecular immunology* 47 (11-12), pp. 2005–2014. DOI: 10.1016/j.molimm.2010.04.015.

Brinkman, Eva K.; Chen, Tao; Amendola, Mario; van Steensel, Bas (2014): Easy quantitative assessment of genome editing by sequence trace decomposition. In *Nucleic acids research* 42 (22), e168. DOI: 10.1093/nar/gku936.

Chang, Ming-Xian; Zou, Jun; Nie, Pin; Huang, Bei; Yu, Zhanglong; Collet, Bertrand; Secombes, Chris J. (2013): Intracellular interferons in fish: a unique means to combat viral infection. In *PLoS pathogens* 9 (11), e1003736. DOI: 10.1371/journal.ppat.1003736.

Chen, Shan Nan; Zou, Peng Fei; Nie, Pin (2017): Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in fish: current knowledge and future perspectives. In *Immunology* 151 (1), pp. 16–25. DOI: 10.1111/imm.12714.

Collet, Bertrand; Munro, Eann S.; Gahlawat, Suresh; Acosta, Felix; Garcia, Jose; Roemelt, Christina et al. (2007): Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. In *Fish & shellfish immunology* 22 (1-2), pp. 44–56. DOI: 10.1016/j.fsi.2006.03.011.

Dahle, Maria K.; Jørgensen, Jorunn B. (2019): Antiviral defense in salmonids - Mission made possible? In *Fish & shellfish immunology* 87, pp. 421–437. DOI: 10.1016/j.fsi.2019.01.043.

Dalskov, Louise; Narita, Ryo; Andersen, Line L.; Jensen, Nanna; Assil, Sonia; Kristensen, Kennith H. et al. (2020): Characterization of distinct molecular interactions responsible for IRF3 and IRF7 phosphorylation and subsequent dimerization. In *Nucleic acids research* 48 (20), pp. 11421–11433. DOI: 10.1093/nar/gkaa873.

Dehler, Carola E.; Boudinot, Pierre; Martin, Samuel A. M.; Collet, Bertrand (2016): Development of an Efficient Genome Editing Method by CRISPR/Cas9 in a Fish Cell Line. In *Marine biotechnology (New York, N.Y.)* 18 (4), pp. 449–452. DOI: 10.1007/s10126-016-9708-6.

Dehler, Carola E.; Lester, Katherine; Della Pelle, Giulia; Jouneau, Luc; Houel, Armel; Collins, Catherine et al. (2019): Viral Resistance and IFN Signaling in STAT2 Knockout Fish Cells. In *Journal of immunology (Baltimore, Md. : 1950)* 203 (2), pp. 465–475. DOI: 10.4049/jimmunol.1801376.

Edvardsen, Rolf B.; Leininger, Sven; Kleppe, Lene; Skaftnesmo, Kai Ove; Wargelius, Anna (2014): Targeted mutagenesis in Atlantic salmon (Salmo salar L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. In *PloS one* 9 (9), e108622. DOI: 10.1371/journal.pone.0108622.

Gahlawat, Suresh K.; Ellis, Anthony E.; Collet, Bertrand (2009): Expression of interferon and interferon--induced genes in Atlantic salmon Salmo salar cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV. In *Fish & shellfish immunology* 26 (4), pp. 672–675. DOI: 10.1016/j.fsi.2009.02.021.

Gratacap, Remi L.; Jin, Ye Hwa; Mantsopoulou, Marina; Houston, Ross D. (2020a): Efficient Genome Editing in Multiple Salmonid Cell Lines Using Ribonucleoprotein Complexes. In *Marine biotechnology (New York, N.Y.)* 22 (5), pp. 717–724. DOI: 10.1007/s10126-020-09995-y.

Gratacap, Remi L.; Regan, Tim; Dehler, Carola E.; Martin, Samuel A. M.; Boudinot, Pierre; Collet, Bertrand; Houston, Ross D. (2020b): Efficient CRISPR/Cas9 genome editing in a salmonid fish cell line using a lentivirus delivery system. In *BMC biotechnology* 20 (1), p. 35. DOI: 10.1186/s12896-020-00626-x.

Hsu, Patrick D.; Lander, Eric S.; Zhang, Feng (2014): Development and applications of CRISPR-Cas9 for genome engineering. In *Cell* 157 (6), pp. 1262–1278. DOI: 10.1016/j.cell.2014.05.010.

Iliev, Dimitar B.; Thim, Hanna; Lagos, Leidy; Olsen, Randi; Jørgensen, Jorunn B. (2013): Homing of Antigen-Presenting Cells in Head Kidney and Spleen - Salmon Head Kidney Hosts Diverse APC Types. In *Frontiers in immunology* 4, p. 137. DOI: 10.3389/fimmu.2013.00137.

Ivashkiv, Lionel B.; Donlin, Laura T. (2014): Regulation of type I interferon responses. In *Nature reviews. Immunology* 14 (1), pp. 36–49. DOI: 10.1038/nri3581.

Jenberie, Shiferaw; Peñaranda, Ma Michelle D.; Thim, Hanna L.; Styrvold, Morten Bay; Strandskog, Guro; Jørgensen, Jorunn B.; Jensen, Ingvill (2020): Salmonid Alphavirus Subtype 3 Induces Prolonged Local B Cell Responses in Atlantic Salmon (Salmo salar) After Intraperitoneal Infection. In *Frontiers in immunology* 11, p. 1682. DOI: 10.3389/fimmu.2020.01682.

Jenberie, Shiferaw; Thim, Hanna L.; Sunyer, J. Oriol; Skjødt, Karsten; Jensen, Ingvill; Jørgensen, Jorunn B. (2018): Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. In *Scientific reports* 8 (1), p. 3565. DOI: 10.1038/s41598-018-21895-9.

Khan, Imran; Maldonado, Emanuel; Silva, Liliana; Almeida, Daniela; Johnson, Warren E.; O'Brien, Stephen J. et al. (2019): The Vertebrate TLR Supergene Family Evolved Dynamically by Gene Gain/Loss and Positive Selection Revealing a Host–Pathogen Arms Race in Birds. In *Diversity* 11 (8), p. 131. DOI: 10.3390/d11080131.

Lauksund, Silje; Greiner-Tollersrud, Linn; Chang, Chia-Jung; Robertsen, Børre (2015): Infectious pancreatic necrosis virus proteins VP2, VP3, VP4 and VP5 antagonize IFNa1 promoter activation while VP1 induces IFNa1. In *Virus research* 196, pp. 113–121. DOI: 10.1016/j.virusres.2014.11.018.

Lauksund, Silje; Svingerud, Tina; Bergan, Veronica; Robertsen, Børre (2009): Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-kappaB and localizes to mitochondria. In *Developmental and comparative immunology* 33 (11), pp. 1196–1204. DOI: 10.1016/j.dci.2009.06.012.

Le Cong; Ran, F. Ann; Cox, David; Lin, Shuailiang; Barretto, Robert; Habib, Naomi et al. (2013): Multiplex genome engineering using CRISPR/Cas systems. In *Science (New York, N.Y.)* 339 (6121), pp. 819–823. DOI: 10.1126/science.1231143.

Li, Chun; Greiner-Tollersrud, Linn; Robertsen, Børre (2016): Infectious salmon anemia virus segment 7 ORF1 and segment 8 ORF2 proteins inhibit IRF mediated activation of the Atlantic salmon IFNa1 promoter. In *Fish & shellfish immunology* 52, pp. 258–262. DOI: 10.1016/j.fsi.2016.03.038.

Liao, Zhiwei; Su, Jianguo (2021): Progresses on three pattern recognition receptor families (TLRs, RLRs and NLRs) in teleost. In *Developmental and comparative immunology* 122, p. 104131. DOI: 10.1016/j.dci.2021.104131.

Loo, Yueh-Ming; Fornek, Jamie; Crochet, Nanette; Bajwa, Gagan; Perwitasari, Olivia; Martinez-Sobrido, Luis et al. (2008): Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. In *Journal of virology* 82 (1), pp. 335–345. DOI: 10.1128/JVI.01080-07.

Monjo, A. L.; Poynter, S. J.; DeWitte-Orr, S. J. (2017): CHSE-214: A model for studying extracellular dsRNA sensing in vitro. In *Fish & shellfish immunology* 68, pp. 266–271. DOI: 10.1016/j.fsi.2017.07.025.

Munir, Danish; Munro, Eann S.; Secombes, Christopher J.; Dooley, Helen (2020): Atlantic salmon kidney (ASK) cells are an effective model to characterise interferon (IFN) and IFN-induced gene expression following salmonid alphavirus infection. In *Fish & shellfish immunology* 106, pp. 792–795. DOI: 10.1016/j.fsi.2020.08.043.

Ooi, Ei Lin; Verjan, Noel; Hirono, Ikuo; Nochi, Tomonori; Kondo, Hidehiro; Aoki, Takashi et al. (2008): Biological characterisation of a recombinant Atlantic salmon type I interferon synthesized in Escherichia coli. In *Fish & shellfish immunology* 24 (5), pp. 506–513. DOI: 10.1016/j.fsi.2007.10.004.

Perry, Stuart T.; Prestwood, Tyler R.; Lada, Steven M.; Benedict, Chris A.; Shresta, Sujan (2009): Cardif-mediated signaling controls the initial innate response to dengue virus in vivo. In *Journal of virology* 83 (16), pp. 8276–8281. DOI: 10.1128/JVI.00365-09.

REED, L. J.; MUENCH, H. (1938): A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. In *American Journal of Epidemiology* 27 (3), pp. 493–497. DOI: 10.1093/oxfordjournals.aje.a118408.

Reyes-Cerpa, Sebastián; Reyes-López, Felipe E.; Toro-Ascuy, Daniela; Ibañez, Jorge; Maisey, Kevin; Sandino, Ana María; Imarai, Mónica (2012): IPNV modulation of pro and anti-inflammatory cytokine expression in Atlantic salmon might help the establishment of infection and persistence. In *Fish & shellfish immunology* 32 (2), pp. 291–300. DOI: 10.1016/j.fsi.2011.11.018.

Robertsen, Børre (2018): The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. In *Developmental and comparative immunology* 80, pp. 41–52. DOI: 10.1016/j.dci.2017.02.005.

Robertsen, Børre; Bergan, Veronica; Røkenes, Torunn; Larsen, Rannveig; Albuquerque, Artur (2003): Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 23 (10), pp. 601–612. DOI: 10.1089/107999003322485107.

Robertsen, Børre; Greiner-Tollersrud, Linn; Jørgensen, Lars Gaute (2019): Analysis of the Atlantic salmon genome reveals a cluster of Mx genes that respond more strongly to IFN gamma than to type I IFN. In *Developmental and comparative immunology* 90, pp. 80–89. DOI: 10.1016/j.dci.2018.09.004.

Schmittgen, Thomas D.; Livak, Kenneth J. (2008): Analyzing real-time PCR data by the comparative C(T) method. In *Nat Protoc* 3 (6), pp. 1101–1108. DOI: 10.1038/nprot.2008.73.

Sena, J. de; Rio, G. J. (1975): Partial purification and characterization of RTG-2 fish cell interferon. In *Infection and immunity* 11 (4), pp. 815–822. DOI: 10.1128/iai.11.4.815-822.1975.

Servant, Marc J.; Tenoever, Benjamin; Lin, Rongtuan (2002): Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 22 (1), pp. 49–58. DOI: 10.1089/107999002753452656.

Seth, Rashu B.; Sun, Lijun; Ea, Chee-Kwee; Chen, Zhijian J. (2005): Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. In *Cell* 122 (5), pp. 669–682. DOI: 10.1016/j.cell.2005.08.012.

Sharma, Sonia; tenOever, Benjamin R.; Grandvaux, Nathalie; Zhou, Guo-Ping; Lin, Rongtuan; Hiscott, John (2003): Triggering the interferon antiviral response through an IKK-related pathway. In *Science (New York, N.Y.)* 300 (5622), pp. 1148–1151. DOI: 10.1126/science.1081315.

Skjesol, Astrid; Skjæveland, Ingrid; Elnæs, Marianne; Timmerhaus, Gerrit; Fredriksen, Børge N.; Jørgensen, Sven Martin et al. (2011): IPNV with high and low virulence: host immune responses

and viral mutations during infection. In *Virology journal* 8, p. 396. DOI: 10.1186/1743-422X-8-396.

Skotheim, Svenn Amund: Co-infection with Norwegian Salmonid Alphavirus (NSAV) and Infectious Pancreatic Necrosis Virus (IPNV) in Chinook Salmon Embryo Cells (CHSE-214). The University of Bergen. Available online at https://bora.uib.no/bora-xmlui/handle/1956/3564.

Sobhkhez, Mehrdad; Joensen, Lisbeth L.; Tollersrud, Linn Greiner; Strandskog, Guro; Thim, Hanna L.; Jørgensen, Jorunn B. (2017): A conserved inhibitory role of suppressor of cytokine signaling 1 (SOCS1) in salmon antiviral immunity. In *Developmental and comparative immunology* 67, pp. 66–76. DOI: 10.1016/j.dci.2016.11.001.

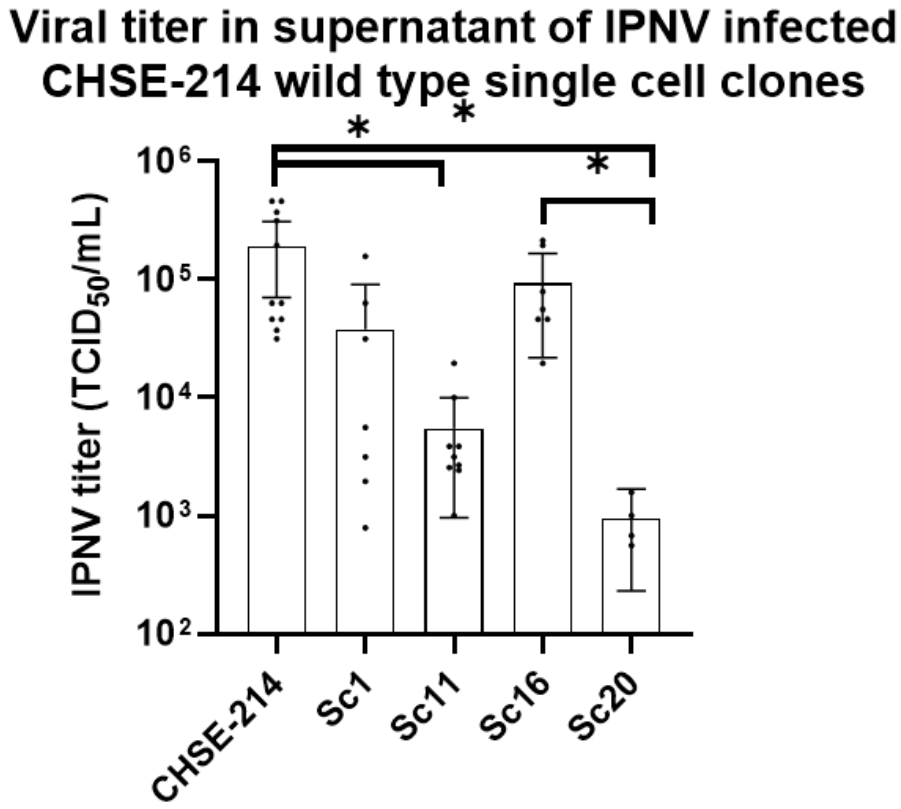
Strandskog, Guro; Villoing, Stéphane; Iliev, Dimitar B.; Thim, Hanna L.; Christie, Karen Elina; Jørgensen, Jorunn B. (2011): Formulations combining CpG containing oliogonucleotides and poly I:C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon. In *Developmental and comparative immunology* 35 (11), pp. 1116–1127. DOI: 10.1016/j.dci.2011.03.016.

Sun, Baojian; Skjæveland, Ingrid; Svingerud, Tina; Zou, Jun; Jørgensen, Jorunn; Robertsen, Børre (2011): Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. In *Journal of virology* 85 (17), pp. 9188–9198. DOI: 10.1128/JVI.00319-11.

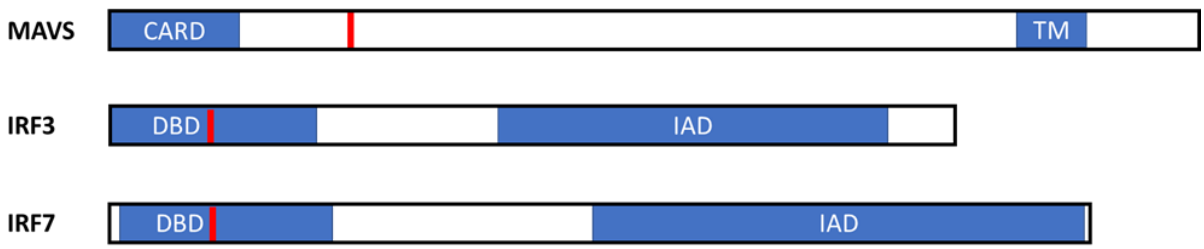
Westermann, Lukas; Li, Yong; Göcmen, Burulca; Niedermoser, Matthias; Rhein, Kilian; Jahn, Johannes et al. (2022): Wildtype heterogeneity contributes to clonal variability in genome edited cells. In *Scientific reports* 12 (1), p. 18211. DOI: 10.1038/s41598-022-22885-8.

Xu, Cheng; Guo, Tz-Chun; Mutoloki, Stephen; Haugland, Øyvind; Marjara, Inderjit S.; Evensen, Øystein (2010): Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro. In *Journal of virology* 84 (17), pp. 8903–8912. DOI: 10.1128/JVI.00851-10.

