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Effect of Salmonid alphavirus infection on housekeeping gene expression, validation by quantitative, real-time PCR

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Content

List of abbreviations

PD	Pancreas disease
SAV	Salmonid alphavirus
SD	Sleeping disease
SPDV	Salmon Pancreas Disease Virus
+ssRNA	Positive-sense single-stranded RNA
EG	Eosinophilic granules
ER lumen	Endoplasmatic reticulum
dNTP	deoxunucleotide
nsP2	Non-structural protein 2
IFN	Interferon
ISGs	IFN-stimulated genes
NLS	Nuclear localization signal
dATP	Deoxyadenosine triphosphate
dTTP	Deoxythymidine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
JAK-STAT	Janus kinase-signal transducer and activator of transcription
FBS	Fetal bovine serum

1. Introduction

In Norwegian salmon farming, approximately 15-20% of the total number of fish transferred to sea do not complete their production cycle. There are an array of factors contributing to this loss, and pancreas disease (PD) caused by Salmonid alphavirus (SAV) is one such important cause. SAV is recognized as a serious pathogen of farmed Atlantic salmon (*Salmo salar* L.) and Rainbow trout (*Oncorhynchus mykiss* Walbaum) in Europe, causing pancreas disease or sleeping disease (SD) respectively. PD or SD outbreaks can lead to significant economic losses due to mortality, retarded growth and reduced slaughter weights. The occurrence of PD in Norway is increasing, with 138 confirmed outbreaks in 2016, compared to 44 outbreaks for the year of 2004 (Norwegian Veterinary Institute, 2016). Economic losses per outbreak site are estimated at over 1.08 million Euro (Gonzales Vecino, et al., 2010).

We know a great deal more about Alphaviruses in general then we do specifically about SAV. One hallmark of Alphavirus infection in vertebrate cells is the ability to shut down host transcription and translation processes without affecting viral protein and nucleic acid synthesis (Fros & Pijlman, 2016). It is of significant interest to find out if SAV shares this characteristic.

The aim of this project is to investigate Salmonid alphavirus' ability to down-regulate host cell transcription. By using a two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for quantitative analysis of different housekeeping genes in non-infected and infected cells, we are hoping to evaluate if SAV3 (Salmonid alphavirus subtype 3) causes down-regulation of transcription. Furthermore, we wish to establish at what time interval down-regulation may occur, and also to what degree transcription is affected. This experiment and its consequent results, will serve as a pilot study to optimize current methodologies and iron out possible mistakes for further experimentation.

2. Literature review

The infectious agent causing pancreas disease in farmed Atlantic salmon was first isolated in Scotland in 1995, later proposed with the name Salmonid Alphavirus (SAV). Other similar diseases were later discovered to be caused by a similar agent; Sleeping Disease in Rainbow trout in France and Norwegian Salmonid Alphavirus in Atlantic Salmon and Rainbow trout in Norway (McLoughlin & Graham, 2007). These diseases are closely related and are now considered subtypes of SAV. There are currently six recognized subtypes of SAV; SAV 1-6, where subtypes SAV2 and SAV3 occur in Norway. In Norway, south of Hustadvika in Møre og Romsdal, SAV3 is defined as an endemic disease causing agent. Marine SAV2 was diagnosed in Norway in 2011 after several outbreaks north of the endemic region. Marine SAV2 spread rapidly, forcing a legislation that defined a second endemic region in 2012 just north of Hustadvika (Jansen, et al., 2015).

2.1. Salmonid Alphavirus

2.1.1 Classification of SAV

Salmonid alphavirus belongs to the genus Alphavirus in the family Togaviridae. The Togaviridae family contains two genera; Alphavirus and Rubivirus, which have a similar genomic organization but phylogenetically are only distantly related (Koonin, et al., 1993). The Alphavirus genus contains about 30 virus species, most of which are maintained through a transmission cycle between vertebrate hosts and mosquito vectors (Chamberlain, 1981). There are, however, exceptions to this vertebrate-mosquito transmission cycle, namely the Buggy Creek virus and Southern Elephant Seal virus, which are transmitted by swallow bugs and seal lice (Fros & Pijlman, 2016). SAV is an exception to other Alphaviruses as it does not require an arthropod vector, rather it can be transmitted horizontally and be maintained for extended periods in seawater (Fiskeri og Havbruksnæringens Forskningsfond, 2012).

Alphaviruses are subdivided based either upon their geographic distribution or their genocomplexes. There are three main genocomplexes; the Venezuelan Eastern Encephalitis-

Eastern Equine Encephalitis (VEE-EEE) group, the Semliki Forest (SF) group and the Western Equine Encephalitis (Sindbis) group (McLoughlin & Graham, 2007). Grouping Alphaviruses based on their geographical distribution factions them into New and Old World Alphaviruses. The New World Alphaviruses include Venezuelan, Western, and Eastern Equine Encephalitis viruses, which are important human pathogens causing encephalitis. Old World alphaviruses typically causes rash, fever and polyarthritis, and include Sindbis virus and Chikungunya virus among others.

Regardless of how one subdivides the Alphaviruses, they all share a common epidemiology, with infection being transmitted between primary avian or mammalian reservoir hosts by hematophagous arthropod vectors (McLoughlin & Graham, 2007). SAV does not have an identified arthropod vector, and horizontal infection of SAV is well recognized (Kongtorp, et al., 2010). However, sequencing of the 3'-end portion of the genome demonstrated that the previously named Salmon Pancreas Disease Virus (SPDV), now called SAV, had a molecular structure consistent with it being a member of the genus Alphavirus, and was classified as the first alphavirus reported in fish in 1999 (Weston, et al., 1999). The causative agent of SD, Sleeping Disease Virus (SDV), which was previously recognized as an evolutionary distinct alphavirus (Villoing, et al., 2000), has now been demonstrated, through comparative genome sequence analysis with SPDV (SAV), to be a closely related member of the same species, now called Salmonid Alphavirus (SAV) (Weston, et al., 2002). Although the name is not yet recognized by the International Committee on the Taxonomy of Viruses, it is commonly used by those who work in this area (Graham, et al., 2008).

2.1.2 SAV Subtypes

Until 2008, SAV strains were categorized into three subtypes: SPDV isolated from Ireland and the United Kingdom (SAV1); freshwater SDV isolated from the UK, France and Germany affecting rainbow trout (SAV2); and the Norwegian salmonid alphavirus (SAV3), which is currently still only found in Norway. Sleeping disease in Rainbow trout was first described in France in 1994 by Boucher and Baudin-Laurencin, first isolated in 1997 by Castric et al., and described as an "Atypical alphavirus" in 1999 by Villoing et al. SPDV had been classified as the first alphavirus reported in fish a few months before by Weston et al.

A comparative genome sequencing study conducted in 2002 by Weston et al. found that SDV and SPDV were closely related members of the same species, which lead them to propose the new name; Salmonid alphavirus (SAV). Norwegian salmonid alphavirus (SAV3) was described as a third subtype in 2005 (Hodneland, et al., 2005).

In 2008, 54 isolated viral strains originating from Ireland, United Kingdom, Norway, France, Spain and Italy were sequenced and compared (Fringuelli, et al., 2008). The results indicated that one could group the SAV viruses into six subtypes, not three, adding SAV subtypes 4, 5, and 6. The SAV2 subtype exists in both fresh water and salt water. Fringuelli et al. (2008) found that the monophyletic nature of the SAV2 freshwater strain suggests that a single introduction of SAV2 occurred at an early point, and that this introduction is likely to have followed from a marine source.

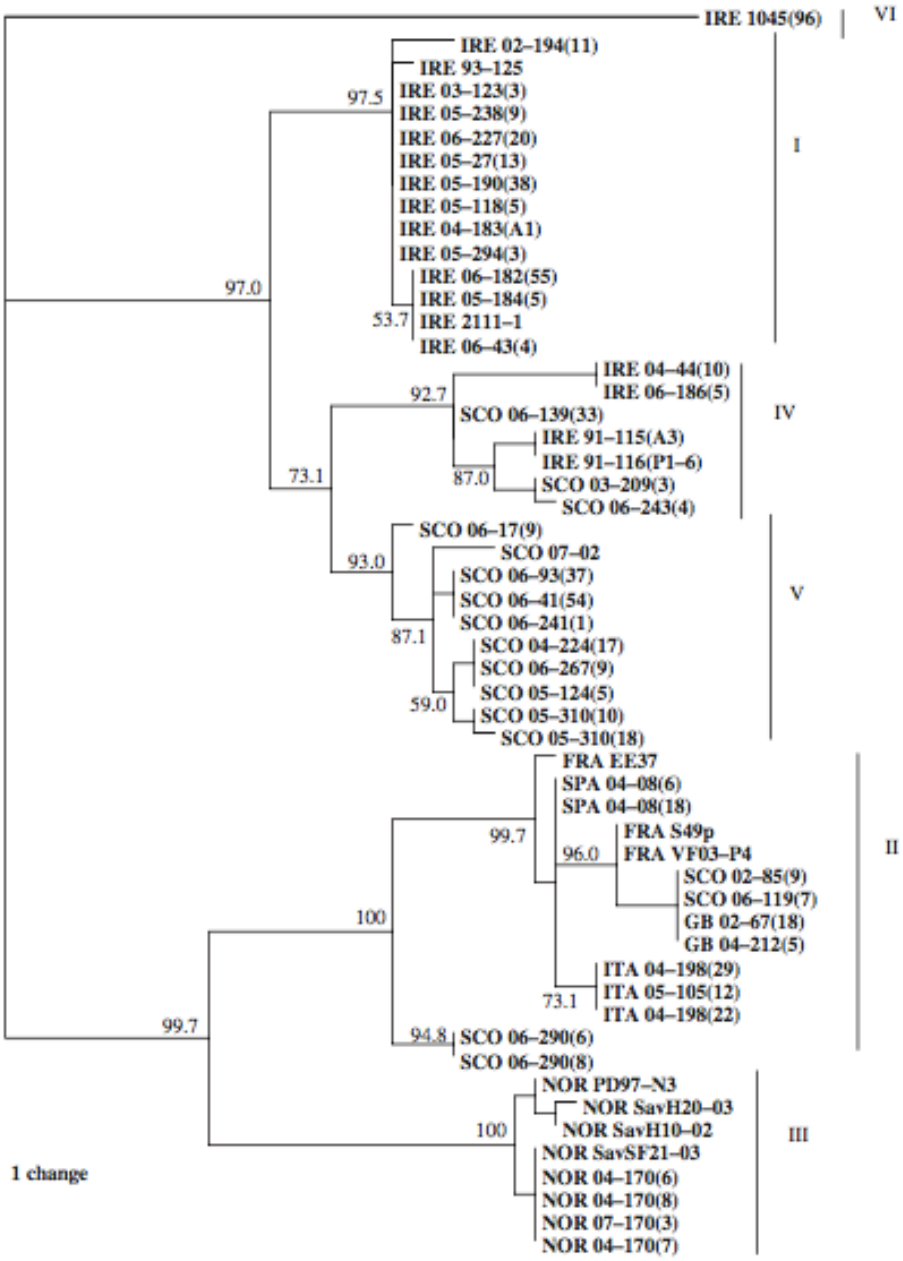

Figure 1: Phylogenetic tree showing the relationship between SAV strains based on nucleotide sequence of the E2 gene fragment. SCO 06-290 representing the Marine SAV2. From (Fringuelli, et al., 2008).

