Assessment of ISAV stability using a novel *in vivo* method with Atlantic salmon fry combined with immunological and histological analysis

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Abstract

Since 1984, Infectious salmon anemia virus (ISAV) has been a great issue in the aquaculture industry, where it today threatens the health and welfare of Atlantic salmon worldwide. The spread and transmission of ISAV is an area of great importance when making epidemiological inferences and further creating and deciding specific control measures for avoiding the disease. Determination of the stability of a virus in its natural environment becomes crucial information for the development of such measures. Current knowledge on ISAV stability in its natural environment is varying, both in results and methodology which poses the need for a standardized method for determination. In this thesis, a novel *in vivo* challenge method using Atlantic salmon fry, is used for determining ISAV stability. In addition, the histopathology, tissue distribution and immunological response was assessed for the ISAV challenged fry.

This thesis show that ISAV retains its infectivity for over 1 day in raw seawater at 10°C and loses infectivity after 7 days. The larger study this thesis was a part of, demonstrates that ISAV remains infective for up until 4 days raw seawater and 7 days in filtered seawater.

No histopathological changes in challenged fry, but ISAV had spread systemically in the challenged salmon fry. A stimulation of the innate immune response was seen in challenged salmon fry, with no stimulation seen in uninfected fry. The degree of innate immune response was found to strongly correlate with the viral load in ISAV challenged fry.

The results contribute to current knowledge of ISAV stability. In addition, the study demonstrates how ISAV challenged Atlantic salmon fry, using this method, is viable for further histopathological, *in situ*-hybridization and immunological measurements. This will hopefully open the possibility for creating better epidemiological models when assessing the spread of ISAV in seawater and potentially other aquatic viruses.

List of abbreviations

AAV	Aquatic Animal Viruses
6FAM:	6-carboxyfluorescein
Bp	nucleic acid base pairs
CARD	Caspase Activation and Recruitment Domains
cDNA	complemantary DNA
CHSE-214	Chinook Salmon Embryo Cells
CNS	Central Nervous System
CPE	Cytopathic effect
CpG	Cytosine and Guanin with phosphodiester bond
Ct	Cycle threshold
Ct_{cal}	Cycle threshold for the calibrator
dig	days incubation of ISAV prior to fry group bath challenge
DIM	Dissolved Inorganic Matter
DNA	deoxyribonucleic acid
DOM	Dissolved Organic Matter
dpc	days post challenge
dpi	days post infection
EL1-α	Elongation Factor 1-α
F	Fusion protein
FFPE	Formalin Fixed Paraffin Embedded
For	Forward
GTPase	Guanin Tri Phosphatase enzyme
HE	Hemagglutinin Esterase
hig	hours incubation of ISAV prior to fry group bath challenge
HPR	Hyper Variable Region
HPR0	non-deleted Hyper Variable Region
HPRΔ	deleted Hyper Variable Region
i.m	intramuscular
i.p	intraperitonal
IFN	interferon
IFN-α	interferon -type I
IFN-β	interferon -β
IFN-γ	interferon type II
IgMs	immunoglobulin M
IKK-β	Inducible Kinase kB
IL-10	interleukin-10
IMR	Institute of Marine Research
IPNV	Infectious Pancreas Necrosis Virus
IPS	Interferon-β Promotor Stimulator
IRAK	IL-1 receptor associated kinases signalling
IRF	Interferon Regulatory Factor
ISA	Infectious Salmon Anaemia

ISAV	Infectious Salmon Anaemia Virus
ISG	Interferon Stimulated Genes
ISH	*in situ* Hybridization
JAK/STAT	Janus Kinases/ Signal Transducer and Activator of Transcription proteins
kb	kilo bases
kDa	kilo Daltons
LRR	Leucine Rich Repeats
M	Matrix protein
MAVS	Mitochondrial Associated Viral Signal
MDA5	Melanoma Differentiation-Associated gene 5
MGBNFQ	Minor Groove Binding Non-Fluorescent Quencher
MHC	Major Histo combability Complex
MID	Minimum Infectious Dose
mRNA	messenger RNA
Mx	Myxovirus resistance protein
My D88	Myeloid Differentiation factor 88
NEP	Nucleic Export Protein
NFSA	Norwegian Food and Safety Authority
NK-κB	Nuclear Factor κB
NOD	Nucleotide binding Oligomerization Domain like receptor
NP	Nuclear Protein
NS	Non-Structural protein
P1,P2,PA	Polymerase proteins
PKR	Protein Kinase A
PMCV	Piscine Myocarditis Virus
PRR	Pattern Recognition Receptor
PRV	Piscine Orthoreovirus
RBC	Red blood cell
RED	RNAscope® 2.5 HD Detection Reagent
Rev	Reverse
RIG-I	Retinoic acid Inducible Gene I
RLRs	RIG-I Like Receptors
RNA	ribonucleic acids
RPL-1	RNA polymerase -1
RT-qPCR	Reverse Transcriptase quantitative Polymerase Chain Reaction
s8ORF2	antagonistic ISAV protein
SAV	Salmonid Alpha Virus
SD	Standard Deviation
ssRNA	single stranded RNA
TCID50	median tissue culture infectious dose
TLRs	Toll Like Receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β
UV	ultra violet
VAMPs	Viral Associated Molecular Patterns
Vip	Viperine

vRNA	viral RNA
vRNA-M1	viral RNA-matrix protein attached

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1. Introduction

1.1 Infectious salmon anemia

The first reports of infectious salmon anemia (ISAV) were made in 1984 from fish farms on the south-western coast of Norway. Several outbreaks were reported in the following years before it was recognized as a transferable disease caused by an *orthomyxo*-like virus called infectious salmon anemia virus (ISAV) (Thorud and Djupvik, 1988). ISA outbreaks continued to increase until they reached a peak in 1991 with 80 outbreaks reported from several geographical locations (Veterinærinstituttet, 2022)(see **Figure 1.1**). Following this peak, several control and safety measures were implemented, leading toa drastic decrease in the number of reported outbreaks (Rimstad and Mjaaland, 2002, Hästein et al., 1999). The number of outbreaks has since averaged approximately 10 per year between 1993 and 2019. However, in 2020 and 2021 more than 20 outbreaks were reported each year, a level not recorded since 1992, which is a concerning development (Veterinærinstituttet, 2022).

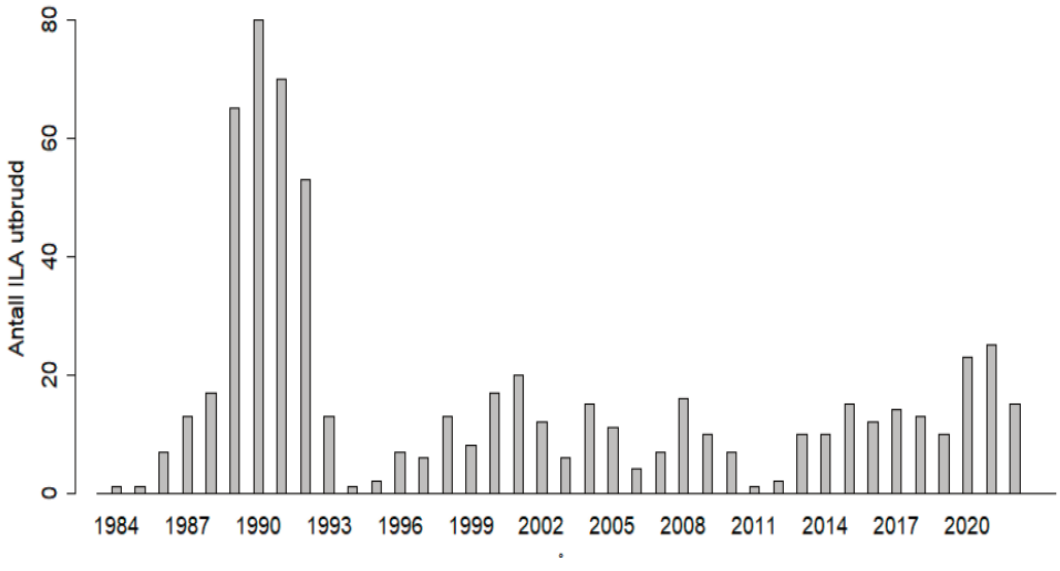

***Figure 1.1**:* Bar chart showing number of outbreaks of ISA each year since 1984 to 2022 (Veterinærinstituttet, 2022)

1.1.2 Structure of ISAV

ISAV virions have a diameter of approximately 100nm with a icosahedral symmetry, though it can also elicit a pleomorphic shape (Dannevig et al., 1995) (see **Figure 1.2**). It is covered with an envelope with small mushroom shaped glycoproteins covering the surface that measure about 10 nm in diameter (Falk, 1997). The genome consists of 8 single stranded RNA segments with negative polarity and are attached to a disk-like ribonucleoprotein via 12 nucleotides, (Falk, 1997, Mjaaland et al., 1997). Each segment is about 1.0-2.3 kb and encode their own specific proteins, where there are 4 structural and 6 non-structural proteins (Mjaaland et al., 1997). Two different surface proteins, namely the hemagglutinin esterase protein (HE) and fusion protein (F), are embedded in a host-derived lipid membrane that surrounds an inner surface consisting of a matrix protein (M). Inside this is the vRNA with 3 polymerase subunits (P1, P2 and PA) and nucleoprotein (NP) attached to the vRNA (Mjaaland et al., 1997). Other important non-structural proteins include two innate anti-viral response antagonists (NS and s8ORF2) and a nuclear export protein (NEP).

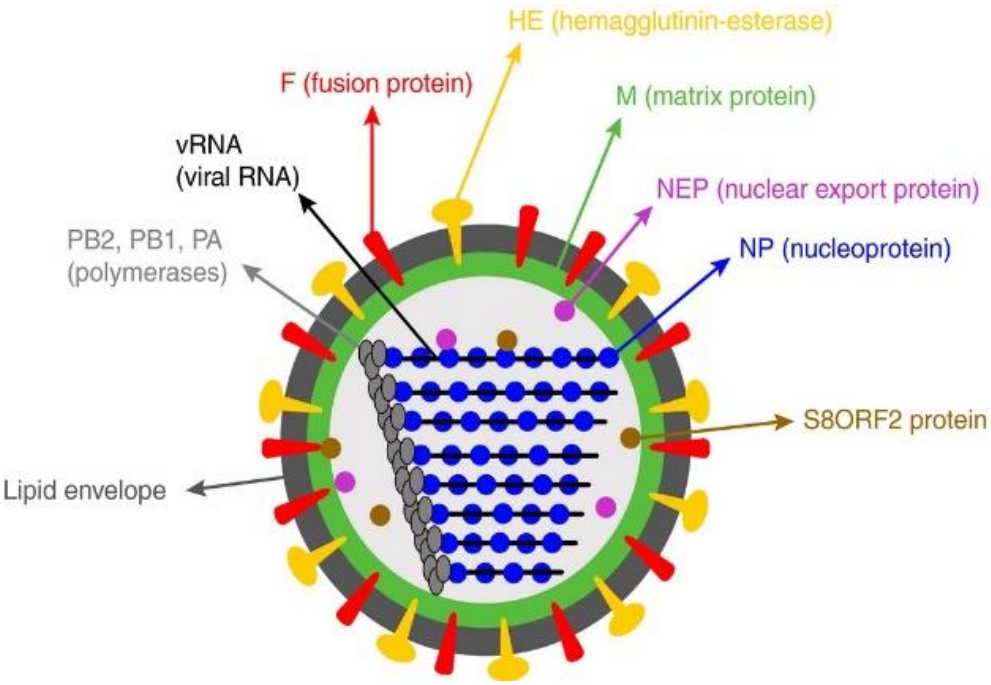

Figure 1.2: Overview of ISAV structure. Figure reproduced from Rimstad and Markussen, 2020 .

1.1.2 Viral entry and cell tropism

The main target cells of ISAV are endothelial cells lining the blood vessels of all organs. ISAV is also able to attach, but not replicate in, red blood cells (RBCs). Despite endothelial cells being determined to be a major target cell for ISAV, any morphological changes is shown to be absent even during later stages of infection (Aamelfot et al., 2012).

4-*O*- acetylated sialic acids are a receptor determinant for ISAV where ISAVs HE- protein binds to the 4-O-acytelated sialic acids covering proteins on target cell and initiates cellular uptake of ISAV (Hellebø et al., 2004). Distribution of 4-0 acetylated receptors seem to correlate with that of endothelial cells and RBCs, but not with other cells and is therefore believed to be important for infection of ISAV (Aamelfot et al., 2012).

By budding out from the endothelial cells into the bloodstream, the virus is further believed to be distributed via the RBCs around the circulatory system, attaching to new endothelial cells and creating a systemic infection (viremia).

The port of entry for ISAV was first believed to be primarily across the gill membrane, but ISAV has also been shown to replicate at mucosal surfaces including the skin and pectoral fins (Aamelfot et al., 2015).

1.1.3 Viral Replication

Infection is initiated by HE binding to the surface receptor of a target cell, initiating endocytosis. The naturally low pH of the endosome will then induce the fusion protein to modify and fuse the viral membrane with the endosomal one, resulting in the release of the virus into the cytosol of the cell. The matrix protein will then dissociate and release the vRNA segments (Eliassen et al., 2000). The four proteins (PB2, PB1, PBA, NP) have nucleus localization motifs that will direct the vRNA into the nucleus. These proteins are less than 40kDa and can thereby diffuse into the nucleus, where the transcription and replication of the ISAV genome occurs (Ramly et al., 2013). Both mRNA and vRNA are transcribed by exploits the hosts transcription machinery. The vRNA steals the caps of host mRNA, and is later

polyadenylated, disguising them as normal host mRNA. This in turn outcompetes the hosts own mRNA and favors the vRNA protein translation (Sandvik et al., 2000). A viral nuclear exporting protein is vital for the export of the vRNA-M1 complex produced inside the nucleus. The exported vRNA-M1 complex is transferred to the outer cellular membrane where newly synthesized virions are assembled and bud out with the host cell membrane (Ramly et al., 2013).

1.1.4 ISA disease

During outbreaks of ISAV the fish can often look sluggish and lethargic with varying degrees of mortality, ranging from 0% to 90%. Clinical signs can also vary, but often include pale gills, exophthalmia, and hemorrhagic petechia in the visceral fat and somatic muscle. (Thorud and Djupvik, 1988, Rimstad and Mjaaland, 2002). Internal signs often include a dark and swollen spleen with small hemorrhagic petechiae lining different organs like the intestine, visceral fat, muscle, and swim bladder (see **Figure 1.3**). Ascites is also a normal finding in affected fish (Thorud and Djupvik, 1988, Rimstad and Mjaaland, 2002). However, these findings can differ, and the most consistent sign for ISA is lowered hematocrit values, often as low as 10%. This results in the observed anemia, which is the most characteristic sign of ISA (Thorud and Djupvik, 1988).

Figure 1.3: Atlantic salmon showing clinical signs of ISA. Picture from the Fish Health Report (Veterinærinstituttet, 2022) and modified for this thesis

Histopathological lesions can include focal conflating hemorrhagic necrosis in the liver and hemorrhagic lesions in kidney and spleen. Congestion of blood vessels and gill lamellae are often seen with congestion and necrosis of hematopoietic tissue. (Evensen et al., 1991, Thorud and Djupvik, 1988).

Why the anemia occurs is still not completely understood, but it is previously reported that the RBCs lifespan is heavily reduced as its fragility is increased when bound by ISAV (Aamelfot et al., 2014). The subsequent hypoxia caused by the decrease in RBCs is believed to cause secondary pathology in necrosis seen in various organs.

There is a strong correlation between the increase of histological lesions and of *in situ* hybridization (ISH) signals. Endothelial cells and RBCs were also previously found to predominantly give positive signals in ISH compared to other organs like liver and kidneys, which concurs with the perception that ISAV has endothelial cells as its main target cell for replication (Moneke et al., 2005).

ISAV will use approximately 5-10 days post infection (dpi) to reach a detectable level in any organ and will reach peak viremia at approximately 15 dpi. A temporary dip in viremia will then follow and the host will either overcome the infection or die (Rimstad and Markussen, 2020).

1.1.5 Two variants of ISAV: HPR0 and HPRΔ

The HE- protein consists of three proteins that make up a trimer that is embedded in the lipid membrane of the virus and is vital for the binding to target cells (see **Figure 1.4, a**). The F-protein is also made up in a trimer that is embedded in the viral membrane (Falk et al., 2004) (see **Figure 1.4, b**). The F- protein is first activated when it is exposed to pH 5.4-5.6, in the endosome. It is then cleaved from its F0 state into F1 and F2 fragments that can fuse with the endosomal membrane and release the virion into the cytoplasm (Aspehaug et al., 2005).

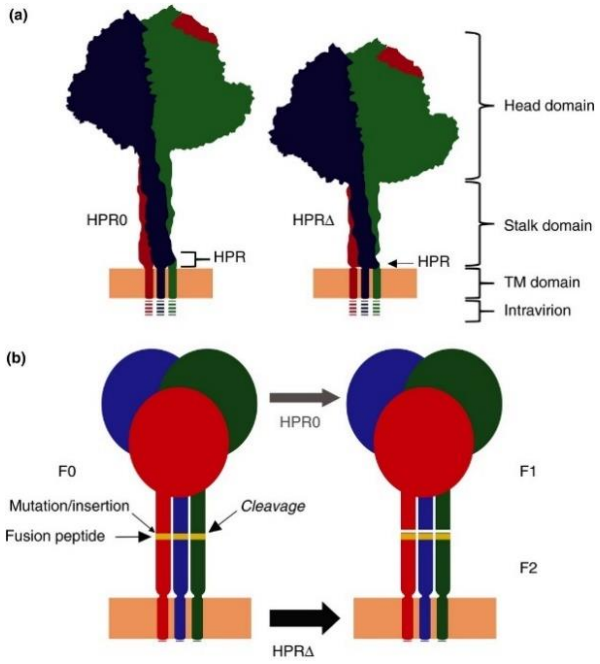

***Figure 1.2*:** Illustration of the showing the difference between the non-virulent (HPR0) and virulent (HPRΔ) hemagglutinin esterase protein (HE). **a)** shows the domains of the HE as well as the hypervariable region (HPR) and its location. **b)** Shows the cleavage area in in the Fusion protein (F) Figure reproduced from Rimstad and Markussen, 2020.

Two variants of ISAV exist, a non-virulent form (HPR0) and a virulent form (HPRΔ). The virulent variant, HPRΔ, is thought to originate from the non-virulent HPR0 by a deletion in the hyper-variable region (HPR) in the hemagglutinin esterase surface protein and 8-9 aa insertions above the cleavage site of the fusion protein (Devold et al., 2001, Christiansen et al., 2017, Markussen et al., 2008, Fourrier et al., 2015).

The nonvirulent HPR0 variant has been shown to be highly prevalent in Norwegian aquaculture as well as in wild Atlantic salmon, but does not seem to elicit any significant virulence, in contrast to the deleted HPRΔ- variant (Lyngstad et al., 2012, Madhun et al., 2019).