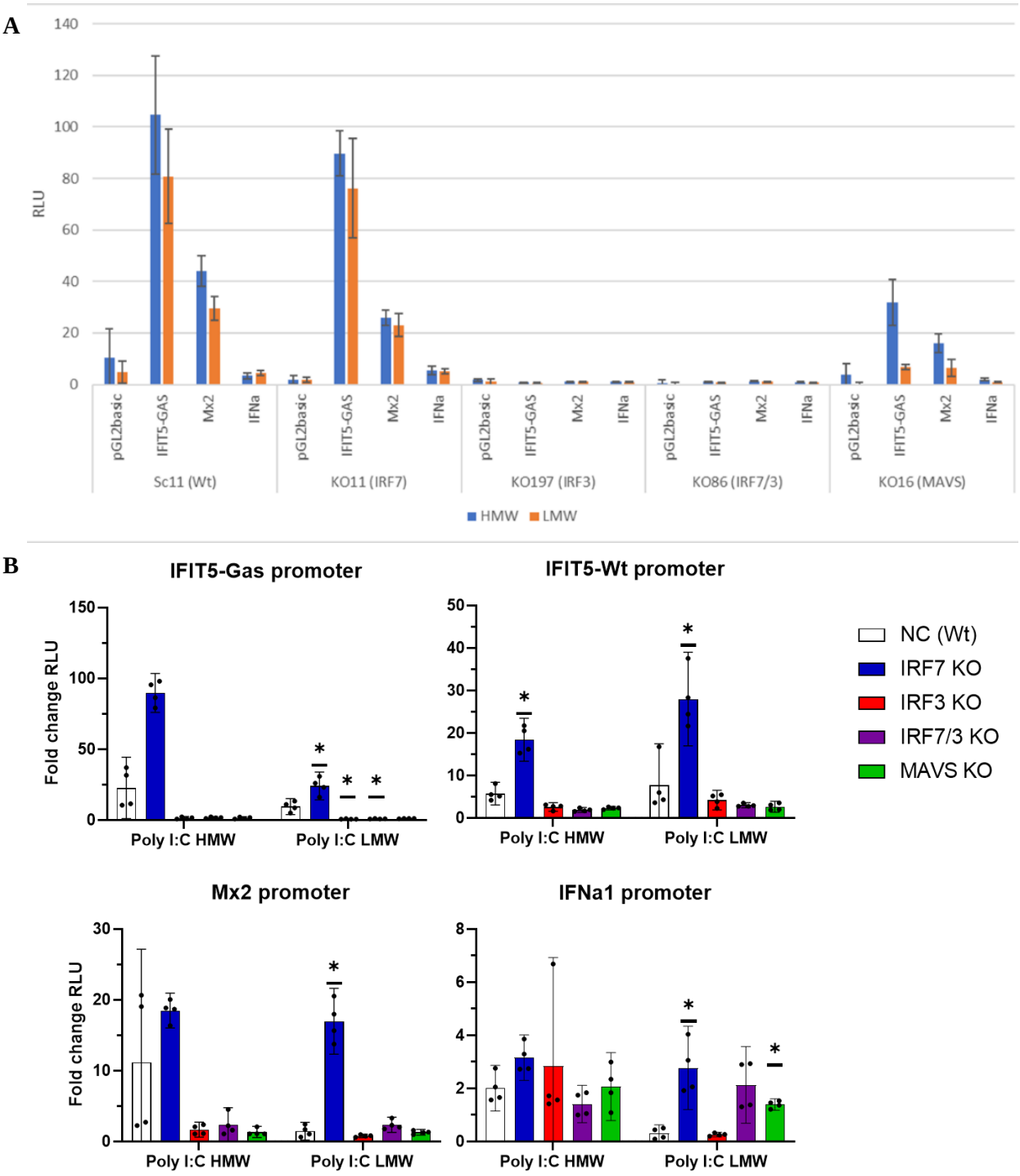
Supplementary material



Supplementary figure 1: IPNV growth on wild type single cell clones of CHSE-214 cells. IPNV titers in supernatants of infected wild type single cell clones 2 days post infection. Values of duplicates or triplicates from two (Sc20), three (Sc1, Sc16), or five (CHSE-214, Sc11) experiments visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant differences between wild type clones and/or original CHSE-214 pool.



Supplementary Figure 2: Schematic locations of domains and introduced mutations in MAVS, IRF3, and IRF7 edited CHSE-214 cells. Blue boxes indicate important functional domains: DNA-binding domain (DBD), caspase activation recruitment domain (CARD), IRF association domain (IAD), and transmembrane domain (TM). The red line indicates the location of the induced mutation in tested single cell clones.

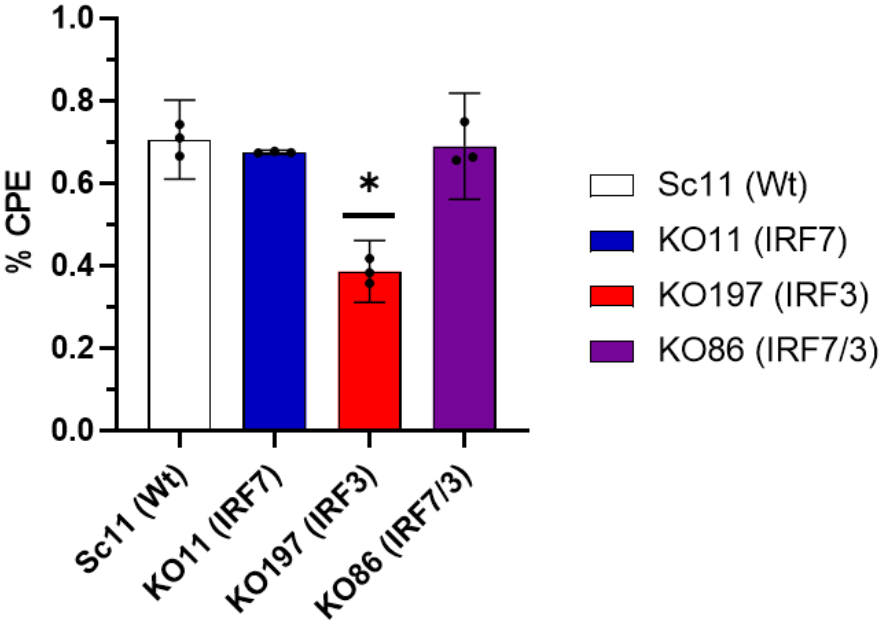


Supplementary figure 3: Promoter activation in IRF7, IRF3, and MAVS KO CHSE-214 cells 48 hours after HMW or LWM poly I:C transfection. A: Alternative representation of data from figure 2. The graph shows the means of the RLU normalized against co-transfected Renilla plasmid for non-stimulated, HMW poly I:C, and LMW poly I:C transfected cells. B: Data from a second promoter activation experiment. The graphs show the fold change of RLU (normalized against co-transfected Renilla plasmid) compared to non poly I:C transfected controls. Values of the quadruplicates visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type NC.

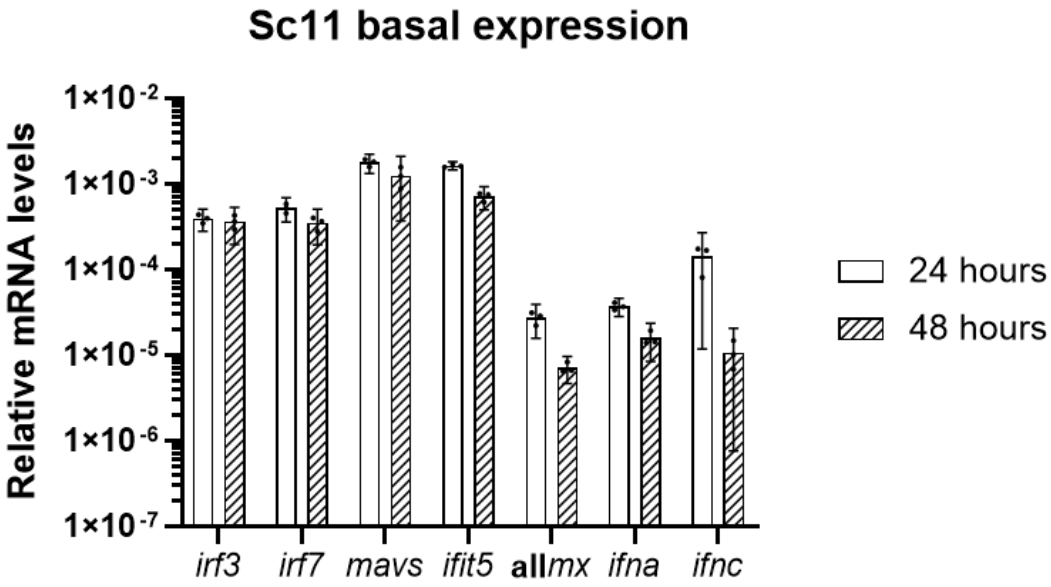
Supplementary table 1: CRISPR/Cas induced mutations and premature stop codons in investigated KO single cell clones.

Single cell clone	Gene/ allele	Indel	Genome sequence at mutation site	Amino acids until (premature) stop codon
Sc11	IRF7	Wt	CACAATTCTAGGAAG GATTGCTCCGAGGA	MQSCKPQFADWLIEQVRTEQYTGLFFIDNNKFRVPWKHNSRKDCSEDDRKIF RAWAVVSGKINEHPTDKAKWKTNFRSALNSLCRRFKMVEDHSKDSNDPHK VYLVINEYNYENPHIEEITLENYGLDCALIPTENTPPGMEHDILNFSNLTLNPL DLNQHTENSIPVHTHHSVPPVLVQQPYYQVNPDALLNLPAAHSSLWDLEITIS YRGSEMRKTQVSGPRVQLHYQCNALEPNTQPLCFPSTDGLPDHKQ
	IRF3	Wt	GCTGCGCGAACAGAT AAATAGTGCCGGT	MSQSKPLLIPWLREQINSGRYPGVTWTNQERTEFCIPWKHALRQDSCSDDVL IFKAWAEVSNGRVQGDHSIWKRNFRSALRAKGFKMLLDNKNDAANPNKLF QWPDEAPTGGSQHPEHDLYQDPSPLQESHGLPCFNDLYLAAEETVYTAEGIS TTINQDILQKCLQGLNIEQTEAIQGYEVPVEELQYPMEGTIGGHVLLGQQQYP VVMEDAVGGAVLPSQPVYPMDGAVGGSHEQQVVEQLTNELSRTMVGENF KTHFRVSVYYRGVKMPEQLVENEAGFRLVYSSVSTRPELTQPLLDPDSGLNL VSLPSPPPVQDETQAKLTQDILALLGEGLEVGASGSIIYGLRKGEIKAFWSLD KFDNSRRPQGVSKCPEPLYQAKDFYG
	MAVS	Wt	sgRNA (2) site: CAGCCCTGGAGCTGT TGGAGCATCAGGAT sgRNA (3) site: CAATTCCTTCTACCA GCTCTGAGGGGTCGC	MSSFTREKLSLHLRRRMGVFVSRVKATELMANLPCLTPSDKEEIQAKKDFSG NYAAMQLLLDYVQKRMNWPEELMSALELLEHQDLADELRAEWNKHNQNN PYPPSPAATTTVRTHVHPIPSTSSEGSPCSLVLPGQPAPPEVAAPPEASLPPEVA PEVLPPPVVAAQPEAPPRSVPKAPMAGSSSKHAPKAAVSPEIASEAAPSPVAA PQAEPQAAPLSPVSVEEPTVISEPPASSQPGSIETVSLEDNLCHSDAPTQMALS ETTPTLSGSHLIPVVSEITPTLPVSHLALSQTESTPTPAALATFQSPERRPVQDT SPHTVKVPTFYQEAVDSDPTQVTEDEQHTEPSQSQHFATAPADTSMNEDDV NFSKPEVLRSEVMDSQPYSGDSTRLQRRMEFLRK*
KO11	IRF7	+1 T	CACAATTCTAGGAAG GATTGCTTCCGAGGA	MQSCKPQFADWLIEQVRTEQYTGLFFIDNNKFRVPWKHNSRKDCFRGRP*
KO197	IRF3 allele 1	-2 GT	GCTGCGCGAACAGAT AAATA--GGCCGGT	MSQSKPLLIPWLREQINRPVSRGYLDQSGANRVLHPLETCFEAGFLQR*
	IRF3 allele 2	+1 T	GCTGCGCGAACAGAT AAATAGTTGGCCGGT	MSQSKPLLIPWLREQINSWPVSRGYLDQSGANRVLHPLETCFEAGFLQR*
KO86	IRF7	+1 T	CACAATTCTAGGAAG GATTGCTTCCGAGGA	MQSCKPQFADWLIEQVRTEQYTGLFFIDNNKFRVPWKHNSRKDCFRGRP*
	IRF3	-2 TG	GCTGCGCGAACAGAT AAATAG--GCCGGT	MSQSKPLLIPWLREQINRPVSRGYLDQSGANRVLHPLETCFEAGFLQR*
KO16	MAVS allele 1	-2 CT	CAATTCCTTCTACCA GCT--GAGGGGTCGC	MSSFTREKLSLHLRRRMGVFVSRVKATELMANLPCLTPSDKEEIQAKKDFSG NYAAMQLLLDYVQKRMNWPEELMSALELLEHQDLADELRAEWNKHNQNN PYPPSPAATTTVRTHVHPIPSTS*
	MAVS allele 2	+1 T	CAATTCCTTCTACCA GCTCTTGAGGGGTCG C	MSSFTREKLSLHLRRRMGVFVSRVKATELMANLPCLTPSDKEEIQAKKDFSG NYAAMQLLLDYVQKRMNWPEELMSALELLEHQDLADELRAEWNKHNQNN PYPPSPAATTTVRTHVHPIPSTSS*

T: insertion, - : deletion, * : premature stopcodon.



Supplementary figure 4: CPE on IPNV infected IRF7 and IRF3 KO CHSE-214 cells 2 days post infection. Crystal violet staining of IPNV infected cell monolayers was measured at OD_{590} as indication of confluence. % CPE was calculated as follows: 1 − OD(infected) / OD(non-infected). Values of single wells from three experiments visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the wild type Sc11.



Supplementary figure 5: Basal expression of *ifit5*, all*mx*, *ifna*, *ifnc*, *irf3*, *irf7*, and *mavs* in wildtype CHSE-214 cells 24 and 48 hours after mock stimulation. The graphs show the mRNA levels relative to *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval.

Supplementary material

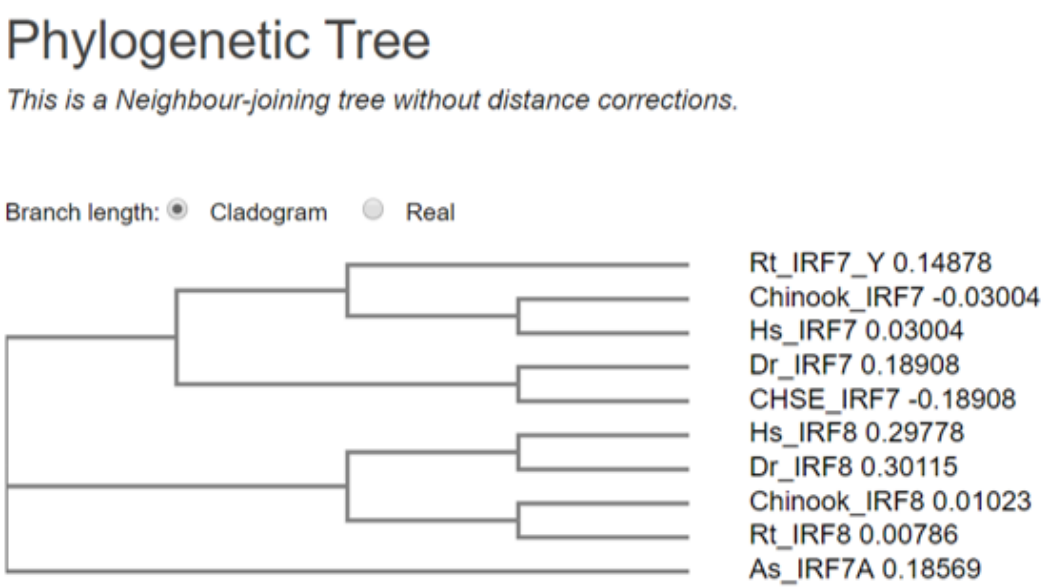
The genes for IRF3, IRF7, and MAVS from rainbow trout and Atlantic salmon were aligned per gene, and sequencing primers were designed in homologous regions. These primers enabled sequencing of the respective genes from the chinook salmon genome as isolated from CHSE-214 cells, and a blast against the chinook salmon genome in ncbi confirmed the genetic locations and sequences.

Table: Locations and predictions of genomic locations

Gene	Sequence	Location	Annotation ncbi	Sequence ID
IRF3	Oncorhynchus tshawytscha isolate Ot180627B linkage group LG24, Otsh_v2.0, whole genome shotgun sequence	5299671 - 5302798	Predicted IRF3	NC_056452.1
IRF7	Oncorhynchus tshawytscha isolate Ot180627B linkage group LG06, Otsh_v2.0	36074493 - 36084407	Predicted IRF7-like	NC_056434.1
MAVS	Oncorhynchus tshawytscha isolate Ot180627B linkage group LG11, Otsh_v2.0	55045023 - 55053498	Predicted Proline-rich protein 36	NC_056439.1

We identified possible duplicate genes by a blast of the coding sequences in the ncbi nucleotide collection. IRF3 only showed homology with IRF3 transcript variants. IRF7 had 80% homology with a predicted mRNA for IRF8, along with being identical to predicted IRF7 mRNA. The IRF8 mRNA was aligned in Clustal omega with IRF7 and IRF8 from *Homo sapiens*, *Danio rerio*, and *Oncorhynchus mykiss* and clustered with IRF8, so is probably the IRF8 mRNA and not a IRF7 duplicate.

Figure: Phylogenetic tree of IRF7 and IRF8 coding sequences



MAVS corresponded to a predicted proline-rich protein 36 mRNA and had 66% homology with the predicted MAVS mRNA. The gene we found while searching for MAVS was also predicted to be PRP36, so we set out to check these predictions. An alignment with the CDSs of *Homo sapiens*, *Mus musculus, Danio rerio*, and *Oncorhynchus mykiss* MAVS and PRP36 showed grouping of both mRNAs with MAVS, with the predicted PRP36 mRNA being closest to the other salmonid MAVS sequences.

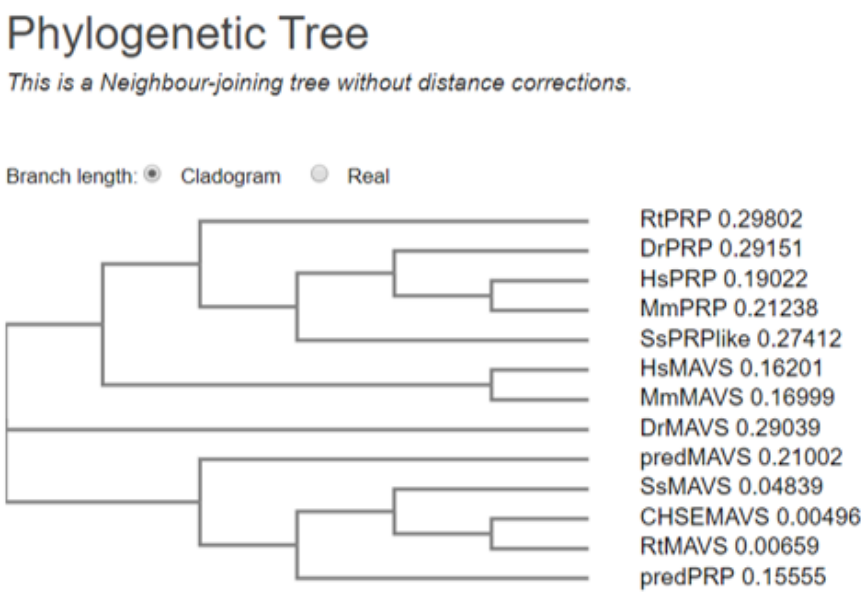


Figure: Phylogenetic tree of MAVS and PRP36 coding sequences

The predicted PRP36 in chinook salmon thus seems to be actually MAVS and will now be called ChMAVSa, while the mRNA predicted to be MAVS could be a duplicate gene and will be called ChMAVSb. These conclusions are corroborated by domain predictions. ChMAVSa has a very clear CARD domain, while in ChMAVSb only the death domain superfamily was recognized. Both proteins have a transmembrane domain at the C terminus.

We blasted both mRNA sequences against the chinook salmon, rainbow trout, and Atlantic salmon ncbi RefSeq Genome Databases to find the corresponding genes and to investigate the gene synteny.