2.1.3 Distribution of SAV in Norway

There are currently two separate endemic zones in Norway. A SAV3 endemic zone was established south of Hustadvika in Møre and Romsdal at the same time as SAV3 was made in notifiable in 2007. The legislation established restrictions on transport and movement of fish from and within this zone, and PD outbreaks occurring outside the zone

were subject to successful eradication and fallowing. Following a PD outbreak north of Hustadvika in 2011, DNA sequencing revealed that the marine SAV2 subtype was the causative agent (Hjortaas, et al., 2012). SAV2, previously undiagnosed in Norway, was probably introduced as early as 2010 by a contaminated well boat that was recently used for the transportation of smolt in the UK. A second endemic zone was established from Hustadvika to the border between Sør-Trøndelag and Nord-Trøndelag.

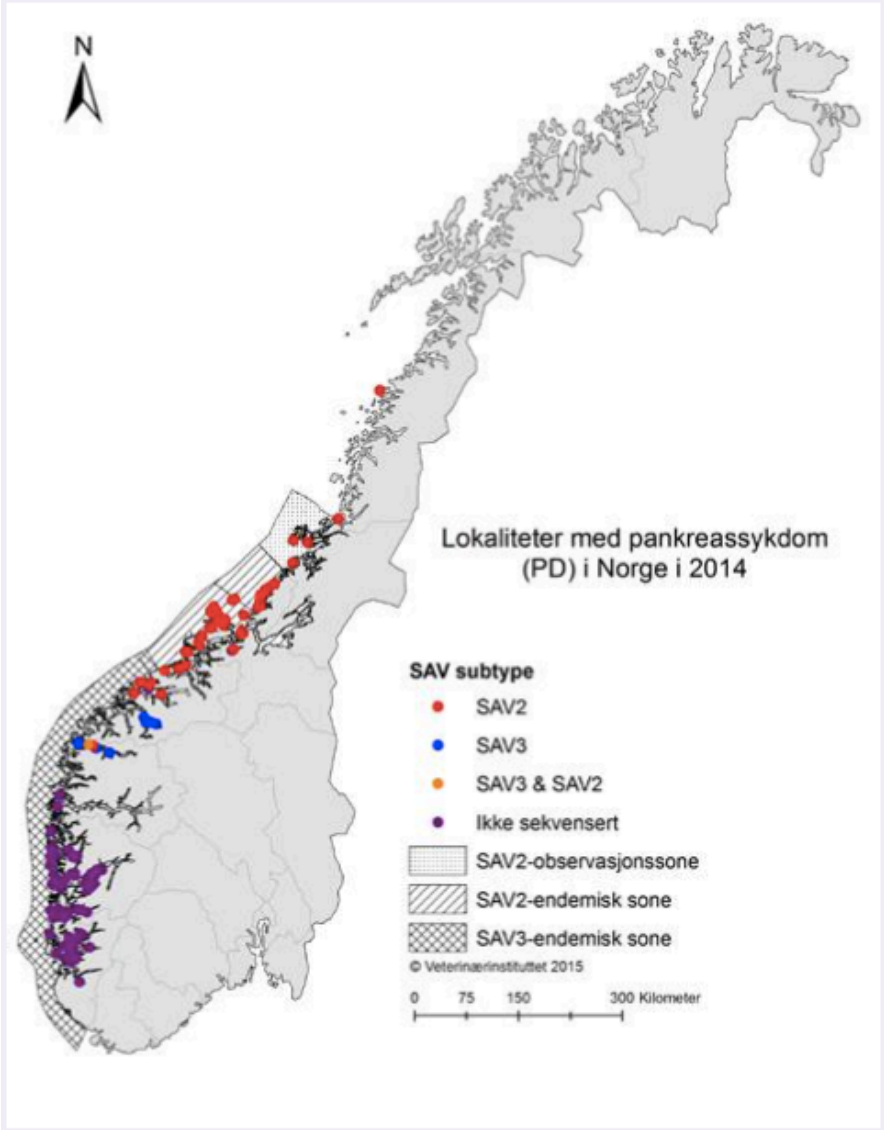

Figure 1: SAV endemic zones in *Norway*. From the bottom; SAV3 endemic zone (squares), SAV2 endemic zone (lines), SAV2 observation zone (dots) (Norwegian Veterinary Institute, 2015).

2.1.4 Distribution of SAV: United Kingdom (Scotland) and Ireland

Pancreas disease (PD) was first described in Scotland in 1976. Here one can find SAV subtypes 1, 2, 4, 5, and Marine SAV2 (see figure 3). By 1986 PD had been reported in 19% of the salmon farming sites in Scotland (Jansen, et al., 2016). A nationwide study conducted in 2006-2007 tested 74 out of approximately 250 active sites with RT-PCR, and found an 18 % prevalence of SAV in Salmonids throughout fish farms in Scotland (Lester, et al., 2011), giving an estimated prevalence of occurrence at that time.

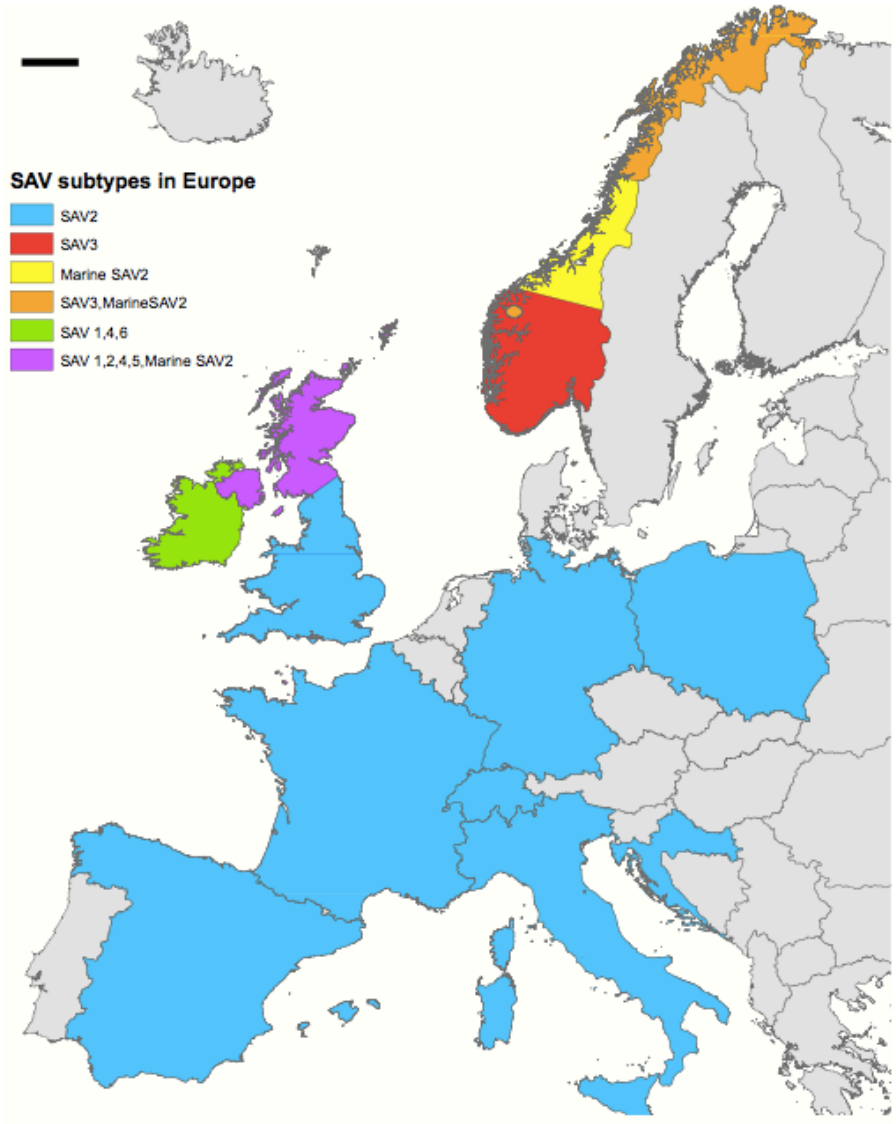

Figure 2: 2016 Distribution of the different subtypes of Salmonid alphavirus (Jansen et al. 2016)

PD disease is considered the most significant infectious disease affecting marine salmon in Ireland, having a great economic impact on Irish fish farms. In 2003 and 2004 it was estimated that PD was responsible for a total loss of 35 and 12 million euros respectively (Fringuelli, et al., 2008). As seen in figure 1, one can find all the SAV subtypes in Ireland except SAV3 (Madhun, et al., 2016).