1.1.6 Suseptible hosts

Atlantic salmon (*Salmo salar*) is regarded as the main host for ISAV, but it has been isolated and shown to propogate in other wild salmonids including sea trout (*Salmo trutta*), arctic char (*Salvinus alpinus*) and rainbow trout (*Onchorynchus mykiss*). It does not however afflict any significant pathogenesis in these species and they can be regarded as asymptomatic carriers of ISAV (Rolland and Winton, 2003). Infection of these species can therefore be regarded as a potenital reservoir for ISAV. All life stages of Atlantic salmon are considered susceptible to ISAV infection and even though it is generally considered an issue in the adult fish, the earlier parr and smolt stages can experience mortalties as high as 100% after infection (Grefsrud et al., 2021).

1.2 Transmission

1.2.1 HPR0→HPRΔ transmission

The spread of ISAV is complicated, where the avirulent and virulent variants of ISAV can both be the source of outbreaks. Currently, there is no active surveillance of ISAV HPR0 in Norway and it is only tested for ISAV HPRΔ when fish show signs of ISA. Great genetic similarity has been shown to exist between the HPR0 and the HPRΔ which suggests that transition between the two are of importance for an outbreak to occur (Lyngstad, 2011, Lyngstad et al., 2012, Vike et al., 2009). A Norwegian surveillance program has recently shown that fish from HPR0 positive hatcheries have been shown to be the origin of later HPRΔ seawater outbreaks (Jansen and Moldal, 2022). However, this route of infection is only thought to be a minor source of the spreading of outbreaks.

1.2.2 Vertical transmission

Vertical transmission of ISAV has also been proven to occur but is regarded as of less significance in the spread of the virulent ISAV, but might be an important route in the spread of avirulent ISAV (Nylund et al., 2019).

1.2.3 Horizontal transmission

ISAV is mainly believed to spread from infected fish-farms to neighboring fish-farms through hydrographic water movements, in addition to various alternative routes, like live fish movements, non-disinfected equipment, and via personnel (Salama and Murray, 2013, Oidtmann et al., 2014, Mardones et al., 2014). From the time an individual is infected until it begins to show visual clinical symptoms, can take up to 4 weeks. This will thus increase the window for the virus to spread to nearby surrounding fish farms. Fish farms in Norway today, are purposefully placed in areas with great movement of surface water, so to avoid an over-concentration of nutritious salts that can negatively affect the local environment (Grefsrud et al., 2021). This, however, can potentially lead to increased spreading of waterborne pathogens like ISAV.

A modelling study done among Norwegian salmon farms predicted that the risk of ISAV outbreaks decreased with increasing distance between the farms (Aldrin et al., 2011). The hydrodynamic environment in each specific area can be determinant for the spread of ISAV virions, where variation in the local topography, precipitation, tidal waves, wind movements and river outlets affect the movement of water. In Norwegian fjords, the stream of sea water generally flows out of the fjord, but sometimes inwards and the variation in water movement can vary between 0-50 km over 24 hours. It is assumed that a virus concentration will be diluted as it is transported (Grefsrud et al., 2021).

1.5 Virus stability and its importance

1.5.1 Viruses and their stability

Several factors can affect the survival of a waterborne virus, where temperature is the one factor that has the greatest impact on viral survival. There is a variation in temperature tolerance among different viruses, but a general fact is that an elevated temperature will decrease survival time, while a low temperature will increase its survival time. Freezing will in the process inactivate a certain share of the virus but will preserve surviving viruses for longer periods until thawing. Higher temperatures will denature various proteins making up

the virus, like surface, capsid or internal proteins and thus destroy its ability to infect a host (Pinon and Vialette, 2018, Shoham et al., 2012).

Solar influence or more specifically, UV-radiation, can also strongly influence the survival of a waterborne virus, where it can damage the nucleic acids in its genome and other parts (Flannery et al., 2013). Viruses thus generally survive longer in dark conditions, unexposed to UV-radiation.

The sterility of water, i.e., absence of microbial organisms, is also shown to consistently increase viruses' survival time. This is believed to be caused by bacteria's ability to produce proteolytic enzymes that can degrade proteins of the virus and leaving it non-infectious (Hawley and Garver, 2008).

Other factors that have negative impact on virus survival can be disinfectants, extreme pH-values, water hardness (i.e., high levels of $Ca^{2+}$and Mg^{2+}) and aeration levels (Shahid et al., 2009).

Waterborne viruses do however have ways to protect themselves from these adverse survival factors. Aggregation, where viruses bind together to each other, creates a collective defense against the various factors explained, where they together are more resilient to external stresses. Adhesion to dissolved organic and inorganic materials (DOMs and DIMs, respectively) has also been shown to provide protection to free waterborne viruses. This can include proteins, cell tissue and dissolved sediments (Gassilloud and Gantzer, 2005).

The survival of a virus is dependent on a range of factors, but different types of viruses can cope with their surroundings differently. Enveloped viruses are generally more prone to inactivation than non-enveloped viruses due to their fragile lipid membrane. On the other hand, Influenza viruses, which are enveloped, are shown to have lower rates of inactivation post-freezing (Pinon and Vialette, 2018).

1.5.2 Stability of ISAV

In the literature review by Oidtman et. al. (2018), the available literature on the persistence of different relevant aquatic animal viruses (AAV's), including ISAV was assessed. Regarding the persistence of ISAV, only a few articles have been published on this subject which all displayed an array of different results in addition to variation in methods applied (Nylund et al., 1994, Tapia et al., 2013, Vike et al., 2014, Rimstad and Mjaaland, 2002). An overview of the literature and results are given in **Table 1.1.** Rimstad and Mjaaland (2002) claimed that ISAV would remain infective for 4 months at 4°C and another study by McLeod et al (2003) claimed it would remain infective for 7 days at both 4-6 and 15°C (Oidtmann et al., 2018). *In vitro* assessments reported ISAV survival from 10 days in sterile seawater to 70 days in sterile fresh water at 10-15°C (Tapia et al., 2013). The experiment by Vike et al. (2014) is mainly focusing on how ultraviolet radiation (UVR) affects ISAV survival in natural and sterile seawater and reports that ISAV loses its infectivity after 3 hours in raw seawater and 24 hours in sterile seawater at 10°C. Nylund et al. (1994) found that it could remain infective in seawater for 20 hours in seawater and 4 days in kidney tissue at 6°C. Both these experiments are using Atlantic salmon smolts as *in vivo* models of assessing ISAV infectivity, but the methods vary as well as the results.

The results from Vike et al. (2014) stands in contrast to other results but are the only one this far to investigate ISAVs stability in natural seawater, whereas Tapia et al. (2013) used sterile seawater and Nylund et al. (1994) used a blood-water mixture as ISAV incubation medium. The reporting from McLeod et al. (2003) and from Rimstad and Mjaaland (2002) lack experimental details and are therefore non-reproducible and difficult to compare.

Increasing temperatures, UV-radiation, dissolved organic matter and oxygen levels are generally regarded to decrease the infectivity of most aquatic viruses, even though there are big differences in how long each virus species can remain infectious These factors are important when assessing the real survival of a virus in the environment. Still, the knowledge regarding ISAV survival is varying in both results and methods applied and is therefore an area that needs further clarification.

Reference	Method	Temperature	ISAV stability/survival			
			Fw		Sw	
			St	Sl	St	Sl
(Nylund et al., 1994)	Intra peritoneal (i.p) injecton in Atlantic salmon (150 gram) Unknown starting virus titre	6°C	nt	nt	Nt	≥20 hours
(Rimstad and Mjaaland, 2002) *Unpublished data*	Endpoint virus titration in cell culture Starting virus titre not indicated	4°C	nt	nt	≥112days	Nt
Data of MacLeod *et al. (*2003) in Rimstad *et al* (2011)	Virus infectivity in cell culture Starting virus titre not indicated	4-6 °C	≥7days	Nt	≥105 days	≥ 7 days
		15°C	≥7days	nt	≥21 days	≥7days
(Tapia et al., 2013)	End point virus titration in cell culture ($1x10^6$ $TCID_{50}$/ml virus, total volume of 10 mL)	5°C	65 days	Nt	9 days	Nt
		10°C	70 days	Nt	10 days	Nt
		15°C	55 days	Nt	8 days	Nt
		20°C	37 days	nt	5 days	Nt
(Vike et al., 2014)	i.p injection	10 °C UV	Nt	Nt	1 day	3 hours
		10 °C no UV	nt	nt	3 hours	3 hours

Table 1.1. Overview of ISAV stability/ survival periods including information of methods used at different temperatures in non-sterile/sterile saline (Sw) or fresh water (Fw)

1.3 Host Immune mechanisms

1.3.1 Innate immune system

Teleosts have innate immune responses, both cellular and humoral that act as a first line of defense to invading viral particles. Physical barriers, inflammatory responses, the complement system, immune cells, and various cytokines are among the important parts of the innate immune system (Magnadóttir, 2006).

1.3.2 Physical barriers

Skin, gut, and gills are the main tissues that act as a physical barrier and are covered with a layer of mucus, crucial for stopping the various invading microbial pathogens including viruses (Magnadóttir, 2006). The mucus layer physically slows down, but also traps invading pathogens allowing other parts of the fish innate immune system, present in the mucus, to act on the pathogen. This can include complement proteins, IgMs, lectins, lysosomes and antimicrobial peptides (Alexander and Ingram, 1992).

1.3.3 Inflammation

Inflammation is the body's response to tissue damage or infiltrating pathogens like viruses and consequently restoration of the tissue. Invading pathogens can encounter cells called macrophages, that are innate immune cells equipped with pathogen recognition receptors (PRRs) (see **section 1.3.4**) that can recognize non-self-particles. Upon recognition, they will engulf the pathogen through the process of phagocytosis, followed by its destruction through an oxidative burst response inside the macrophage (Grayfer and Belosevic, 2012).

Following the detection of the pathogen, the macrophage can also release soluble pro-inflammatory cytokines and chemokines like IFN-γ, type 1 IFN or IL-10 (*discussed below*) that will recruit other immune cells to the place of infection through chemotactic attraction.

1.3.4 PRRs

The innate immune system contains certain receptors that can recognize pattern associated molecular patterns (PAMPs) like lipopolysaccharides on bacteria or viral RNA/DNA from viruses. These are called pattern recognition receptors (PRRs) (Gulati et al., 2018). They serve as an essential step in the initial response to an invading viral particle, where it will upon contact induce the cells production of various pro-inflammatory cytokines that can later induce other immune cells to react (Koyama et al., 2008). These PRRs recognize pathogen associated molecular patterns (PAMPs) or for viruses, viral associated molecular patterns (VAMPs) (Takeuchi and Akira, 2009).

Three such families of PRRs have been described to exist in the innate immune system and include toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (Takeuchi and Akira, 2009)

1.3.5 Toll -like receptors

TLRs are a key receptor in the innate immune system. Some can identify VAMPs at the surface of the cell, whilst others have been discovered to remain in the endosomal membranes in the cytosol of the cell and thereby detect viruses that use the endocytic pathway (Takeuchi and Akira, 2009). There are over 13 different families of TLRs in mammalian cells, with many also conserved in teleost fish, including salmonids (Purcell et al., 2006, Uematsu and Akira, 2007). These receptors can detect a wide range of microorganisms like bacteria, fungi, protozoans and viruses and each TLR, except TLR3, activates a common signaling pathway leading to the production of proinflammatory cytokines (Uematsu and Akira, 2007). TLR3, TLR7 and TLR9 for instance all detect viral RNA, but in different forms, whereas TLR3 detects dsRNA, TLR7 ssRNA and TLR9 unmethylated DNA with CpG motifs.

1.3.6 RIG-I–like receptors

The RLRs are an important set of receptors that reside in the cytoplasm of the cell and consist of three different types, namely Retinoic inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2) (Takeuchi and Akira, 2009). RIG-I has a helicase domain and a caspase recruitment domain (CARD), where the helicase domain can recognize dsRNA and 5'phospates of viral RNAs, while the CARD domain is initiating the downstream signaling pathway for expression of both proinflammatory cytokines and type 1 IFN (Takeuchi and Akira, 2009).

Upon recognition of viral RNA, the toll receptors or RIG-I receptors can activate signaling molecules (IKK-*i*, IKK-α/β). Phosphorylation of interferon regulatory factor (IRF) 7 and 3 can be activated via these signaling molecules and initiate interferon type-I transcription. Activation of nuclear factor- κB (NF- κB) can also be initiated by these signaling molecules, where it will translocate to the nucleus and initiate transcription of various genes, including pro-inflammatory cytokines (Takeuchi and Akira, 2009, Liu et al., 2017). **Figure 1.5** gives an overview of this activation pathway.

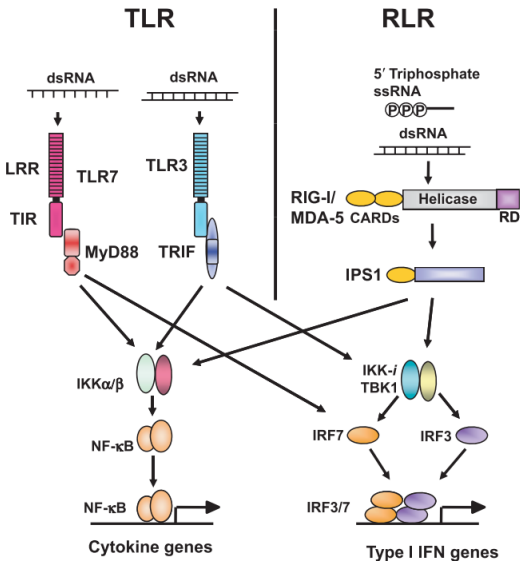

Figure 1.5: Activation pathways of cytokine gene expression and Type I IFN gene expression. For details see main text. Figure reproduced from Takeuchi and Akira (2009) and adapted for this thesis.

1.3.7 Type I interferons

Interferons (IFN) are cytokines that can regulate cell growth and are especially important as a way for an organism to adapt and defend itself against a viral infection. These can be subcategorized into three subfamilies; type I, II and III, where each of them differs in the receptors they bind to and the response they elicit. In teleost's the type I and II interferon system is shown to be present and play an important role in the hosts antiviral defense (Zou and Secombes, 2011).

Within the type I interferon system, three groups have been described in vertebrates (group 1,2,3) where group I and II are shown to be present in teleost's (Sun et al., 2009). Type-I group 1 interferons are that which is most studied of the two, and is shown to stimulate the expression of Mx, Viperin, ISG15 and PKR genes that are proteins associated with antiviral activity (Zou and Secombes, 2011). In addition to elevated expression of immune genes, type -I group-1 IFN stimulation leads to expression of immunoglobulins, chemokines and cytokines including their own family of IFNs (Martin et al., 2007).

These self-expressed IFNs have been shown to activate other neighboring cells by binding to IFN-receptors, expressed on the cells' surface. This activates a JAK/STAT (Janus Associated Kinase/Signal Transducers and Activators of Transcription) pathway that leads to an upregulation of interferon stimulated genes (ISGs), transcribed in the nucleus of the cell. They can also induce the transcription of more IFNs, resulting in a positive feedback loop, which amplifies the antiviral response (Collet, 2014, Collet and Secombes, 2002).

1.3.8 Type II Interferons

Interferon type II or INF-γ is known to be present in teleosts, including Atlantic salmon, where they have been shown to activate ISGs, cytokine and chemokine expression and enhancement of antigen presentation in macrophages (Martin et al., 2007). However, the cytokine is weaker compared to the type I interferon where a greater amount is needed to elicit the equivalent response. Type II IFNs has been shown to elicit similar responses to type I IFNs, where they can activate the transcription of antiviral proteins like Mx and Viperin (Jørgensen et al., 2007,

Grayfer and Belosevic, 2009). It is however thought to be of less significance in activating the innate immune response and rather compliment the type-I IFN (Zou et al., 2007).

1.3.9 *IL-10*

Interleukin 10 (IL-10) is a cytokine that is a pleiotropic cytokine (i.e., several functions) that has important immunoregulatory functions (Piazzon et al., 2016). It is however an anti-inflammatory cytokine, where it inhibits differentiation of monocytes, impairs phagocytosis and suppress genes encoding MHC class I and II molecules as well as other pro-inflammatory cytokines (Opal and DePalo, 2000). This serves to regulate the immune response and limit the degree of inflammation initiated by other pro-inflammatory cytokines that itself may be harmful to the host if left unbalanced (Rebl and Goldammer, 2018). The congruent expression of IL-10, among other anti-inflammatory cytokines during an infection is therefore vital for preserving homeostasis in the host. IL-10 has been shown to be expressed in salmonids and have elevated expression during later stages of ISAV infection (Inoue et al., 2005, Collet et al., 2015).

1.3.10 Mx proteins

Mx proteins are known from mammalian vertebrates as great antiviral compounds, where they are shown to be pivotable in mice immune protection when challenged with influenza-C. They are also shown to be induced by type I IFN (Haller et al., 1998, Haller et al., 1979). Mx proteins are proteins of 70-80 kDa with GTPase activity, which by inducing the GTPases make them involved in important cellular functions like endocytosis, intracellular vesicle transport and mitochondria distribution (MacMicking, 2004). Their antiviral function is still not completely understood but has shown to be able to block viral nucleocapsids' on intracellular transport by binding to them. They thereby prevent them from replicating inside the nucleus of the host, which is crucial for *Orthomyxoviruses* like ISAV (Haller and Kochs, 2002). Atlantic salmon have 3 genes for Mx proteins which have shown to be able to have antiviral effects towards IPNV and ISAV. In Mx1 expressing CHSE-214 cells infected with ISAV a delayed and reduced cytopathic effect (CPE) as well as a 10-fold reduction in virus was seen, confirming the Mx antiviral effects in Atlantic salmon (Kibenge et al., 2005). Mx can be induced by type-I IFN and other cytokines during an ISAV infection, but has also been shown to be expressed

in cells without type-I IFN present, indicating that they can be expressed directly during an ISAV infection (Kileng et al., 2007).

1.3.11 Viperin proteins

Viperin is another antiviral protein found in a range of vertebrates, including teleosts and is induced by both type I and type II interferons (Chin and Cresswell, 2001). Upon induction, Viperin is translocated to the endoplasmic reticulum where it is able to prevent viruses budding from the plasma membrane by interfering with specific enzymes involved in lipid biosynthesis (Wang et al., 2007).

Viperin is also shown to be present in Atlantic salmon as well, where it was shown to be up-regulated during an ISAV infection, indicating it having an important role in antiviral defense (Workenhe et al., 2009).

1.3.12 ISAVs ability to evade the immune response

Despite the antiviral responses elicited by the host, infection is often still achieved by the invading ISAV virions as they are able to replicate so rapidly. In addition, they can also have antagonizing mechanisms towards the host immune defense, called virulence factors. Important virulence factors in ISAV are two proteins encoded by the 7^{th} and 8^{th} segments of the ISAV genome that both are capable of interfering with the type-I IFN response (García-Rosado et al., 2008). Support for this have been found in other studies where the ISAV is shown IFN- type I stimulated cells does not protect against a subsequent ISAV infection (Kileng et al., 2007).