Table: Genomic locations of genes corresponding to ChMAVSa and ChMAVSb

Name used here	Species	Predicted in ncbi	Location	Accession no. ncbi
ChMAVSa				XM_042330395
ChMAVSa	Chinook salmon	proline-rich protein 36	LG11 - end	LOC112236223
RtMAVSa	Rainbow trout	MAVS	Chr. 19 - end	100499614 (mavs)
AsIPS-1	Atlantic salmon	IPS-1 (MAVS)	Chr. 01 - start	LOC100316613
ChMAVSb				XM_042326218
ChMAVSb	Chinook salmon	MAVS	LG08 - start	112237596 (mavs)
RtMAVSb	Rainbow trout	mRNA-nascent polypeptide-associated complex subunit alpha, muscle-specific form	Chr. 25 - end	LOC110504282
AsMAVSb	Atlantic salmon	MAVS-like / mucin-6-like	Chr. 9 - 1/3	LOC106611470

Synteny for ChMAVSa compares very well between all three species, indicating that ChMAVSa is actually MAVS as described for Atlantic salmon (Lauksund 2009, Biacchesi 2009). The synteny of ChMAVSb compares well with the RtMAVSb gene (predicted nascent polypeptide-associated complex subunit alpha, muscle-specific form), and to a lesser extend to the AsMAVSb gene (predicted MAVS-like or mucin-6-like). Interestingly, AsMAVSb is predicted to be MAVS-like, has two genes in the vicinity that are near the actual ChMAVSa, and is on a location of chromosome 9 that was duplicated from the start of chromosome 1, where the AsIPS-1 (MAVS) gene is situated. This all suggests that this gene might be a duplicate of MAVS. The ChMAVSb and AsMAVSb proteins both contain a death domain superfamily domain that indicates a possible CARD domain at the N terminus and a transmembrane domain at the C terminus, suggesting a function similar to MAVS. The RtMAVSb protein lacks these domains, however, and will not have a MAVS-like function.

Figure: Synteny of chinook salmon, rainbow trout, and Atlantic salmon genes corresponding to ChMAVSa and ChMAVSb

MAVS gene/duplicate
Strong synteny in the three species
Synteny in chinook salmon and rainbow trout
Synteny in Atlantic salmon and rainbow trout
Synteny in Atlantic salmon and chinook salmon
Present near Atlantic salmon MAVS duplicate and chinook salmon MAVS gene
Genes with largely conserved gene order

Legend

Paper IV

Piscirickettsia salmonis growth in the salmonid cell line CHSE-214 is affected by IRF7, IRF3, and MAVS knockouts

Authors

Yorick A. van der Wal[1,2], Allan Akandwanaho[1], Henriette Nordli[2], Linn Greiner-Tollersrud[2], Jaap Kool[1], Jorunn B. Jørgensen[2]

1 Vaxxinova Research & Development GmbH, Münster, Germany

2 Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries & Economics, UiT The Arctic University of Norway, Tromsø, Norway

Author contributions

YvW and JJ conceived and designed research. YvW, AA, HN, and LGT performed research and analyzed data. JK helped design experiments. YvW, JK, and JJ wrote the paper. All authors reviewed and approved the manuscript.

Abstract

Host interferon (IFN) responses are important to reduce the effects of viral infections to the host and have been shown to affect intracellular bacteria as well. Still, their effects on infections with the intracellular bacterium *Piscirickettsia salmonis*, a problematic pathogen of salmonids, have barely been studied. We investigated the induction of IFN responses in CHSE-214 cells after *P. salmonis* infection, as well as the effect of knocking-out the IFN signaling molecules interferon regulatory factor (IRF) 3, IRF7, and mitochondrial antiviral signaling protein (MAVS) by gene-editing on *P. salmonis* growth on this cell line. Although *P. salmonis* infection does not induce IFN type I responses in wild type CHSE-214 cells (NC), the IRF3 knock out (KO) resulted in a minor, non-significant, elevation of IFN and interferon stimulated gene expressions. Interestingly, the *P. salmonis* growth was significantly affected by the different KOs. The NC and IRF7 KO, both with intact PRR signaling, yielded higher bacterial titers than the IRF7/3 and MAVS KOs, both of which have disrupted signaling. Surprisingly, the IRF3 KO resulted in increased (though not significantly) bacterial titers. These initial experiments show that IFN responses play a role in *P. salmonis* infections, which could help with vaccine development.

Introduction

Intracellular pathogens remain a major problem for salmon aquaculture around the globe, with vaccines against these pathogens generally having moderate protection at best (Adams 2019). Most intracellular pathogens in salmonids are viruses, but some intracellular bacteria are also known to cause disease in salmonids. A particularly problematic bacterium is *Piscirickettsia salmonis*, the causative agent of Salmonid Rickettsial Septicaemia (SRS) (Fryer et al. 1992). *P. salmonis* is a facultative intracellular bacterium that preferentially infects macrophages and is related to the *Coxiella* and *Francisella* genera

(Rozas and Enríquez 2014). SRS is a major threat to the Chilean aquaculture industry, the second largest producer of salmonids (Vargas et al. 2021), and it causes annual losses of over of $700 million USD in Chile (Maisey et al. 2017). Although several experimental vaccines have been tested and over 25 commercial vaccines are available, long term protection in the field remains an issue (Evensen 2016).

Innate immune responses at the cellular level are an important part of protective responses against intracellular pathogens and might be targeted to increase vaccine efficacy. Many of these responses are induced by interferons (IFNs), a group of cytokines with antiviral activity, which are expressed after pattern recognition receptor (PRR) activation (Abbas et al. 2020). Several families of PRRs have been identified, such as membrane bound and endosomal toll-like receptors (TLRs), the cytosolic RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). Different PRRs recognize different pattern associated molecular patterns (PAMPs), such as LPS, flagellin, unmethylated DNA, and RNA (ss or ds) (Liao and Su 2021). Dependent on cell type, ligation of PRRs by their PAMPs will initiate a signaling cascade: for RLRs this involves mitochondrial antiviral signaling protein (MAVS), and for TLRs this involves MyD88, TRIF, and/or TRAM as key components (Abbas et al. 2020). The engagement of the signaling pathways leads to the activation of transcription factors such as interferon regulatory factor (IRF) 3, IRF7, and/or nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and finally the transcription of IFNs. IFNs are cytokines that can have a paracrine or endocrine signaling effect through extracellular IFN receptors on other cells (Abbas et al. 2020). In rainbow trout, an autocrine route was suggested, where alternatively spliced IFN mRNA stayed in the cell and activated intracellular IFN receptors (Chang et al. 2013). IFN receptor activation leads to expression of interferon stimulated genes (ISGs), usually through the JAK-STAT pathway. ISGs have diverse functions, but most of them contribute to an antiviral state in the cell (Robertsen 2018).

Although type I IFN responses are mainly described as antiviral, they can be induced after recognition of intracellular bacteria (Perry et al. 2005). In mammals, type I IFN responses have been shown to be protective against some bacteria, such as the extracellular *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (Parker and Prince 2011) and the intracellular *Legionella pneumophila* (Snyder et al. 2017), while increasing damage induced by others (*Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*) (Martin et al. 2009; Mayer-Barber et al. 2014; O'Connell et al. 2004). For *Coxiella burnetti*, an intracellular bacterium related to *P. salmonis*, the effect of type I IFNs on infection was tissue dependent (Hedges et al. 2016). The effect of IFN type I responses on intracellular bacterial infection of salmonids has not been investigated yet, although there are indications that *Renibacterium salmoninarum* infection affects type I responses in chinook salmon (Rhodes et al. 2009) and Atlantic salmon (Eslamloo et al. 2020).

Investigations of our group have shown minor upregulation of IFNs and/or ISGs after in vivo *P. salmonis* infection (Svenning et al. 2019; Bela-Ong et al. 2020). In contrast, (Tacchi et al. 2011)

observed a downregulation of *ifn type I* in muscle of *P. salmonis* infected Atlantic salmon, but not in other organs (Tacchi et al. 2011) or in other transcriptome experiments (Tacchi et al. 2011; Pulgar et al. 2015; Rozas-Serri et al. 2018a; Rozas-Serri et al. 2018b). *Ifn type I* expression after vaccination with life-attenuated *P. salmonis* was reported by Vargas et al. 2021. The expression was upregulated 5 dpi, while at later timepoints it was no longer upregulated, similar to the other investigated genes (Vargas et al. 2021).

We investigated the induction and effect of type I IFN responses in *P. salmonis* infected CHSE-214 cells and the role of IRF3, IRF7, and MAVS in these responses. To this end, we infected earlier developed IRF3, IRF7, and MAVS KO CHSE-214 clones, as well as a wildtype clone, with *P. salmonis* and measured IFN and ISG expression along with bacterial titers. Since we observed slight modulations of IFN expression *in vivo* (Svenning et al. 2019; Bela-Ong et al. 2020), we investigated whether *P. salmonis* would also induce IFN responses in CHSE-214 cells. In addition, we examined whether the disruption of IFN response induction in IRF3 and MAVS KO clones would affect intracellular *P. salmonis* growth. To our knowledge, this study is the first to investigate the effect of type I IFN responses on *P. salmonis* infection.

Materials & Methods

2.1 Culture of cells and pathogens

CHSE-214 cells (kindly provided by Bjørn Krossøy, Vaxxinova Norway AS) were grown in growth medium (L15 (PanBiotech) with 1% L-glutamine and 10% FBS (HyClone)) at 20°C. KO clones of IRF7, IRF3, MAVS, and a double KO of IRF7/3 were developed as described in van der Wal et al., manuscript in preparation and cultivated as described for the CHSE-214 cells.

Piscirickettsia salmonis strain LF-89 (ATCC VR-1361) was propagated on CHSE-214 cells at 18°C. Supernatant was harvested after 14 days at extensive cytopathic effect (CPE), titrated, and frozen with 5% DMSO (Roth) in 1 mL aliquots at -80°C until use in infection experiments.

2.2 P. salmonis infection and titration

NC (negative control cells, the wt single cell clone of CHSE-214 used to develop the KO clones) (van der Wal et al., manuscript in preparation) and KO cells were seeded in 24 well plates with 125.000 cells/well in 1 mL infection medium (L15 with 1% L-glutamine and 2% FCS) one day before infection. For each cell line, the number of cells per well was counted to calculate the number of bacteria for the inoculum. *P. salmonis* was added at an MOI of 0.01 and the cells were incubated at 18°C. CPE was observed and supernatant for titration sampled at 14 dpi. *P. salmonis* supernatant samples were titrated

by end-point titration on CHSE-214 cells with 8 wells per dilution, and CPE was scored after 14 days. $TCID_{50}$/mL was calculated following the method of Reed and Muench (REED and MUENCH 1938).

A similar infection was performed for expression analysis of immune genes, with the exception that NC and KO cells were seeded at a density of 250.000 cells/24-well. The cell layer was lysed in RLT buffer for RNA isolation after 24 and 48 hours.

2.3 qPCR

The RNeasy kit (Qiagen) was used to isolate RNA from cell lysates according to manufacturer's instructions, and the QuantiTect RT kit (Qiagen) was used for cDNA synthesis according to manufacturer's instructions with 500 ng RNA per 20 µL reaction.

qPCR reactions contained 6 µL cDNA (diluted 1:5), 7,5 µL 2× Fast SYBR® Green Master Mix (Applied Biosystems), and 0,8 µL of each primer (5 µM stock). Taqman PCR reactions consisted of 6 µL cDNA, 7,5 µL 2x TaqMan universal master mix (Applied Biosystems), 0,18 µL of each primer (100 µM), 0,05 µL probe (100 µM, 6FAM-BHQ1), and 1,09 µL water. All qPCR reactions were performed in 384-well plates under the following conditions: 95 °C for 5 minutes and 45 cycles of 95 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds (QuantStudio 6, Applied Biosystems). Melting curves were included as quality control for SYBR Green PCR, and the primers have previously been published (see table 1 for primers and references). We calculated the relative expression compared to NC using the delta ct method with *elf2a* as a reference gene (Schmittgen and Livak 2008).

Table 1. List of primers used in this study with references to original publications of the primers.

Target	FW/RV	Sequence	Efficiency	Published in:
Elf2a	FW	TGCCCCTCCAGGATGTCTAC	2.24	(Iliev et al. 2013)
	RV	CACGGCCCACAGGTACTG		
AllMx	FW	TGCAACCACAGAGGCTTTGAA	2.06	(Robertsen et al. 2019)
	RV	GGCTTGGTCAGGATGCCTAAT		
IFIT5	FW	GCTGGGAAGAAGCTTAAGCAGAT	2.05	(Bela-Ong et al. 2020)
	RV	TCAGAGGCCTCGCCAACT		
IFNa (IFNa1-2)	FW	TGACTGGATCCGACACCACT	2.06	(van der Wal et al.,
	RV	ATCTCCTCCCATCTGGTCCA		manuscript in preparation)
	Probe	AGCGCAGAATACCTTTCCCT		
IFNc (IFNc1-4)	FW	ATACCGCCAGATTGAAGAGAG	2.04	(van der Wal et al.,
	RV	CAGTCCTTCTGTCCTGATGAGATA		manuscript in preparation)
	Probe	GGGCAGTGTGGATACCAGTG		

2.4 Statistics

We performed statistical tests in GraphPad Prism version 8.4.1. Welch's ANOVA with Dunnett's T3 multiple comparisons test (α=0.05) was performed to find significant differences between the NC and the KOs.

Results & Discussion

This study investigated the effect of IFN type I responses on *P. salmonis* infection of CHSE-214 cells. We did not observe a change in *ifna*, *ifnc*, *ifit5*, and *mx* expression after Ps infection in NC cells 24 and 48 hours after infection (fig. 1). Although *P. salmonis* infection did not induce IFN and ISG expression in CHSE-214 cells and transcriptome investigations likewise did not report IFN type I responses in infected Atlantic salmon (Tacchi et al. 2011; Pulgar et al. 2015; Rozas-Serri et al. 2018b), moderate IFN or ISG induction was observed through qPCR in infected (Bela-Ong et al. 2020; Svenning et al. 2019) and vaccinated Atlantic salmon (Vargas et al. 2021) tissues, most notably in immune organs. It thus seems that *P. salmonis* only induces IFN type I responses in some cell types, probably professional immune cells.

Even though *P. salmonis* did not elevate expression of the investigated IFNs and ISGs in wt cells, it did result in a minor increase of *ifna*, *ifnc*, and at 48 hours, *ifit5* expression in the IRF3 KO (fig. 1). This increase was not significant, but the trend is persistent in both investigated IFN genes and at both time points. The induction of IFN expression in an IRF3 KO is surprising, since IFN induction after poly I:C stimulation or viral infections is inhibited in this KO cell line (van der Wal et al., manuscript in preparation). Since IRF3 is an integral part of several PRR signaling routes and it has several possibilities to interact with other proteins (Servant et al. 2002), it is conceivable that it takes part in regulating IFN responses. The IRF3 KO might release other minor pathways to induce IFN expression, possibly dependent on IRF7, since the IRF7/3 double KO does not show this effect.

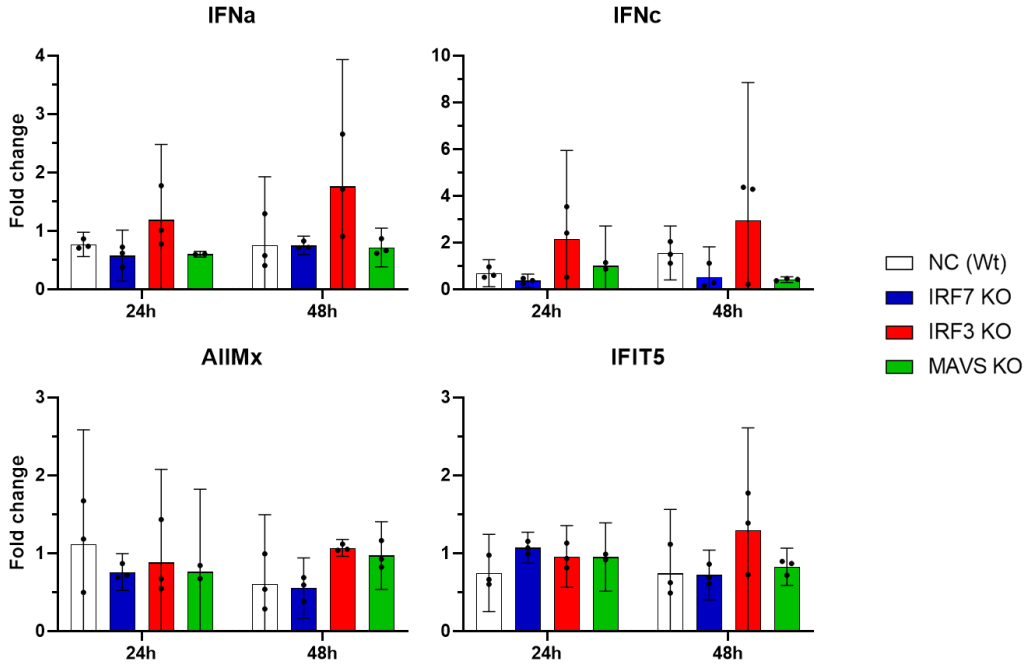


Figure 1: Expression in IRF7, IRF3, and MAVS KO CHSE-214 cells 24 or 48 hours after *P. salmonis* infection. The graphs show the fold change of expression compared to non-infected controls and normalized against *elf2a*. Values of the triplicates visualized as dots, and error bars indicate 95% confidence interval.

Finally, the effect of the IRF3, IRF7, and MAVS KOs on the growth of *P. salmonis* was investigated. Supernatants were harvested from infected cells at 14 dpi and titrated on CHSE-214 cells. *P. salmonis* titers in the IRF3/7 double KO were significantly reduced, while titers were not changed in the IRF7 KO (fig. 2F). We did observe increased titers on the IRF3 KO, although not a significant difference. The MAVS KO resulted in significantly reduced bacterial titers, presenting the clearest phenotypical change. Visual observation of CPE showed a similar trend, high CPE in the IRF3 KO and reduced CPE in the IRF7/3 and MAVS KOs (fig. 2A-E).