2.1.5 Distribution of SAV: Europe and North America

PD and SD prevalence in the rest of Europe can be seen in table 1. A PD-like condition has been described once outside Europe, in North America in 1987, but no virus was detected (McLoughlin & Graham, 2007).

SAV2, causing sleeping disease in freshwater rainbow trout, was first described in France, and has since been demonstrated in England, Scotland, Germany, Poland, Italy and Spain, as seen in table 1.

Table 1 Salmonid alphavirus genotype group and distribution (OIE, 2016)

SAV subtype	**Host and environment**	Country
SAV 1 (PD)	Atlantic salmon (sea water) Rainbow trout (fresh water)	Ireland, UK (Northern Ireland, Scotland)
SAV 2 FW (SD)	Rainbow trout (fresh water) Atlantic salmon (sea water)	France, Germany, Italy, Spain, Switzerland, Poland, UK (England, Scotland)
SAV 2 Marine (PD)	Atlantic salmon (sea water)	Norway, UK (Scotland)
SAV 3 (PD)	Rainbow trout (sea water) Atlantic salmon (sea water)	Norway
SAV 4 (PD)	Atlantic salmon (sea water)	Ireland, UK (Northern Ireland, Scotland)
SAV 5 (PD)	Atlantic salmon (sea water)	UK (Scotland)
SAV 6 (PD)	Atlantic salmon (sea water)	Ireland

2.1.6 Pathology, Histopathology, and Clinical Manifestation of SAV Infection

Clinical signs of PD may be sudden inappetence with high morbidity in the affected cages 1-2 weeks before the detection of a disease outbreak, while lethargy, increased

mortality, and insufficient weight gain follow (McLoughlin, et al., 2002). The affected fish will often crowd in dense numbers near to the waters surface against the current, or, alternatively, rest at the bottom of the cage. Salmon with decreased weight gain (picture 1), are seldom observed during the initial outbreak of clinical signs, but are reported several months later when the majority of surviving fish appear clinically healthy (Taksdal, et al., 2007).

Picture 1: Healthy farmed salmon (top) and a same age farmed salmon with PD, called "Runts" (bottom) (Press, 2013).

In the early acute stage of the disease, few gross pathological lesions can be observed. Most notably there is absence of food in the gut, presence of yellow fecal casts and a reduced body fat measurement (McLoughlin, et al., 2002). Occasionally petechial hemorrhages can be detected over the surface of the pyloric caeca and the surrounding fat tissue (McLoughlin & Graham, 2007).

In cases of chronic PD, histopathological lesions can be observed in several organs. Necrosis of the pancreatic acinar cells disrupts the ability to produce digestive enzymes which, together with muscle damage of the esophagus due to myopathy, explains the decreased weight gain. In a study by Taksdal et al. in 2007 which described clinical signs and pathological findings of PD in Atlantic salmon and Rainbow trout in Norway, heart lesions appeared concurrently or slightly later than the pancreatic lesions, and included both cardiomyocytic necrosis and inflammatory cellular infiltration in both the compact and spongious layer of the ventricle. About half the sample population had only mild or no noticeable heart lesions, indicating the processes of recovery, while pancreatic lesions were

more persistent. Skeletal muscle lesions could be found as degeneration and inflammation in both the red and white muscles in a large percentage of SAV infected fish. Cells packed with eosinophilic granules (EG) lining the kidney sinusoids could be found in 64 % of the diseased fish that presented with myositis, and similar cells in macrophage-like cells in the spleen could be found in 21 % of diseased fish presented with myositis. The damage of heart and skeletal muscles can affect the circulatory system and concurrently the swimming pattern of the fish. Furthermore, the damage to muscle integrity may affect the slaughter quality in recovered fish.

2.2 Alphavirus characteristics

2.2.1 Alphavirus Structure

The Alphavirus genome consists of a single positive stranded RNA (+ssRNA), approximately 12 kb, enclosed in a protein capsid surrounded by a lipid envelope. The envelope is composed of a lipid bilayer embedded with 240 copies of two structural glycosylated proteins, E2 and E1. E2 and E1 form heterodimers, and three E2-E1 heterodimers interact to form 80 spikes that are found on the virus surface, arranged in a T=4 (triangulation number) lattice (Strauss & Strauss, 1994). The carboxy-termini (-COOH) of E2 and E1 interact with the capsid, and the amino termini of E2 and E1 face outward from the lipid membrane. The E2 glycoprotein is responsible for receptor binding and E1 for membrane fusion. It has been demonstrated that a single amino acid change in the E2 glycoprotein can have a drastic impact on virulence, which has further been noted for SAV (Merour, et al., 2013). The nucleocapsid contains the +ssRNA genome complexed with 240 copies of the capsid protein (Strauss & Strauss, 1994). The capsid proteins are arranged as pentamers and hexamers to form a T=4 icosahedral symmetry (Cheng, et al., 1995).

The +ssRNA Alphavirus genome is shown schematically in figure 4, showing the 5' cap, the 5' two thirds of the genome that codes for the non-structural proteins nsP1-4, the 3' one third that codes for the structural proteins, and the polyadenylated tail (Strauss & Strauss, 1994).

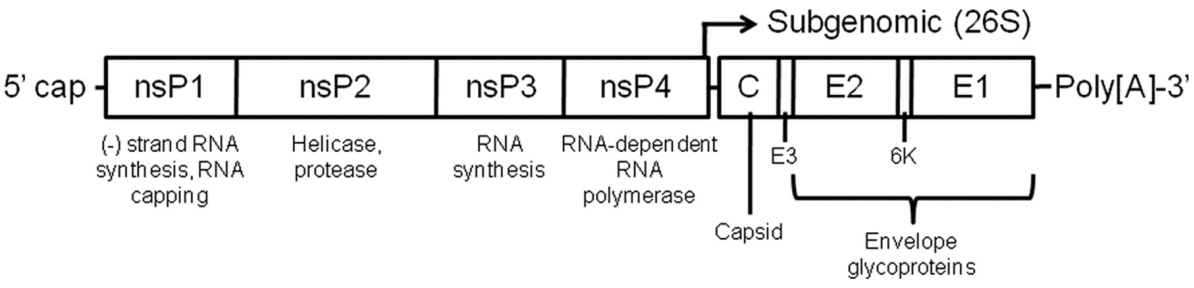

Figure 3: Schematic of the Alphavirus genome (Sherman & Weaver, 2010)

2.2.2 Replication Cycle

Alphaviruses enter the cell through receptor mediated endocytosis, facilitated by glycoprotein E2 receptor binding to the host cell. The lowered pH in the endosome triggers the membrane fusion activity of E1, and the nucleocapsid is released into the cytoplasm and starts dissociation. The nonstructural region of the +ssRNA gets translated directly into polyprotein P1234, which is then cleaved into a replicase complex of P123 and nsP4. This replicase complex will function as a polymerase complex that will transcribe the +ssRNA genome into the complementary negative sense RNA (Strauss & Strauss, 1994).

A buildup of P123 and nsP4 in the host cell a few hours after the infection leads to instability in the P123-nsP4 replicase complex, and the P123 is further cleaved into nsP1, nsP2, and nsP3, by nsP2-protease activity (Hodneland, 2006). The now four non-structural proteins make a replicase complex that produces the (+)RNA genomes and the subgenomic 26S mRNAs from the complementary negative sense RNA (Strauss & Strauss, 1994). The subgenomic mRNA is translated into the structural polyprotein capsid-p62-6K-E1. The capsid is autoproteolytically cleaved from this polyprotein, and will become complexed with the genomic mRNAs to form the nucleocapsid of the new virus particles.

The remaining structural polyprotein p62-6K-E1 translocates to the endoplasmatic reticulum (ER), to undergo cleavage into p62, 6K and E1 (Liljestrom & Garoff, 1991). Carbohydrate chains are added to the structural proteins in the ER lumen (Strauss & Strauss, 1994). During transport through the ER and the Golgi complex p62 is oligomerized into E2 and E3, and finally the structural proteins accumulate in the host cells' membrane awaiting the nucleocapsid for ultimate virus assembly, where finally budding of progeny viruses will occur. Alphavirus infection is relatively fast, resulting in acute disease in vertebrate hosts (Fros & Pijlman, 2016).

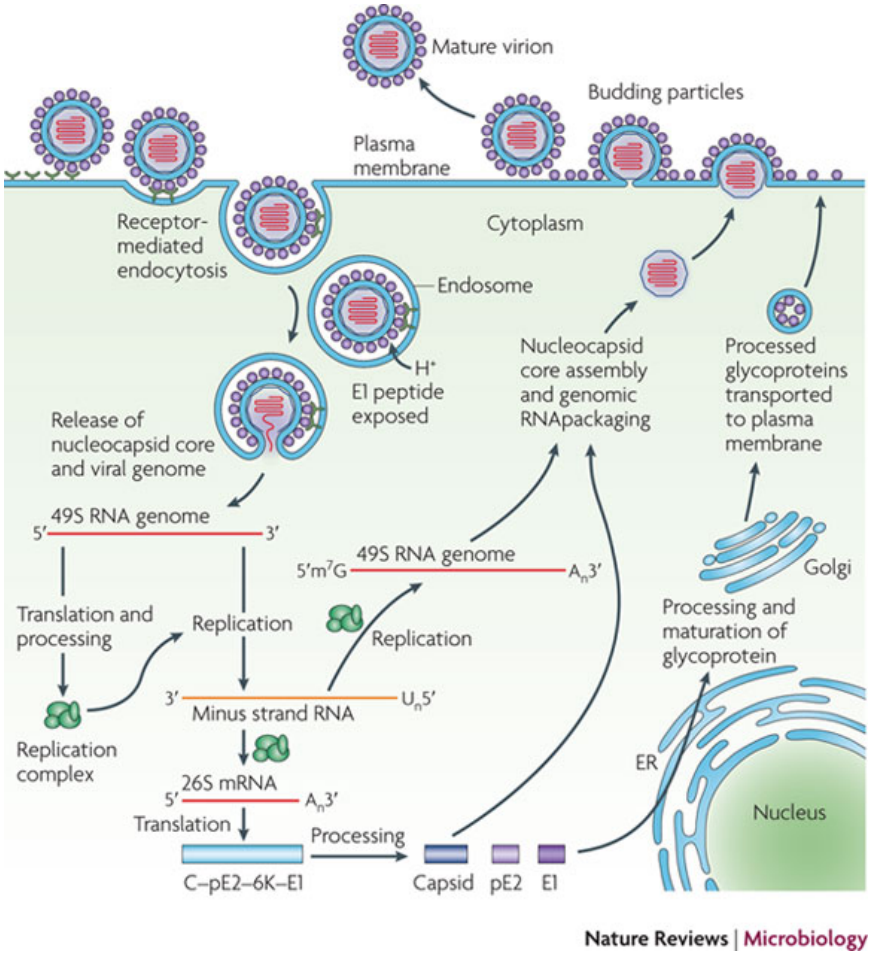

Figure 4: Alphavirus life cycle (Schwartz & Albert, 2010)

2.2.3 Alphavirus Host Cell Shut-Off and Inhibition of Antiviral Responses

Alphaviruses have evolved to counteract the vertebrate immune system through obstructing antiviral responses of the host cell, mainly by inhibiting certain signaling pathways and modulating general cellular processes. The most important strategy is the host cell shut-off, which is caused by the inhibition of general cellular transcription and/or translation (Fros & Pijlman, 2016). The inhibition of translation has a strong down regulatory effect on the translation of cellular mRNAs, while the translation of viral RNA remains efficient (Gorchakov, et al., 2004).