Differences in the mortality caused by ISAV infection have been found to be correlated with differences in gene expression, where high mortalities were seen in fish with high viral loads, and strong expression of innate immune genes. Low mortalities on the other hand were seen in fish that avoided a strong early inflammatory response. It has been suggested that a stressing inflammation might not fight the ISAV infection, but on the contrary, it may weaken the host, making it more susceptible to further infection (Jørgensen et al., 2008a). This might

explain why stress is often associated with high mortality outbreaks of ISAV (Nylund et al., 2003). Fish that overcame infection had managed to elicit an adaptive immune response consisting of Ig-related transcripts in gills and various organs and manage to clear out the virus systemically. Their findings suggest that for the host to survive an ISAV infection, it is necessary to survive long enough (several weeks) so that an effective adaptive immune response is elicited and that early innate immune response is not suited to fight and clear out infection (Jørgensen et al., 2008b).

1.4 Control measures

The main control measure for preventing the ISAV spread, is the establishment of a restriction zone around afflicted fish farms, set by the Norwegian Food Safety Authority (NFSA) (Aldrin et al., 2021). How long the infected fish can remain in the fish farm is up to the NFSA to decide for each specific location and is a risk-based decision based on how likely it is for the virus to spread to neighboring fish-farms. The risk of virulent ISAV to spread between fish farms is therefore present, even after a restriction zone has been established. The risk for the spreading of ISAV from these zones, becomes highly dependent on how long the virus could remain infective in the surrounding sea water i.e., horizontal transmission.

Vaccines are available and are currently used in USA, Scotland, Norway and Chile, but none of the available vaccines seems to offer a complete protection against ISAV (Falk, 2014). Possibilities for more effective vaccines are however promising, with recombinant DNA vaccines being bound to improve the efficiency of ISAV-vaccines (Kibenge and Kibenge, 2016).

1.6 Aims of this study

The aim of this thesis is to determine ISAV stability/survival outside its host in its natural environment using a novel *in vivo* experimental design. Furthermore, we will investigate the elicited immune response as well as histopathological changes in ISAV challenged salmon fry to see if the degree of immune response corresponds to the amount of ISAV measured in each fry. These results will hopefully help to better understand how long virulent ISAV strains remain infective in between fish farms and thereby create a better basis for how to improve existing control measures in ISAV. It will in addition reveal if the Atlantic salmon fry, as an *in vivo* model, is suited for reliable histological, *in situ* -hybridization and immunological analysis.

2. Material and methods

2. 1. ISAV stability study- overview

The ISAV stability study was conducted in three stages: **1**. MID experiment, **2**. Virus incubation and **3**. *In vivo* bath challenge (see **Figure 2.1**).

Prior to thesis work, an ISAV infection trial was done to determine minimal infectious dose (MID) and sampling time points for the main ISAV stability experiment (*Peñaranda* et al. unpublished).

Based on the results from the MID experiment the selected dose of a virulent strain of ISAV was added to both raw and filtered seawater and then incubated at 10°C for a specific period, ranging from 35 days to 0 minutes prior to a bath challenge

Third part consisted of bath challenging several groups of Atlantic salmon fry (*Salmo salar*), each with an ISAV-inoculated raw /filtered seawater sample from a specific time point to test the infectivity of the different ISAV-inoculated seawater samples.

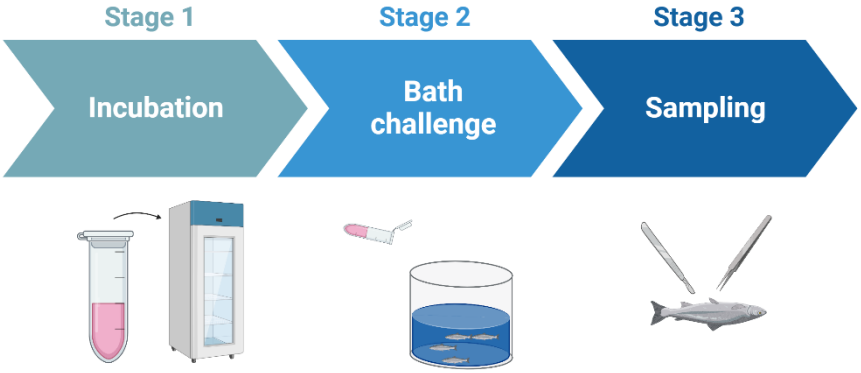

Figure 2.3: Overview with illustrations of the different stages in the ISAV stability study.

2.1.1 Virus

ISAV Glesvær 2/90 strain propagated in the ASK cell line were procured from NVI, Ås. The virus harvested in L15 medium was stored at -80°C until the experiment.

2.1.2. MID

Prior to thesis work, an ISAV infection trial was done at IMR to determine minimal infectious dose (MID) and sampling time points for the ISAV stability experiment (*Peñaranda* et al, unpublished). The following dilutions were used in a bath challenge method - $2.5x10^4$, $5.0x10^3$, $1.0x10^3$, $2.0x10^2$, $4.0x10^1$, $8.0x10^0$, $1.6x10^0$, $3.2x10^{-1}$, $6.4x10^{-2}$ TCID50/mL. Based on the MID experiment, the optimal viral dose for bath challenge was determined to be $1x10^4$ TCID50/mL (2 times the dilution 2 ($5.0x10^3$).

1mL of the Glesvær 2/90 virus diluted in Leibovitz L15 medium to the titer of $2x10^6$ TCID50/mL) stored at -80 °C, was thereby used as virus inoculate for the ISAV stability study.

2.1.3. Incubation of ISAV-inoculated seawater

Raw seawater (salinity - 35 ppt) from IMR facilities in Nordnes, Bergen was used in the experiment. In brief 10mL tubes were filled with 9 mL of raw seawater and stored at -80°C.

ISAV virus stock (1mL) ($TCID_{50}$/ml) was added into tubes starting seven days prior to the ISAV bath challenge. On the day of virus incubation, a tube containing raw seawater was first thawed in a 20°C water bath for 5 minutes. Then 1 mL ISAV virus stock was collected from the freezer and subsequently thawed in a 20°C water bath for 2 minutes, spun down and then pipetted into each raw seawater tube, resulting in a approx. $1x10^4$ TCID50/mL ISAV -inoculated raw seawater sample. The tube was then kept at 10°C in a shaker until the bath-challenge. This procedure was performed at selected time points prior to the ISAV bath challenge. The overview of all the incubation time is shown in **Table 2.1**.

For histological and immunological assays, an additional parallel incubation for five virus incubation time points, 7 days, 1 day, 6 hours, 1 hour and 0 hours prior to the ISAV bath challenge was set up. This study was done in a similar manner parallel to the main ISAV stability study, but on a smaller scale. The goal of this study was to assess the histopathological changes as well as the elicited immune response in ISAV infected fry. In this experiment, 5 groups of fry were each challenged with a specific incubated ISAV- inoculated raw seawater sample. Half of the fry (n=10) in each group was sampled for histopathological changes and

the other half (n-10) for measuring of viral load as well as the measuring of a selected set of immune genes.

Table.2.1: Overview of ISAV incubation time-points before bath challenge. ***dig****= days incubation of ISAV prior to fry group bath challenge,* ***hig****= hours incubation of ISAV prior to fry group bath challenge*

Raw seawater incubation	**Filtered seawater incubation**	**Seawater incubation (viral load, histological, an immunological analysis**
35 dig, **20** dig, **10** dig, **7** dig, **6** dig, **5** dig, **4** dig, **3** dig, **2** dig, **1** dig **12** hig, **6** hig, **4** hig, **2** hig **1** hig, **0.5** hig**, 0** hig	**35** dig **20** dig, **14** dig, **7** dig, **5** dig, **4** dig **1** dig **0** hig	**7** dig, **1** dig **12** hig, **6** hig, **1** hig, **0** hig

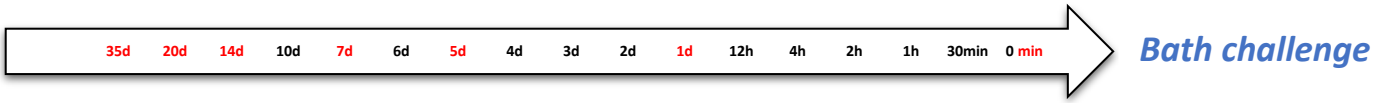

Figure 4.2: Timeline of ISAV incubations prior to batch challenge. Time points marked with red color indicates where parallel filtered seawater incubation where placed. d=days, h=hours, min= minutes.

2.1.4 Fry

Atlantic salmon fry (*Salmo salar*) was purchased from ILAB, Bergen, Norway and transported to the Institute of Marine Research (IMR) facilities prior to the bath challenge. The salmon fry were acclimatized in a common acclimation tank with a temperature fluctuating between 11.8 and 13,6°C and a waterflow of 400 L/h until the bath infection trial. The light/dark regime was 12/12. The fish was regularly fed with 0.8mm Skretting Nutra Spirit pellets. The average size of the fry was ~0.5grams.

Before the trial, 16 fish were screened for common salmonid viruses which included ISAV, PRV, IPNV, SAV and PMCV by sampling the heart and organ package to determine the presence by qPCR (See Taq-man One step qPCR). No fry tested positive for the selected viruses.

2.1.5 Bath challenge

The infection trial outlined for this experiment was approved by the NFSA (FOTS 27817).

To save virus inoculum, the bath challenge was done in separate 1L boxes. On the day of bath challenge, each box was filled with 190mL of freshwater before the ISAV- inoculated seawater was added, adding altogether up to 200mL. A 1:20 relationship between the raw seawater incubation and freshwater bath was chosen to keep the salinity concentration as low as possible, as well as the bath volume.

20 fry was randomly selected, and bath challenged for 4 hours in 200 mL freshwater in 1 L containers, and air was continually bubbled through the water in the container.

After the bath challenge the 20 fry were transferred into a separate 3.5L tank with a 20-24L/h water flow and kept at 10-12 °C. The fry was regularly fed with 0.8mm Skretting Nutra Spirit pellets to satiety, and excess food was removed regularly. Here the fry were kept for 12 days prior to sampling to get a successful infection.

Every group of fry challenged with ISAV-inoculated raw or filtered seawater from a specific incubation time point was added into separate 3.5L tanks for 12 days until sampling. **Figure 2.3** gives an overview of the tank set-up.

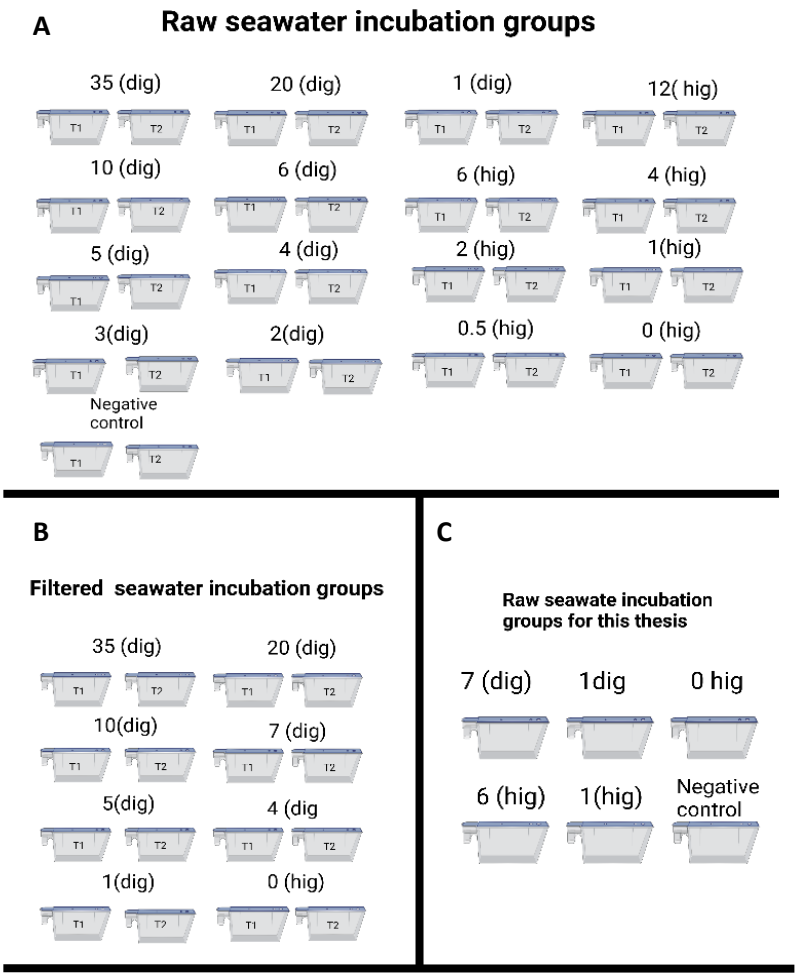

Figure 2.3: Illustration of tank set up for the different groups of fry (n=20) challenged with a specific ISAV- incubated seawater sample. *Peñeranda* et al, unpublished **A**: Groups (in duplicates) challenged with ISAV incubated in raw seawater. **B:** Groups (in duplicates) challenged with ISAV incubated for a in filtered seawater. For the master thesis work **C:** Groups challenged with ISAV incubated in raw seawater, meant for histological and immunological analysis.

2.1.6 Sampling

After 12 days the fry where euthanized groupwise and sampled heart and organ package of each individual fry using scalpel and forceps. The specimens that had died prior to the sampling was stored in a freezer and later sampled at the time its respective group were sampled. The negative control groups were sampled first to avoid contamination. The groups were then sampled in descending order from 7 dig to 0 hig.

Each fish was placed on a fresh plastic cover paper for each dissection. The scalpel blades were sterilized in 80% EtOH solution and sterilized between each fry. The blades were replaced between each group, in addition to gloves, needles and aluminum foil, covering the work bench.

The fish was collected using a net that was replaced for each group to avoid cross contamination. 5 fish were euthanized at a time by an overdose of Tricaine mesylate. The anesthetic solution bath was changed and disinfected between each group.

Both heart and organ package were sampled into a tube containing lysis buffer with a steel bead and were subsequently homogenized with a tissue lyser before being stored at -80°C for later processing.

For the viral load/immunological testing, 10 fish per group were sampled for standard qPCR testing and the other 10 for histological screening. For histology fixation, the tail was cut off, the abdomen slit open, and the gill arches were removed placed in histological cassettes and fixed in a formalin bath 4% paraformaldehyde (PFA) solution. One fish for each group was weighed and measured.

2.2 RNA isolation

Samples were taken out from a -80°C freezer and kept on ice. A Promega Maxwell© HT simplyRNA kit and protocol as well as the use of the Biomek® 4000 automated laboratory workstation (Beckman Coulter, Brea, California, United States) was used to isolate the RNA from each sample. 200 µL of each sample were added into a new Deep-well plate using a

multi-pipette. Between each of the groups it was added a negative control well to identify if there is any contamination during the RNA isolation procedure.

The RNA concentration and quality of the samples were then measured using a nanodrop - sspectrophotometer (Nanodrop ND-1000, Thermo Scientific™ by Thermo Fisher Scientific, Waltham, Massachusetts, United States). 1.8µL of each sample were pipetted into the nanodrop socket and measured.

Using the results from the nanodrop, the individual samples were diluted with nuclease free water, to achieve an equal concentration in every sample. The diluted RNA samples were then stored at -80°C until further processing.

2.2.1 The viral load estimation by RT-qPCR

A one step RT-qPCR was chosen to quantify the viral load of each individual fry. For the quantification of the viral load in the samples, AgPath-ID™ One-step RT-PCR Kit (Applied Biosystems kit, Thermo Fisher Scientific) was used. ISAV segment 8 (ISAV S8) (Plarre et al., 2005) was targeted for quantifying ISAV in individual samples. The Atlantic salmon house-keeping gene, EL1-α (Olsvik et al., 2005) was used as endogenous control (see **Table 2.2**).

Table 2.2: Primers and probes used One step qPCR Abbreviations; **For**: Forward, **Rev:** Reverse, **bp**: basepair, **S8**: Segment eight, **MGBNFQ**: minor groove binding non-fluorescent quencher, **6FAM**: 6-carboxyfluorescein

Primer	**Direction**	**Sequence (5`-3`)**	**Amplicon (bp)**	**Reference**
ISAV S8	For	CGACGATGACTCTCTACTGTGTGAT	63	(Plarre et al., 2005)
	Rev	TCATCAGTGTCGCCATGCTT		
	Probe	6FAM-ACGGTGGATCTTTC-MGBNFQ		
EL1-α	For	CCC CTC CAG GAC GTT TAC AAA	57	(Olsvik et al., 2005)
	Rev	CAC ACG GCC CAC AGG TAC A		
	Probe	FAM-ATC GGT GGT ATT GGA AC-MGBNFQ		

Two runs were set up, one for ISAV segment 8 (ISAV S8) and one for the elongation factor reaction (EL1-α). The isolated RNA from each sample was diluted to 50 ng/ µL RNA using a Biomek® 4000 automated laboratory workstation (Beckman Coulter, Brea, California, United States). 8µL of master mix and 2µL of diluted sample (100ng/µL) were added into separate wells in the qPCR plate. The samples were run in a thermal cycler (Quantstudios 5, Applied biosystems™) with the program described in **Table 2.4**.

Volumes required for one sample is shown in **Table 2.3**.

Table 2.3: Reagents and their volume for one RT-qPCR reaction

Reagent	**Volume (µL)**	**Final concentration**
RT-qPCR buffer	5.0	1x
Enzyme-mix	O.4	1x
ILAV For (10 µM)	0.4	400 nM
ILAV Rev (10 µM)	0.4	400 nM
Taq Man probe (10 µM)	0.16	160 nM
RNA	2	
Nuclease free water	≤10	

Table 2.4: Overview of qPCR reaction conditions

Temperature	**Time**	**Cycle (s)**
45	10	1
95	10	1
95	15	40
60	45	
4	∞	

2.3 Immune gene analysis

2.3.1 Two step RT-qPCR SYBR-GREEN®

The same fry that was analyzed for the ISAV viral load was also tested for the expression of selected immunogens by a two step-RT-qPCR SYBR-GREEN- method.

2.3.2 cDNA-synthesis

Isolated RNA samples (see RNA isolation) were diluted to 100 ng/µL and converted into cDNA with the use of a SuperScript®VILO™ cDNA Syntesis Kit with protocol as shown in **Table 2.5**. All samples were run in duplicates with some RT negative samples to monitor the presence of genomic DNA. The samples were incubated in a 2720 Thermal cycler Applied biosystems at a Vilo program (details shown **in Table 2.6**) The cDNA samples were stored at -20°C until further use.