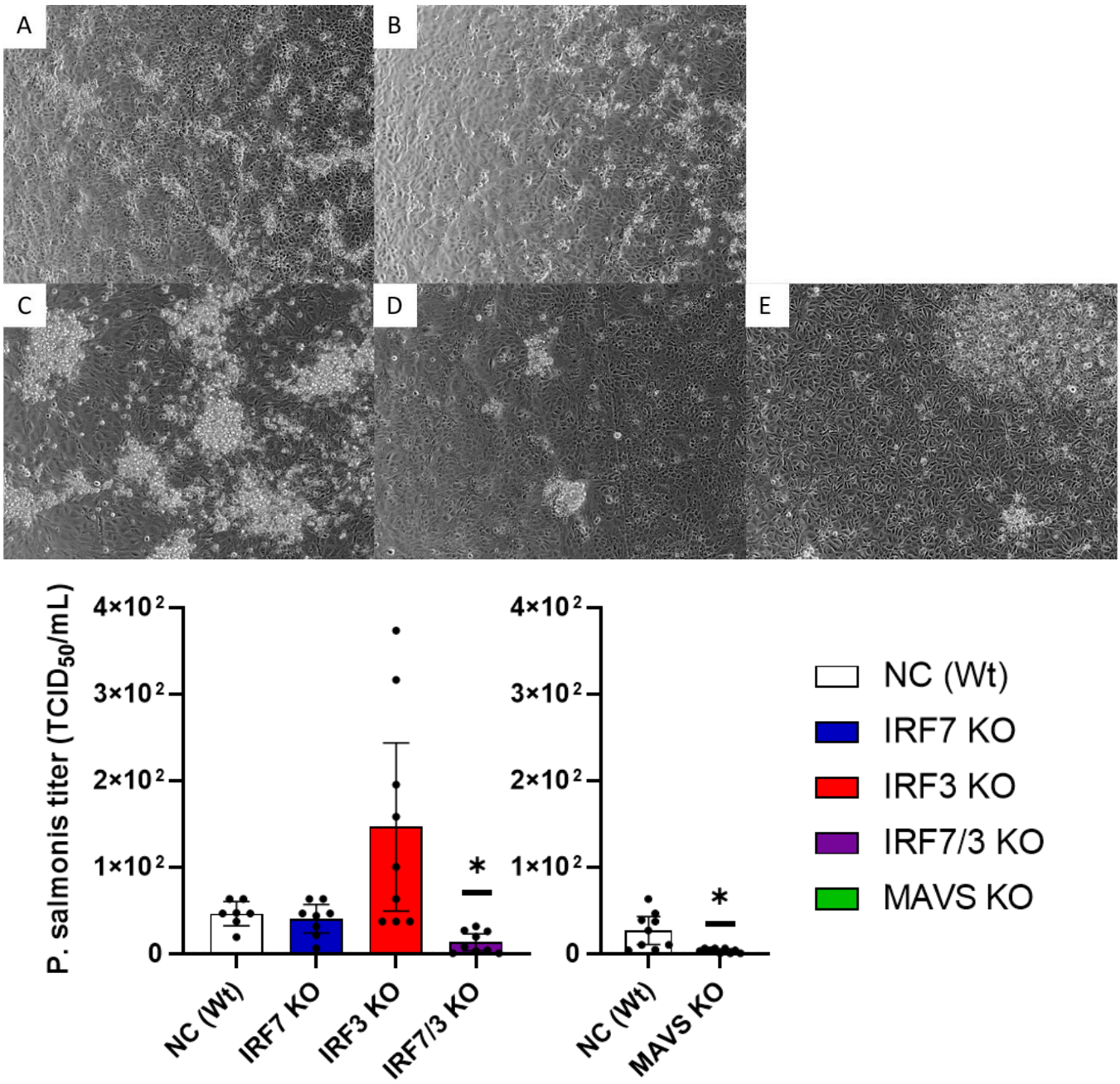


Figure 2: *P. salmonis* growth on KO CHSE-214 cells. A-E: pictures of CPE on *P. salmonis* infected cells 14 dpi: NC (A), IRF7 KO (B), IRF3 KO (C), IRF7/3 KO (D), and MAVS KO (E). F: *P. salmonis* titers in supernatants of infected KO cells 14 dpi. Values of triplicates from three experiments visualized as dots, and error bars indicate 95% confidence interval. (*) Statistically significant different from the NC.

The reduced growth of *P. salmonis* in the IRF7/3 and MAVS KO clones was comparable to what we found earlier for IPNV (van der Wal et al., manuscript in preparation). Although no evidence is available for a direct effect of *P. salmonis* on PRR signaling, we theorize that if *P. salmonis* affects PRR signaling through direct interactions with signaling molecules, it might have become partially dependent on these interactions. Removing one of these interacting signaling molecules could thus impair *P. salmonis* growth. Since *P. salmonis* is known to have a major effect on infected cells and is a more complex organism than IPNV, it is conceivable that it affects PRR signaling pathways. Furthermore, it is expected that successful intracellular pathogens influence innate responses, and the IFN-mediated responses are obvious targets. Combined with our findings, this all suggests that *P. salmonis* could inhibit PRR signaling and might be partially dependent on it, like we suggested for IPNV (van der Wal et al., manuscript in preparation). This could explain the reduced growth in KOs with disrupted PRR signaling and would fit with our observation that only the IRF3 KO, the only KO with a minor increase of IFN expression, accommodated a slight increase of *P. salmonis* growth. Future studies are required to investigate and eventually confirm this.

One interesting find was that the IRF3 KO did not yield the same results as the IRF7/3 double KO after *P. salmonis* infection, while in earlier experiments with poly I:C stimulation and viral infections (van der Wal et al., manuscript in preparation), these clones yielded similar results. The IRF3 KO showed slightly increased, albeit not significant, bacterial titers, whereas the IRF7/3 KO had significantly reduced *P. salmonis* titers. Even though the same wild-type clone (NC) was used to generate both these KO clones, some differences could have occurred in the processes. The two most obvious events would be an off-target effect from the CRISPR-Cas editing or a (minor) differentiation in one of the clonal lines. The CRISPR format used to induce the KOs is suggested to have lower changes of off-target effects because of the limited presence of the Cas9 protein in the cells (Elke Lorbach May 2018). That, combined with low matches of possible off-target locations, suggests that chances of off-target effects affecting the investigated PRR signaling are very small. However, there still is a chance that some other pathway would be affected by an off-target effect or differentiation of the clonal line. That such an effect would be observed after *P. salmonis* infection and not after specific investigation into PRR signaling or viral infections as described by van der Wal et al., manuscript in preparation is not surprising, since *P. salmonis* is a more complex organism and would affect and be affected by more different pathways than a virus. Finally, it is interesting to mention that the IRF3 KO clonal line shows a slightly different morphology from the other clonal lines at medium confluence. This could be an indication of a differentiation effect in the IRF3 KO and explain the difference in *P. salmonis* growth. It would be interesting to repeat these experiments while including different Sc clones of the KOs to obtain more solid data to further elucidate the effects of the different signaling molecules on *P. salmonis* infection.

A similar investigation in Atlantic salmon macrophages would be very interesting because *P. salmonis*, like several other intracellular bacteria, infects macrophages (McCarthy et al. 2008). Since the effect of IFN type I responses on *C. burnetti* infection differ between tissues, it would be good to check the effect on *P. salmonis* infection of their preferential host cells. Priming macrophages with IFN type I before infection could already give some answers. A second reason why IRF3, IRF7, and MAVS KOs in macrophages would be interesting is that macrophages have more diverse PRR pathways compared to CHSE-214 cells. In addition, their responses to PRR signaling probably have more effect on infection outcome than most regular host cells due to their central function in immune responses.

In conclusion, we show that *P. salmonis* infection does not induce IFN type I responses in CHSE-214 cells and that only an IRF3 KO might have a minor effect on the expression of IFNs and ISGs. Different KOs in PRR signaling did, however, affect *P. salmonis* growth: clones with intact PRR signaling (NC and IRF7) yielded higher bacterial titers than two clones (IRF7/3 and MAVS KOs) with disturbed signaling, which show similarities to previous results for IPNV. Interestingly, the IRF3 KO resulted in increased (though not significantly) bacterial titers. Additional effort will be required to elucidate the innate responses of host cells to *P. salmonis* infection, with macrophages being especially interesting.

Publication bibliography

Abbas, Abul K.; Lichtman, Andrew H.; Pillai, Shiv (2020): Basic immunology. Functions and disorders of the immune system. With assistance of David L. Baker, Alexandra Baker. 6th edition. Philadelphia: Elsevier (Medical Textbooks Immunology).

Adams, Alexandra (2019): Progress, challenges and opportunities in fish vaccine development. In *Fish & shellfish immunology* 90, pp. 210–214. DOI: 10.1016/j.fsi.2019.04.066.

Bela-Ong, Dennis Berbulla; Greiner-Tollersrud, Linn; van der Andreas Wal, Yorick; Jensen, Ingvill; Seternes, Ole Morten; Jørgensen, Jorunn B. (2020): Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene ifit5 in a teleost fish. In *Developmental and comparative immunology* 111, p. 103746. DOI: 10.1016/j.dci.2020.103746.

Chang, Ming-Xian; Zou, Jun; Nie, Pin; Huang, Bei; Yu, Zhanglong; Collet, Bertrand; Secombes, Chris J. (2013): Intracellular interferons in fish: a unique means to combat viral infection. In *PLoS pathogens* 9 (11), e1003736. DOI: 10.1371/journal.ppat.1003736.

Elke Lorbach (2018): CRISPR. Tips and Tricks for Efficient Delivery into Primary Cells and Cell Lines. Lonza webinar. Lonza. Online, May 2018, checked on May 2018.

Eslamloo, Khalil; Caballero-Solares, Albert; Inkpen, Sabrina M.; Emam, Mohamed; Kumar, Surendra; Bouniot, Camila et al. (2020): Transcriptomic Profiling of the Adaptive and Innate Immune Responses of Atlantic Salmon to Renibacterium salmoninarum Infection. In *Frontiers in immunology* 11, p. 567838. DOI: 10.3389/fimmu.2020.567838.

Evensen, Øystein (2016): Immunization Strategies against Piscirickettsia salmonis Infections: Review of Vaccination Approaches and Modalities and Their Associated Immune Response Profiles. In *Frontiers in immunology* 7, p. 482. DOI: 10.3389/fimmu.2016.00482.

Fryer, J. L.; Lannan, C. N.; Giovannoni, S. J.; Wood, N. D. (1992): Piscirickettsia salmonis gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. In *International journal of systematic bacteriology* 42 (1), pp. 120–126. DOI: 10.1099/00207713-42-1-120.

Hedges, Jodi F.; Robison, Amanda; Kimmel, Emily; Christensen, Kelly; Lucas, Erin; Ramstead, Andrew; Jutila, Mark A. (2016): Type I Interferon Counters or Promotes Coxiella burnetii Replication Dependent on Tissue. In *Infection and immunity* 84 (6), pp. 1815–1825. DOI: 10.1128/IAI.01540-15.

Liao, Zhiwei; Su, Jianguo (2021): Progresses on three pattern recognition receptor families (TLRs, RLRs and NLRs) in teleost. In *Developmental and comparative immunology* 122, p. 104131. DOI: 10.1016/j.dci.2021.104131.

Maisey, Kevin; Montero, Ruth; Christodoulides, Myron (2017): Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. In *Expert review of vaccines* 16 (3), pp. 215–228. DOI: 10.1080/14760584.2017.1244483.

Martin, Francis J.; Gomez, Marisa I.; Wetzel, Dawn M.; Memmi, Guido; O'Seaghdha, Maghnus; Soong, Grace et al. (2009): Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. In *The Journal of clinical investigation* 119 (7), pp. 1931–1939. DOI: 10.1172/JCI35879.

Mayer-Barber, Katrin D.; Andrade, Bruno B.; Oland, Sandra D.; Amaral, Eduardo P.; Barber, Daniel L.; Gonzales, Jacqueline et al. (2014): Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. In *Nature* 511 (7507), pp. 99–103. DOI: 10.1038/nature13489.

McCarthy, Una M.; Bron, James E.; Brown, Linton; Pourahmad, Fazel; Bricknell, Ian R.; Thompson, Kim D. et al. (2008): Survival and replication of Piscirickettsia salmonis in rainbow trout head kidney macrophages. In *Fish & shellfish immunology* 25 (5), pp. 477–484. DOI: 10.1016/j.fsi.2008.07.005.

O'Connell, Ryan M.; Saha, Supriya K.; Vaidya, Sagar A.; Bruhn, Kevin W.; Miranda, Gustavo A.; Zarnegar, Brian et al. (2004): Type I interferon production enhances susceptibility to Listeria monocytogenes infection. In *The Journal of experimental medicine* 200 (4), pp. 437–445. DOI: 10.1084/jem.20040712.

Parker, Dane; Prince, Alice (2011): Type I interferon response to extracellular bacteria in the airway epithelium. In *Trends in immunology* 32 (12), pp. 582–588. DOI: 10.1016/j.it.2011.09.003.

Perry, Andrea K.; Chen, Gang; Zheng, Dahai; Tang, Hong; Cheng, Genhong (2005): The host type I interferon response to viral and bacterial infections. In *Cell research* 15 (6), pp. 407–422. DOI: 10.1038/sj.cr.7290309.

Pulgar, Rodrigo; Hödar, Christian; Travisany, Dante; Zuñiga, Alejandro; Domínguez, Calixto; Maass, Alejandro et al. (2015): Transcriptional response of Atlantic salmon families to Piscirickettsia salmonis infection highlights the relevance of the iron-deprivation defence system. In *BMC genomics* 16, p. 495. DOI: 10.1186/s12864-015-1716-9.

REED, L. J.; MUENCH, H. (1938): A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. In *American Journal of Epidemiology* 27 (3), pp. 493–497. DOI: 10.1093/oxfordjournals.aje.a118408.

Rhodes, Linda D.; Wallis, Steviebrooke; Demlow, S. Ellen (2009): Genes associated with an effective host response by Chinook salmon to Renibacterium salmoninarum. In *Developmental and comparative immunology* 33 (2), pp. 176–186. DOI: 10.1016/j.dci.2008.08.006.

Robertsen, Børre (2018): The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. In *Developmental and comparative immunology* 80, pp. 41–52. DOI: 10.1016/j.dci.2017.02.005.

Rozas, M.; Enríquez, R. (2014): Piscirickettsiosis and Piscirickettsia salmonis in fish: a review. In *Journal of fish diseases* 37 (3), pp. 163–188. DOI: 10.1111/jfd.12211.

Rozas-Serri, M.; Peña, A.; Arriagada, G.; Enríquez, R.; Maldonado, L. (2018a): Comparison of gene expression in post-smolt Atlantic salmon challenged by LF-89-like and EM-90-like Piscirickettsia salmonis isolates reveals differences in the immune response associated with pathogenicity. In *Journal of fish diseases* 41 (3), pp. 539–552. DOI: 10.1111/jfd.12756.

Rozas-Serri, Marco; Peña, Andrea; Maldonado, Lucerina (2018b): Transcriptomic profiles of post-smolt Atlantic salmon challenged with Piscirickettsia salmonis reveal a strategy to evade the adaptive immune response and modify cell-autonomous immunity. In *Developmental and comparative immunology* 81, pp. 348–362. DOI: 10.1016/j.dci.2017.12.023.

Schmittgen, Thomas D.; Livak, Kenneth J. (2008): Analyzing real-time PCR data by the comparative C(T) method. In *Nat Protoc* 3 (6), pp. 1101–1108. DOI: 10.1038/nprot.2008.73.

Servant, Marc J.; Tenoever, Benjamin; Lin, Rongtuan (2002): Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. In *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 22 (1), pp. 49–58. DOI: 10.1089/107999002753452656.

Snyder, Deann T.; Hedges, Jodi F.; Jutila, Mark A. (2017): Getting "Inside" Type I IFNs: Type I IFNs in Intracellular Bacterial Infections. In *Journal of immunology research* 2017, p. 9361802. DOI: 10.1155/2017/9361802.

Svenning, Steingrim; Gondek-Wyrozemska, Agata T.; van der Wal, Yorick Andreas; Robertsen, Børre; Jensen, Ingvill; Jørgensen, Jorunn B.; Edholm, Eva-Stina (2019): Microbial Danger Signals Control Transcriptional Induction of Distinct MHC Class I L Lineage Genes in Atlantic Salmon. In *Frontiers in immunology* 10, p. 2425. DOI: 10.3389/fimmu.2019.02425.

Tacchi, Luca; Bron, James E.; Taggart, John B.; Secombes, Christopher J.; Bickerdike, Ralph; Adler, Michael A. et al. (2011): Multiple tissue transcriptomic responses to Piscirickettsia salmonis in Atlantic salmon (Salmo salar). In *Physiological genomics* 43 (21), pp. 1241–1254. DOI: 10.1152/physiolgenomics.00086.2011.

Vargas, Deborah; Vallejos-Vidal, Eva; Reyes-Cerpa, Sebastián; Oyarzún-Arrau, Aarón; Acuña-Castillo, Claudio; Imarai, Mónica et al. (2021): The Analysis of Live-Attenuated Piscirickettsia salmonis Vaccine Reveals the Short-Term Upregulation of Innate and Adaptive Immune Genes in Atlantic Salmon (Salmo salar): An In Situ Open-Sea Cages Study. In *Microorganisms* 9 (4). DOI: 10.3390/microorganisms9040703.

Appendix

Atlantic salmon & Chinook salmon IFN regions

Literature & bioinformatics study - Yorick van der Wal

Abstract

We performed a bioinformatic investigation to identify IFN genes in the current NCBI GenBank assemblies of Atlantic salmon and Chinook salmon. This was combined with a literature study of publications on IFNs in these species to present an overview on the described IFN genes and the used primers. This led to the identification of additional putative IFN genes, especially in the Chinook salmon IFN rich regions from the most recent assembly (Otsh v2.0). In addition, we coupled different accession numbers and names to the identified genes, based on the assembly loci. Finally, we present an overview of the primers used for expression analyses of IFN type I genes in Atlantic salmon and Chinook salmon including the IFN genes that they possibly bind. The results show that most of the primers have the potential to bind and amplify multiple IFN genes of the same type.

Abbreviations

BAC	Bacterial artificial chromosome	ISG	Interferon stimulated gene
CDS	Coding sequence(s)	NCBI	National Center for Biotechnology Information
CRISPR	Clustered regularly interspaced short palindromic repeats	NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
GH	Growth hormone	PRR	Pattern recognition receptor
IFN	Interferon	rIFN	Recombinant interferon
IRF	Interferon regulatory factor	RLR	Rig-I-like receptor
IPNV	Infectious pancreatic necrosis virus	TLR	Toll-like receptor
IPS-1	Mitochondrial IFN-beta promoter stimulator-1	SAV	Salmon alphavirus
ISAV	Infectious salmon anaemia virus	UTR	Untranslated region

1 Introduction

1.1 IFN general

The aquatic environment enables easy access of pathogens to aquatic animals such as salmonid fish. Coupled with a less pronounced adaptive immune system than mammals, this means that salmonids and other fish rely more on their innate immune system (Uribe et al. 2011). The salmonid innate immune system is well developed and complex, as illustrated by the many type I interferon (IFN) genes found in Atlantic salmon and rainbow trout (Sun et al. 2009; Zou et al. 2014). Type I IFNs are cytokines that are an important part of the innate signalling after pathogen recognition.

Cells recognize invading pathogens mainly through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Rig-I-like receptors (RLRs), and Nod-like receptors. These receptors are germline encoded, can be located on the outer membrane or intracellularly, and recognize, among others, pathogen associated molecular patterns (PAMPs). After binding to a PAMP, the PRR activates a signalling cascade that leads to the activation of transcription factors such as IRF3, IRF7, and NFkB and consequently the transcription of IFN genes with corresponding promoter elements (Abbas et al. 2020). The translated IFNs then bind IFN receptors on neighbouring cells or, as demonstrated in rainbow trout (Chang et al. 2013), intracellular IFN receptors in the same cell. Binding of the IFN receptor leads to activation of a second signalling pathway and transcription of IFN stimulated genes (ISGs), such as Mx and IFIT genes (Bela-Ong et al. 2020). ISGs consist of many genes with differing functions, but in general they lead to an anti-viral state in the cell by, among others, inhibiting viral entry and replication. This makes the type I IFN genes an important part of the innate immune system at a cellular level.

There are three types of IFNs in mammalians: type I (IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, and IFNτ), type II (IFNγ), and type III (IFNλ1, IFNλ2, and IFNλ3). Type I and III IFNs are part of the innate system signalling as described above, while the type II IFNγ is also part of the adaptive immune system (Pestka et al. 2004). Type I and II IFNs have been found in teleost fish, although the type I IFNs in fish have an intron structure similar to type III IFNs, while mammalian type I IFNs do not have introns (Zou et al. 2014). The protein structure of teleost type I IFNs is still clearly most similar to mammalian type I IFNs (Robertsen et al. 2003).