Transcriptional Shut-off

The Old-World Alphaviruses have been showed to reduce host cell transcription via the nonstructural protein nsP2. This protein is a multifunctional molecule which plays a role in the aforementioned replicase complex together with other non-structural proteins. In

addition, it has protease and helicase activity (Kumas Das, et al., 2014). The protease domain is important for autoproteolytic cleavage of the non-structural polyprotein, and helicases are enzymes that utilize energy from the hydrolysis of NTP or dNTP to unwind double stranded RNA to single stranded RNA. Furthermore, nsP2 has been demonstrated to induce cellular shut-off and reduce interferon (IFN) responses by obstructing the expression of IFN-stimulated genes (ISGs), as well as binding several different host cell proteins (Bourai, et al., 2012). The transcriptional shut-off is induced by nsP2 translocating to the nucleus using a self-contained nuclear localization signal (NLS) (Peranen, et al., 1990), and then degrading host cell RNA polymerase II subunit Rpb1 (Akhrymuk, et al., 2012), which results in general host-cell transcriptional shut-off.

The New-World Alphaviruses on the other hand, have evolved to use a different strategy and protein to inhibit host cell transcription. The viral capsid protein forms a complex with nuclear export and import factors, exportin and importin, which obstruct the nuclear pores and thus limit nucleocytoplasmic trafficking (Atasheva, et al., 2010).

There are different opinions on whether SAV deploys the strategy of Old or New-World Alphaviruses to induce transcriptional shut-off. A study done by Løvoll, et al. in 2011, revealed that the transcription of reference genes is indeed affected by SAV infection. Another study indicates that the SAV capsid has the potential to interfere with vital host-cell processes, leading to cytotoxicity and the inhibition of cellular proliferation (Karlsen, et al., 2010).

Translational Shut-Off

One important antiviral strategy of the eukaryotic cell is the down regulation of translation when sensing cellular stress This occurs through phosphorylation of the α-subunit of eukaryotic translation initiation factor 2α (eIF2α), rendering it unable for recycling back to its active form (Harding, et al., 2000). This strategy aims at blocking the translation of viral mRNAs, but ultimately also blocks the translation of host cell mRNA as well. The eIF2α is phosphorylated by four kinases; Protein kinase R (PKR) senses double stranded RNA (dsRNA); PKR-like ER kinase (PERK) senses unfolded proteins in the ER; GCN2 senses nutrient starvation; and HRI senses heme deficiency (Harding, et al., 2000).

PKR is induced by interferons and activated by dsRNA, an intermediate of RNA

virus replication. Many viruses have therefore developed to either prevent activation of PKR and/or phosphorylation of eIFα. It has been reported that the nonstructural protein, nsP4, of the Alphavirus chinkungunya does indeed suppress eIF2α phosphorylation (Rathore, et al., 2013), but there is also established evidence suggesting that Alphaviruses are unaffected by phosphorylation of eIF2α, and that the translation of viral structural proteins from subgenomic mRNA is not restricted by modification of eIF2α (Ventoso, et al., 2006). Furthermore, host-cell translational shut-off induced by Alphavirus infection has been shown to be independent of PKR and eIF2α activation, suggesting that there must be another mechanism by which Alphaviruses induce translational shut-off (Fros & Pijlman, 2016).

Interferon Response

Interferons (IFN) elicit distinct antiviral effects, notably IFN alpha and beta (IFNα/β) which, when activated, trigger the transcription of "antiviral" genes termed IFN-Stimulated Genes (ISGs) (Randall & Goodbourn, 2008). IFNs can be induced by the presence of dsRNA and through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Once PRRs recognize viral elements, IFNs activate neighboring cells via the janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, leading to the upregulation of ISG transcription. Generally, Alphavirus induced host-cell shut-off also affects the expression of IFNs and ISGs. Furthermore, Alphavirus infection is resistant to IFNs, showing no reduction in viral titers and replicon RNA replication (Fros & Pijlman, 2016). It has been shown that the nsP2 inhibits the JAK-STAT signaling (Fros, et al., 2010), thus impeding the host-cell interferon response via both general host-cell shut-off and inhibition of the JAK-STAT pathway.

Unfolded Protein Response

The Unfolded Protein Response (UPR) is activated when unfolded or misfolded proteins are present in the ER. As mentioned earlier, the Alphaviruses structural proteins translocate to the ER and undergo maturation whist travelling through the ER to the Golgi complex and onwards to the host cell membrane. In the presence of unfolded proteins, activation of certain signaling pathways induces an inhibition of general cellular translation and an increase in the production of several proteins responsible for protein folding, degradation and apoptosis. (Fros, et al., 2015).

3. Goals/Questions

In this experiment, CHSE-214 cells (Chinook salmon embryo cells) were infected with SAV3 H10/02. Mock treated CHSE-214 cells were used as a negative control. These, and infected cells, were sampled at 0, 4, 12, 24, 48, 72, and 96 hours post infection (hpi). RNA extraction and cDNA synthesis was done before real-time qPCR (real time quantitative polymerase chain reaction). The expression of housekeeping genes EF1aB (elongation factor 1 alpha B), B-actin, and 18S rRNA was measured. Expression of SAV protein nsP1 was also measured for confirmation of infection.

The goal of this research, as a pilot study, is to give insight into the ability of Salmonid alphavirus to regulate host cell transcription post-infection, but also to optimize the materials and methods for future larger scale studies.

4. Materials and Methods

4.1 Cells

CHSE-214 cells (Chinook salmon embryo) were cultivated in two 175 cm^2 cell culture flasks in Leibovitz-15 medium (Life Technologies), supplemented with 10% heat inactivated fetal bovine serum (FBS), L-glutamine, mercaptoethanol, and gentamycin-sulphate. Before seeding the cells over to plates, the cell number was counted with the Countess Cell Counter to ensure adequate cell numbers. 400 000 cells were transferred to each well, and the plates where incubated at 20°C until the cell cultures showed at least 80% confluency (~48 hours).

4.2 Infection and Harvesting of Cell Cultures

A 1:1000 virus suspension of SAV3 H20/02 was added to two of the wells on each plate. One well per plate housed the negative control (mock treated).

Once a sample is harvested, RNA becomes unstable. RNA is degraded by ribonuclease enzymes (RNases) from the cells. The inhibition of RNases was achieved by adding Qiazol Lysis Reagent (QIAGEN), a monophasic solution of phenol and guanidine thiocyanate, which facilitates lysis of the cells and inhibits RNases. Guanidine thiocyanate aids in the denaturation of proteins that strongly bind nucleic acids and those who degrade RNA. The 0 hpi cell cultures were harvested after 60 minutes of virus suspension exposure. The remaining samples were harvested at 4, 12, 24, 48, 72 and 96 hpi. Samples were harvested with Qiazol Lysis Reagent and stored at minus 80°C.

4.3 RNA Extraction

For the RNA extraction, the QIAGEN RNeasy Mini kit was used (QIAGEN). Chloroform was first added to the sample which then separated into an aqueous phase and a lower organic phase, RNA partitions into the aqueous phase, while protein into the organic phase. 70% ethanol was subsequently added to the aqueous phase containing the RNA, forming a binding solution, which was then placed in spin columns containing a silica

membrane. Upon centrifugation, the solution was forced through the membrane while nucleic acid (RNA) bound to the membrane. Through serial steps of washing and centrifuging with different buffers, impurities were removed. In the last step the nucleic acid was eluted from the membrane with RNase free water. The RNA concentration was measured with NanoDrop spectrophotometer, results shown in table 2.