Table 2.5: Shows reagents for one reaction using the SuperScript®VILO™ cDNA Syntesis Kit

Reagent	**Volume**
5X VILO® Reaction mix	4 µL
10X SuperScript®Enzyme Mix	2 µL
RNA template (100ng/µL)	5 µL
Nuclease free water	9 µL

Table 2.6 Shows details for Vilo cDNA program

Time (minutes)	**Temperature (°C)**
10	25
60	42
5	85
4	∞

2.3.3 qPCR

The isolated cDNA was diluted 1:20 with nuclease free water before being used in a qPCR reaction. A Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix kit was used for qPCR. The 1.0 mM reference dye used was diluted 1:500 in nuclease free water. A master mix was

prepared for each of the genes. **Table 2.7** shows the primers used in the qPCR reactions. **Table 2.8** shows reagents for one qPCR reaction, with the qPCR program given in **Table 2.9** and melt curve program in **Table 2.10**.

Table 2.7: List of primers used in the qPCR

Name	**Direction**	**Sequence**	**Amplicon (bp)**	**Reference**
RPL1	Fwd	ACTATGGCTGTCGAGAAGGTGCT	118	(Ugelvik et al., 2022)
	Rev	TGTACTCGAACAGTCGTGGGTCA		
EL1-α	Fwd	CACCACCGGCCATCTGATCTACAA	77	(Øvergård et al., 2018)
	Rev	TCAGCAGCCTCCTTCTCGAACTTC		
Mx	Fwd	GGTGGTTGTGCCATGCAA	100	(Moore et al., 2018)
	Rev	TGGTCAGGATGCCTAATGTC		
Viperin	Fwd	AGCAATGGCAGCATGATCAG	101	(Grove et al., 2013)
	Rev	TGGTTGGTGTCCTCGTCAAAG		
IFN-α	Fwd	CCTGTGTATCACCTGCCATGAA	82	(Moore et al., 2018)
	Rev	GCCTGTGCACTGTAGTTCATTT		
IFN-γ	Fwd	GGTCCACTATAAGATCTCCAAGGA	133	(Moore et al., 2017)
	Rev	CTGGCAAGATACTCCGATACAC		
IL10	Fwd	GCTATGGACAGCATCCTGAAGTT	76	(Ugelvik et al., 2022)
	Rev	GGTTGTTCTGCGTTCTGTTGTT		
IRF3	Fwd	CACAGCAGAGGGGATCTCAA	143	(Iliev et al., 2011)
	Rev	CCTGAATAGCCTCTGTTGGAGA		

Table 2.8: List of reagents and volumes required for one qPCR reaction.

Reagent	**Volume (µL)**
2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix	3,5
Fwd primer	0,28
Rev primer	0,28
Ref dye (1:500)	0,10
cDNA (1:20)	2.0
Nuclease free water	0.84
Total	7

Table 2.9: qPCR program

Cycles	**Duration**	**Temperature (°C)**
1	3 (min)	95
40	5 (sec)	95
	20 (sec)	60

Table 2.10: Melt curve program

Cycles	**Duration**	**Temperature**
	1 (sec)	**95**
	20 (sec)	**60**
	1 (sec)	**95**

2.4 Histopathology

2.4.1 *Embedding*

The entire fry was sampled and its tail cut off and placed in a histology cassette. 10 fry from each of the 6 groups (0 hig, 1 hig, 6 hig, 1 dig, 7 dig and negative control) were sampled, altogether 60 fry. Samples were stored in 4% formalin fixative. After 48 hours, they were transferred to 70% ethanol. The tissue processing was performed using a Leica TP1020. The fish samples were embedded in paraffin using Kunz instruments Histowax™ by adding paraffin into the histology cassette containing the samples placed in pre-heated metal -plates orienting the sample with the head pointing left, allowing for later sectioning in a vertical sagittal angle. They were then cooled down over approximately 30 minutes for the paraffin to harden and subsequently archived until later processing.

2.4.2 Sectioning

The paraffin embedded fish where sectioned using a Thermo Scientific™ Micron HM355S with Accu edge 4686™ microtome blades at a 10° angle, 3µm thick. Each of the section were placed on a 26x76 mm,1-1.2 mm STARK (Medite™) cover-glass and inspected in an Olympus CX31® light-microscope where the gill, heart and liver were the main focus of detection. The sections were placed in a holder to dry overnight.

2.4.3 Staining

Before staining of the sections, they were preheated for 30 minutes in a Labnet mini incubator™ at 56,6±2,0°C. The sections were stained using HES, hematoxylin (Instant hematoxolyn, Thermo Scientific™), erythtrocin (Merck™), Saffron (Saffron alcoholic, Masson Walderck™) where they were stained in accordance to the protocol, given in **Table 2.11**. The sections were then stored for drying over approximately 36hrs before fitting them with a 24x32 mm coverlid (VWR®).

Table.2.11: Deparaffinization and staining regime for selected tissue samples

Deparaffinization		
Container (number)	**Solution**	**Time**
1	Xylene	10 min
2	100% EtOH	5 min
3	100% EtOH	5 min
4	96% EtOH	5 min
5	80% EtOH	5 min
6	50% EtOH	5 min
Water container with running tap water	Tap water	5 min
Staining		
Container	**Filtered haematoxylin**	**Time**
Water container with running tap water	Tap water	4 min
Separate container	1% Erythrosine	1,5 min
Water container with running tap water	96% EtOH	Dipping the section 3-5 times
8	96 % EtOH	45 sec
9	100 % EtOH	1 min
Separate container	Saffron	10 sec
10	100% EtOH	1 min
11	Xylene	5 min (minimum)
12	Xylene	5 min (minimum)

2.4.4 Scanning and imaging

Scanning of the sections was done using a Hamamatsu Nanozoomer S60™ and then transferred to a personal hard drive for storing and later analysis. Selected sections were photographed using a Flexacam C1 12, Leica.

2.5 In-situ hybridization

The *in-situ* hybridization was done according to the user manual provided by Advanced Cell Diagnostics, Inc, with some alterations at IMR facilities (Wang and Flanagan, 2015). The process took 3 days, where sectioning was performed on day 1, formalin-fixed paraffin-embedded (FFPE) sample preparation and pretreatment on day 2 and RNAscope® 2.5 HD detection reagent (RED) took place on day 3. Two already paraffin-embedded individual fry were selected from the negative control group, 0 hig, 1 dig and two positive controls provided by IMR.

2.5.1 Day 1

Sectioning

One FFPE slide for each individual fish was prepared on a SuperFrost®Plus slide by sectioning the paraffin-embedded individual fish in a sagittal cross section. The slides were then dried at 40 °C on a warming plate for 30 min and covered with aluminum foil and stored in a refrigerator at 4-6 °C overnight.

2.5.2 Day 2

Preparation for the RNAscope® 2.5 Assay

Pretreatment of samples

Prepared FFPE slides were baked at 60°C for one hour in a drying oven before being deparaffinized under a fume hood. Two tissue Tek® Clearing agents' dishes were filled with 200ml fresh Xylene (1st, 2nd) and two more with 200 mL 100% alcohol. FFPE slides were placed in a Slide rack and submerged in the prepared dishes according to the flow chart given in **Figure 2.4**.

***Figure 2.5**:* Deparaffinization flow chart of FFPE tissue slides

200 mL of 1X RNAscope ® Target retrieval reagents was prepared by mixing 180 mL distilled water with 20 mL of 10X Target Retrieval Reagents for later use, done according to user manual. The samples were then treated with RNAscope® Hydrogen peroxide at RT for 10 minutes.

Target retrieval

A 3L glass beaker with distilled water was heated on a hot plate for antigen retrieval. Two plastic trays, one filled with 200mL with RNA scope® Target Retrieval Reagent, while the other was filled with 200 mL of distilled water were added to the glass beaker. The entire beaker was then warmed to ~99°C. The sections were dipped in distilled water and moved to antigen retrieval buffer for 15 minutes. Afterwards, slides were moved to 100% EtOH for 3 minutes and then dried at 60°C.

The tissue slides were marked with a hydrophobic barrier using a Immedge™ hydrophobic barrier pen and left to air dry overnight at room temperature.

2.5.3 Day 3

Hybridization of the probe

The sections were pre-warmed at 40°C for 2hrs in an oven before the hybridization of the probe was initiated. A slide rack box with a manually created humid environment was used to place the tissue slides. The humidity was regularly checked and regulated throughout the process.

A RNAscope™ Probe-V-Salmon-Isavirus (CAT No:847521) and a RNAscope™ Probe-Ssa-ppib (CAT No:494421), hereafter referred to as ISA-probe and ppib-probe respectively, provided by Advanced Cell Diagnostics Inc. were used as appropriate probes for the *in-situ* hybridization. The ppib-probe was applied to its own section as a positive control probe that would target a common housekeeping gene and the ISA-probe was applied to the rest of the tissue slides as a target probe.

The hybridization was performed in accordance with the user manual.

Hybridzation of AMP1-6

For the amplification steps the RNAscope® 2.5 HD detection reagent (RED) was used according to the user manual. These amplification steps were done to build greater attachment areas for with probes that can be detected using the fast RED substrate.

Detection of signal

For the detection of the signal, 100 µL of RED-B was mixed with 600 µL of RED-A in a 2mL Eppendorf tube. This mixture was then applied to the tissue slides according to the user manual. The signal created from this procedure would be seen as a red color in the tissue slides.

Counterstaining of slides

Each slide was counterstained using gills hematoxylin, Thermo Scientific ™, followed by washing in tap water for then to be stained in a 0,02% Ammonia water. This was done to create a blue color to the cells in the tissue samples, so the red color from the signal detection could be seen more easily. The finished slides were then dried and mounted with a cover slip using xylene followed by a drop of EcoMount. Sectioning and imaging were done as described before for the histology sections.

2.5.4 Statistical Analysis

A cycle threshold of 0.2 was set in program and Ct-values was collected from the QuantStudio™ 5 Real-Time PCR System Design & Analysis software (Applied Biosystems™ by Thermo Fisher Scientific). Ct-values exceeding 40 was regarded as negative. Since the variables were assumed to be normally distributed and independent, a one-way ANOVA was used to test the significant difference between fry groups viral load and fold changes in innate immune responses using Graph Pad PRISM 8. This was followed up by a *post hoc* Tukey's test for multiple comparisons of fry group Ct-values.

Ct-values from EL1-α and RPL-1 was used as housekeeping genes for the calibrator (i.e, negative control fry) and samples of interest (ISAV-challenged fry). The fold change was calculated for each immune gene tested with the formula:

$$\Delta Ct_{cal} = [Ct_{negative\ control} - Ct_{housekeeping\ genes}]$$

$$\Delta Ct_{sample} = [Ct_{ISAV\ challanged\ fry} - Ct_{housekeeping\ genes}]$$

$$Fold\ change = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{sample} - mean\ of\ \Delta Ct_{cal})}$$

The fold change values were imported to Graph Pad Prism 8 for analysis. One-way ANOVA was used to test the significant difference between fry groups using Graph Pad PRISM 8. This was followed up by a Dunnets test for comparing of fry group fold change against the negative control fry groups fold change.

A Pearsons *r* correlation test between Ct-values of fry ISAV S8 and immune gene fold change ($2^{-(\Delta\Delta Ct)}$) was conducted for each of the genes tested using Graph Pad PRISM 8.

3. Results

3.1 Prevalence and viral load

All fry in the negative control group tested negative (Ct>40) for ISAV S8. There was no mortality in the fry tanks meant for histological and immunological analysis. Details of viral load for each incubation group is given below.

0 hours incubation of ISAV prior to bath challenge of fry group (0 hig)

All fry challenged with ISAV incubated in seawater 0 hours before bath challenge tested positive for ISAV S8 (Ct<40). 8 out of 10 fry challenged had Ct value above 20, while the remaining two had Ct- values below 30 (see **Figure 3.1 A, B**). The Ct-mean for the 0 hig group was 19.44 with a standard deviation (SD) of 6.38, statistically significant compared to the control group, as well as the 7 dig ($p<0,0001$). There was no statistical significance between 0 hig and the other groups. The Ct- mean and SD for 0 hig fry with Ct-values below 30 was 16.43 and 0.76 respectively.

1 hour incubation of ISAV prior to bath challenge of fry group (1 hig)

9 fry challenged with ISAV incubated in seawater 1 hour before bath challenged tested positive for ISAV S8 (Ct<40) and one fry tested negative (Ct>40). 6 out of 10 fry challenged had Ct-values below 30 (see **Figure 3.1 A, B**). The Ct- mean for the 1 hig was 26.15 with a SD of 9.04 and was significantly different from 7 dig ($p<0.0011$), but not against other fry groups. The Ct-mean and SD for 1 hig fry with Ct-values <30 was 19.71 and 3.92 respectively.

6 hours incubation of ISAV prior to bath challenge of fry group (6 hig) All fry challenged with ISAV incubated in seawater 6 hours before bath challenge tested positive for ISAV S8 (Ct<40). 7 out of 10 fry challenged had Ct value below 30, while the remaining three had Ct- values above 30 (see **Figure 3.1 A, B**). The Ct-mean for the 6 dig group 16.62 with a SD of 8.12 and was significantly different from the 7 (dig) ($p<0.0001$), but not against other fry groups. The Ct- mean and SD for 6 hig fry with Ct-values <30 was 16.92 and 2.72 respectively.

1 day incubation of ISAV prior to bath challenge of fry group (1 dig)

9 fry challenged with ISAV incubated in seawater 1 day before bath challenge tested positive for ISAV S8 (Ct<40), while 1 fry tested negative (Ct>40). 5 out of 10 fries had Ct values below 30, with 2 individuals showing Ct-values below 20 (see **Figure 3.1 A, B**). The Ct-mean for the 1 (dig) group was 28.29 with a SD of 9.00 and was significantly different from the 7 dig ($p<0.0074$), but not against other fry groups. The Ct- mean and SD for 1 dig fry with Ct<30 was 20.37 and 4.15 respectively.

7 days incubation of ISAV prior to bath challenge of fry group (7 dig)

No fry in challenged with ISAV incubated in seawater 7 days before bath challenged tested positive for ISAV S8 and was all negative (Ct>40) (see **Figure 3.1 A, B**).

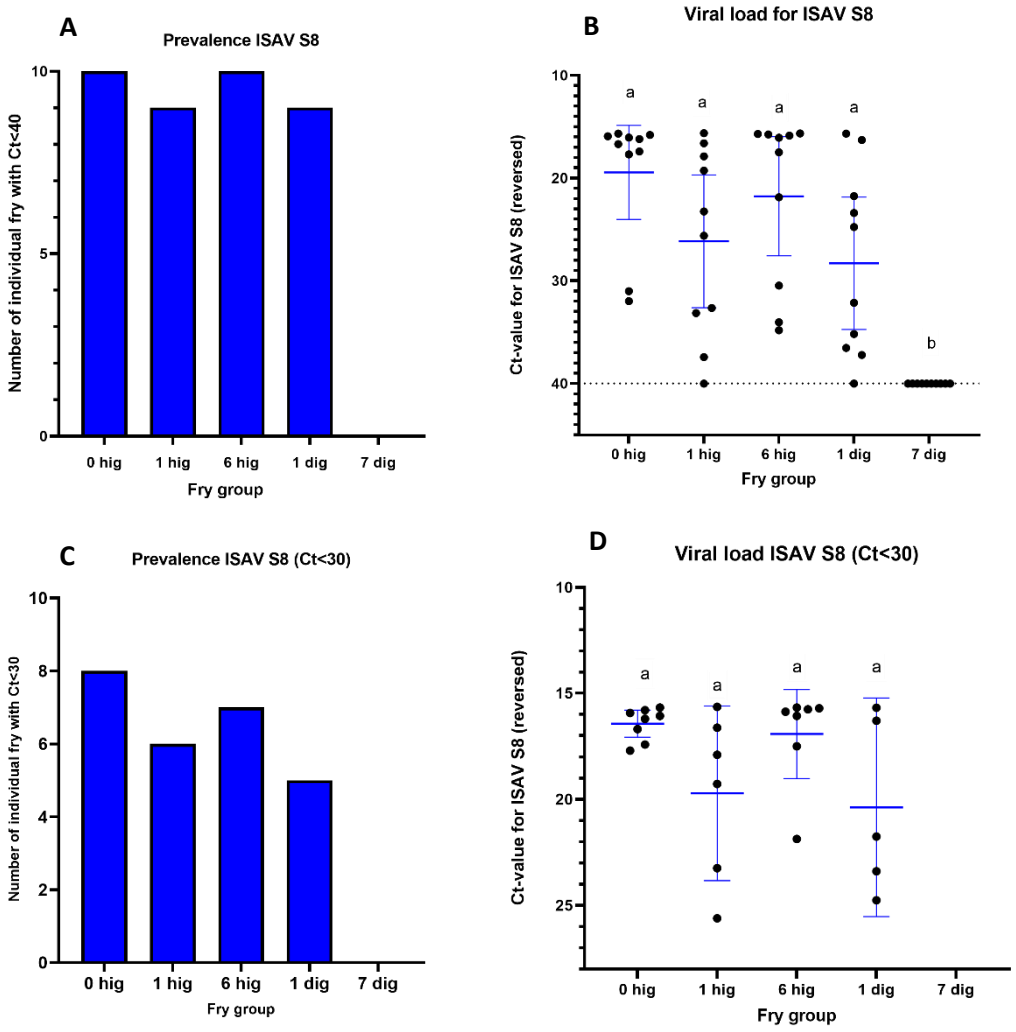

Figure 3.1: **Prevalence and RT-qPCR analysis of ISAV-challenged Atlantic salmon fry.** Figure **A** and **C** shows the prevalence of ISAV infection in challenged fry, with a cut off Ct-value of 40 and <30 respectively. Figure **B** and **D** display Ct-value for each fry in each fry group. Means and variance with 95% confidence interval were added in GraphPad. One way ANOVA was used for assessing the significance of the results.

3.2 Histology

All the fish in the ISAV infected groups, (0 hig, 1 hig, 6 hig, 1dig, 7 dig) showed normal histology.

The heart tissue in both negative control fry and ISAV challenged fry both showed normal histology without any signs of pathology (see **Figure 3.2**). Compactum and spongiosum are intact as well as the endocardium. There was no visible infiltration of immune cells in any parts of the heart tissue.

The gill tissue in both negative control fry and ISAV challenged fry also showed normal histology with no signs of histopathological changes. The squamous epithelial layer is unchanged as well as the branchial vessel. (see **Figure 3.2**).

The liver tissue as well as the kidney tissue in both negative control and challenged fry, also showed normal histology with no signs of pathological changes (see **Figure 3.3**).