1.2 IFN investigations in Atlantic salmon

The first IFNs described in Atlantic salmon were SasaIFNα1 and SasaIFNα2 (Robertsen et al. 2003), later named IFNa1 and IFNa2, although IFN like activity was also described for supernatants of stimulated cells (Johansen et al. 2004). The anti-viral activity of these IFNs was then investigated using recombinant proteins. Recombinant IFNa1 and IFNa2, produced in HEK cells or in *Escherichia coli*, activate ISG promoters, induce anti-viral gene expression, and protect against several viral infections in

multiple systems: TO cells (Robertsen et al. 2003; Røkenes et al. 2007; Bergan et al. 2008; Berg et al. 2009; Kileng et al. 2009; Bergan et al. 2010; Xu et al. 2010; Sun et al. 2011; Svingerud et al. 2012; Skjesol et al. 2014), CHSE-214 cells (Robertsen et al. 2003; Jørgensen et al. 2007a; Ooi et al. 2008b; Skjesol et al. 2009; Xu et al. 2010), ASK cells (Sun et al. 2011; Svingerud et al. 2012; Svingerud et al. 2013; Chang et al. 2016), Ssp9 cells (Bela-Ong et al. 2020), primary leukocytes (Svenning et al. 2019; Bela-Ong et al. 2020), Rainbow trout (Ooi et al. 2008a), and Atlantic salmon (Chang et al. 2015). In addition, rIFN type I from rainbow trout induced expression of several genes in SHK-1 cells (Martin et al. 2007).

The promoters of IFNa1 and IFNa2 contain two regulatory regions resulting in two transcripts, one of which has a long ‘5-UTR (Bergan et al. 2006), and have been used to develop luciferase assays for promoter activation. The IFNa1 promoter can be activated by Poly I:C (a synthetic dsRNA mimic and immunostimulant), ISAV, IRF1, IRF3, IRF7, and IPS-1, and activation by IPS-1 can be inhibited by IPNV proteins (Bergan et al. 2006; Kileng et al. 2009; Lauksund et al. 2009; Bergan et al. 2010; Lauksund et al. 2015; Li et al. 2016).

Additional type I IFN genes were later identified in salmonids: IFNb, IFNc, IFNd, IFNe, and IFNf (Sun et al. 2009; Chang et al. 2009; Zou et al. 2014). These IFN genes can be separated into two groups: IFNa, IFNd, and IFNe have two cysteines for one disulfide bond, while IFNb, IFNc, and IFNf have four cysteines and thus two disulfide bonds (Zou et al. 2014; Robertsen 2018). Expression plasmids containing IFNa1, IFNb, or IFNc showed immunostimulating effects in Atlantic salmon after intramuscular injection (Chang et al. 2014; Chang et al. 2015; Robertsen et al. 2016). The IFNa1 effect was mainly localized at the injection site, while IFNb and IFNc also upregulated immune genes in HK and liver. The plasmids offered protection against ISAV and SAV challenge in live fish: IFNb minor, IFNc major, and IFNa1 no protection (Chang et al. 2014; Chang et al. 2016). These plasmids furthermore increased the protection against ISAV challenge from a DNA vaccine based on the HE protein (Chang et al. 2015; Robertsen et al. 2016).

Finally, the expression of several IFN genes has been investigated in Atlantic salmon, Chinook salmon, or their cultured cells in over 40 publications (see table 12). IFN expression is induced by stimulation with poly I:C (RLR, TLR3, TLR22 ligand), S-27609 (TLR7 ligand), rIFNa1, rIFNγ, or R848 (TLR7 ligand) and infection by SAV, aquabirnaviruses, ISAV, or PRV (Robertsen et al. 2003; Bergan et al. 2006; Sun et al. 2009; Skotheim 2009; Svingerud et al. 2012; Sun et al. 2011; Gamil et al. 2015; Dahle et al. 2015). IPNV infection generally inhibits IFNa expression in cell lines but can induce expression in vivo (Skotheim 2009; McBeath et al. 2007; Skjesol et al. 2011; Reyes-Cerpa et al. 2012). The antagonistic effects of IPNV proteins on IFNa expression have been determined by Lauksund et al. 2015. Finally, *Piscirickettsia salmonis* infection in Atlantic salmon has only small effects on IFN

expression: IFNa and IFNc were reduced in the liver at 2 dpi, while IFNa was increased in the liver and head kidney at 7 and/or 14 dpi (Svenning et al. 2019).

1.3 Aims of the investigation

While investigating possible primers for IFN gene expression analysis in CHSE-214 cells, we found that primer pairs described in publications were mainly borrowed from Atlantic salmon or rainbow trout and that a lot of different primer pairs were described for IFN gene expression. Since many IFN genes had been described for salmonids, we decided to generate a current overview of IFN genes and their locations in Chinook salmon and Atlantic salmon. This overview was then used to evaluate the IFN genes and the primers used for analysis of their expression in current literature. We limited the scope of the investigation to Atlantic salmon and Chinook salmon and on the IFN rich regions in the current assemblies, thus we will mostly ignore IFNd, which is found in another location. Finally, we look at the naming systems that have been used or suggested for Atlantic salmon IFN genes.

2 Materials & Methods

2.1 Identification of IFN genes in predicted loci

The coding sequence (CDS) of the Atlantic salmon IFNa1 (100137019), IFNa2 (100136436), IFNb (LOC101448042), and IFNc (LOC101448043) genes were blasted against the Chinook and Atlantic salmon nucleotide collections at NCBI GenBank to identify possible IFN loci. Any IFN genes described in previous literature were also added to the pool of found genes.

2.2 Identification of additional IFN (pseudo)genes in IFN regions

Genscan (webtool) and Augustus (Genious plug-in) were used to find additional possible coding sequences in the Chinook salmon growth hormone (GH) - IFN regions on chromosomes 9 and 27. Identified IFN genes for Atlantic salmon and Chinook salmon were grouped based on described genes and multiple alignments were made of each pair of IFN types (IFNa-IFNb, IFNa-IFNc, etc.). Consensus sequences (20-70 bp) with high numbers of identical bases in all aligned sequences were transformed into motifs by substituting non-identical bases with ‘N’. These motifs were used to search the IFN regions for additional possible IFN (pseudo)gene locations. Genscan was used on the regions around motifs without previously predicted CDS to identify possible IFN exons.

2.3 Evaluation of possible IFN genes

All identified CDS, including the Genscan/Augustus predictions and remaining Chinook salmon loci (NCBI GenBank) in the GH-IFN regions, were aligned several times in different constellations to identify the IFN genes, IFN pseudogenes, and other genes. This was repeated with the protein sequences.

For possible pseudogenes, the Expasy webtools was used to find possible translations. The NCBI GenBank conserved domain tool was used to identify the conserved IFN domain in the putative proteins.

2.4 Determination of genomic positions

Any genes from literature that were not connected with a locus in the used assemblies were aligned with the genes with known positions to find whether they matched any of those. We manually searched for the genes that did not match in the IFN containing regions, exon by exon.

Locations and orientation of all genes in the IFN rich regions (Chinook salmon chromosome 9 and 27 and Atlantic salmon chromosome 3, 6, and 28) were annotated in Geneious prime and compiled in tables. Any non-IFN genes in these regions were checked for conserved domains to include them in a synteny comparison.

2.5 Literature: accession numbers and primers

IFN accession numbers and primer sequences from all found papers mentioning IFN genes and/or their expression in Atlantic salmon or Chinook salmon were compiled and coupled to the genes compiled here. The primers were blasted against a local database (Geneious) of IFN genes and matches with less than 3 mismatches per primer (location of mismatches was ignored) were noted as possible binding sites. Primers without local blast matches were blasted in NCBI GenBank to find other binding sites. Matches from forward and reverse primers, including any probes, were combined to identify possible amplified genes per pair.

2.6 Assemblies and bioinformatic tools

Chinook salmon: Otsh_v2.0

Atlantic salmon: ICSASG_v2

Genious prime 2019.2.1

Genscan: http://argonaute.mit.edu/GENSCAN.html

Expasy: https://web.expasy.org/translate/

Conserved domains (NCBI GenBank): https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Blast (NCBI GenBank): https://blast.ncbi.nlm.nih.gov/Blast.cgi

3 Results

3.1 IFN genes identified – accession numbers, types, and names

All IFN genes identified through blasting, investigations of IFN rich loci, and literature search were categorized in tables 1 and 2. For Chinook salmon, IFN genes and pseudogenes were grouped. One group of four highly similar genes, predicted in the assembly, formed a separate group in the phylogenetic trees, and they were designated IFN-like genes. A long DNA motif (AGGAACANAGCGCATGNGCATNNGAGNTCNNNNGAGNGNAGNTTCGCNACAANCTGGT GCAGTTNNANAAATTCCTNGACANCANNGTCAAGCNNTGA) that was shared by these genes was found in 5 more locations in the same region. The predicted genes contained conserved IFN domains and were most similar to IFNb but could be a different type of IFN. These predicted IFN like genes contain stop codons in the middle, which indicates pseudogenes, but several possible exons can be identified in their locations, and we might not have found the correct CDS. A more detailed evaluation could indicate if and how these genes are transcribed. The other motifs from these IFN-like genes were in possible CDS but are probably pseudogenes. The IFN genes described by Liu et al. (2020) were all connected to found genes through alignment and synteny comparisons.

The Atlantic salmon IFN genes were distributed in four categories: full genes on chromosomes 3, 6, or 28, unplaced full genes (according to the used assembly), genes or accession numbers with partial/missing sequences, and pseudogenes. The Atlantic salmon IFN genes described by Liu et al. (2020) were also all connected to found genes through alignment and synteny comparisons. Furthermore, IFN genes described by Sun et al. (2009) were aligned to the found genes, but IFNc3 was not found. It aligned best with the locus that also aligned with IFNc2, but the alignment with IFNc2 was better. Since the assemblies are based on a single individual, they do not account for allelic variance, which can result in issues while matching very similar genes. In addition, the gene order of the different IFN genes between the bacterial artificial chromosome (BAC) sequenced by Sun et al. (2009) does not match that of the assembly.

3.2 IFN rich regions in Chinook and Atlantic salmon show synteny including growth hormone and globoside alpha-1,3-N-acetylgalactosaminyltransferase 1

IFN genes and other interesting elements were annotated on the two IFN rich regions in Chinook salmon on chromosomes 9 and 27 and described in tables 3 and 4. Both these regions were flanked by a version of GH and contained a gene with a conserved glycosyl transferase domain. In addition, conserved reverse transcriptase domains were found in pseudogenes or non-IFN genes in both regions. The IFN-like genes and the additional locations of their large, shared motif were found in the centre of the region on chromosome 27. This region seems to be very repetitive, which could either be an indication of

sequencing errors or of gene duplications. In the event of sequence errors, the four IFN-like genes might actually only be one.

The two Atlantic salmon regions with GH and IFN genes were also annotated, in addition to a small region on chromosome 28 with some IFN genes. The genes and elements of these regions are described in tables 5, 6, and 7. The GH flanked IFN rich regions of Atlantic salmon also contain some (pseudo)genes with conserved elements related to genome rearrangements (reverse transcriptase, integrin, and transposase). One of the two regions also contains a gene with a conserved glycosyl transferase domain, where the other contains the globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 gene with the same domain. Alignment of all four genes, two from Atlantic salmon and two from Chinook salmon, indicates that they are all versions of globoside alpha-1,3-N-acetylgalactosaminyltransferase 1, confirmed by synteny.

The general synteny between the IFN regions of Chinook salmon chromosome 9 and Atlantic salmon chromosome 3 and between Chinook salmon chromosome 27 and Atlantic salmon chromosome 6, is quite good (figures 1 and 2). The most striking difference is that in both Chinook salmon regions an insert containing several IFN genes seems to be present.

3.3 Publications mostly refer to the same few IFN genes

We found around 50 publications that covered Chinook salmon and/or Atlantic salmon IFN gene expression, recombinant proteins, plasmids, or reviews. Publications on the IFN promoters and their activity were not included here. It is very striking that most accession numbers on the investigated genes correspond to a small selection of IFN genes, mainly the first described ones (see tables 8-10). Although this is not surprising, it means that the effects and expression of a lot more IFN genes can be investigated. Accession numbers mentioned in publications on IFN expression in Chinook salmon refer to a rainbow trout gene (RtIFN-a1) or to an Atlantic salmon gene (AsIFNa2) (table 8). Functional investigations in Atlantic salmon focused almost exclusively on the first two described IFNa genes (AsIFNa1 and AsIFNa2) and on the first described IFNb (AsIFNb2) and IFNc (AsIFNc4) genes (table 9). Accession numbers in publications on IFN gene expression in Atlantic salmon corresponded mainly with the first described IFNa genes (AsIFNa1 and AsIFNa2), while a recent publication mentioned 5 other IFNa genes (table 10, Munir et al. 2020).

3.4 Primers used for expression analysis probably amplify several genes

Possible binding of primers described in literature for expression analysis of IFN genes was evaluated after blasting the primers against our local collection of IFN genes. An overview of possible amplification, based on amounts of mismatches, is given in table 11, with the corresponding publications listed in table 12.

The four primer pairs described for IFN gene expression in Chinook salmon show very different specificities: the primers used by Gamil et al. (2015) bind CsIFNa1 exactly and CsIFNa2 with a few mismatches, the primers used in Waterloo and Auckland (Monjo et al. 2017; Semple et al. 2018; Lulijwa et al. 2020a, 2020b) bind several genes well, the primer-probe combination from Skotheim (2009) only binds the found IFN genes with several mismatches, and the primer pair described by Rajanbabu and Chen (2011), actually binds both GH genes and not IFN genes. Expression primers used in Atlantic salmon generally bind to several IFN genes, with two exceptions: the primer pair described by Reyes-Cerpa et al. (2012) do not seem to bind any of the genes we described here since one primer binds only to AsIFNa7, while the other binds only to other genes. One of the primers in the second pair only binds a possible pseudogene, a sequence with missing sequence, or three genes with an 18 out of 24 bp stretch. One final primer pair of interest is the pair that was designed for the long transcript of AsIFNa2 by Bergan et al. (2006) which seems to bind in the ‘5-UTR of three IFNa genes.

4 Discussion

4.1 Limitations: sequence quality and predictive value

The main limitation of this investigation is the dependency on the used assemblies (current reference assemblies in NCBI GenBank). These assemblies will probably still contain errors, as illustrated by the discrepancy between the IFN gene order found in the Atlantic salmon BACs by Sun et al. (2009) and the current assembly. The number of additional identified IFN genes in the new Chinook salmon assembly compared to the previous assembly used by Liu et al. (2020) also illustrates this. The current assemblies cannot take allelic variation into account since they are based on a single individual, which could introduce some issues for highly similar genes. Still, for the scope of this study, the assemblies were sufficient to compare the IFN genes and primers covered in the literature. For a true overview of the IFN genes in Chinook salmon and Atlantic salmon, a directed sequencing effort would be necessary. To get long reads specifically from the IFN gene locations, the recently developed CRISPR/Cas directed nanopore sequencing could be a great asset (Schultzhaus et al. 2021). This allows selective sequencing without the need for amplification at CRISPR/Cas cut sites, which can be developed for very specific locations such as certain IFN genes. Multiple long reads can thus be obtained with high quality even in genomic regions rich in repeats that affect amplification, like the IFN regions are.

Another limitation of the current study is that we only focused on (predicted) CDS to determine possible IFN genes and did not include an evaluation of promoter regions. If the CDS would translate to a seemingly functional IFN protein, we classified it as a putative active IFN gene, without taking any promoter regions into account. In addition, the CDS prediction algorithms are mostly not optimized for these species. This means that the predicted CDS could be wrong and that we might have missed possible CDSs. A future investigation should therefore include the IFN gene promoter regions and their

putative (in silico) or actual (in vitro) activities. Targeted sequencing of putative IFN transcripts in RNA pools from different cells, tissue types, and stimulations will also help to identify the active IFN genes and the requirements for their transcription.

4.2 IFN genes in literature

Publications on IFN genes in Chinook and Atlantic salmon are not always easy to compare due to differences in names used for the genes and the different accession numbers, some of which have not been located on the current assemblies yet.

The first described IFN genes in Atlantic salmon were named SasaIFNα1 and SasaIFNα2 (Robertsen et al. 2003), and these have been used most extensively in salmon IFN research. When it became apparent that the teleost IFNs were not orthologues of the mammalian IFNs, SasaIFNα was renamed IFNa and additional IFN types were named b, c, d, e, and f (Sun et al. 2009; Zou et al. 2014). In large yellow croaker, an IFNh has been identified recently (Ding et al. 2016). This change from IFNα to IFNa was not adopted by all publications of a later date, but most publications did use it. This nomenclature does seem to be the best option, since it indicates the distinction with the mammalian nomenclature.

The initial numbering of the Atlantic salmon IFN genes was based on the first two IFNs described and later expanded by Sun et al. (2009) to follow the location on the sequenced BAC, although the initial numbering for IFNa1 and IFNa2 was kept. Liu et al. (2020) suggested a new terminology, where the IFN genes per type got two numbers, one for the genomic region they were located in (1 or 2) and a second one counted the copies of one type inside the region, based on the location (1, 2, 3, 4, 5, etc.). However, this method has a few downsides. First, this numbering is different from the established and commonly used nomenclature, possibly leading to more confusion while comparing papers that use the different nomenclatures. Secondly, if new sequence data and/or assemblies lead to identification of additional genes, they may be located between the earlier annotated genes and either warrant renaming of the earlier named genes or get names that are not in line with the proposed nomenclature. Thus, we have followed the nomenclature as started by Sun et al. (2009) and used this for the identified genes in Chinook salmon and Atlantic salmon. However, we prioritized similarity over location within a region since these locations might still shift when new sequence information is published.

Over the years, several primer pairs have been used to investigate the expression of IFNs in Atlantic salmon and Chinook salmon. Several groups have used earlier published primer pairs, resulting in multiple publications with the same primer pairs, but others have developed their own primers. The diversity of primers used in Chinook salmon is particularly interesting since most used primers have been borrowed from Atlantic salmon or rainbow trout investigations. The only primer that was described to be designed specifically for Chinook salmon IFN actually seems to amplify the gh1 and gh2 genes of Chinook salmon (Rajanbabu and Chen 2011). This indicates the challenges of designing primers based

on a gene assembly with very few confirmed annotations. Based on the number of possible binding matches for many of the investigated primer pairs, one should be very careful in claiming to measure the expression of a single IFN locus. Still, for most publications the used primers still support the conclusions, since the general IFN response was investigated. It is interesting to mention that Svingerud et al. (2012) specifically mention the use of primers that amplify all IFNb or IFNc genes in their investigation.

4.3 Salmonid IFN gene numbers and diversity

Salmonids have a very high number of IFN genes compared to other teleost fish (Liu et al. 2020), which is supported by the findings presented here. The additional whole genome duplication that salmonids underwent most probably enabled this expansion of the IFN gene numbers, but transposases also could have played a role (Secombes und Zou 2017). The presence of several transposase/reverse transcriptase domains in the IFN rich regions and the theory that introns have been lost in the type I IFN genes of mammals due to integration of cDNA in the genome are arguments for the role of transposases (Secombes und Zou 2017; Zou et al. 2014).