Table 2 Nanodrop results. A 260/280 ratio of ~2.0 is generally accepted as "pure" for RNA

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
0 hpi brønn 1	Default	17.08.2015	13:09	137,90	3,448	1,609	2,14	1,67	40,00	230	2,064	0,051
0 hpi brønn 2	Default	17.08.2015	13:10	108,35	2,709	1,314	2,06	0,86	40,00	230	3,140	0,184
0 hpi neg ctrl	Default	17.08.2015	13:11	121,09	3,027	1,433	2,11	2,04	40,00	230	1,483	-0,073
4 hpi brønn 1	Default	17.08.2015	13:12	80,20	2,005	0,967	2,07	1,72	40,00	230	1,165	0,101
4 hpi brønn 2	Default	17.08.2015	13:13	79,04	1,976	0,978	2,02	1,79	40,00	230	1,106	0,292
4 hpi neg ctrl	Default	17.08.2015	13:13	113,43	2,836	1,328	2,14	2,11	40,00	230	1,341	0,150
12 hpi brønn 1	Default	18.08.2015	11:58	81,29	2,032	0,946	2,15	0,63	40,00	230	3,226	-0,008
12 hpi brønn 2	Default	18.08.2015	11:58	77,93	1,948	0,938	2,08	1,63	40,00	230	1,196	0,246
12 hpi neg ctrl	Default	18.08.2015	11:59	62,42	1,560	0,727	2,15	0,73	40,00	230	2,147	0,086
24 hpi brønn 1	Default	18.08.2015	12:00	142,07	3,552	1,646	2,16	1,69	40,00	230	2,101	0,112
24 hpi brønn 2	Default	18.08.2015	12:01	139,58	3,490	1,647	2,12	1,92	40,00	230	1,816	0,339
24 hpi neg ctrl	Default	18.08.2015	12:01	127,69	3,192	1,536	2,08	1,91	40,00	230	1,674	0,307
48 hpi brønn 1	Default	18.08.2015	12:03	182,72	4,568	2,148	2,13	2,15	40,00	230	2,121	0,108
48 hpi brønn 2	Default	18.08.2015	12:04	155,34	3,883	1,842	2,11	1,89	40,00	230	2,057	0,219
48 hpi neg ctrl	Default	18.08.2015	12:05	150,36	3,759	1,754	2,14	2,15	40,00	230	1,745	0,109
72 hpi brønn 1	Default	20.08.2015	12:44	231,54	4,631	2,269	2,04	2,15	50,00	230	2,157	0,105
72 hpi brønn 2	Default	20.08.2015	12:45	211,72	4,234	2,032	2,08	2,20	50,00	230	1,927	0,056
72 hpi neg ktrl	Default	20.08.2015	12:46	212,94	4,259	2,061	2,07	1,76	50,00	230	2,415	0,160
96 hpi brønn 1	Default	20.08.2015	12:47	156,27	3,125	1,537	2,03	2,05	50,00	230	1,527	0,202
96 hpi brønn 2	Default	20.08.2015	12:47	146,69	2,934	1,426	2,06	1,44	50,00	230	2,032	0,081
96 hpi neg ktrl	Default	20.08.2015	12:48	176,42	3,528	1,741	2,03	1,62	50,00	230	2,172	0,139

4.4 cDNA Synthesis

In the cDNA synthesis of 0 and 4 hpi samples a human error was initially made, therefore this procedure will be described separately.

4.4.1 cDNA Synthesis of 12, 24, 48, 72, 96 hpi Samples

For the cDNA (complementary DNA) synthesis, QIAGEN Quantitect Reverse Transcription (QIAGEN u.d.) was used. This kit contains reverse transcriptase (RT), random primers, poly-T oligonucleotides, various buffers, and deoxynucleotide triphosphates

(dNTPs: dATP, dTTP, dCTP, dGTP). In eukaryotic cells, DNA is transcribed into pre-mRNA, which is then processed by removing the introns and adding a polyadenylated (polyA) tail and a 5' Methyl-Guanine cap. One can therefore use a poly-T (poly thymine) oligonucleotide as a primer. The poly-T oligonucleotide primer gets annealed to the 3'-end of the mRNA, and the RT extends the primer by using the mRNA as a template, adding the correct complementary dNTPs at the correct locations.

A Reverse Transcription master mix was prepared beforehand following the formula in table 3.

Table 3. Master mix setup

Quantiscript Reverse Trascriptase	1 µl
Quantiscript RT Buffer 5x	4 µl
RT Primer Mix	1 µl
Total	6 µl

The gDNA (genomic DNA) elimination reaction was prepared on ice using the following scheme in table 4 to ensure 750 ng of template RNA per reaction; 6 µl master mix was added to gDNA eliminated template RNA, to reach the final volume of 20 µl.

Table 4 Elimination reaction setup

gDNA Wipeout Buffer, 7x	2 µl	
Template RNA	750 ng : (ng/µl)*	*Values from NanoDrop
RNase-free water	Variable	
Total Volume	14 µl	

4.4.2 cDNA Synthesis of 0 and 4 hpi Samples

The 0 and 4 hpi samples were not calculated to contain 750 ng template RNA in each reaction, rather a calculation was done for the highest A_{260} value (0 hpi well 1) to contain close to 1 µg RNA template (965,3 ng). A 7 µl sample was used from the template

RNA from all samples when mixing the genomic DNA elimination reactions, therefore yielding different amount of RNA for each sample. For all 6 reactions the formula in table 4 was used; 7 µl template RNA, 2 µl gDNA wipeout buffer, 5 µl RNase-free water, up to the total volume of 14 µl. gDNA eliminated template RNA was added to each of the tubes containing the 6 µl master mix, reaching final volumes of 20 µl. Upon realizing the mistake, the cDNA 0 and 4 hpi samples where diluted with RNase-free water when preparing the final qPCR mix. In theory, the samples should contain the same amount of cDNA as the starting concentration of RNA, assuming the reverse transcription reaction was 100% effective. The cDNA samples from 0 and 4 hpi therefore had to be diluted with RNase-free water when preparing the PCR assays that each sample contained 3 µl cDNA.

4.4.3 Real Time Quantitative PCR

The real-time quantitative PCR assays were performed in triplets with an AriaMx detection system in the following conditions: 50°C/2 minutes, 95°C/10 minutes, 40 cycles of 95°C/15 seconds and 60°C/60 seconds. Data were captured using AriaMx qPCR software (Agilent Technologies). Each reaction contained 2x Master Mix (TaqMan, ThermoFisher), primers (300 nM), TaqMan MGB probe (200 nM), 15 ng cDNA (0.15 ng cDNA for 18S cDNA), and DEPC-water (Diethylpyrocarbonate) to a final volume of 13 µl. The cDNA for 18S cDNA was diluted as the expression of this gene is very high in cells. Transcription levels of the housekeeping genes elongation factor 1αß (EF1αß), β-actin, and 18S rRNA, as well as the Salmonid alphavirus gene nsP1 were obtained for 0, 4, 12, 24, 48, 72, and 96 hpi. The primers and probe for nsP1 were obtained from Hodneland et al. (Hodneland & Endresen, 2006), table 5. Primer and probe sequences for β-actin, EF1αß, and 18S rRNA were obtained from Olsvik et al. (Olsvik, et al., 2005), table 5. A negative control, a no template control (NTC), was run in triplets for all genes. NTCs provide a mechanism to control for external contamination or other factors that may result in a non-specific increase in the fluorescent signal.

Four real-time qPCR assays were run as one qPCR plate can only hold 96 samples. A positive calibrator of the nsP1 gene with known C_t values was run for every assay, to ensure that all plates ran under the same conditions. For the first assay, run for 0 and 4 hpi, 18S cDNA was not diluted. These samples where run again with the proper dilution on the

last assay together with the 96 hpi samples, except the 0 hpi 18S negative control. It was decided that since the nsP1 expression did not occur until 24 hpi, the 0 hpi negative control would not yield results that would greatly differ compared to the 0 hpi infected samples.

Table 5. Oligonucleotide sequences, amplicon lengths, GenBank accession numbers and standard curve evaluation for qPCR assays (Løvoll, et al., 2011)

Gene	Oligonucleotide sequence (5′-3′)	Amplicon (bp)	GenBank acc. no	Slope	R^2	Efficiency (*E*)*
EF1αB	TGCCCCTCCAGGATGTCTAC	57	BG933897	3.460	0.979	1.94
	CACGGCCCACAGGTACTG					
	6FAM-AAATCGGCGGTATTGG-MGBNFQ					
RPS20	GCAGACCTTATCCGTGGAGCTA	85	BG93667	3.055	0.979	2.12
	TGGTGATGCGCAGAGTCTTG					
	6FAM-CCTCAAGGTGAAGGGA-MGBNFQ					
β-actin	CCAAAGCCAACAGGGAGAAG	91	BG933897	3.173	0.996	2.06
	AGGGACAACACTGCCTGGAT					
	6FAM-TGACCCAGATCATGTTT-MGBNFQ					
18S rRNA	CCCCGTAATTGGAATGAGTACACTTT	98	AJ427629	3.092	0.978	2.10
	ACGCTATTGGAGCTGGAATTACC					
	6FAM-CTTTAACGAGGATCCATTGG-MGBNFQ					
SPDV	CCGGCCCTGAACCAGTT	107	AY604235			
	GTAGCCAAGTGGGAGAAAGCT					
	6FAM-CTGGCCACCACTTCGA-MGBNFQ					

* The amplification efficiency of each primer set was assessed according to the equation $E = 10^{(1/-\text{slope})}$.

5. Results

In quantitative real-time PCR, the amount of amplified product is linked to fluorescent intensity using a fluorescent reporter molecule. The intensity of the reporter molecule is measured after each cycle by sending out excitatory light and detecting the feedback light intensity. The Ct (Cycle threshold) value is defined as the number of cycles needed for the fluorescence to reach a specific threshold level of detection ("threshold fluorescence"), and is inversely correlated with the amount of template nucleic acid present in the reaction, that is to say; high Ct values reflects a low target concentration and vice versa. The threshold value was set manually to ensure it would be the same for all four assays. It was set to a fluorescent value of 100, a region in which all plots where at their log linear-phase in all samples.

A Ct cut-off value was determined as 35 (CC (cycle cut-off) = 35) for the reference genes. Samples with higher Ct values were considered unreliable. A CC value was set as 37.5 for the nsP1 gene following the methodology of a previous study, where they did two-fold dilutions (30 replicates) of a viral cDNA-stock, and it denotes the mean Ct value in the highest dilution for which all 30 replicates were positive (Hodneland & Endresen, 2006).

The Ct values for all samples can be seen in table 7 and 8. The standard deviation (SD) was determined for all triplets, not accepting SD values of or above 0.25. SD is the most common measure of precision. To be able to discriminate between a 2-fold dilution in more than 95% of cases, the SD has to be ≤0.250. Running triplicates of each samples allows for removing one of the triplicate results if the SD ≥0.25. SD values above 0.25 were excluded when calculating the mean value of the triplets, marked in red table 7 and 8.