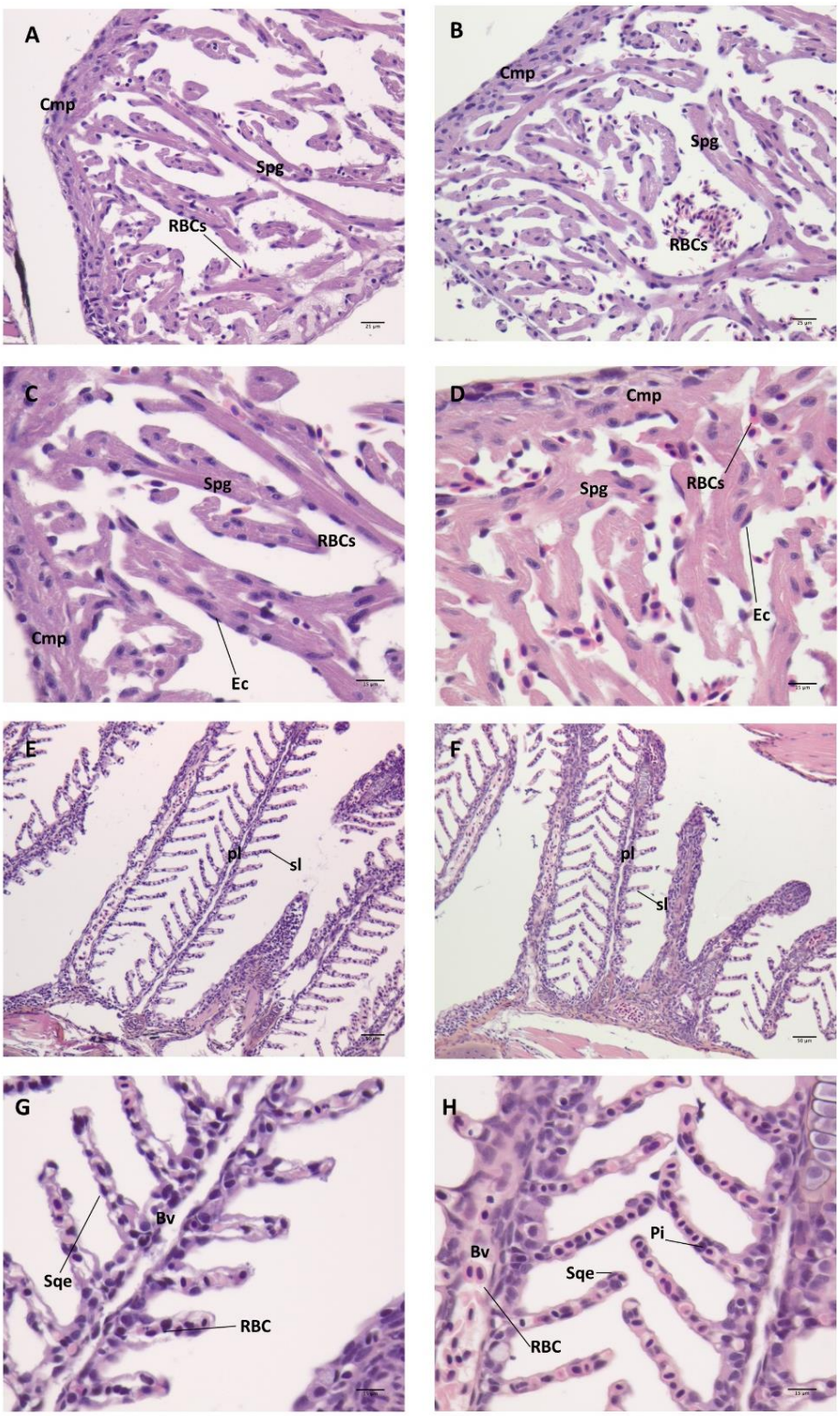

Figure 3.2: HES-stained sagittal sectioned fry from the negative control group (Uninfected) and 0 hours incubation of ISAV challenged fry (0 hig). A and **C** displays heart tissue from un-infected fry. **E** and **G** displays gill tissue from un-infected fry. **B** and **D**; 0 displays heart tissue from 0 hig challenged fry. **F** and **H** displays gill tissue from 0 hig challenged fry. No histopathological changes were evident in any of the challenged fry. *For all pictures,* ***Cmp****: Compactum,* ***Spg****: Spongiosum,* ***RBCs****: Red blood cells,* ***Ec:*** *endocardium,* ***pl****: primary lamella,* ***sl:*** *secondary lamella****, sqe****, squamous epithelia,* ***Bv****: branchial vessel,* ***Pi****: Pillar cell*

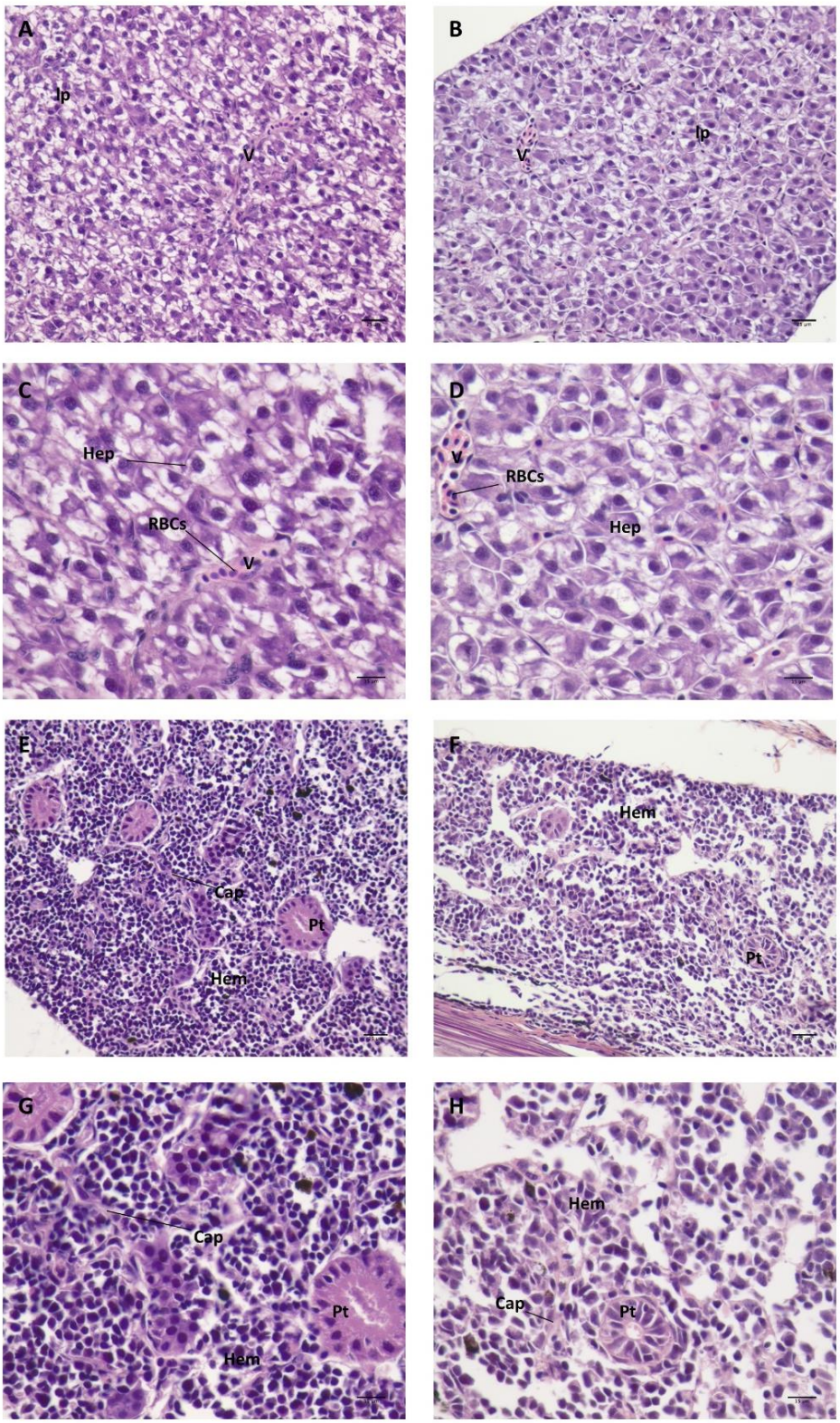

Figure 3.3: HES stained sagittal sectioned fry from the negative control group (Uninfected) and 0 hours incubation of ISAV prior to fry group bath challenge (0 hig). **A** and **C;** Display liver tissue from uninfected fry. **E** and **G**; displays kidney tissue from uninfected fry. **B** and **D**; displays liver tissue from 0 hig challenged fry. **F** and **H;** displays kidney tissue from 0 hig challenged fry. No histopathological changes were evident in any of the challenged fry. *For all pictures,* ***lp****: liver parenchyma,* ***v****: vein,* ***Hep****: hepatocyte,* ***Cap****, capillary,* ***Pt****: Proximal tubuli****, Hem****, Hematopoietic tissue*

3.3 In situ hybridization

In-situ hybridization (ISH) of ISAV on Atlantic salmon fry sagittal tissue sections revealed positive signals from most organs across ISAV challenged fry. The negative control fry showed weak signal in some of the nuclei of the cells but was significantly weaker than that of ISAV challenged fry and deemed fit for comparison. Only parts of the kidney tissue were deemed fit for parallel HES-stained comparison (see **Figure 3.4**) for identifying cell types in ISH tissues.

Kidney

Evenly distributed signal, mostly in the hematopoietic tissue, was clearly visible in the kidney (see **Figure 3.4 A-E**). Comparison of parallel HES stain of the kidney tissue revealed that RBCs elicits positive signal, indicating ISAV's presence on these cells. ISAV also appears to have infected sinusoidal capillaries in their endothelial cells. The proximal tubuli did not elicit any signal and thus did not seem to be infected by ISAV. The kidney was the organ with the strongest and densest number of signals in the challenged fry, when compared to other organs.

Heart

The heart tissue from ISAV challenged fry showed clear signals for ISAV infection (see **Figure 3.4, A-D**). The signals are mainly limited to the endocardium in spongiosum layer and to a less extent in parts of the epicardium and compactum layer. Signals from RBCs are also visible. Overall, the endothelial cells and RBCs seem to be the infected parts in the heart tissue.

Liver

Liver tissue from ISAV challenged fry showed clear signals for ISAV infection (see **Figure 3.5, E-H**). Here the signals were evenly distributed throughout the liver parenchyma. RBCs and capillaries seem to be the cells eliciting positive signal. The signal is limited to the areas around the hepatocytes in a somewhat string-like fashion.

Gills

The gills from ISAV challenged fry gave clear signals from especially the branchial blood vessels, central in the primary lamella of the gills (see **Figure 3.6**, **A, B**) Some signals can also be seen in outer parts of secondary lamella as well. It is most likely RBCs that elicit positive signal from the branchial blood vessels in the gills.

Artery/endothelium

Clear signals from the arteries near the gills could be identified in ISAV challenged fry both inside the lumen and further out in the endothelial tissue. It looks that both RBCs as well as endothelial cells are eliciting signal for ISAV (see **Figure 3.6 C, D**).

Eye tissue

Clear signals from the choroid plexus in near the retina was visible in ISAV challenged fry, where the RBCs are the cells eliciting positive signal (see **Figure 3.6, E, F**). Signals could also be seen in RBCs closer to the retina. The retina itself did not elicit any signal.

Central nervous system (CNS)

The CNS from ISAV challenged fry gave of clear signal in parts of the molecular layer and the granular layer (see **Figure 3.6, G, H**). This is most likely originating from blood vessels and RBCs located in both the molecular layer and granular layer of the CNS. The amount of signal was low and significantly spread out compared to other tissues.

Mid-intestine

The gut in the mid intestine from ISAV challenged fry gave of clear signal from the lamina propria (see **Figure 3.7, A, B**). Signal could also be viewed in the basal areas of the lamina propria as well as the submucosal layer. The signal most likely originates from RBCs or endothelial cells.

Pyloric caeca

Signal was also visible in the lamina propria in the pyloric caeca from ISAV challenged fry (see **Figure 3.7, C, D**). The signal most likely originates from RBCs or endothelial cells.

Skeletal muscle

Skeletal musculature from ISAV challenged fry gave of clear signal (see **Figure 3.7**). There were little and highly dispersed signals from the white skeletal muscle compared to other tissues. The signals seem to originate from the capillaries in the white skeletal muscles.

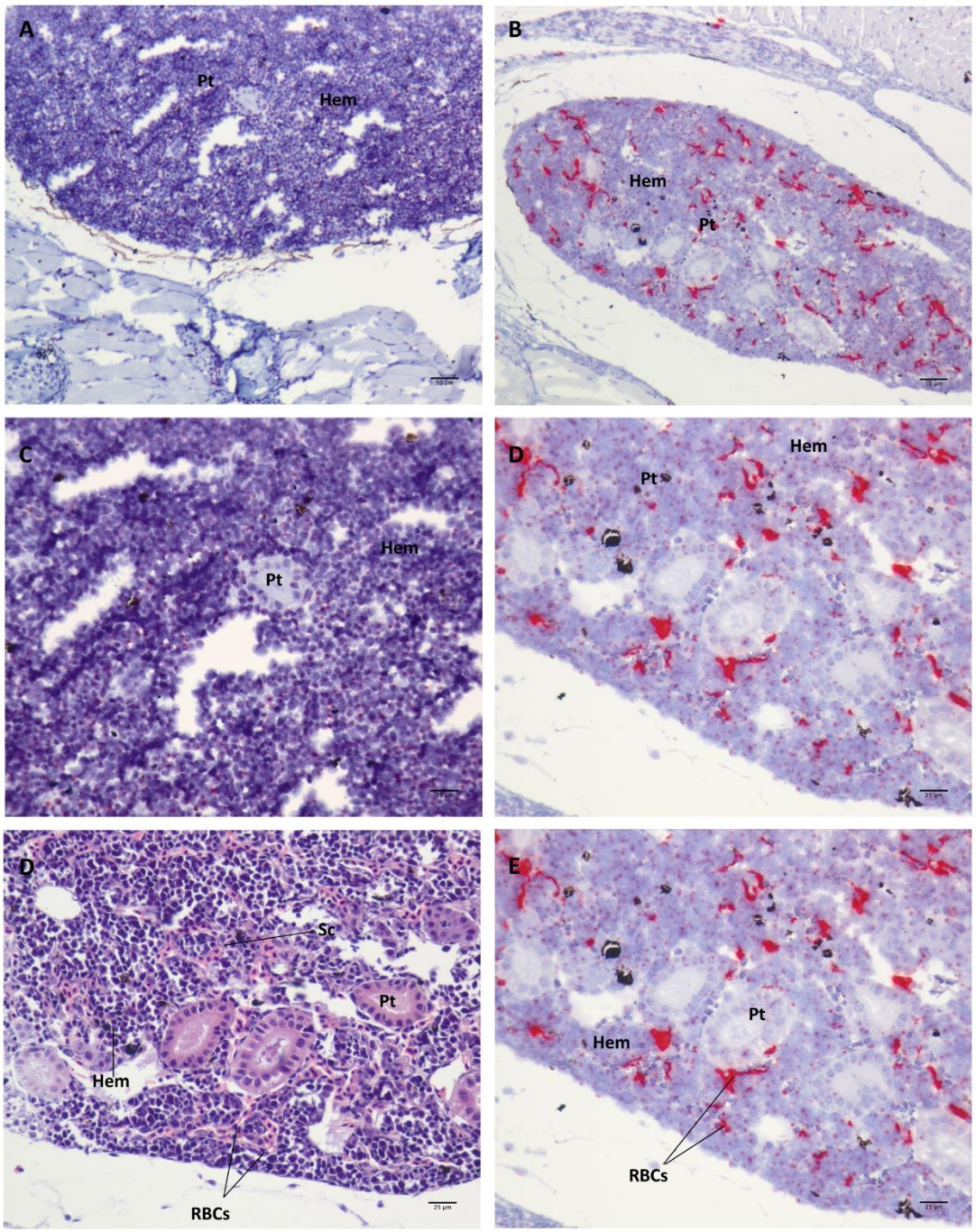

Figure 3.4: ***In-situ hybridization*** **sections including a HES stain of sagittal sectioned fry head-kidney from 1 day incubation of ISAV prior to fry group bath challenge (1 dig) and negative control fry**. Red colored parts indicate a positive signal. **A** and **C**; displays head kidney/ frontal kidney from negative control fry. **B** and **D**; displays head kidney/ frontal kidney from 1dig infected fry. **E**: HES stain displaying kidney tissue from 1d (dig) challenged fry. **F**; Same as **D**. *For all pictures,* ***Pt****: Proximal tubuli,* ***Hem****, hematopoietic tissue,* ***Sc****; Sinusoid capillary.*

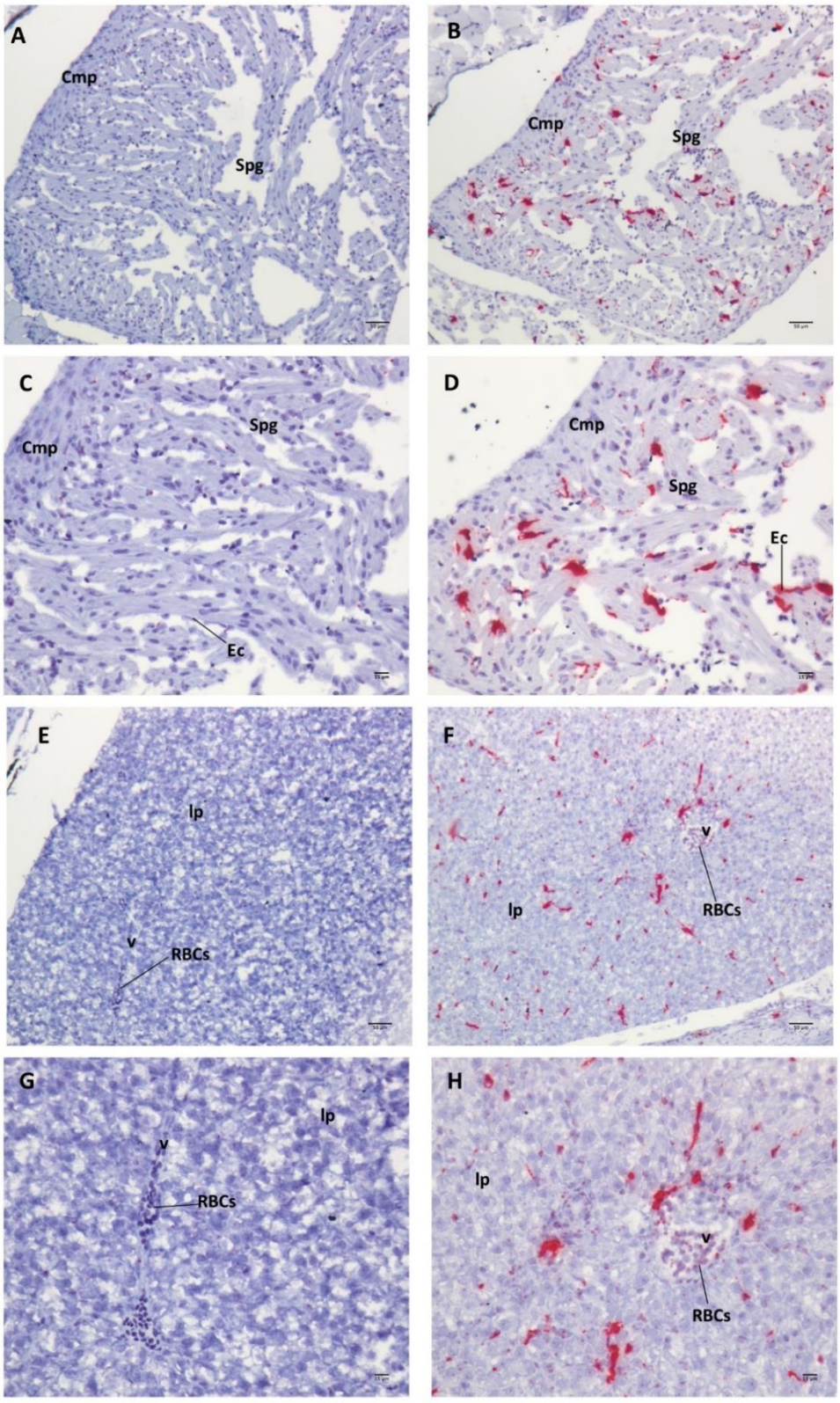

Figure 3.5: *In-situ hybridization* sections of sagittal sectioned fry including heart and liver from 1 day incubation of ISAV prior to fry group bath challenge (1 dig) and negative control fry. Red colored parts indicate a positive signal. A and **C**; displays heart tissue from negative control fry. **B** and **D**; Displays heart tissue from 1 dig challenged fry. **E** and **G**; Liver tissue from negative control fry. **F** and **H**; Liver tissue from 1 dig challenged fry. *For all pictures, **Cmp**: compactum, **Spg**: Spongiosum, **Ec**: Endocardium, **lp**: liver parenchyma, **v:** vein, **RBCs**: Red blood cells*