Aside from the original duplication, a gene should also have a specific function in order to be kept. The number of IFN pseudogenes in the IFN rich regions attests that those surplus genes without specific functions will be lost during evolution. Still, we can observe a rather large number of seemingly intact IFN genes, spread out over the different IFN groups, suggesting that they obtained a difference in function. Although a difference in expression and/or effects has been shown for the different groups (Svingerud et al. 2012; Chang et al. 2014) and for IFNa1&2 and IFNa3 (Sun et al. 2009), for most genes within a group there is little data on differences in expression or function. A differential cell type or tissue specific expression of these genes is very probable, based on the above-mentioned observations and the identification of cell type specific transcription factor binding sites in different rainbow trout IFN promoters (Zou et al. 2014). To confirm whether the genes identified here are all active and have different expression patterns as we hypothesize, an in-silico promoter analysis could be the first step. However, expression analysis of different cell types, tissues, and conditions using primers specific to the identified IFN loci will yield even more convincing answers.

5 Conclusion

The bioinformatic investigation of the IFN rich regions in the current assemblies of Chinook salmon and Atlantic salmon identified additional putative IFN genes and highlights the possibilities to finding new (IFN) genes with a focused effort. IFN genes described in earlier publications were coupled to the identified genes. An overview of the described IFN genes shows that most publications have been investigating the first described IFNs, IFNa1 and/or IFNa2.

Analysis of used primer pairs reveals a potential of most primer pairs to bind cDNA from several putative genes within each IFN subtype. This highlights the need for studies on the expression of these separate genes using gene specific primers. These will enable identification of IFN genes that are expressed in Chinook salmon and Atlantic salmon.

6 Tables and figures

Table 1: Overview of IFN genes found in Chinook salmon with corresponding accession numbers

Name	Otsh v2.0 loci	Chromosome	# of exons	# of cysteins	Accession numbers			Name Liu et al., 2020
IFNa1	LOC112258507	LG09	6	6	XM_024432926.1	XM_024432927.2	GGDU01704706.1	IFNa2.4
IFNa2	LOC112258508	LG09	6	6	XM_024432928.1	XM_024432929.2		IFNa2.5
IFNa3	LOC121847206	LG09	5	6	XM_042327308.1			
IFNa4	LOC121847204	LG09	5	6	XM_042327307.1			
IFNa5	LOC112258512	LG09	5	6	XM_024432932.1			IFNa2.3
IFNa6	LOC112259403	LG09	5	7	XM_024434108.1			IFNa2.1
IFNa7	LOC112259172	LG09	9	8	XM_024433920.1	XM_042327871.1		IFNa2.2
IFNa8	LOC112225816	LG27	6	7	XM_024389909.1	XM_024389910.2	GGDU01227959.1	IFNa1.1
IFNb1	LOC112258504	LG09	5	6	XM_024432924.1			IFNb2.3
IFNb2	LOC112259406	LG09	5	6	XM_024434111.1			IFNb2.2
IFNb3	LOC112259405	LG09	5	6	XM_024434110.1			IFNb2.1
IFNc1	LOC112259404	LG09	5	6	XM_024434109.1			IFNc2.3
IFNc2	LOC121847202	LG09	5	6	XM_042327306.1			
IFNc3	LOC112258510	LG09	5	6	XM_024432931.1			IFNc2.4
IFNc4	LOC121847201	LG09	5	6	XM_042327305.1			
IFNc5	LOC112259401	LG09	5	7	XM_024434105.1			IFNc2.1
IFNc6	LOC112259402	LG09	5	6	XM_024434106.1	XM_042327870.1		IFNc2.2
IFNc7	LOC121841123	LG27	5	5	XM_042307513.1			
IFNc8	LOC121841121	LG27	5	5	XM_042307512.1			
IFNc9	LOC121841120	LG27	5	5	XM_042307511.1			
IFNc10	LOC121841118	LG27	5	5	XM_042307509.1			
IFNc11	LOC121841115	LG27	5	5	XM_042307507.1			
IFNc12	LOC112225815	LG27	5	5	XM_024389908.1			IFNc1.5
IFNc13	LOC121841106	LG27	5	5	XM_042307463.1			
IFNc14	LOC121841114	LG27	5	5	XM_042307506.1			
IFNc15	LOC112225814	LG27	5	5	XM_024389907.1			IFNc1.4
IFNc16	LOC112225813	LG27	5	5	XM_024389905.1	XM_024389905.2		IFNc1.1
IFNe1	LOC121847315	LG09	5	4	XM_042327925.1			IFNe2.1
IFNe2	LOC112259408	LG09	5	4	XM_024434112.1	XM_042312779.1		IFNe2.2
IFNe3	LOC112258514	LG09	5	4	XM_024432933.1	XM_024432933.2		IFNe2.3
IFNe4	LOC121847316	LG09	5	4	XM_042327926.1			
IFNe5	LOC121847317	LG09	5	4	XM_042327927.1			
IFNf1	LOC112258505	LG09	5	5	XM_024432925.1	XM_024432925.2	GGDU01391346.1	IFNf2.1
IFNf2		LG09	5	5				IFNf2.2
IFNf3		LG27	5	5				IFNf1.1
Pseudogenes								
pIFNa1	LOC121838839	LG09	4 / 7	12	XM_042327309.1	GGDU01227956.1		
pIFNa2	LOC121847205	LG09	5	1				
pIFNa3	LOC112258516	LG09	7	0				
pIFNb1		LG27	5	21				
pIFNb2		LG27	2	2				
pIFNc1		LG09	2	3				
pIFNc2	LOC121847203	LG09	5	4				
pIFNe1	LOC112258511	LG09	5	1				
pIFNe2	LOC112225711	LG27	5	3				IFNe1.1
pIFNf1	LOC121847207	LG09	3	1	XR_006084148.1			
pIFNf2		LG09	2	0				
IFNlike group								
IFNlike1	LOC121841054	LG27	5	3	XM_042307093.1			
IFNlike2	LOC121841055	LG27	5	3	XM_042307094.1			
IFNlike3	LOC121841056	LG27	6	1	XM_042307095.1			
IFNlike4	LOC121841057	LG27	5	0	XM_042307096.1			
IFNlike_motif1		LG27	1	2				
IFNlike_motif2		LG27	1	3				
IFNlike_motif3		LG27	1	2				
IFNlike_motif4		LG27	1	3				
IFNlike_motif5		LG27	1	2				
IFNlike_CDS1		LG27	4	5				
IFNlike_CDS2		LG27	4	5				

Table 2: Overview of IFN genes found in Atlantic salmon with corresponding accession numbers

Name	ICSASG_v2 loci	Chromosome	# of exons	# of cysteins	Accession numbers				Name Liu et al., 2020	Name Sun et al., 2009
IFNa1	100137019	ssa28	5	7	NM_001123710.1	AY216594	EU768890.1	DQ354152.1		**IFNa1**
IFNa3	LOC106600964	ssa03	6	7	XM_014192772.1	XM_014192773.1			IFNa2.2	IFNa3
IFNa4	LOC106600963	ssa03	6	7	XM_014192770.1	XM_014192771.1	GBRB01066895.1	AY327546.1	IFNa2.4	
IFNa5	LOC106600865	ssa03	7	6	XM_014192595.1	XM_014192596.1			IFNa2.3	
IFNa6	LOC106600969	ssa03	6	7	XM_014192776.1	XM_014192777.1	XM_014192778.1		IFNa2.1	
IFNa7	LOC106607463	ssa06	6	6	XM_014204440.1	XM_014204439.1	JT836113.1		IFNa1.1	
IFNb1	LOC106600866	ssa03	5	6	XM_014192597.1				IFNb2.3	*IFNb1*
IFNb2	LOC101448042	ssa03	5	6	NM_001279095.1	JX524152.1			IFNb2.2	*IFNb2*
IFNb4	LOC106600967	ssa03	5	6	XR_001327924.1	XR_001327923.1			IFNb2.1	*IFNb4*
IFNc1		ssa03	5	8		EU735544			IFNc2.3	**IFNc1**
IFNc2	LOC106600965	ssa03	6	6	XM_014192774.1	EU768890.1			IFNc2.4	**IFNc2**
IFNc4	LOC101448043	ssa03	5	6	NM_001279097.1	JX524153.1	EU735549		IFNc2.2	IFNc4
IFNc5	LOC106600970	ssa03	6	6	XM_014192779.1				IFNc2.1	IFNc5
IFNc6	LOC106607529	ssa06	5	5	XM_014204560.1				IFNc1.3	
IFNc7	LOC106607528	ssa06	5	5	XM_014204559.1				IFNc1.2	
IFNc8	LOC106607525	ssa06	5	5	XM_014204556.1				IFNc1.1	
IFNe1	LOC106600780	ssa03	5	5	XM_014192432.1				IFNe2.1	
IFNe2	LOC106600867	ssa03	5	4	XM_014192598.1				IFNe2.2	
IFNe3	LOC106600962	ssa03	5	4	XM_014192769.1				IFNe2.3	
IFNe4	LOC106607407	ssa06	5	3					IFNe1.1	
IFNe5	LOC106589965	ssa28	5	4	XM_014180435.1					
IFNf1	106600961	ssa03	5	5	XM_014192768.1	GBRB01049694.1			IFNf2.1	
IFNf2		ssa03	5	7					IFNf2.2	
IFNf3	LOC106607408	ssa06	5	5	XM_014204335.1				IFNf1.1	
Unplaced										
IFNa2	100136436	unplaced	2	5	NM_001123570.1 DQ354154.1	AY216595	NP_001117042.1	DQ354153.1		IFNa2
IFNa8	LOC106596334	unplaced	5	5	XM_014187640.1	XM_014187639.1				
IFNc9	LOC106594534	unplaced	5	5	XM_014185901.1					
IFNc10	LOC106597742	unplaced	5	5	XM_014188889.1					
IFNc11	LOC106594533	unplaced	5	5	XM_014185900.1					
IFNc12	LOC106597870	unplaced	5	5	XM_014188992.1					
IFNc13	LOC106597883	unplaced	5	5	XM_014189004.1					
IFNc14	LOC106595256	unplaced	5	5	XM_014186631.1					
Missing/partial sequence	Corresponding gene								Comment	
part1	LOC106600783	ssa03			XM_014192435.1					
part2	LOC106590949	unplaced			XM_014182127.1					
part3	As3, As5				AY327544.1				IFN intron 1	
part4	As6, As7, As10				AY327545.1				IFN exon 4, 5, part CDS	
part5	As11 snp				EU735552.1				partial CDS	
part6	As14 snp				EU735545				partial CDS	
part7	As14 snp				EU735547				partial CDS	
part8	As14 snp				EU735548				partial CDS	
part9	As14 snp				EU735550				partial CDS	
Pseudogenes										
pIFNa1	LOC106600966	ssa03	6	7	EU621898.1					
pIFNa2	LOC106589964	ssa28	6	9	DQ354155.1					
pIFNc1		ssa03	6	6						
pIFNf1		ssa03	8	7						
pIFNf2		ssa03	3	3						

Table 3: genes found on the IFN rich region on Chinook salmon chromosome 9

Locus annotation in Otsh V2.0	Gene name	Location start	Location end	Orientation
112258502	gh1	68702700	68706923	forward
112258503	rdm1	68712582	68718604	forward
LOC112259401	IFNc5	68727946	68729120	reverse
LOC112259402	IFNc6	68734335	68738392	reverse
LOC112259403	IFNa6	68741221	68742788	reverse
LOC112259404	IFNc1	68747318	68748314	reverse
LOC112259405	IFNb3	68754821	68756819	reverse
LOC112259406	IFNb2	68762435	68764176	reverse
LOC112259172	IFNa7	68768077	68916252	reverse
-	pIFNc1	68773099	68777576	forward
LOC112258504	IFNb1	68779388	68781112	forward
LOC112258510	IFNc3	68788790	68789781	forward
LOC112258512	IFNa5	68799041	68800578	forward
LOC121847203	pIFNc2	68804123	68805109	forward
LOC121847204	IFNa4	68814367	68815934	forward
LOC121847202	IFNc2	68819488	68820477	forward
LOC121847205	pIFNa2	68825709	68827287	forward
LOC121847201	IFNc4	68830833	68831820	forward
LOC121847206	IFNa3	68841078	68842647	forward
LOC112258505	IFNf1	68853372	68854678	reverse
LOC112258507	IFNa1	68867073	68871229	forward
-	IFNe motifs	68878732	68878764	forward
LOC112258508	IFNa2	68885967	68890060	forward
LOC112258511	pIFNe1	68905329	68908568	reverse
LOC112258513	ncRNA	68911505	68914146	reverse
LOC112259408	IFNe2	68919718	68921562	reverse
LOC121847315	IFNe1	68932555	68934027	reverse
LOC121847316	IFNe4	68943677	68944807	reverse
LOC121847207	pIFNf1	68946131	68949923	reverse
LOC112258516	pIFNa3	68954291	68988551	reverse
LOC121838839	pIFNa1	68990622	68994542	reverse
LOC121847317	IFNe5	68999012	69000468	reverse
LOC121847318	IFNe3	69010489	69011620	reverse
-	pIFNf2	69013360	69015520	reverse
-	IFNf2	69021007	69022266	reverse
LOC112258518	Glycosyl transferase	69032183	69046469	reverse

Table 4: genes found on the IFN rich region on Chinook salmon chromosome 27

Locus annotation in Otsh V2.0	Gene name	Location start	Location end	Orientation
LOC112225899	gh2	4019191	4022796	forward
LOC112225710	Other	4023768	4036306	forward
LOC112225813	IFNc16	4043929	4044985	reverse
-	IFNc motifs	4059615	4059635	forward
LOC112225814	IFNc15	4062865	4064013	forward
-	IFNb motif	4066730	4066750	reverse
LOC121841115	IFNc11	4069527	4070675	reverse
-	IFNlike_motif1	4072332	4072631	forward
-	IFNlike_CDS1	4072468	4077483	reverse
-	pIFNb1	4073788	4079067	reverse
LOC121841114	IFNc14	4080246	4081394	reverse
-	IFNlike_motif2	4083056	4083370	forward
LOC121841054	IFNlike1	4084926	4092090	reverse
LOC121841123	IFNc7	4087794	4088942	reverse
-	IFNlike_motif3	4090610	4090909	forward
LOC121841055	IFNlike2	4092480	4099583	reverse
LOC121841120	IFNc9	4095363	4096511	reverse
-	IFNlike_motif4	4098103	4098402	forward
LOC121841056	IFNlike3	4099973	4111154	reverse
LOC121841121	IFNc8	4102841	4103989	reverse
-	IFNlike_motif5	4105660	4105959	forward
-	IFNlike_CDS2	4105796	4110870	reverse
LOC121841118	IFNc10	4113674	4114822	reverse
LOC121841057	IFNlike4	4118364	4131852	reverse
LOC121841106	IFNc13	4121241	4122389	reverse
-	pIFNb2	4124341	4126047	reverse
LOC112225815	IFNc12	4127281	4128429	reverse
-	IFNlike motif no CDS	4130181	4130278	reverse
LOC112225711	pIFNe2	4149400	4150792	reverse
LOC112225816	IFNa8	4155884	4162063	forward
LOC112225900	Glycosyl transferase	4174116	4186892	reverse
LOC121841069	Transposase / reverse transcriptase	4207390	4239209	forward
-	IFNf3	4212801	4214130	forward

Table 5: genes found on the IFN rich region on Atlantic salmon chromosome 3

Locus annotation in ICSASG_v2	Gene name	Location start	Location end	Orientation
LOC100136588	gh1	55678562	55682490	forward
100196558	rdm1	55686098	55695815	forward
-	Integrase	55700602	55701508	reverse
-	Reverse transcriptase	55702073	55706651	forward
-	pIFNc1	55707961	55712318	reverse
LOC106600970	IFNc5	55716190	55717328	reverse
LOC106600969	IFNa6	55727454	55729466	reverse
LOC101448043	IFNc4	55731238	55732228	reverse
LOC106600967	IFNb4	55747457	55749239	reverse
LOC106600966	pIFNa1	55756787	55758934	reverse
-	IFNc1	55760100	55761269	reverse
LOC101448042	IFNb2	55772855	55774641	reverse
LOC106600965	IFNc2	55796179	55797317	forward
LOC106600964	IFNa3	55799175	55801097	forward
LOC106600783	part1	55801386	55824844	forward
LOC106600866	IFNb1	55832742	55834529	forward
LOC106600782	Myosin	55836608	55847673	reverse
LOC106600781	Myosin / tegument	55847722	55855436	forward
LOC106600780	IFNe1	55861389	55862513	forward
LOC106600867	IFNe2	55870612	55872012	forward
LOC106600865	IFNa5	55875156	55879301	forward
-	pIFNf2	55905905	55906769	reverse
LOC106600963	IFNa4	55922799	55926694	reverse
LOC106600962	IFNe3	55929815	55932230	reverse
-	IFNf2	55947106	55949280	reverse
106600961	IFNf1	55951725	55952826	forward
100194757	gbgt1	55965832	56037519	reverse
	pIFNf1	56016635	56022728	forward

Table 6: genes found on the IFN rich region on Atlantic salmon chromosome 6

Locus annotation in ICSASG_v2	Gene name	Location start	Location end	Orientation
LOC106607410	No	42038089	42048449	reverse
LOC106607409	Integrin partial	42047557	42058525	reverse
LOC106607408	IFNf3	42070186	42071293	forward
LOC106607464	Glycosyl transferase	42097014	42120441	forward
LOC106607463	IFNa7	42127627	42133799	reverse
-	IFNb/e motif	42150451	42150473	forward
LOC106607407	IFNe4	42161193	42162731	forward
-	IFNc/f motif	42178132	42178161	forward
LOC106607529	IFNc6	42183269	42184420	forward
LOC106607528	IFNc7	42189379	42190530	forward
LOC106607526	Transposase	42208138	42209492	reverse
LOC106607525	IFNc8	42214623	42215598	forward
LOC106607524	No	42227269	42234236	reverse
LOC106607462	gh2	42238585	42242242	reverse

Table 7: genes found on the IFN rich region on Atlantic salmon chromosome 6

Locus annotation in ICSASG_v2	Gene name	Location start	Location end	Orientation
LOC106589966	Immunoglobulin	36825026	36833853	reverse
LOC106589969	No	36816824	36875858	reverse
LOC106589964	pIFNa2	36879098	36882369	reverse
LOC106589965	IFNe5	36885549	36887007	reverse
100137019	IFNa1	36891016	36891836	forward
LOC106589972	TACC / DNA polymerase III	36908261	36956650	forward

Chinook salmon chromosome 9		Atlantic salmon chromosome 3	
Gene name	Orientation	Gene name	Orientation
gh1	forward	gh1	forward
rdm1	forward	rdm1	forward
-	-	Integrase	reverse
-	-	Reverse transcriptase	forward
IFNc5	reverse	pIFNc1	reverse
IFNc6	reverse	IFNc5	reverse
IFNa6	reverse	IFNa6	reverse
IFNc1	reverse	IFNc4	reverse
IFNb3	reverse	IFNb3	reverse
IFNb2	reverse	-	-
IFNa7	reverse	pIFNa1	reverse
pIFNc1	forward	IFNc1	reverse
IFNb1	forward	IFNb2	reverse
IFNc3	forward	IFNc2	forward
IFNa5	forward	IFNa3	forward
pIFNc2	forward	-	-
IFNa4	forward	part1	forward
IFNc2	forward	-	-
pIFNa2	forward	-	-
IFNc4	forward	-	-
IFNa3	forward	-	-
IFNf1	reverse	-	-
IFNa1	forward	-	-
IFNe motifs	forward	-	-
IFNa2	forward	-	-
pIFNe1	reverse	-	-
-	-	IFNb1	forward
-	-	Myosin	reverse
ncRNA	reverse	Myosin / tegument	forward
IFNe2	reverse	IFNe1	forward
IFNe1	reverse	IFNe2	forward
IFNe4	reverse	-	-
-	-	IFNa5	forward
pIFNf1	reverse	pIFNf2	reverse
pIFNa3	reverse	-	-
pIFNa1	reverse	IFNa4	reverse
IFNe5	reverse	IFNe3	reverse
IFNe3	reverse	-	-
pIFNf2	reverse	IFNf2	reverse
IFNf2	reverse	IFNf1	forward
Glycosyl transferase	reverse	gbgt1	reverse
-	-	pIFNf1	forward