The positive calibrator of the nsP1 gene with known Ct values did not display variation in values greater than a standard deviation of 0.25, which is within the acceptable level of variance. This indicated that all assays were run under similar conditions. The NTC (non-template control) did not indicate any external contamination.

Table 6. Calibrator results for all four assays

	0 and 4 hpi	**12 and 24 hpi**	**48 and 72 hpi**	**96 hpi**
	28,12	28,2	28,37	28,52
SD				0,178582381
Average				28,3025

Table 7. Ct results for Actin and EF1aB, showing the mean and SD. Triplets showing a SD of more than 0.25 was excluded from the mean

0 hpi		4 hpi		12 hpi		24 hpi		48 hpi		72 hpi		96 hpi	
Actin	**Ct**	**Actin**	**Ct**	**Actin**	**Ct**	**Actin**	**Ct**	**Actin**	**Ct**	**Actin**	**Ct**	**Actin**	**Ct**
Well 1	21,87	Well 1	21,26	Well 1	23,37	Well 1	24,45	Well 1	27,41	Well 1	24,79	Well 1	25,87
Well 1	21,79	Well 1	21,23	Well 1	23,45	Well 1	24,46	Well 1	27,33	Well 1	24,63	Well 1	25,91
Well 1	21,78	Well 1	21,47	Well 1	23,43	Well 1	24,56	Well 1	27,35	Well 1	24,49	Well 1	25,86
21,813	**0,049**	**21,32**	**0,131**	**23,417**	**0,042**	**24,49**	**0,061**	**27,363**	**0,042**	**24,637**	**0,150**	**25,88**	**0,026**
Well 2	21,84	Well 2	20,7	Well 2	24,38	Well 2	24,61	Well 2	24,88	Well 2	25,26	Well 2	25,11
Well 2	21,98	Well 2	20,67	Well 2	24,57	Well 2	24,67	Well 2	24,92	Well 2	25,34	Well 2	25,38
Well 2	22,01	Well 2	20,71	Well 2	24,49	Well 2	24,77	Well 2	24,8	Well 2	25,4	Well 2	25,05
21,943	**0,091**	**20,693**	**0,021**	**24,48**	**0,095**	**24,683**	**0,081**	**24,867**	**0,061**	**25,333**	**0,070**	**25,18**	**0,176**
Neg. Ctrl	22,61	Neg. Ctrl	21,17	Neg. Ctrl	25,61	Neg. Ctrl	24,41	Neg. Ctrl	27,55	Neg. Ctrl	24,49	Neg. Ctrl	23,79
Neg. Ctrl	22,72	Neg. Ctrl	20,95	Neg. Ctrl	25,81	Neg. Ctrl	24,68	Neg. Ctrl	27,6	Neg. Ctrl	24,63	Neg. Ctrl	23,74
Neg. Ctrl	22,54	Neg. Ctrl	21,22	Neg. Ctrl	25,46	Neg. Ctrl	24,71	Neg. Ctrl	27,49	Neg. Ctrl	24,33	Neg. Ctrl	24,11
22,623	**0,091**	**21,113**	**0,144**	**25,627**	**0,176**	**24,6**	**0,165**	**27,547**	**0,055**	**24,483**	**0,150**	**23,88**	**0,201**

0 hpi		4 hpi		12 hpi		24 hpi		48 hpi		72 hpi		96 hpi	
EF1aB		**EF1aB**		**EF1aB**		**EF1aB**		**EF1aB**		**EF1aB**		**EF1aB**	
Well 1	20,88	Well 1	20,1	Well 1	22,93	Well 1	22,8	Well 1	24,23	Well 1	22,56	Well 1	23,87
Well 1	20,4	Well 1	20,58	Well 1	22,35	Well 1	22,71	Well 1	23,93	Well 1	22,39	Well 1	23,72
Well 1	20,19	Well 1	20,14	Well 1	22,14	Well 1	22,58	Well 1	23,76	Well 1	22,5	Well 1	23,6
20,295	**0,148**	**20,12**	**0,028**	**22,245**	**0,148**	**22,697**	**0,111**	**23,973**	**0,238**	**22,483**	**0,086**	**23,73**	**0,135**
Well 2	20,32	Well 2	19,91	Well 2	22,47	Well 2	22,14	Well 2	22,4	Well 2	22,99	Well 2	22,82
Well 2	20,45	Well 2	19,6	Well 2	22,38	Well 2	22,02	Well 2	22,36	Well 2	22,95	Well 2	22,65
Well 2	20,44	Well 2	19,82	Well 2	22,35	Well 2	21,96	Well 2	22,26	Well 2	22,82	Well 2	22,68
20,403	**0,072**	**19,777**	**0,159**	**22,4**	**0,062**	**22,04**	**0,092**	**22,34**	**0,072**	**22,92**	**0,089**	**22,717**	**0,091**
Neg. Ctrl	21,46	Neg. Ctrl	20,68	Neg. Ctrl	22,71	Neg. Ctrl	22,28	Neg. Ctrl	23,71	Neg. Ctrl	21,75	Neg. Ctrl	21,1
Neg. Ctrl	21,24	Neg. Ctrl	20,39	Neg. Ctrl	22,52	Neg. Ctrl	22,19	Neg. Ctrl	23,66	Neg. Ctrl	22,12	Neg. Ctrl	20,77
Neg. Ctrl	21,44	Neg. Ctrl	20,36	Neg. Ctrl	22,54	Neg. Ctrl	22,06	Neg. Ctrl	23,7	Neg. Ctrl	21,5	Neg. Ctrl	20,95
21,38	**0,122**	**20,477**	**0,177**	**22,59**	**0,104**	**22,177**	**0,111**	**23,69**	**0,026**	**21,625**	**0,177**	**20,94**	**0,165**

Table 8. Ct results for 18S and nsP1, showing the mean and SD. Triplets showing a SD of more than 0.25 was excluded from the mean

0 hpi	
18S	**Ct**
Well 1	22,01
Well 1	22
Well 1	22,28
22,097	**0,159**
Well 2	22,43
Well 2	22,48
Well 2	22,61
22,507	**0,093**
Neg. Ctrl	
Neg. Ctrl	
Neg. Ctrl	

4 hpi	
18S	**Ct**
Well 1	21,02
Well 1	20,82
Well 1	20,67
20,837	**0,176**
Well 2	20,31
Well 2	20,23
Well 2	20,22
20,253	**0,049**
Neg. Ctrl	20,48
Neg. Ctrl	20,47
Neg. Ctrl	20,4
20,45	**0,044**

12 hpi	
18S	**Ct**
Well 1	26,74
Well 1	26,71
Well 1	26,8
26,75	**0,046**
Well 2	27,98
Well 2	27,7
Well 2	27,84
27,84	**0,14**
Neg. Ctrl	28,62
Neg. Ctrl	28,65
Neg. Ctrl	28,52
28,597	**0,068**

24 hpi	
18S	**Ct**
Well 1	25,41
Well 1	25,28
Well 1	25,09
25,26	**0,161**
Well 2	25,86
Well 2	25,71
Well 2	25,63
25,733	**0,117**
Neg. Ctrl	24,84
Neg. Ctrl	24,83
Neg. Ctrl	24,72
24,797	**0,067**

48 hpi	
18S	**Ct**
Well 1	27,5
Well 1	27,23
Well 1	27,28
27,337	**0,144**
Well 2	24,1
Well 2	23,96
Well 2	23,82
23,96	**0,14**
Neg. Ctrl	28,64
Neg. Ctrl	28,43
Neg. Ctrl	28,34
28,47	**0,154**

72 hpi	
18S	**Ct**
Well 1	22,45
Well 1	22,16
Well 1	21,98
22,197	**0,237**
Well 2	24,88
Well 2	22,74
Well 2	22,6
22,67	**0,099**
Neg. Ctrl	23,23
Neg. Ctrl	22,95
Neg. Ctrl	22,99
23,057	**0,151**

96 hpi	
18S	**Ct**
Well 1	23,43
Well 1	23,06
Well 1	23,12
23,203	**0,199**
Well 2	22,78
Well 2	22,72
Well 2	22,39
22,63	**0,21**
Neg. Ctrl	22,51
Neg. Ctrl	22,28
Neg. Ctrl	21,99
22,395	**0,163**

0 hpi	
nsP1	
Well 1	No Cq
Well 1	No Cq
Well 1	No Cq
Well 2	No Cq
Well 2	No Cq
Well 2	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	38,6

4 hpi	
nsP1	
Well 1	37,75
Well 1	37,67
Well 1	37,36
37,593	**0,206**
Well 2	37,64
Well 2	36,12
Well 2	37,88
37,76	**0,170**
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq

12 hpi	
nsP1	
Well 1	No Cq
Well 1	No Cq
Well 1	37,98
Well 2	No Cq
Well 2	No Cq
Well 2	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq

24 hpi	
nsP1	
Well 1	33,53
Well 1	33,28
Well 1	32,77
33,405	**0,177**
Well 2	33,09
Well 2	32,57
Well 2	32,77
32,81	**0,262**
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq

48 hpi	
nsP1	
Well 1	28,38
Well 1	28,58
Well 1	28,37
28,443	**0,118**
Well 2	24,96
Well 2	24,89
Well 2	25,69
24,925	**0,049**
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq

72 hpi	
nsP1	
Well 1	19,88
Well 1	19,9
Well 1	19,84
19,873	**0,031**
Well 2	20,96
Well 2	20,59
Well 2	20,87
20,807	**0,193**
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq
Neg. Ctrl	No Cq

96 hpi	
nsP1	
Well 1	20,46
Well 1	20,55
Well 1	20,55
20,52	**0,052**
Well 2	19,93
Well 2	19,73
Well 2	19,88
19,847	**0,104**
Neg. Ctrl	No Cq
Neg. Ctrl	37,47
Neg. Ctrl	38,48

If SAV causes the downregulation of transcription within host cells, we could have expected to see the Ct value for actin, EF1aB and 18S to increase over time. The negative control was a representation of normal gene expression, as these cells were not infected and therefore should not display signs of host cell shut-off or transcription downregulation. There was, notably, a Ct increase of the negative control of Actin towards 48 hpi (see table 9 and figure 6). At this point, the Ct value was similar to the Ct values of the infected well 1. The increase in Ct value between 0 hpi and 96 hpi in well 1 and well 2 is 4,07 and 3,24 cycles, respectively, compared to a Ct value increase of the negative control of 1,26 cycles.