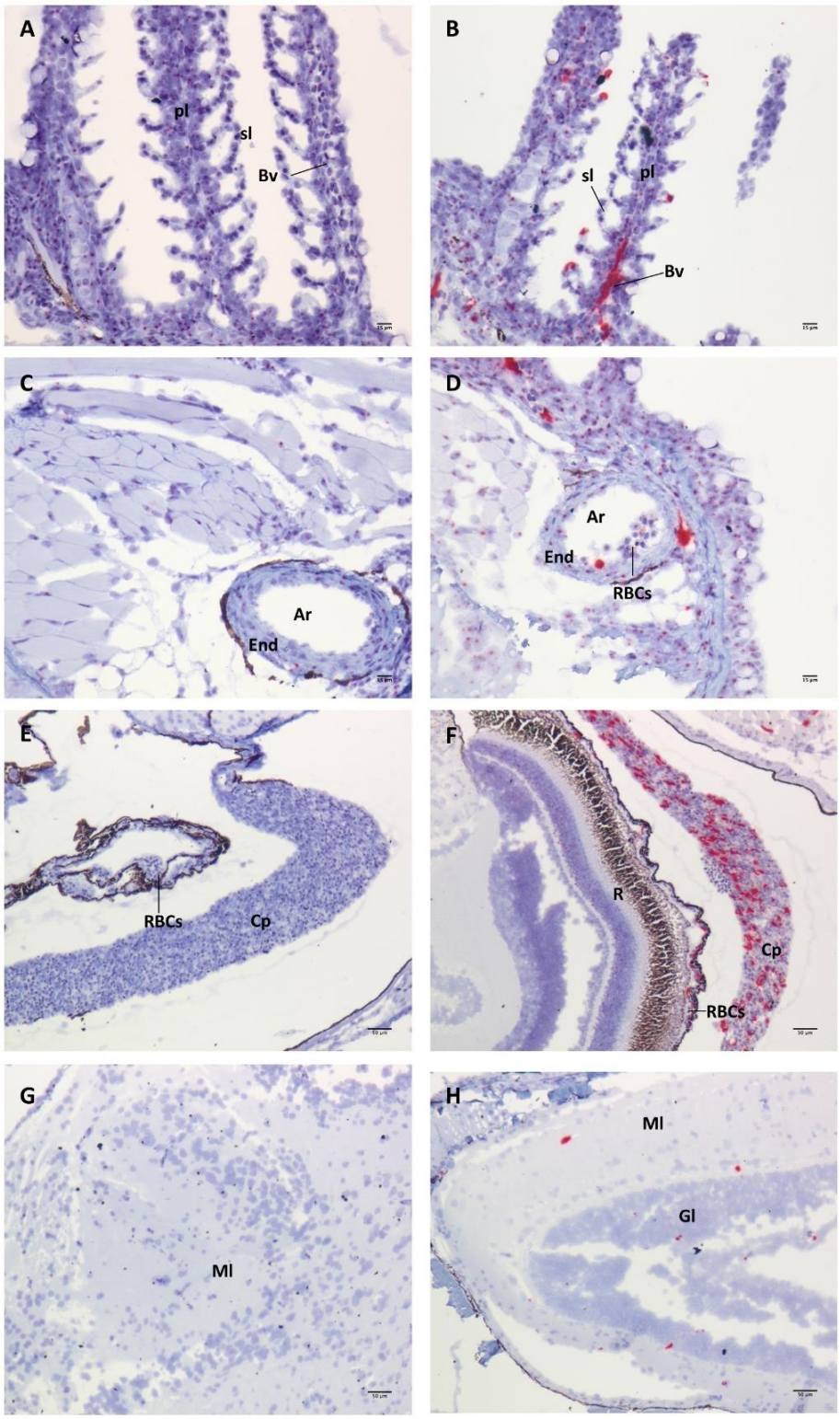

Figure 3.6: *In-situ hybridization* sections of sagittal sectioned fry including gills, endothelia, eye tissue and CNS from 1 day incubation of ISAV prior to fry group bath challenge (1 dig) and negative control fry. Red colored parts indicates a positive signal. A displays gill tissue from un-infected fry and **B** from 1 (dig) challenged fry. **C** displays artery near the gills from un-infected fry and **D** from 1 (dig) challenged fry. **E** displays choroid plexus from un-infected fry and **F** from 1 (dig) challenged fry, **G** displays CNS-tissue from un-infected fry and **H** from 1 (dig) challenged fry. *For all pictures, **pl**: primary lamella, **sl**: secondary lamella, **Bv**: blood vessel, **Ar**: Artery, **RBCs**: Red blood cells, **Cp**: Choroid plexus, **R**: Retina, **Ml**: Molecular layer, **Gl**: Granular layer*

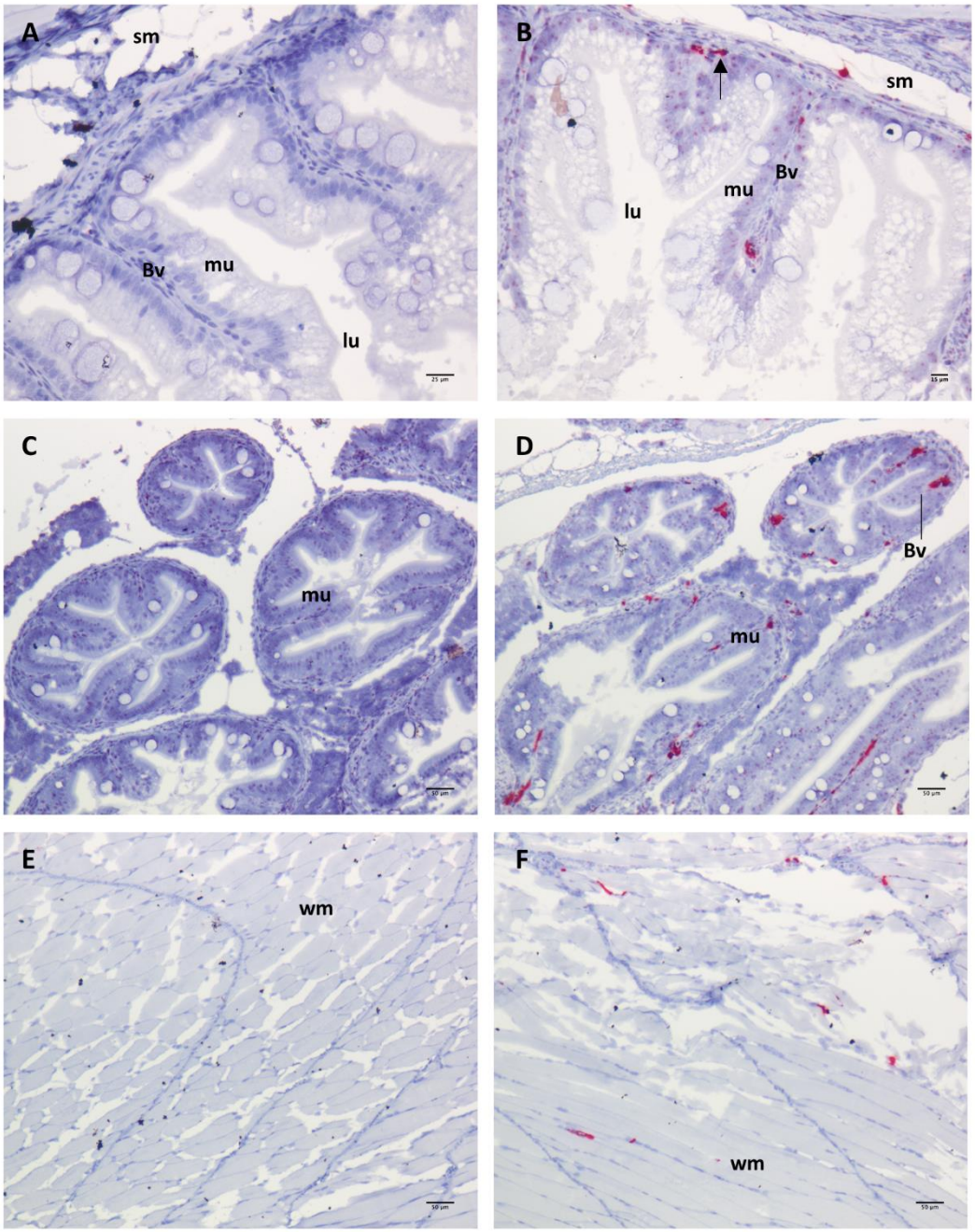

Figure 3.7: *In-situ hybridization* sections of sagittal sectioned fry including mid intestine, pyloric caeca, and skeletal musculature from 1 day incubation of ISAV prior to fry group bath challenge (1 dig) and negative control fry of sagittal sectioned fry from 1 (dig) challenged fry and negative control fry get tissues into title. Red colored parts indicate a positive signal. A displays mid-intestine tissue from un-infected fry and **B** from 1 (dig) challenged fry. **C** displays pyloric caeca from un-infected fry and **D** from 1 (dig) challenged fry. **E** displays skeletal muscle from un-infected fry and **F** from 1d ipc challenged fry. *For all pictures, **lu**: lumen, **mu**: mucosa, **Bv**: Blood vessel, **sm**: sub mucosa, **wm**: white musculature*

3.4 Immune response

Mx protein (Mx), Viperin (Vip), interferon type I (IFN-α), interferon type II (IFN- γ), interleukin-10 (IL-10) and interferon regulatory factor -3 (IRF-3) gene transcripts was selected as parameters for measuring of the innate and antiviral immune response.

3.4.1 Antiviral proteins

Mx

Mx -mRNA transcript fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values (log-transformed) with means and 95% confidence interval are given in **Figure 3.8, A.** 0 hig 1 hig and 6 hig had a significant degree fold change compared to the control group ($p<0,0011$, <0.0217 and <0.0055, respectively)**.** Two fry in 0 hig had a fold change less than 10.0, 4 in the 1 hig and 3 fry in the 6 hig. There was however no significant difference in fold-change compared to the negative control group in the 1 dig or 7 dig. All fry in 7 dig had a fold change less than 10, while 4 fry in both 1hig and 1 dig had a fold change less than 10.

Viperin

Vip -mRNA transcript fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values(log-transformed) with means and 95% confidence interval are given in **figure 3.8, B.** There was a significant Vip fold change increase in 0 hig, 1 hig, and 6 hig compared to the control group as well as the 7 dig. There was no significant Vip fold change increase in the 1 dig or 7 dig compared to the negative control. 2 fry in 0 hig, 3 in 1 hig, 3 in 6 hig, 4 in 1 dig and 10 in 7 dig had a Viperine fold change less than 10.

3.4.2 Cytokines

IFN-α

IFN-α -mRNA transcript fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values (log-transformed) with means and 95% confidence interval are given in **Figure 3.8, C.** There was a significant IFN-α fold change increase in both 0 hig and 6 hig compared to the control group. There was no significant IFN-α fold change increase in the 1 hig, 1 dig or 7 dig compared to the negative control. 2 fry in 1 hig and 3 in 6 hig had an IFN-α fold change less than 10, while 6 fry in 1 hig had a IFN-α fold change less than 10 and 7 in 1 dig.

IFN-γ

IFN-γ -mRNA transcripts fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values(log-transformed) with means and 95% confidence interval are given in **Figure 3.8, D.** The 0 hig and 6 hig showed a significant fold -change in IFN-γ Ct-values compared to the control group as well as the 7 dig, The mean fold -change for 1 hig, 1 dig and 7 dig was not significantly different from negative control. There was four fry in the 6 hig with fold-change values above 30, while only one fry in the 0 hig.

IL-10

IL-10 mRNA transcript fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values (log-transformed) with means and 95% confidence interval are given in **Figure 3.8, E.** There was a statistically significant difference in Ct -mean fold -change of IL-10 in the 0 hig ($p<0.059$) and the 6 hig ($p<0.0017$) compared to the negative control group. There was no significant difference among the other groups Ct fold change-means, but large SDs, where 5 fry in the 1 hig and 3 in the 1 dig had Ct-fold change above 20. All fry in the 7 dig had Ct fold change values less than 2.

3.4.3 Cellular transcription factor

IRF-3

IRF-3 mRNA transcript fold change means and SD are given in **Table 3.1** for all fry groups. Individual fold-change values (log-transformed) with means and 95% confidence interval are given in **Figure 3.8, E.** For IRF there was a significant mean-fold change in 0 hig ($p<0.0059$) and 6 hig ($p<0.0017$) compared to the control group. There was no significant difference in the 1 hig, 1 dig or 7 dig compared to the control group.

Table 3.1: Mean fold change ($2^{-(\Delta\Delta Ct)}$) and SD for each immune gene in the fry groups normalized against a negative control fry group (non-log transformed). Data are colored according to the gene fold change relative to each other. *Dark-brown= High, Brown= medium, light brown= low, white-brown= non-significant.* Data was processed and received from PRISM 8.

Fry group	Mx	Vip	IFN-α	IFN-γ	IL-10	IRF-3
0h dbc	51.49±26.87	269.6±159.0	24.47±18.48	14.89±8.862	54.98±33.22	8.78±4.266
1h dbc	32.34±27.03	166.7±151.1	10.59±11.89	10.50±9.275	27.69±29.43	8.90±6.253
6h dbc	53.08±35.99	252.8±182.3	22.28±18.40	20.00±17.68	63.30±47.61	13.93±11.31
1d dbc	26.81±30.50	115.2±142.8	8.421±10.45	13.62±15.62	32.91±57.21	6.63±5.499
7d dbc	1.17±0.31	1.047±0.63	1.1534±0.41	1.60± 0.31	1.134±0.44	1.17±0.28

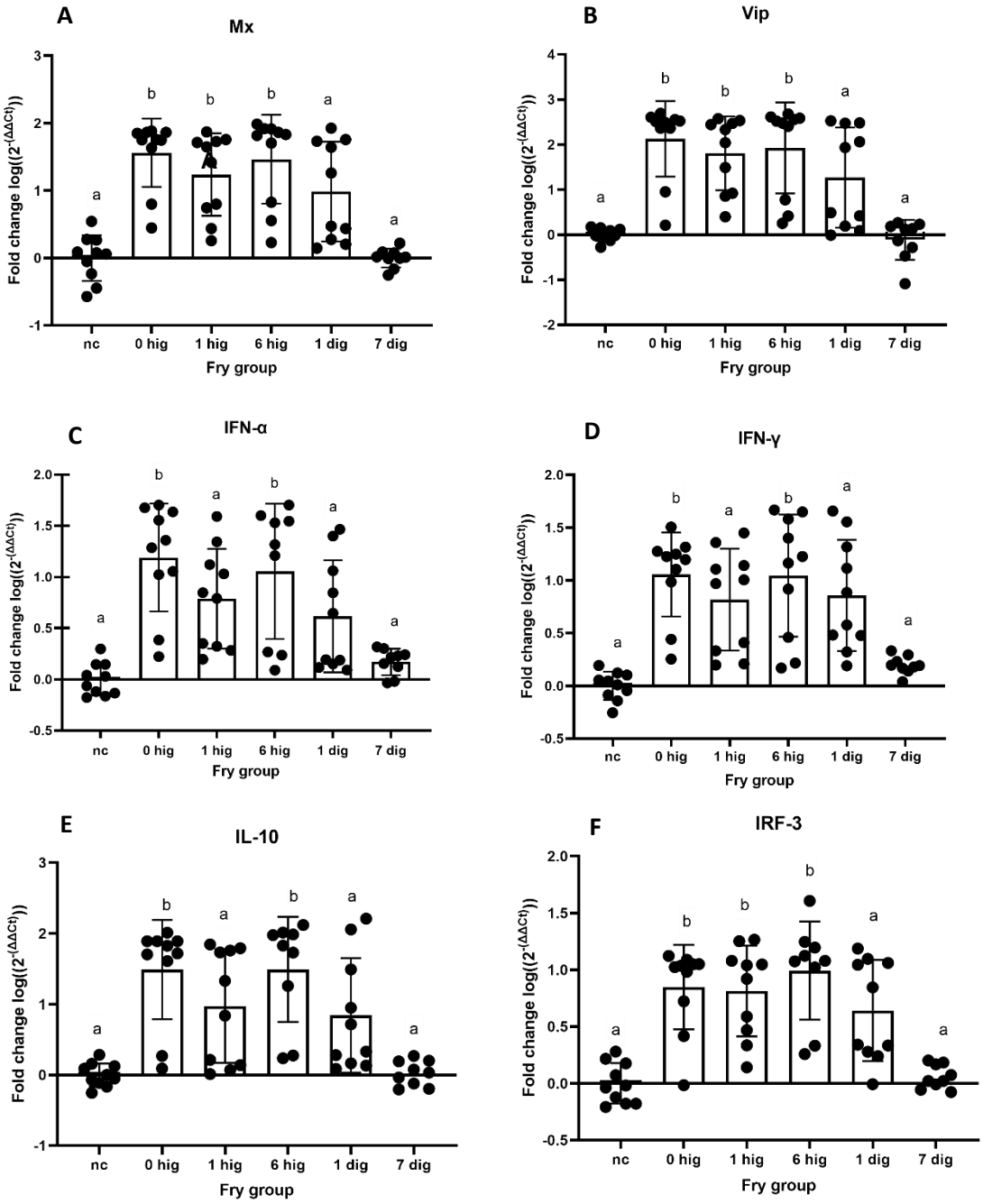

Figure 3.8: Graphs displaying the fold change (log-transformed) for selected immune genes in ISAV challenge fry normalized against negative control fry. A: Mx gene, **B**: Viperin, **C**: Interferon-α, **D**: Interferon- γ, **E**: Interlaukin-10, **F**: interferon regulatory factor- 3. *A One-way ANOVA, Dunnett's multiple comparison test was used to compare the group means (non-log transformed). p<0,05 was considered significant. Significance between the negative control means and the challenged fry groups are highlighted by a compact letter display. Means not sharing the same letter are significantly different.*

3.5 Correlation between the viral load and immune gene fold change in each fry

An apparent pattern between group Ct- means of ISAV S8 and innate immune (Antiviral, cytokines and cellular transcription factors) gene fold change means was shown, and their association was further analyzed.