Strong match
Weak match
Minor mismatch
Major mismatch

Figure 1: syntheny between the IFN rich regions of Chinook salmon chromosome 9 and Atlantic salmon chromosome 3

Chinook salmon chromosome 27		Atlantic salmon chromosome 6	
Gene name	**Orientation**	**Gene**	**Orientation**
gh2	forward	gh2	forward
Other	forward	No	forward
IFNc16	reverse	IFNc8	reverse
IFNc motifs	forward	-	-
-	-	Transposase	forward
IFNc15	forward	IFNc7	reverse
IFNb motif	reverse	-	-
IFNc11	reverse	IFNc6	reverse
IFNlike_motif1	forward	-	-
IFNlike_CDS1	reverse	-	-
pIFNb1	reverse	-	-
IFNc14	reverse	-	-
IFNlike_motif2	forward	-	-
IFNlike1	reverse	-	-
IFNc7	reverse	-	-
IFNlike_motif3	forward	-	-
IFNlike2	reverse	-	-
IFNc9	reverse	-	-
IFNlike_motif4	forward	-	-
IFNlike3	reverse	-	-
IFNc8	reverse	-	-
IFNlike_motif5	forward	-	-
IFNlike_CDS2	reverse	-	-
IFNc10	reverse	-	-
IFNlike4	reverse	-	-
IFNc13	reverse	-	-
pIFNb2	reverse	-	-
IFNc12	reverse	IFNc/f motif	reverse
IFN like motif, no CDS	reverse	-	-
pIFNe2	reverse	IFNe4	reverse
-	-	IFNb/e motif	reverse
IFNa8	forward	IFNa7	forward
Glycosyl transferase	reverse	Glycosyl transferase	reversse
-	-	IFNf3	reverse
Transposase / reverse transcriptase	forward	Integrin partial	forward
IFNf3	forward	-	-

Figure 2: syntheny between the IFN rich regions of Chinook salmon chromosome 27 and Atlantic salmon chromosome 6 (inverted)

Strong match
Weak match
Minor mismatch
Major mismatch
Inversion

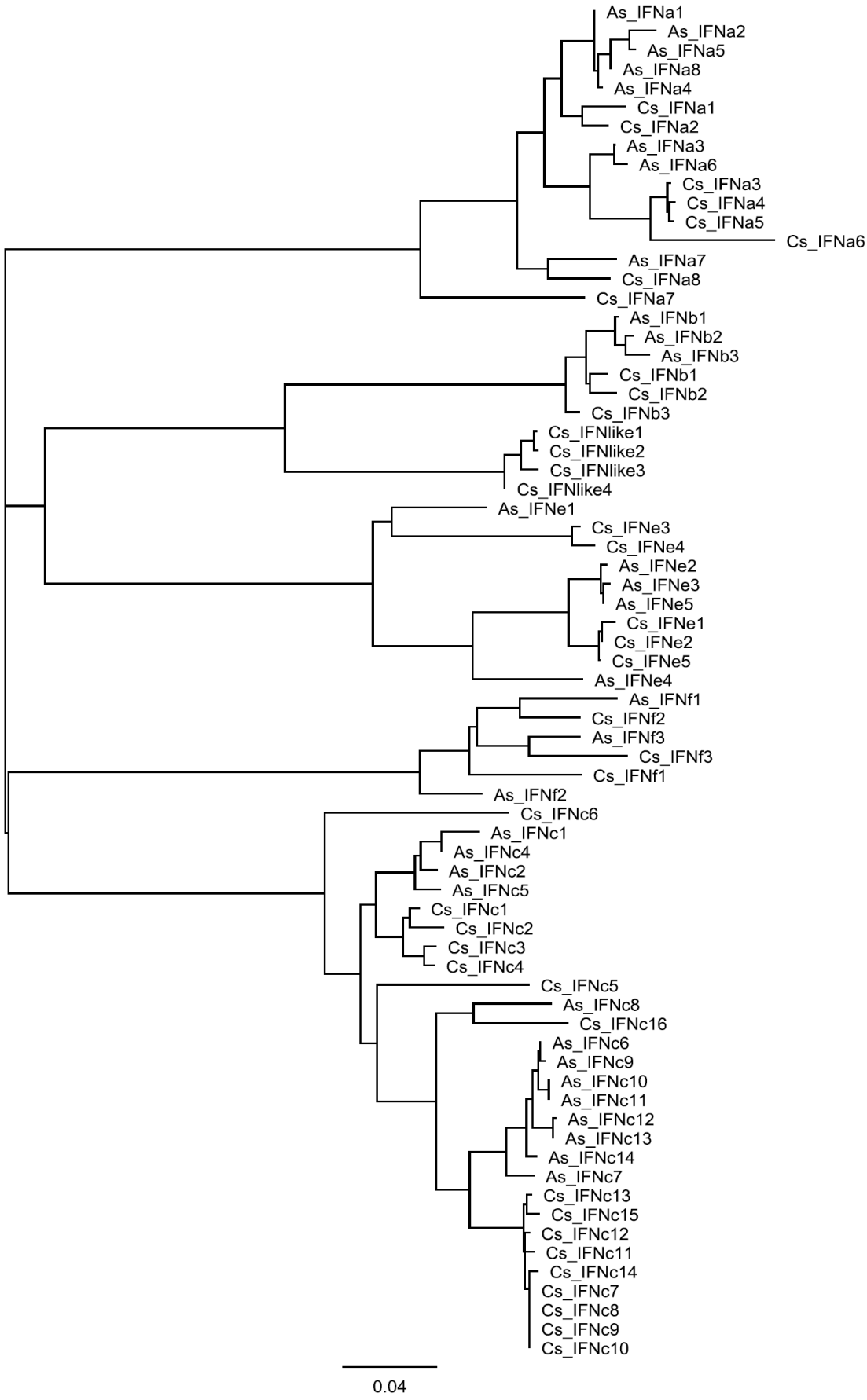


Figure 3: Phylogenetic tree of the IFN genes identified in Chinook salmon and Atlantic salmon

Table 8: publications on expression of IFN genes in Chinook salmon mentioning accession numbers

Chinook salmon expression publications	
Rt_IFN-a1 (IFN1)	**As_IFNa2**
(Lulijwa et al. 2020a)	(Gamil et al. 2015)
(Lulijwa et al. 2020b)	
(Monjo et al. 2017)	
(Semple et al. 2018)	

Table 9: publications on functional investigations of IFN genes in Atlantic salmon mentioning accession numbers

Atlantic salmon other publications			
As_IFNa1	**As_IFNa2**	**As_IFNb2**	**As_IFNc4**
(Berg et al. 2009)	(Bergan et al. 2008)	(Chang et al. 2014)	(Chang et al. 2014)
(Bergan et al. 2006)	(Krasnov et al. 2011)	(Chang et al. 2015)	(Chang et al. 2015)
(Bergan et al. 2008)	(Ooi et al. 2008a)	(Chang et al. 2016)	(Chang et al. 2016)
(Bergan et al. 2010)	(Ooi et al. 2008b)	(Svingerud et al. 2012)	(Robertsen et al. 2016)
(Chang et al. 2014)	(Robertsen et al. 2003)	(Svingerud et al. 2013)	(Svingerud et al. 2012)
(Chang et al. 2015)	(Xu et al. 2016)		(Svingerud et al. 2013)
(Chang et al. 2016)			
(Jørgensen et al. 2007a)			
(Kileng et al. 2007)			
(Kileng et al. 2009)			
(Robertsen et al. 2003)			
(Robertsen et al. 2016)			
(Skjesol et al. 2009)			
(Skjesol et al. 2014)			
(Sun et al. 2011)			
(Svingerud et al. 2012)			
(Svingerud et al. 2013)			
(Xu et al. 2010)			

Table 10: publications on expression of IFN genes in Atlantic salmon mentioning accession numbers

Atlantic salmon expression publications				
As_IFNa1	**As_IFNa1&2**	**As_IFNa2**	**As_IFNa3-7**	**As_IFNd**
(Andresen et al. 2020)	(Fourrier et al. 2009)	(Bergan et al. 2006)	(Munir et al. 2020)	(Svingerud et al. 2012)
(Bela-Ong et al. 2020)	(Gahlawat et al. 2009)	(Dahle et al. 2015)		(Svenning et al. 2019)
(Bergan et al. 2006)	(McBeath et al. 2006)	(Das et al. 2007)		
(Chang et al. 2014)	(Monjo et al. 2017)	(Wessel et al. 2015)		
(Herath et al. 2013)	(Robertsen et al. 2003)			
(Jørgensen et al. 2006)	(Skjesol et al. 2011)			
(Jørgensen et al. 2007b)				
(Kileng et al. 2007)				
(Kileng et al. 2008)				
(Kileng et al. 2009)				
(Lauksund et al. 2009)				
(Lauksund et al. 2015)				
(Sun et al. 2009)				
(Sun et al. 2011)				
(Svenning et al. 2019)				
(Svingerud et al. 2012)				
(Workenhe et al. 2008)				
(Xu et al. 2010)				
(Xu et al. 2012)				

Table 11: possible binding sites for Chinook and Atlantic salmon IFN expression analysis primers described in publications. The number of mismatches per primer pair is indicated for genes with less than 5 mismatches, **bold** indicates complete lack of mismatches and thus a perfect fit.

Chinook salmon primer pairs		
Pair #	**Mismatches (0, 1)**	**Mismatches (2,3,4)**
1	GH1&2	-
2	CsIFNa1, **CsIFNa2**, CsIFNa3, CsIFNa4, CsIFNa5	CsIFNa6, CsIFNa8
3	**CsIFNa1**	CsIFNa2
4	-	CsIFNa2, CsIFNa3, CsIFNa4, CsIFNa5, CsIFNa6, CsIFNa7
Atlantic salmon primer pairs		
Pair #	**Mismatches (0, 1)**	**Mismatches (2,3,4)**
5	AsIFNa1, **AsIFNa2**, AsIFNa4, AsIFNa8, As_part2	AsIFNa5
6	**AsIFNa1, AsIFNa2, AsIFNa4, AsIFNa5**, AsIFNa7, AsIFNa8	As_part2
7	**AsIFNa1, AsIFNa2, AsIFNa4,** AsIFNa5, AsIFNa7, AsIFNa8, As_part2	AsIFNa3, AsIFNa6, As_part1
8	**AsIFNa1, AsIFNa2, AsIFNa3, AsIFNa4**, **AsIFNa5, AsIFNa6, AsIFNa7, AsIFNa8**	-
9	**AsIFNa1**, AsIFNa5, AsIFNa8, As_part2	AsIFNa2, AsIFNa3, AsIFNa4, AsIFNa6, AsIFNa7, As_part1
10	**AsIFNa1, AsIFNa2, AsIFNa4,** AsIFNa5, AsIFNa8, As_part2	AsIFNa3, AsIFNa6, As_part1
11	No	-
12	**AsIFNb1, AsIFNb2, AsIFNb4**	-
13	**AsIFNc2, AsIFNc4, AsIFNc5**	AsIFNc8
14	**AsIFNa1, AsIFNa2, AsIFNa4,** AsIFNa3, AsIFNa5, AsIFNa6, AsIFNa8	-
15	**AsIFNc1, AsIFNc2, AsIFNc4,** AsIFNc5	-
16	-	AsIFNa3, AsIFNa6, As_part1 (lacking 6 of 24 bp)
17	**AsIFNa1, AsIFNa3, AsIFNa4, AsIFNa5, AsIFNa6, AsIFNa7**	AsIFNa2, AsIFNa8
18	**AsIFNa1, AsIFNa4, AsIFNa8**	-
19	**AsIFNc1, AsIFNc2, AsIFNc4, AsIFNc5**	AsIFNc6, AsIFNc7, AsIFNc9, AsIFNc10, AsIFNc11, AsIFNc12, AsIFNc13, AsIFNc14
20	**AsIFNa1, AsIFNa2, AsIFNa4,** AsIFNa3, AsIFNa5, AsIFNa6, AsIFNa8	-
21	**AsIFNa1, AsIFNa2, AsIFNa4,** AsIFNa5**, AsIFNa8, As_part2**	AsIFNa3, AsIFNa6, As_part1
Chinook salmon & Atlantic salmon primer pairs		
Pair #	**Mismatches (0, 1)**	**Mismatches (2,3,4)**
22	**CsIFNa3,** CsIFNa4, **AsIFNa6, As_part1**	CsIFNa1, CsIFNa2, CsIFNa5, CsIFNa7, AsIFNa1, AsIFNa2, AsIFNa3, AsIFNa4, AsIFNa5, AsIFNa7, AsIFNa8, As_part2
23	CsIFNa1	AsIFNa4
24	CsIFNa8, AsIFNa2, AsIFNa3, AsIFNa4, AsIFNa5, AsIFNa6, AsIFNa8	CsIFNa1
Atlantic salmon primer-probe combinations		
	Full matches	**Partial matches**
7	**AsIFNa1, AsIFNa2, AsIFNa4, AsIFNa8, As_part2**	AsIFNa3, AsIFNa5, AsIFNa6, AsIFNa7, As_part1
19	AsIFNc1, AsIFNc2, AsIFNc4	AsIFNc5
20	AsIFNa1, AsIFNa2, AsIFNa4, AsIFNa8	AsIFNa3, AsIFNa5, AsIFNa6

Table 12: publications using the investigated primer pairs

Primer pair	Papers					
1	(Rajanbabu und Chen 2011)					
2	(Lulijwa et al. 2020a)	(Lulijwa et al. 2020b)	(Monjo et al. 2017)	(Semple et al. 2018)		
3	(Gamil et al. 2015)					
4	(Skotheim 2009)					
5	(Robertsen et al. 2003)					
6	(Xu et al. 2010)	(Xu et al. 2012)				
7	(Fourrier et al. 2009)	(Gahlawat et al. 2009)	(McBeath et al. 2006)	(McBeath et al. 2007)		
8	(Andresen et al. 2020)	(Bela-Ong et al. 2020)	(Chang et al. 2014)	(Herath et al. 2013)	(Kileng et al. 2007)	
"	(Kileng et al. 2009)	(Lauksund et al. 2009)	(Sun et al. 2009)	(Sun et al. 2011)	(Svingerud et al. 2012)	(Workenhe et al. 2008)
9	(Andresen et al. 2020)	(Jørgensen et al. 2006)	(Jørgensen et al. 2007a)			
10	(Munir et al. 2020)					
11	(Reyes-Cerpa et al. 2012)					
12	(Lauksund et al. 2015)	(Sun et al. 2009)	(Svenning et al. 2019)	(Svingerud et al. 2012)		
13	(Lauksund et al. 2015)	(Svenning et al. 2019)	(Svingerud et al. 2012)			
14	(Svenning et al. 2019)					
15	(Sun et al. 2009)					
16	(Sun et al. 2009)					
17	(Bergan et al. 2006)	(Lauksund et al. 2015)				
18	(Bergan et al. 2006)					
19	(Skjesol et al. 2011)					
20	(Skjesol et al. 2011)					
21	(Dahle et al. 2015)	(Wessel et al. 2015)				
22	(Schalburg et al. 2008)					
23	(Ooi et al. 2008a)					
24	(Collet et al. 2007)					

References

Abbas, Abul K.; Lichtman, Andrew H.; Pillai, Shiv (2020): Basic immunology. Functions and disorders of the immune system. Unter Mitarbeit von David L. Baker und Alexandra Baker. 6th edition. Philadelphia: Elsevier (Medical Textbooks Immunology).

Andresen, Adriana Magalhães Santos; Boudinot, Pierre; Gjøen, Tor (2020): Kinetics of transcriptional response against poly (I:C) and infectious salmon anemia virus (ISAV) in Atlantic salmon kidney (ASK) cell line. In: *Developmental and comparative immunology* 110, S. 103716. DOI: 10.1016/j.dci.2020.103716.

Bela-Ong, Dennis Berbulla; Greiner-Tollersrud, Linn; van der Andreas Wal, Yorick; Jensen, Ingvill; Seternes, Ole Morten; Jørgensen, Jorunn B. (2020): Infection and microbial molecular motifs modulate transcription of the interferon-inducible gene ifit5 in a teleost fish. In: *Developmental and comparative immunology* 111, S. 103746. DOI: 10.1016/j.dci.2020.103746.

Berg, Kristel; Svingerud, Tina; Sun, Baojian; Robertsen, Børre (2009): An antiserum against Atlantic salmon IFNa1 detects IFN and neutralizes antiviral activity produced by poly I:C stimulated cells. In: *Developmental and comparative immunology* 33 (4), S. 638–645. DOI: 10.1016/j.dci.2008.11.005.

Bergan, Veronica; Jagus, Rosemary; Lauksund, Silje; Kileng, Øyvind; Robertsen, Børre (2008): The Atlantic salmon Z-DNA binding protein kinase phosphorylates translation initiation factor 2 alpha and constitutes a unique orthologue to the mammalian dsRNA-activated protein kinase R. In: *The FEBS journal* 275 (1), S. 184–197. DOI: 10.1111/j.1742-4658.2007.06188.x.

Bergan, Veronica; Kileng, Øyvind; Sun, Baojian; Robertsen, Børre (2010): Regulation and function of interferon regulatory factors of Atlantic salmon. In: *Molecular immunology* 47 (11-12), S. 2005–2014. DOI: 10.1016/j.molimm.2010.04.015.

Bergan, Veronica; Steinsvik, Silje; Xu, Hao; Kileng, Øyvind; Robertsen, Børre (2006): Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions. In: *The FEBS journal* 273 (17), S. 3893–3906. DOI: 10.1111/j.1742-4658.2006.05382.x.

Chang, Chia-Jung; Jenssen, Iris; Robertsen, Børre (2016): Protection of Atlantic salmon against salmonid alphavirus infection by type I interferons IFNa, IFNb and IFNc. In: *Fish & shellfish immunology* 57, S. 35–40. DOI: 10.1016/j.fsi.2016.08.020.

Chang, Chia-Jung; Robertsen, Camilla; Sun, Baojian; Robertsen, Børre (2014): Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid. In: *Vaccine* 32 (36), S. 4695–4702. DOI: 10.1016/j.vaccine.2014.05.059.

Chang, Chia-Jung; Sun, Baojian; Robertsen, Børre (2015): Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model. In: *Vaccine* 33 (21), S. 2442–2448. DOI: 10.1016/j.vaccine.2015.03.093.

Chang, Mingxian; Nie, Pin; Collet, Bertrand; Secombes, Christopher J.; Zou, Jun (2009): Identification of an additional two-cysteine containing type I interferon in rainbow trout Oncorhynchus mykiss provides evidence of a major gene duplication event within this gene family in teleosts. In: *Immunogenetics* 61 (4), S. 315–325. DOI: 10.1007/s00251-009-0366-y.