Table 9. Mean Ct results for Actin

ACTIN	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	72 hpi	96 hpi
Well 1	21,81	21,32	23,42	24,49	27,36	24,64	25,88
Well 2	21,94	20,69	24,48	24,68	24,87	25,33	25,18
Neg. Ctrl	22,62	21,11	25,63	24,6	27,55	24,48	23,88

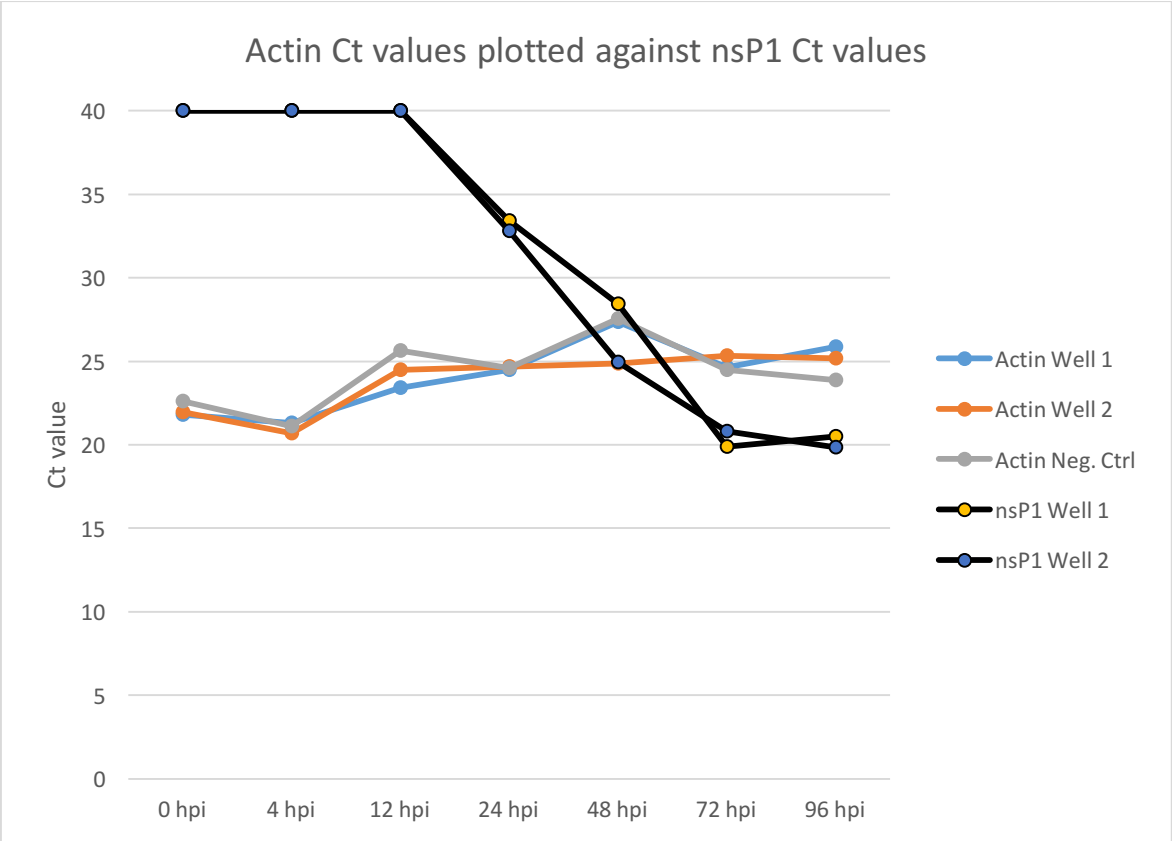

Figure 5: Actin Ct values over the different time points in relation to nsP1 expression. The curve for nsP1 between 0 and 12 hpi is set to 40 for the purpose of making this diagram, real value is “No Cq”

EF1aB Ct value increased by 3,43 cycles for well 1, and 2,32 cycles for well 2 between 0 and 96 hpi, while the negative control decreased by 0,44 cycles (table 10).

Table 10. Mean Ct results for EF1aB

EF1aB	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	72 hpi	96 hpi
Well 1	20,3	20,12	22,25	22,7	23,97	22,48	23,73
Well 2	20,4	19,78	22,4	22,04	22,34	22,92	22,72
Neg. Ctrl	21,38	20,48	22,59	22,18	23,69	21,63	20,94

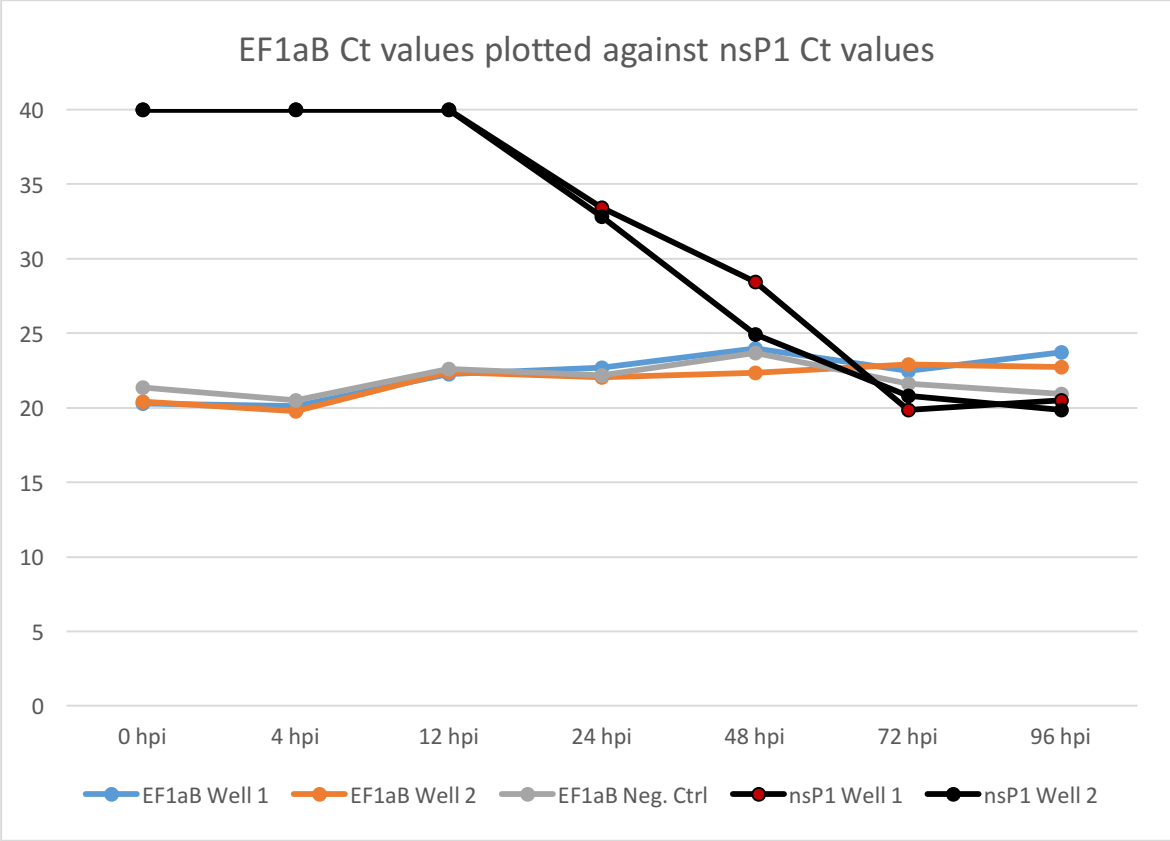

Figure 6: EF1aB Ct values over the different time points in relation to nsP1 expression. The curve for nsP1 between 0 and 12 hpi is set to 40 for the purpose of making this diagram, real value is "No Cq".

The 18S gene, table 11, showed less difference between the negative control and the infected wells. As the negative control sample was not run at 0 hpi again, the following calculation was made between 4 hpi and 96 hpi. Well 1 and 2 increased in Ct values with 2,36 and 2,38 cycles, respectively, while the negative control increased with 1,95 cycles.

Table 11. Mean Ct results for 18S

18S	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	72 hpi	96 hpi
Well 1	22,1	20,84	26,75	25,26	27,34	22,2	23,2
Well 2	22,51	20,25	27,84	25,73	23,96	22,67	22,63
Neg. Ctrl		20,45	28,6	24,8	28,47	23,06	22,4

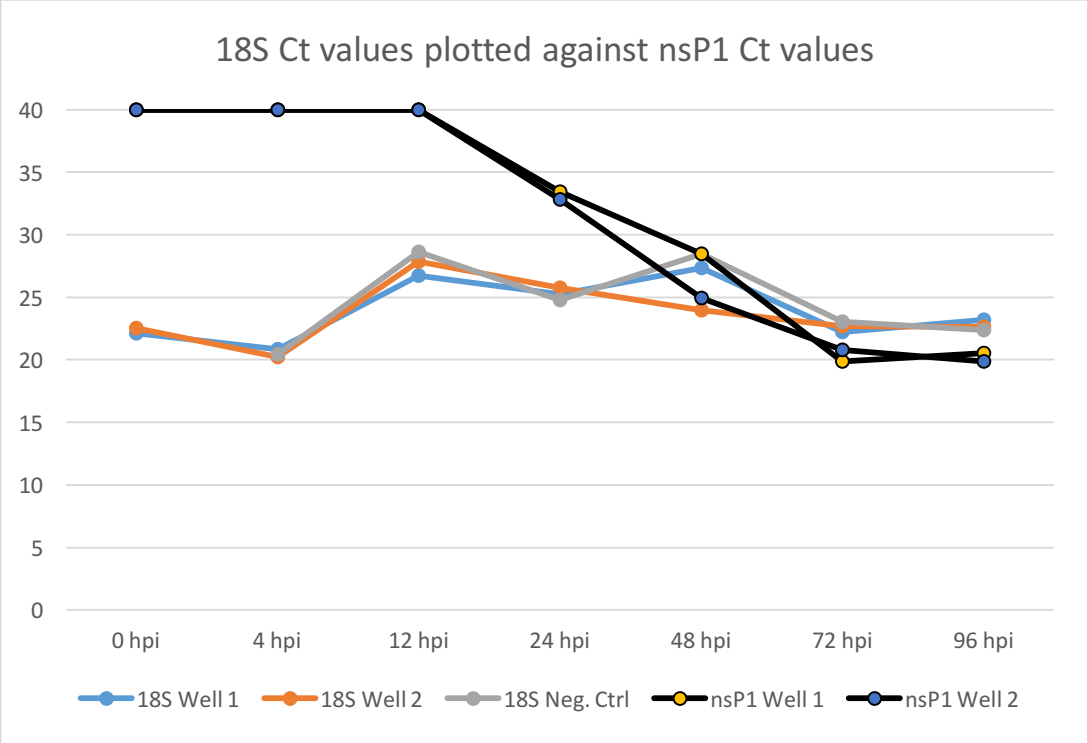

Figure 7: 18S Ct values in relation to nsP1 expression. The curve for nsP1 between 0 and 12 hpi is set to 40 for the purpose of making this diagram, real value is "No Cq"