A *Pearsons r* correlation test showed a strong correlation between the amount of ISAV S8 (Ct) and the fold change ($2^{-(\Delta\Delta Ct)}$) of each respective gene (see **Table 3.2**).

Individual fry from all ISAV challenged groups (0 hig, 1 hig, 6 hig, 1 dig, 7 dig) were compared up against each respective innate immune gene fold change ($2^{-(\Delta\Delta Ct}$).

The antiviral protein Mx showed a strong correlation (R^2=0.87) as well as the Viperin protein (R^2=0.84) (see **Figure 3.9 A** and **B**).

There was a weaker correlation shown within the cytokines compared to the antiviral proteins (**see Figure C, D** and **E)**, but would still be considered as strong correlation (IFN-α: R^2=0.64, IFN-γ: R^2=0.60 and IL-10= R^2=0.70).

The transcriptional factor IRF-3 showed the weakest correlation when compared to the other genes tested (IRF: R^2=0.29) (see **Figure 3.9 F**). This would be considered as a weak association, in contrast to the other innate immune gene measured.

Figure 3.10 shows the overview of all the ISAV S8-fold change for all innate immune genes measured in each fry.

Table 3.2: Shows the correlation parameters. R^2 represents the correlation coefficient. Strong correlation is marked with dark brown color and typical is marked with light brown.

Correlation parameters	**Mx**	**Vip**	**IFN-α**	**IFN-γ**	**IL-10**	**IRF-3**
R	-0,9348	-0,9211	-0,8056	-0,7786	-0,8387	-0,5391
R²	0,8738	0,8484	0,6491	0,6062	0,7034	0,2906
Number of XY pairs	49	49	48	49	48	48
p-value (two tailed)	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001

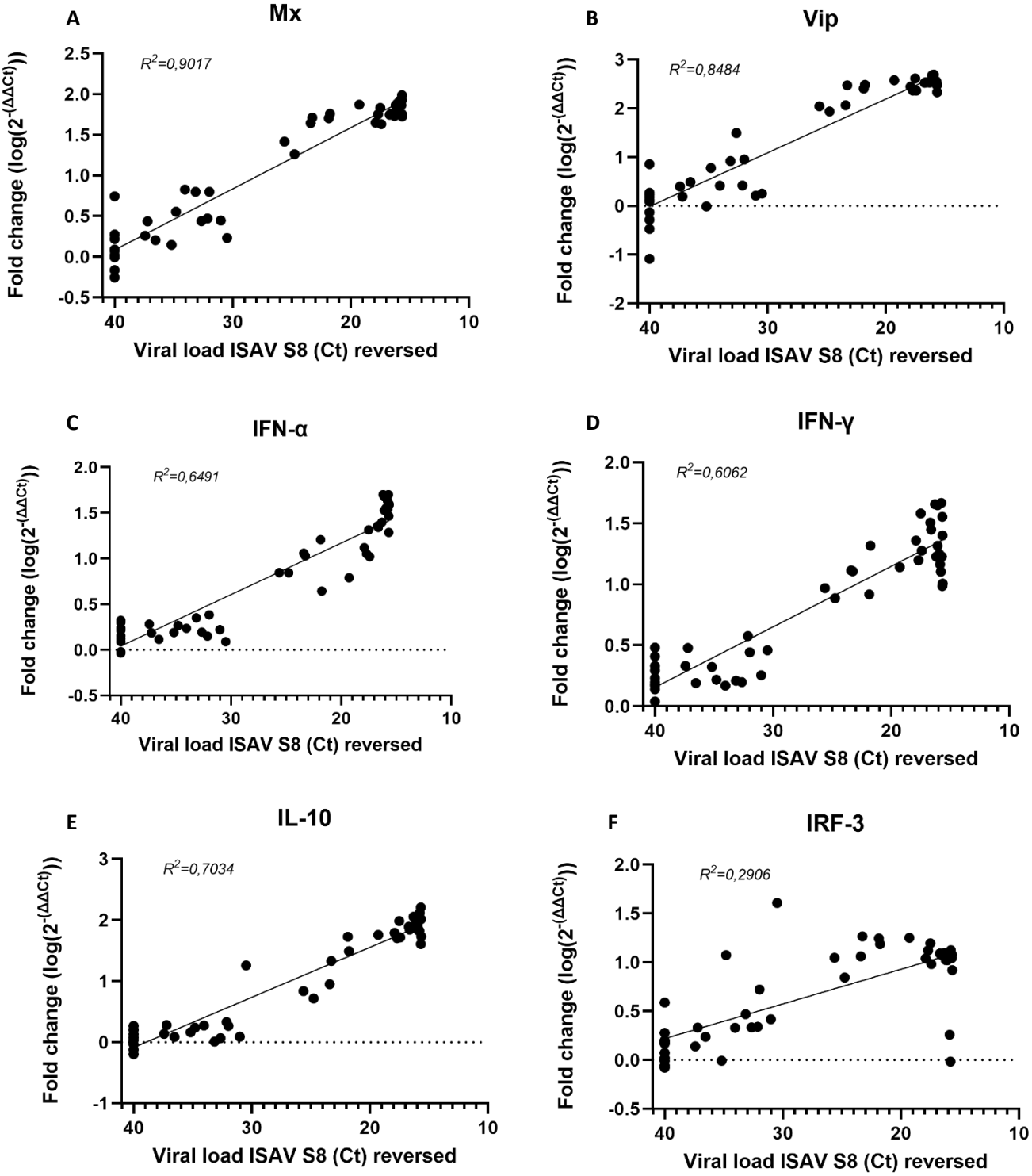

Figure 3.9: Graphs displaying the correlation between viral load and gene fold change in challenged fry. R^2s (Pearsons r) is noted in each graph. ***A****: Correlation of Mx gene,* ***B:*** *Viperin,* ***C:*** *Interferon-α,* ***D:*** *Interferon- γ,* ***E:*** *Interleukin-10,* ***F:*** *interferon regulatory factor- 3. A positive correlation exists between the reversed amount of viral load and gene fold change in all the selected genes across all fry groups.*

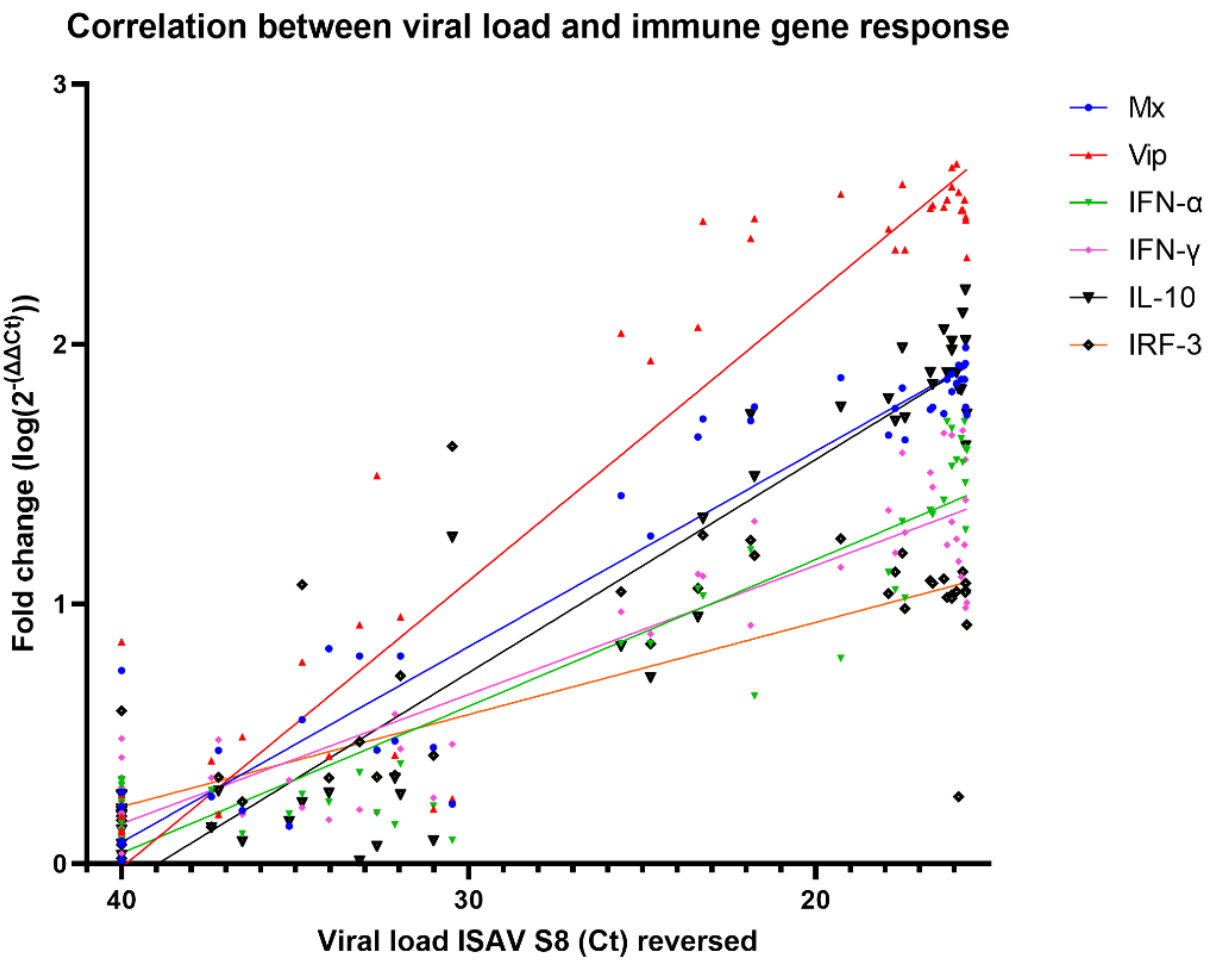

Figure 8: Graphs displaying the correlation between viral load and a fold change of all genes tested in ISAV challenged fry. Correlation lines for each specific gene is color coded.

4 Discussion

This thesis was a part of a larger study carried out to determine the stability of ISAV in seawater using a novel *in vivo* method with Atlantic salmon fry. By measuring the viral load in each individual fry using a RT-qPCR method, ISAV infectivity could be assessed. Histopathological, *in-situ* hybridization and RT-qPCR measurements of selected innate immune genes in ISAV challenged fry was also carried out.

The results from the main ISAV stability study showed that ISAV remains stable in seawater up until 1 day before it quickly loses its infectivity on day 2 and becomes non-infectious after 4 days (*Peñaranda et al. unpublished)*. The results from this thesis concur with these results to some extent, where ISAV loses its infectivity somewhere in between 1 day and 7 days in raw seawater.

This *in-vivo* bath challenge method using Atlantic salmon fry as model animal, proved not to yield any histopathological changes in challenged fry at the sampled time point 12 DPC. It did however manage to infect the challenged fry sufficiently, where *in-situ* hybridization revealed a systemic infection of RBCs and endothelia. Analysis of the immune response in ISAV challenged fry showed that the relationship between viral load and innate immune response is strongly associated.

4.1 ISAV survival

4.1.1 Results from the main experiment

The results from the main experiment are given in **Figure 4.1** and **4.2**, where the viral load for each fry in each time-specific incubation group is plotted. For the raw seawater incubation groups, the plot shows a dramatic decrease in measured ISAV S8 Ct-values in fry after 2 days and is below detectable levels after 4 days. The fry in the 1 dig have a mean Ct-value below 30, while the fry in the 2 dig have a mean Ct-value below 35. This indicates that a great proportion of ISAV -virions loses the ability to sufficiently infect the host after 2 days at 10°C.

Enough ISAV do remain infectious up until 4 days of incubation at 10 °C to infect a few individuals in one of the replicate tanks but must be regarded as being significantly reduced.

A pilot study done prior to our main study, confirmed possible bacterial growth in 10 mL flasks with sampled seawater, when incubated for a longer period (10-35 days). We therefore added filtered samples of ISAV inoculate in our experiment to remove the possible inactivation of virus by bacteria present in the seawater samples. These filtered seawater samples would reduce the chance for bacterial inactivation of incubated ISAV if it reached such late time points (10-35 days).

The filtered seawater ISAV incubation groups showed positive Ct-values for longer incubation time-points than raw seawater. There was no parallel 2 dig group for filtered ISAV incubations, but a 3 dig showed a Ct- mean above 30. 4 individuals in the filtered 3 dig had Ct-values below 25. Positive Ct-values could also be observed in the 5 dig and even 7 dig.

The apparent prolonging of ISAV stability in filtered seawater highlights the effects that microbes can have on virus inactivation and is in agreement with what is previously known (Pinon and Vialette, 2018). If the survival of ISAV was to move on to the later timepoints (10-35 dig), the filtered samples could prove to be useful as the bacterial growth in a limited environment (raw seawater tube) would not sufficiently mimic natural environment conditions. However, this was not the case, as the raw seawater incubations of ISAV lost its infectivity before 10 days post incubation, when bacterial growth would become prominent.

Reviewing the Ct-mean values for the fry of the six selected incubation groups (1 hig- 7 dig) reserved for immunology and histological examinations, ISAV looks to retain its infectivity up until 1 day, where all individuals at 7 dig were negative for ISAV. There was no significant difference in the mean viral load in the 1 dig compared to 0 hig, 1 hig and 6 hig group. There is still a visually increasing spread in Ct-values from 0 hig to 1 dig presented in **Figure 3.1** with an increasing SD. A greater significance between groups would perhaps have been apparent if there had been more salmon fry in each group. Therefore, the results from the main experiments outweigh these results significantly, where ISAV seems to be stable after 1 day post incubation.

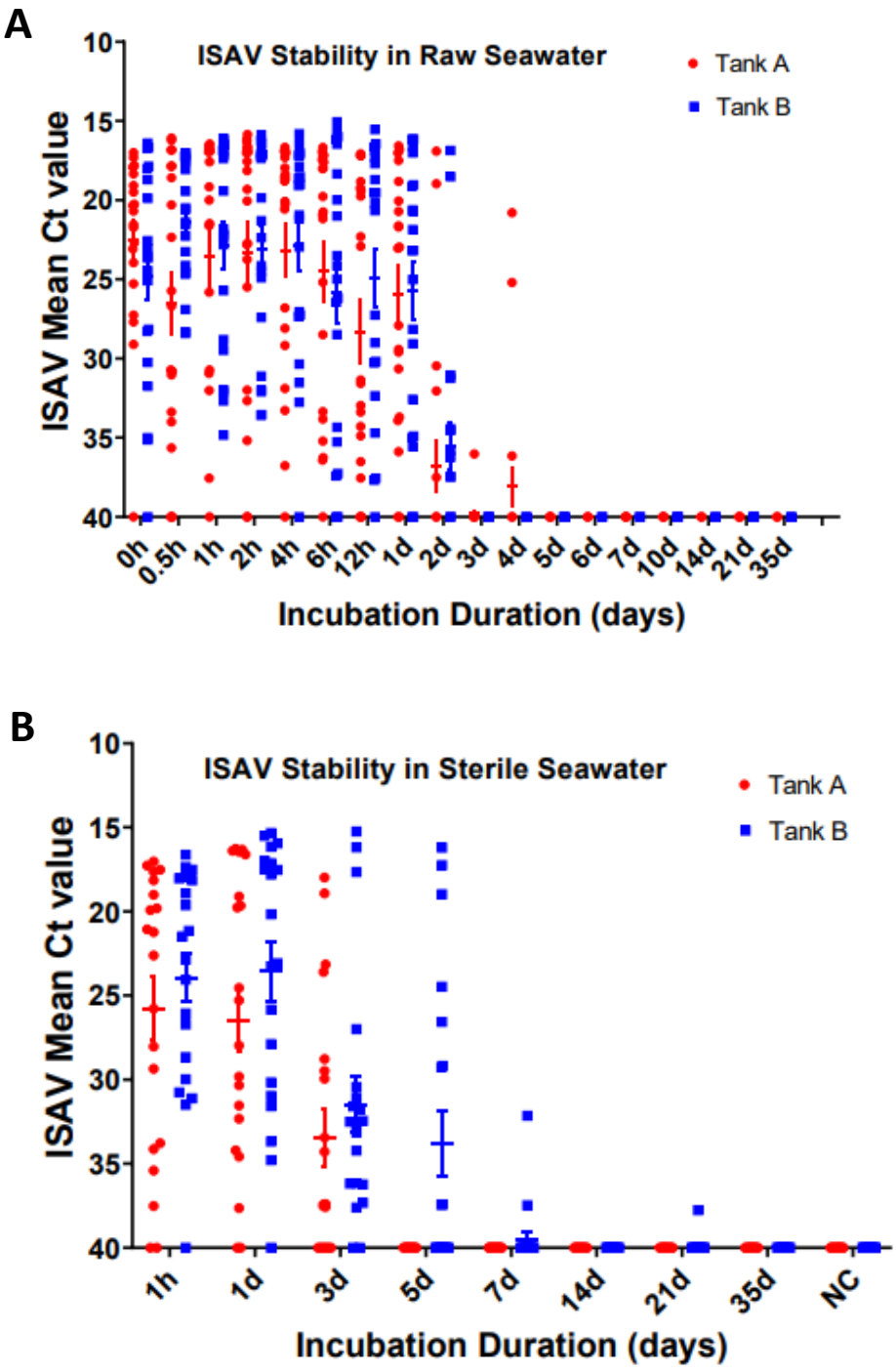

Figure 4.1: Scatterplot of ISAV Ct values for each fry in each incubation group: A shows Ct values for ISAV incubated in raw seawater. **B** shows values for ISAV incubated in filtered/sterile seawater. CT -mean with 25th and 75th percentile are added for each group. Figures are borrowed from *Peñereada et al. unpublished*

4.1.2 Experimental design

Challenge method

Our experimental design differs from the ones previously mentioned (see **section 1.5.2**) where we have used Atlantic salmon fry as an *in vivo* model for measurement of ISAV infectivity. The experiments by Nylund *et al*. (1994) and Vike *et al.* (2014) are in both cases using Atlantic salmon as an *in vivo* model but use larger individuals (44g and 150g respectively). Larger sizes of Atlantic salmon better represent the natural target of ISAV that is of concern today, but reports show that smaller sizes of Atlantic salmon is as susceptible for ISAV infection as larger (Grefsrud et al., 2021) (Søren Grove, personal communication).