Chang, Ming-Xian; Zou, Jun; Nie, Pin; Huang, Bei; Yu, Zhanglong; Collet, Bertrand; Secombes, Chris J. (2013): Intracellular interferons in fish: a unique means to combat viral infection. In: *PLoS pathogens* 9 (11), e1003736. DOI: 10.1371/journal.ppat.1003736.

Collet, Bertrand; Munro, Eann S.; Gahlawat, Suresh; Acosta, Felix; Garcia, Jose; Roemelt, Christina et al. (2007): Infectious pancreatic necrosis virus suppresses type I interferon signalling in

rainbow trout gonad cell line but not in Atlantic salmon macrophages. In: *Fish & shellfish immunology* 22 (1-2), S. 44–56. DOI: 10.1016/j.fsi.2006.03.011.

Dahle, Maria Krudtaa; Wessel, Øystein; Timmerhaus, Gerrit; Nyman, Ingvild Berg; Jørgensen, Sven Martin; Rimstad, Espen; Krasnov, Aleksei (2015): Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV). In: *Fish & shellfish immunology* 45 (2), S. 780–790. DOI: 10.1016/j.fsi.2015.05.049.

Das, B. K.; Collet, B.; Snow, M.; Ellis, A. E. (2007): Expression of interferon type I and II, Mx and gammaIP genes in the kidney of Atlantic salmon, Salmo salar, is induced during smolting. In: *Fish & shellfish immunology* 23 (3), S. 514–520. DOI: 10.1016/j.fsi.2006.10.010.

Ding, Yang; Ao, Jingqun; Huang, Xiaohong; Chen, Xinhua (2016): Identification of Two Subgroups of Type I IFNs in Perciforme Fish Large Yellow Croaker Larimichthys crocea Provides Novel Insights into Function and Regulation of Fish Type I IFNs. In: *Frontiers in immunology* 7, S. 343. DOI: 10.3389/fimmu.2016.00343.

Fourrier, M. C. S.; Arnold, M. F. F.; Collet, B.; Munro, E. S. (2009): The effect of sub-culturing on the basal level of type I interferon (IFN) gene expression in the Salmon Head Kidney (SHK-1) cell line. In: *Fish & shellfish immunology* 27 (3), S. 535–538. DOI: 10.1016/j.fsi.2009.06.017.

Gahlawat, Suresh K.; Ellis, Anthony E.; Collet, Bertrand (2009): Expression of interferon and interferon--induced genes in Atlantic salmon Salmo salar cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV. In: *Fish & shellfish immunology* 26 (4), S. 672–675. DOI: 10.1016/j.fsi.2009.02.021.

Gamil, Amr A. A.; Evensen, Øystein; Mutoloki, Stephen (2015): Infection Profiles of Selected Aquabirnavirus Isolates in CHSE Cells. In: *PloS one* 10 (8), e0134173. DOI: 10.1371/journal.pone.0134173.

Herath, Tharangani K.; Thompson, Kim D.; Adams, Alexandra; Richards, Randolph H. (2013): Interferon-mediated host response in experimentally induced salmonid alphavirus 1 infection in Atlantic salmon (Salmo salar L.). In: *Veterinary immunology and immunopathology* 155 (1-2), S. 9–20. DOI: 10.1016/j.vetimm.2013.05.011.

Johansen, Audny; Collet, Bertrand; Sandaker, Elin; Secombes, Christopher J.; Jørgensen, Jorunn B. (2004): Quantification of Atlantic salmon type-I interferon using an Mx1 promoter reporter gene assay. In: *Fish & shellfish immunology* 16 (2), S. 173–184. DOI: 10.1016/S1050-4648(03)00060-3.

Jørgensen, Jorunn B.; Johansen, Audny; Hegseth, Marit N.; Zou, Jun; Robertsen, Børre; Collet, Bertrand; Secombes, Christopher J. (2007a): A recombinant CHSE-214 cell line expressing an Mx1 promoter-reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. In: *Fish & shellfish immunology* 23 (6), S. 1294–1303. DOI: 10.1016/j.fsi.2007.07.008.

Jørgensen, Sven Martin; Hetland, Dyveke Lem; Press, Charles McL; Grimholt, Unni; Gjøen, Tor (2007b): Effect of early infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes and type I and II interferon in Atlantic salmon (Salmo salar L.) tissues. In: *Fish & shellfish immunology* 23 (3), S. 576–588. DOI: 10.1016/j.fsi.2007.01.005.

Jørgensen, Sven Martin; Syvertsen, Berit Lyng; Lyng-Syvertsen, Berit; Lukacs, Morten; Grimholt, Unni; Gjøen, Tor (2006): Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic salmon (Salmo salar L.) cells. In: *Fish & shellfish immunology* 21 (5), S. 548–560. DOI: 10.1016/j.fsi.2006.03.004.

Kileng, Oyvind; Bergan, Veronica; Workenhe, Samuel Tekeste; Robertsen, Børre (2009): Structural and functional studies of an IRF-7-like gene from Atlantic salmon. In: *Developmental and comparative immunology* 33 (1), S. 18–27. DOI: 10.1016/j.dci.2008.07.020.

Kileng, Øyvind; Albuquerque, Artur; Robertsen, Børre (2008): Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for Toll-like receptor 7. In: *Fish & shellfish immunology* 24 (5), S. 514–522. DOI: 10.1016/j.fsi.2007.10.005.

Kileng, Øyvind; Brundtland, Marthe Iren; Robertsen, Børre (2007): Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon. In: *Fish & shellfish immunology* 23 (2), S. 378–389. DOI: 10.1016/j.fsi.2006.11.011.

Krasnov, Aleksei; Timmerhaus, Gerrit; Schiøtz, Berit Lyng; Torgersen, Jacob; Afanasyev, Sergey; Iliev, Dimitar et al. (2011): Genomic survey of early responses to viruses in Atlantic salmon, Salmo salar L. In: *Molecular immunology* 49 (1-2), S. 163–174. DOI: 10.1016/j.molimm.2011.08.007.

Lauksund, Silje; Greiner-Tollersrud, Linn; Chang, Chia-Jung; Robertsen, Børre (2015): Infectious pancreatic necrosis virus proteins VP2, VP3, VP4 and VP5 antagonize IFNa1 promoter activation while VP1 induces IFNa1. In: *Virus research* 196, S. 113–121. DOI: 10.1016/j.virusres.2014.11.018.

Lauksund, Silje; Svingerud, Tina; Bergan, Veronica; Robertsen, Børre (2009): Atlantic salmon IPS-1 mediates induction of IFNa1 and activation of NF-kappaB and localizes to mitochondria. In: *Developmental and comparative immunology* 33 (11), S. 1196–1204. DOI: 10.1016/j.dci.2009.06.012.

Li, Chun; Greiner-Tollersrud, Linn; Robertsen, Børre (2016): Infectious salmon anemia virus segment 7 ORF1 and segment 8 ORF2 proteins inhibit IRF mediated activation of the Atlantic salmon IFNa1 promoter. In: *Fish & shellfish immunology* 52, S. 258–262. DOI: 10.1016/j.fsi.2016.03.038.

Liu, Fuguo; Wang, Tiehui; Petit, Jules; Forlenza, Maria; Chen, Xinhua; Chen, Liangbiao et al. (2020): Evolution of IFN subgroups in bony fish - 2. analysis of subgroup appearance and expansion in teleost fish with a focus on salmonids. In: *Fish & shellfish immunology* 98, S. 564–573. DOI: 10.1016/j.fsi.2020.01.039.

Lulijwa, Ronald; Alfaro, Andrea C.; Merien, Fabrice; Burdass, Mark; Meyer, Jill; Venter, Leonie; Young, Tim (2020a): Metabolic and immune responses of Chinook salmon (Oncorhynchus tshawytscha) smolts to a short-term poly (I:C) challenge. In: *Journal of fish biology* 96 (3), S. 731–746. DOI: 10.1111/jfb.14266.

Lulijwa, Ronald; Alfaro, Andrea C.; Merien, Fabrice; Burdass, Mark; Meyer, Jill; Venter, Leonie; Young, Tim (2020b): Polyinosinic:polycytidylic acid in vivo enhances Chinook salmon (Oncorhynchus tshawytscha) immunity and alters the fish metabolome. In: *Aquacult Int* 28 (6), S. 2437–2463. DOI: 10.1007/s10499-020-00599-w.

Martin, S. A. M.; Taggart, J. B.; Seear, P.; Bron, J. E.; Talbot, R.; Teale, A. J. et al. (2007): Interferon type I and type II responses in an Atlantic salmon (Salmo salar) SHK-1 cell line by the salmon TRAITS/SGP microarray. In: *Physiological genomics* 32 (1), S. 33–44. DOI: 10.1152/physiolgenomics.00064.2007.

McBeath, A. J. A.; Snow, M.; Secombes, C. J.; Ellis, A. E.; Collet, B. (2007): Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (Salmo salar) following infection

with infectious pancreatic necrosis virus and infectious salmon anaemia virus. In: *Fish & shellfish immunology* 22 (3), S. 230–241. DOI: 10.1016/j.fsi.2006.05.004.

McBeath, Alastair J. A.; Collet, Bertrand; Paley, Richard; Duraffour, Sophie; Aspehaug, Vidar; Biering, Eirik et al. (2006): Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. In: *Virus research* 115 (2), S. 176–184. DOI: 10.1016/j.virusres.2005.08.005.

Monjo, A. L.; Poynter, S. J.; DeWitte-Orr, S. J. (2017): CHSE-214: A model for studying extracellular dsRNA sensing in vitro. In: *Fish & shellfish immunology* 68, S. 266–271. DOI: 10.1016/j.fsi.2017.07.025.

Munir, Danish; Munro, Eann S.; Secombes, Christopher J.; Dooley, Helen (2020): Atlantic salmon kidney (ASK) cells are an effective model to characterise interferon (IFN) and IFN-induced gene expression following salmonid alphavirus infection. In: *Fish & shellfish immunology* 106, S. 792–795. DOI: 10.1016/j.fsi.2020.08.043.

Ooi, Ei Lin; Verjan, Noel; Haraguchi, Ikumi; Oshima, Takeo; Kondo, Hidehiro; Hirono, Ikuo et al. (2008a): Innate immunomodulation with recombinant interferon-alpha enhances resistance of rainbow trout (Oncorhynchus mykiss) to infectious hematopoietic necrosis virus. In: *Developmental and comparative immunology* 32 (10), S. 1211–1220. DOI: 10.1016/j.dci.2008.03.010.

Ooi, Ei Lin; Verjan, Noel; Hirono, Ikuo; Nochi, Tomonori; Kondo, Hidehiro; Aoki, Takashi et al. (2008b): Biological characterisation of a recombinant Atlantic salmon type I interferon synthesized in Escherichia coli. In: *Fish & shellfish immunology* 24 (5), S. 506–513. DOI: 10.1016/j.fsi.2007.10.004.

Pestka, Sidney; Krause, Christopher D.; Walter, Mark R. (2004): Interferons, interferon-like cytokines, and their receptors. In: *Immunological reviews* 202, S. 8–32. DOI: 10.1111/j.0105-2896.2004.00204.x.

Rajanbabu, Venugopal; Chen, Jyh-Yih (2011): Antiviral function of tilapia hepcidin 1-5 and its modulation of immune-related gene expressions against infectious pancreatic necrosis virus (IPNV) in Chinook salmon embryo (CHSE)-214 cells. In: *Fish & shellfish immunology* 30 (1), S. 39–44. DOI: 10.1016/j.fsi.2010.09.005.

Reyes-Cerpa, Sebastián; Reyes-López, Felipe E.; Toro-Ascuy, Daniela; Ibañez, Jorge; Maisey, Kevin; Sandino, Ana María; Imarai, Mónica (2012): IPNV modulation of pro and anti-inflammatory cytokine expression in Atlantic salmon might help the establishment of infection and persistence. In: *Fish & shellfish immunology* 32 (2), S. 291–300. DOI: 10.1016/j.fsi.2011.11.018.

Robertsen, Børre (2018): The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. In: *Developmental and comparative immunology* 80, S. 41–52. DOI: 10.1016/j.dci.2017.02.005.

Robertsen, Børre; Bergan, Veronica; Røkenes, Torunn; Larsen, Rannveig; Albuquerque, Artur (2003): Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. In: *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 23 (10), S. 601–612. DOI: 10.1089/107999003322485107.

Robertsen, Børre; Chang, Chia-Jung; Bratland, Lisa (2016): IFN-adjuvanted DNA vaccine against infectious salmon anemia virus: Antibody kinetics and longevity of IFN expression. In: *Fish & shellfish immunology* 54, S. 328–332. DOI: 10.1016/j.fsi.2016.04.027.

Røkenes, Torunn P.; Larsen, Rannveig; Robertsen, Børre (2007): Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. In: *Molecular immunology* 44 (5), S. 950–959. DOI: 10.1016/j.molimm.2006.03.016.

Schalburg, Kristian R. von; Yazawa, Ryosuke; Boer, Johan de; Lubieniecki, Krzysztof P.; Goh, Benjamin; Straub, Christopher A. et al. (2008): Isolation, characterization and comparison of Atlantic and Chinook salmon growth hormone 1 and 2. In: *BMC genomics* 9, S. 522. DOI: 10.1186/1471-2164-9-522.

Schultzhaus, Zachary; Wang, Zheng; Stenger, David (2021): CRISPR-based enrichment strategies for targeted sequencing. In: *Biotechnology advances* 46, S. 107672. DOI: 10.1016/j.biotechadv.2020.107672.

Secombes, Chris J.; Zou, Jun (2017): Evolution of Interferons and Interferon Receptors. In: *Frontiers in immunology* 8, S. 209. DOI: 10.3389/fimmu.2017.00209.

Semple, S. L.; Vo, N. T. K.; Poynter, S. J.; Li, M.; Heath, D. D.; DeWitte-Orr, S. J.; Dixon, B. (2018): Extracellular dsRNA induces a type I interferon response mediated via class A scavenger receptors in a novel Chinook salmon derived spleen cell line. In: *Developmental and comparative immunology* 89, S. 93–101. DOI: 10.1016/j.dci.2018.08.010.

Skjesol, Astrid; Aamo, Toril; Hegseth, Marit Nøst; Robertsen, Børre; Jørgensen, Jorunn B. (2009): The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signaling is inhibited by IPNV infection. In: *Virus research* 143 (1), S. 53–60. DOI: 10.1016/j.virusres.2009.03.004.

Skjesol, Astrid; Liebe, Theresa; Iliev, Dimitar B.; Thomassen, Ernst I. S.; Tollersrud, Linn Greiner; Sobhkhez, Mehrdad et al. (2014): Functional conservation of suppressors of cytokine signaling proteins between teleosts and mammals: Atlantic salmon SOCS1 binds to JAK/STAT family members and suppresses type I and II IFN signaling. In: *Developmental and comparative immunology* 45 (1), S. 177–189. DOI: 10.1016/j.dci.2014.02.009.

Skjesol, Astrid; Skjæveland, Ingrid; Elnæs, Marianne; Timmerhaus, Gerrit; Fredriksen, Børge N.; Jørgensen, Sven Martin et al. (2011): IPNV with high and low virulence: host immune responses and viral mutations during infection. In: *Virology journal* 8, S. 396. DOI: 10.1186/1743-422X-8-396.

Skotheim, Svenn Amund: Co-infection with Norwegian Salmonid Alphavirus (NSAV) and Infectious Pancreatic Necrosis Virus (IPNV) in Chinook Salmon Embryo Cells (CHSE-214). The University of Bergen. Online verfügbar unter https://bora.uib.no/bora-xmlui/handle/1956/3564.

Sun, Baojian; Robertsen, Børre; Wang, Zhiqiang; Liu, Bin (2009): Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. In: *Developmental and comparative immunology* 33 (4), S. 547–558. DOI: 10.1016/j.dci.2008.10.001.

Sun, Baojian; Skjæveland, Ingrid; Svingerud, Tina; Zou, Jun; Jørgensen, Jorunn; Robertsen, Børre (2011): Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. In: *Journal of virology* 85 (17), S. 9188–9198. DOI: 10.1128/JVI.00319-11.

Svenning, Steingrim; Gondek-Wyrozemska, Agata T.; van der Wal, Yorick Andreas; Robertsen, Børre; Jensen, Ingvill; Jørgensen, Jorunn B.; Edholm, Eva-Stina (2019): Microbial Danger Signals

Control Transcriptional Induction of Distinct MHC Class I L Lineage Genes in Atlantic Salmon. In: *Frontiers in immunology* 10, S. 2425. DOI: 10.3389/fimmu.2019.02425.

Svingerud, Tina; Holand, Jenni Kristin; Robertsen, Børre (2013): Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture. In: *Virus research* 177 (2), S. 163–170. DOI: 10.1016/j.virusres.2013.08.004.

Svingerud, Tina; Solstad, Terese; Sun, Baojian; Nyrud, May Liss J.; Kileng, Øyvind; Greiner-Tollersrud, Linn; Robertsen, Børre (2012): Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues. In: *Journal of immunology (Baltimore, Md. : 1950)* 189 (12), S. 5912–5923. DOI: 10.4049/jimmunol.1201188.

Uribe, C.; Folch, H.; Enriquez, R.; Moran, G. (2011): Innate and adaptive immunity in teleost fish: a review. In: *Veterinarni Medicina* 56 (No. 10), S. 486–503. DOI: 10.17221/3294-VETMED.

Wessel, Øystein; Olsen, Christel Moræus; Rimstad, Espen; Dahle, Maria Krudtaa (2015): Piscine orthoreovirus (PRV) replicates in Atlantic salmon (Salmo salar L.) erythrocytes ex vivo. In: *Veterinary research* 46, S. 26. DOI: 10.1186/s13567-015-0154-7.

Workenhe, Samuel T.; Kibenge, Molly J. T.; Wright, Glenda M.; Wadowska, Dorota W.; Groman, David B.; Kibenge, Frederick S. B. (2008): Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes. In: *Virology journal* 5, S. 36. DOI: 10.1186/1743-422X-5-36.

Xu, Cheng; Evensen, Øystein; Munang'andu, Hetron (2016): De Novo Transcriptome Analysis Shows That SAV-3 Infection Upregulates Pattern Recognition Receptors of the Endosomal Toll-Like and RIG-I-Like Receptor Signaling Pathways in Macrophage/Dendritic Like TO-Cells. In: *Viruses* 8 (4), S. 114. DOI: 10.3390/v8040114.

Xu, Cheng; Guo, Tz-Chun; Mutoloki, Stephen; Haugland, Oyvind; Evensen, Oystein (2012): Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. In: *Veterinary research* 43, S. 78. DOI: 10.1186/1297-9716-43-78.

Xu, Cheng; Guo, Tz-Chun; Mutoloki, Stephen; Haugland, Øyvind; Marjara, Inderjit S.; Evensen, Øystein (2010): Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro. In: *Journal of virology* 84 (17), S. 8903–8912. DOI: 10.1128/JVI.00851-10.

Zou, Jun; Gorgoglione, Bartolomeo; Taylor, Nicholas G. H.; Summathed, Thitiya; Lee, Po-Tsang; Panigrahi, Akshaya et al. (2014): Salmonids have an extraordinary complex type I IFN system: characterization of the IFN locus in rainbow trout oncorhynchus mykiss reveals two novel IFN subgroups. In: *Journal of immunology (Baltimore, Md. : 1950)* 193 (5), S. 2273–2286. DOI: 10.4049/jimmunol.1301796.