Seawater used for the ISAV incubation samples was collected at the IMR facilities from a direct seawater inlet containing a sand filter. A more optimal alternative could have been to collect samples further away, directly from the open waters. This was however not taken into consideration in this experiment.

The practical advantages with using smaller Atlantic salmon fry than larger fish, includes the possibility for larger samples sizes in limited facilities and is also more cost efficient. This gives a more powerful statistical result per bath challenge fry group, compared to what previous experimental designs propose (see **section 1.5.2**). The use of added individuals in a bath challenge infection will also provide a picture of the proportion of fry infected when being exposed for a certain incubated dose.

Our use of a bath challenge as a method of infection, will also better mimic the natural route of infection, which is through the gills and other mucosal surfaces (Aamelfot et al., 2015). Nylund et al. (1994) and Vike et al. (2014) in which both inject the ISAV inoculate i.p. in a group of fish and further by cohabitation infection, which mimics the natural route of infection to a lesser extent. The disadvantage of i.p administration is that it does not reflect the natural route of infection and thus an incubated dose of ISAV injected might infect the host although the dose would not have been sufficient to infect through mucosal surfaces. Furthermore, infection by cohabitation is disadvantageous as the timing of the infection cannot be

controlled and there may be both intra-tank and inter-tank variation in viral load of infected fish.

Another weaknesses with using bath immersion as a way of infection, is that it is harder to keep a controlled and standardized infection of all test animals involved, compared to e.g., i.p. injection (Nordmo, 1997). For this study, a 1:20 ISAV incubation:freshwater relationship was used as a bath challenge solution. This relationship was decided upon as an optimal trade-off between ensuring viral exposure while preserving low salinity levels as not to stress the fresh water adapted fry. This is likely to increase some of the inter group variation seen in fry measured for ISAV S8 Ct-values, where some individuals can possibly have avoided ISAV infection during the bath challenge. The natural infective progression of ISAV, where a drop in viral load is seen after 5-10 days and later followed by a second rise in viral load, can also be a source of inter-individual Ct-value variation (Rimstad et al., 1999). Since the fry were incubated for 12 days after the bath challenge, there might have been some individual variation in terms of developed viremia in this experiment. For instance, the 1 hig group was initially believed to have a greater number of infected individuals than later incubation groups, although it turned out to have a lower degree of infection compared to 6 hig.

Indications, however, suggests that salmon fry reduced physiological complexity compared to larger salmon leads to reduced variation in physiological readings between individuals (Craig Morton, personal communication). More homogeneity within the fish population tested, will in turn yield more significant statistical results for a smaller group of fish. However, larger salmon in the marine phase, is currently the main target for ISAV infection, and the results gained from smaller freshwater phase salmonids, might not reflect the true nature of ISAV infectivity.

In vitro viral titration methods used for confirming ISAV infectivity used by Tapia et al (2009) investigated the time it took for inoculated virus on an ASK monolayer cell culture to reach 99,99% reduction in viral titer. Still, this method only measures the presence of ISAV genome via RT-qPCR in viable ASK cell culture, and not its ability to infect an actual host. Although the virus viability was accounted for by a viral end point titration, it does not reflect ISAV infectivity as an *in vivo* model will. In addition, it has only been tested for sterile seawater in this experiment, which only reflects a natural environment to a limited extent. In Vike et al. (2014) there was also performed a parallel infection trial on ASK cell culture with incubated ISAV

virus, but this trial failed, as the beef extract used in ISAV samples was toxic to the ASK cell culture.

Another disadvantage with *in vitro* cell culture infection methods is that several other current viruses, in which plague the Atlantic salmon farming industry today, are currently non-cultivable. These viruses include Piscine orthoreovirus (PRV), that cause heart and skeletal muscle inflammation (HSMB), and Piscine myocarditis virus (PMCV), which causes cardiomyopathy syndrome (CMS) (Pham et al., 2020, Garseth et al., 2018).

The use of an *in vivo* models with death as an endpoint should be avoided if possible[1]. This current study used an increased number of Atlantic salmon, compared to previous ISAV survival studies. From an ethical perspective, *in vitro* models which use cell cultures to assess virus infectivity, is to be preferred in this regard. If it the scientific or societally gain outweighs the sacrifice and the experimental design cannot be replaced by *in vitro* models, the use of *in vivo* models can be justified.

If this method proposed in this study was to be applied with other viruses, a preliminary MID experiment as well as a confirmation of fry as a suitable *in vivo* model, would be necessary.

Detection method

Nylund et al (1994) used live smolts as an *in vivo* model injected i.p with blood containing ISAV and seawater in a 1:1 relationship for assessing ISAV infectivity. By evaluating hematorcrit values, clinical signs and performing histological examinations, presence of ISAV infection could be determined. The study concludes that ISAV could survive for at least 20 hours in a 1:1 blood:seawater mixture.

Tapia et al (2013) used different viral titres of ISAV, incubated in salt, fresh and brackish water, all steriliezed by antibiotics, boiling and filtering treatments. ISAV infectivity was assessed by viral end point titratrion every 7-15 day over 7 months, where viral survival was measured from the time of inoculation until it showed 99,99% reduction in viral load. ISAV survival was reported for up until 10 days in seawater at 10 °C.

Vike et al (2014) used beakers of sampled seawater inoculated with concentrated ISAV and treated either with or without UV radiation and incubated over 72 hours. ISAV presence and

[1] Forskrift om bruk av dyr i forsøk. *I 2015 hefte 7.* Norge: Landbruks- og matdepartementet.

infectivity in incubation beakers was measured through RT-qPCR and i.p injection of live Atlantic salmon smolts. The gills, heart and kidney tissue were sampled at 7 and 14 days post challenge (dpc) and tested for ISAV via RT-qPCR. The results could only confirm the infectivity of ISAV after 12 hours in non-UV treated sterile seawater, and that ISAV would remain infective in natural seawater for less than 3 hours by both IP and RT-qPCR.

Assessing infectivity of ISAV may be a challenge as there is strengths and weaknesses with any method. RT-qPCR is known for having high sensitvity and specificity in the detection of both HPRΔ and HPR0 ISAV. However, the method does not tell anything about the current infective abilty of the virus in question.By using cell cultures, one may confirm the infectivty by assessing the cytopathic effect (CPE), but this method is not susceptible for all viruses and their use is limited. The use of live Atlantic salmon solves this problem, but is more costly and should be avoided if possible with regrads to animal welfare. Clincal signs of ISAV infection and gross pathology of tissues should always be combined with a RT-qPCR or IFAT/in-situ hybridization for confirming positive infection.

Our experimental set up resembles that of Vike et al. (2014) the most, but our results differ greatly. They used a different ISAV isolate from ISA-infected salmon from Chile (CH35/09), but with a similar consentration ($5x10^4$ $TCID_{50}$/ml) to our study.

It is difficult to simulate the exact environment, in which a virus will be exposed to under experimental conditions. Therefore, the most important factors that affect virus stability (mentioned in **section 1.5.1**) must be compared between the individual experiments. However, these experiments need to have a standardized challenge and detection method for the results to be fit for comparison.

4.2 Histology

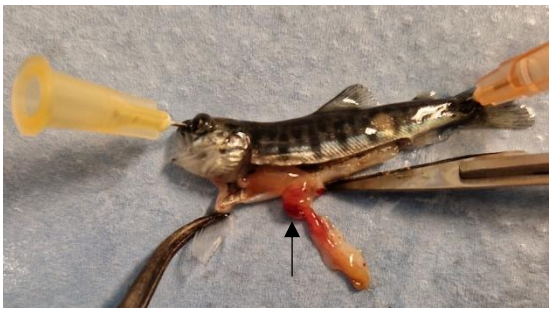

Figure 4.2: Atlantic salmon fry challenged with ISAV during dissection for heart and organ package. Clinical signs reveal bleedings on the intestine and surrounding areas (black arrow).

Atlantic salmon fry, measuring 3.0-3.5 cm, have a quite diffuse set of organs, in which are not easily distinguished from each other upon visual examination. There were a few selections of individuals that showed hemorrhagic lesions in the skin surrounding the anal and intestine tissue (see **Figure 4.2**). These individuals originated from the main experiment and were not sampled for histological examination.

Histopathological lesions caused by ISAV will usually include a hemorrhagic lesion with confocal necrosis around the veins in the liver tissue. Severe hemorrhagic lesions with tubular necrosis in kidney tissue (Thorud and Djupvik, 1988). The histopathological changes are variable in diseased fish, where factors like age, temperature immune status and infective dose of the given virus strain are deciding for the disease development.

A great advantage that this experimental model possesses is the size of the Atlantic salmon fry (3.0-3.5 cm). These sizes are small enough to yield histological sections in a sagittal plane, which have practical implications for histopathological and ISH analysis (see **section 4.3**). This way, several organs can be examined in one section, and ISH analysis can elucidate how a virus spreads in the host. This requires however, that the fry is as susceptible as the later developmental stages.

The histological examination showed no histopathological changes in any of the analyzed fry, where 60 individual fry was analyzed visually by light microscopy. Potential bleedings in the liver and kidney tissue were primarily focused on when assessing the histological tissues, as well as bleedings in the heart and gill tissue.

Our lack of histopathological finding was however not all too unexpected when previous literature reports of pathological signs first being apparent after 15 days post challenge (Rimstad et al., 1999). The reasoning for not incubating the fry longer than 12 days was to reach a high enough viremia while not killing the fry, in addition to avoid shedding-induced

secondary infection in naïve fish. The virus titre and incubation period were decided upon through a MID infection trial (see **section, 2.1.2**), done prior the main ISAV experiment.

4.3 *In-situ* hybridization

The *in-situ* hybridization gave strong signals from most organs in the infected fry, thus confirming the presence of ISAV RNA. The negative controls showed weak signals from mostly the nucleus of cells in different tissues. These signals were significantly weaker than that seen in the infected samples. Others have reported similar unspecific staining of the nucleus when using the RNAscope® 2.5 HD Detection reagent (RED). The sectioning of a parallel HES section to the ISH section partially failed and made the two sections too far apart, and thereby mostly incomparable. However, parts of the kidney were comparable, and signals could be confirmed to originate from RBCs, and most likely from sinusoidal capillaries.

Previous findings, via IHC and *in situ* hybridization, shows ISAV present in heart, kidney, liver, gills, spleen, pyloric caeca and mid-gut (Gregory, 2002). More specifically, ISAV has been found to primarily localize to endothelial cells, RBCs and leukocytes, as well as gill epithelial cells in the early stages of infection (Aamelfot et al., 2012). It is believed that ISAV infect endothelial cells and bud out in the luminary side, for then to attach the surface of circulating RBCs, thus spreading throughout the circulatory system, infecting new endothelial cells (Aamelfot et al., 2014). The distribution of 4-*O* acetylated acid covering proteins on cellular surfaces is shown to correlate well with ISAV tropism, making it an important receptor determinant for ISAV distribution (Aamelfot et al., 2012).

From our results, positive hybridization signals can be detected in most organs, especially in kidney, heart, and liver. The signals in these organs were mostly originating from RBCs, but also from endothelial cells.

Interestingly, this type of sagittal sectioning in Atlantic salmon fry had the possibility to reveal ISAV presence in RBCs and endothelial cells in organs, such as brain, choroid plexus, and skeletal muscle. It is to our knowledge the first time ISAV presence in the RBCs and endothelial cells have been identified in these organs and corresponds with the idea stating that ISAV moves systemically in the host.

The fry selected for the *in-situ* hybridization was collected from the 1 dig based on the suitability of the section. There was not done *in-situ* hybridization of other groups of ISAV challenged fry due to project limitations. Our results demonstrate that ISAV, incubated over 1 day in raw seawater, is able to infect Atlantic salmon systemically during a 12 day challenge period.

Using Atlantic salmon fry allows for ISH-examination of the entire sagittal plane of the fry. This is useful, not only for assessing ISAV tissue distribution, but also for assessing other salmonid viruses tissue distribution, such as PRV, PMCV or SAV. Several questions regarding PMCVs route of entry, their transmission, how they behave in the host and how disease develops has yet to be answered (Polinski et al., 2020). Sagittal ISH sections of fry infected with PMCV could perhaps help to reveal some of its early pathogenesis.

4.4 Immune response

Mx protein is found in mammalian as well as teleost species and have been shown to exhibit antiviral effects against several orthomyxoviruses, including ISAV (Kibenge et al., 2005). Its exact function is unknown, but is believed to prevent the viral genome entrance into the nucleus of the host's target cell (Haller and Kochs, 2002).

The Mx genes in infected fry was significantly upregulated in 0 hig, 6 hig and 1 dig while it was insignificant upregulated dig compared to the control group. The relationship between viral load and Mx fold change in challenged fry was strongly correlated ($R^2=0.9017$). There was an apparent pattern of the same fry, in which did not show elevated levels of Mx, most likely reflecting the absence of ISAV infection. The Mx gene transcription appears to be dependent on the viral load in Atlantic salmon fry.

Viperin is another antiviral protein that is shown to exhibit protection against viral infection, where it can prevent virus from budding from the endoplasmic reticulum during their replication, by inhibiting certain lipid synthesis enzymes (Seo et al., 2011). Several challenged fry showed an increased upregulation of Viperin, correlating well with the measured viral load of ISAV ($R^2=0.84$).

IFN-α or interferon type-I is an important antiviral cytokine expressed upon viral recognition. It will then inform neighboring cells of the viral infection via interferon receptors and make these cells induce interferon stimulated genes (ISGs) that leads to an antiviral state (Zou and Secombes, 2011). These were upregulated in ISAV infected fry and correlated well with the viral load of ISAV (R^2*=0.64*). The induction and expression of IFN-α is pivotal for an innate immune response for several fish viruses. ISAV has however been shown to possess IFN-antagonizing factors, which include two proteins, s7ORF1 and s8ORF2, which can inhibit the cells expression of IFN-α upon infection (García-Rosado et al., 2008).

IFN-γ is a type II-interferon and is more strongly associated with the connection with the adaptive immune system than IFN-α, where it can induce increased antigen presentation on infected cells (Zou and Secombes, 2011). Challenged fry seemed to have similar degrees of up-regulation of IFN-γ and was also correlated with the viral load of ISAV.

IL-10 is an anti-inflammatory cytokine, in contrast to pro-inflammatory cytokines, reduces the inflammatory effects and thus, helping the regulation and preservation of the host homeostasis (Opal and DePalo, 2000, Rebl and Goldammer, 2018). Several challenged fry showed increased levels of IL-10 transcripts and was well correlated with the viral load of ISAV (R^2=0.70).

IRF-3 is a cellular protein responsible for the induction of the cell's expression of cytokines and chemokines upon virus recognition (Takeuchi and Akira, 2009). This was upregulated in challenged fry, but not as well correlated with the viral load of ISAV (R^2=0.29) as with the other genes. This might be due to the progression of the innate immune response, where a higher IRF-3 expression would be observed in the earlier stages of infection (Bergan et al., 2010).

The innate immune response is of great importance during viral infections in vertebrates, where a strong response in some cases can clear out the virus (Collet, 2014). This seems not to be the case with ISAV, where a full viral clearance appears to be dependent on it is ability to mount an adaptive immune response (Jørgensen et al., 2008b). The relationship between the viral dose and elicited immune response can have great consequences for how a virus replicates and spreads to naïve hosts. Orthomyxoviruses have for instance been shown to have an inverse relationship between viral dose and immune response, which can have consequences for how ISAV infects and spreads (Weli et al., 2021).

There was no upregulation for any of the genes without a detection of ISAV viral RNA. This shows that bath challenged fry do not activate the innate immune response without viral RNA present, at least after 12 dpc. It was discussed prior to the experiment that fry challenged with incubations of ISAV would be able to avoid infection and clear potential viral RNA through an effective innate immune response. This however seemed not to be the case as the immune genes and viral load of each challenged fry was well correlated.

The method of infection has previously been shown to affect the timing of the innate immune response during salmonid alphavirus (SAV) infection in Atlantic salmon (Moore et al., 2017). The intra musculature (i.m) infection method gave a stronger and faster innate immune response than the ones challenged using a bath immersion method. The same could be true for this experiment, where the immune response would be weaker and slower than that of i.p injection of ISAV. Since bath challenge better simulate the natural route of infection, the measured immune response might reflect the natural conditions better.

Previous measurements of innate immune genes via a RT-qPCR method, during ISAV infection, have used lysed tissues from specific organs like head kidney, heart or gills (LeBlanc et al., 2010, Valenzuela-Miranda et al., 2015). In this study, we demonstrated the detection of specific innate immune gene mRNA transcripts by RT-qPCR of the entire organ package and its potential use. This confirms that the use of fry in infection trial of both ISAV and possibly other salmonid viruses, is practically viable for studying the viral kinetics and immune response development during viral infection.

4.5 Limitations of the study

The current study was originally planned to include a parallel stability study of salmonid alphavirus (SAV), but it was excluded due to wet lab capacity issues.

It would have been interesting to have performed additional *in-situ* hybridizations of fry in the 1 dig and 7 dig to reveal a potential presence of ISAV. This was not done due to the lack of materials and time.

We were able to test only a limited selection of genes associated with the innate immune response and one time point (12 dpc) in this experiment. It would have been interesting to test for more time points and genes.

4.6 Future prospects

Our findings provide valuable information regarding the stability of ISAV in seawater, and our data will be useful in the development of creating risk assessment of ISAV spread from infected aquaculture facilities. Such risk assessments need to contain information on rate of viral shedding, MID needed for naïve target host, prevalence of shedding in the infected population, and site specific water flow rates and the viral survival parameters (Oidtmann et al., 2018). Developing more accurate risk assessments will in turn give way for the implementation of more effective control measures to prevent horizontal spread of ISAV in the marine environment.

Our results show that this *in vivo* challenge of Atlantic salmon fry is viable for detecting not only viral RNA, but also specific immune genes via RT-qPCR method by sampling the entire organ package. This model could be used for assessing both the stability and immune response for other salmonid viruses, such a PMCV, PRV or SAV.

5. Conclusion

In this thesis it is shown that ISAV remain infectious for more than 1 day in raw seawater at 10 °C and loses its infectivity by 7 days post incubation. The larger study this thesis was a part of, shows that ISAV remains infectious for past 4 days in raw seawater, and 7 days in filtered seawater (*Peñarada* et al. unpublished), using a novel *in vivo* challenge method. The study demonstrates that this method is viable for assessing histopathology and *in situ* hybridization of ISAV infected fry. It is shown that ISAV is present in RBCs and endothelial cells in challenged Atlantic salmon fry, 12 dpc. It also shows that specific innate immune genes can be isolated and detected from the entire organ package of Atlantic salmon fry by an RT-qPCR method and that the viral load of ISAV and innate immune gene response is well correlated.

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