The nsP1 gene ran for all time out-takes represents presence of virus in the cells, indicating viral replication processes. When considering the cut-off value of 37,5, expression of the nsP1 gene seems to occur between 12 and 24 hours post infection, with a steady increase in starting replicon as the hours progresses. The negative control of 96 hpi gave Ct values in two of the triplets, with SD ≥ 0.25.

Table 12. Mean Ct results for nsP1. *Not applicable

nsP1	0 hpi	4 hpi	12 hpi	24 hpi	48 hpi	72 hpi	96 hpi
Well 1	No Cq	37,59	No Cq	33,41	28,44	19,87	20,52
Well 2	No Cq	37,76	No Cq	32,81	24,93	20,81	19,85
Neg. Ctrl	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	n.a.*

6. Discussion

The initial error made when synthesizing cDNA from 0 and 4 hpi samples, may have impacted the results. The initial stock from which we synthesized the cDNA, had been frozen at -80ºC, so we could have thawed the samples and redone the whole process and synthesized cDNA from these time out-takes using the same procedure and calculations as the other time out-take samples. All the other samples would be treated in the same manner; freezing at -80ºC and redoing the cDNA synthesis, in order to treat all samples the same. The process of freezing and thawing the samples imposes a great risk of RNA degradation. Since the 0 and 4 hpi samples were not the most important ones with respect to the time outtakes, made evident by the fact that the nsp1 gene was not expressed until 24 hpi, we chose to dilute the samples as explained in the "Materials and methods" chapter. Therefore, the extent this error had on the results is questionable. Nevertheless, in an ideal experiment all samples should be treated and processed with consistent methodology, under the same conditions.

When planning this project, the use of two different cell lines were intended, the CHSE-214 cell line (Chinook Salmon Embryo) and the ASK cell line (Atlantic Salmon Kidney). The ASK cell line did not reach sufficient confluency during cell culturing, and at the start date of infection and harvesting there were simply not enough ASK cells for it to be included in further steps.. Having more than one cell-line would have given more substantial results, in that we could compare different cell lines, perhaps enabling us to answer whether the increase in Ct value was due to the virus or simply the cell line. The CHSE-214 cell line was derived from Chinook salmon, a species within the same family as Atlantic salmon (Salmonidae), but they do not belong to the same genus. In our study, we used a SAV3 strain collected from diseased Atlantic salmon, and although it has previously been demonstrated that SAV is able to grow in a variety of non-Atlantic salmon cells, it might be useful and relevant to use cells that match the species of the original sample.

The Ct results for B-actin and EF1aB showed an increase when comparing 0 and 96 hpi, as well as showing higher Ct value increase for the infected wells compared to the negative control. Ct value of B-actin increased at 48 hpi, and the negative control increased as much as the infected well 1. This may indicate that the Ct value variation represents a normal fluctuation in gene expression in the cells, or even represents fluctuation due to experimental conditions. There is increasing evidence that expression of many housekeeping genes varies

between different tissues, developmental stages, different physiological stressors, and varying experimental conditions (incubation period, temperature, etc). Therefore, we could not make any conclusions regarding the increase in Ct value between 0 and 96 hpi at this time, especially when the observation of high fluctuations in the negative control was considered.

The Ct values of 18S rRNA showed the least variance between 4 and 96 hpi, as well as showing the least variance between the negative control and the infected wells. The Ct value increased also here at 48 hpi of the negative control and in one of the infected wells.

The results of the nsP1 gene gave Ct values at 4 hpi. Although higher than the cut-off value, thid might be explained by that at 0 hpi, the virus is in the supernatant but has not yet attached to the cells, thereby being washed out in the harvesting process. At 4 hpi the virus might already be attached to the cells, avoiding being washed out, but has not yet entered the cells to start replication processes, therefore still giving a Ct value. This is one of the reasons why determining a cut-off value is important, because it is unlikely that this expression at 4 hpi represents any real replicative process in the cells. At 12 hpi the virus has most likely entered the cells. The genome is in the process of being broken down so that the specific genes can start the replication processes. However, these processes have not yet come far enough to yield any or enough nsP1 genes for expression in Ct values yet.

The Ct values obtained for the negative control at 96 hpi can be explained by a possible contamination between wells during incubation. The infected samples and the negative control were incubated on the same plate in neighbouring wells. SAV tends to "jump" between wells, especially during longer incubations, through aerosol or condensation. This points to a weakness in the plate set-up, and a different approach has to be considered in future studies.

It is interesting to consider how in vitro results relate to in vivo conditions. Although commonly used in experiments with SAV, CHSE-214 cells showed geometric mean titers of SAV significantly lower after 2 or 4 passages relative to that observed after initial isolation, and lower compared to the same number of passages through different cell lines (Atlantic salmon head kidney leucocytes TO-cells and Blue fin-2 cells) (Graham, et al., 2008). Ideally, the use of TO-cells should have been used in this study as well as CHSE-214, as TO-cells originate from Atlantic salmon. Furthermore, it was made clear by Graham,

et al., in their 2008 study, that this cell line offered considerable advantages over those conventionally used. Other cell lines in the same study also showed a decrease in geometric mean titers during more passages, but did not decrease to the same extent as CHSE-214. The exact reason for the decrease in titers after multiple passages in all the cell lines tested remains unknown. The authors of that study hypostatized that the observed effect might be due to mutations in the genomes of the SAV strains during several passages resulting in reduced replicative ability. Another hypothesis was the fact that alphaviruses typically exist as quasispecies, and that under certain conditions only a proportion of viral particles initially present are capable of entering host cells and efficiently completing replication cycles (Graham, et al., 2008). The virus strain used in our study was passed through TO-cells twice, and it raises the question if perhaps the virus had lost some of its effectivity during these passages through possible mutations of the strain. Another possibility, if SAV also exists in quasispecies as other alphaviruses, is if the passage through TO-cells has selected for quasispecies most suitable for, or more effective in, infecting this specific type of cell, ultimately being less effective in CHSE-214 cells. The use of a wild type (wt) virus, a virus passaged in CHSE-214 cells, or the use of a different cell line may have yielded different results.

In retrospect, it was clear that it would have been relevant, more useful and more interesting to extend the period in which the cells were exposed to the virus. As expression of the nsP1 gene does not happen until somewhere between 12 and 24 hpi, in future experiments, fewer time outtakes should be made in the early stages of infection, as it does not yield any valuable information. A schedule of sampling at 0 hpi, then every 24 hours for 10 days would perhaps give more relevant and significant information.

7. Summary

Pancreas disease is responsible for major economic losses in the European salmonid farming industry, due to mortality, diminished growth rate and reduced slaughter weights. The infectious agent causing pancreas disease in Atlantic salmon was first isolated in Scotland in 1995 and later classified as an Alphavirus, the first Alphavirus to be isolated that affects fish. One hallmark of Alphavirus infection in vertebrate cells is the ability of the pathogen to shut down host transcription and translation processes without affecting viral protein and nucleic acid synthesis. It is of interest to find out if SAV shares this characteristic. The goal of this project was to investigate Salmonid alphaviruses ability to down-regulate host cell transcription. Through using a two-step quantitative PCR for analysis of different housekeeping genes in non-infected and infected cells, we hoped to evaluate if SAV3 causes down-regulation of transcription. If so, at what time interval it occurred, and to what degree. This study and its incurred results served as a pilot study, to optimize and fine-tune the methodology for further experimentation.

CHSE-214 cells (Chinook salmon embryo cells) were infected with SAV3 H10/02. Mock treated CHSE-214 cells were used as a negative control. Mock treated and infected cells were sampled at 0, 4, 12, 24, 48, 72, and 96 hpi (hours post infection). RNA extraction and cDNA synthesis were done before real-time qPCR. The expression of housekeeping genes EF1aB, B-actin, and 18S rRNA were measured with the AriaMx detection system and software (Agilent Technologies). Expression of SAV protein nsP1 was also measured for confirmation of infection.

The results indicated that more than one cell-line should be used for future experiments as a recommendation, as well as prolonging the time period in which the cells are incubated after infection, ideally harvesting the samples at 0 hpi and every 24 hours for 10 days. nsP1 gene expression did not occur until after 12 hpi, indicating that less time outtakes are required in early infection. Furthermore, one should consider using a wild-type virus instead of a strain that has undergone several passages. An increase in Ct values could be seen for B-Actin and EF1aB, though no conclusions could be made regarding the significance of these results. The 18S rRNA results, however, did not yield a significant increase in Ct values between the infected and non-infected cells.

8. Bibliography

Akhrymuk, I., Kulemzin, S. V. & Frolova, E. I., 2012. Evasion of the Innate Immune Response: the Old World Alphavirus nsP2 Protein Induces Rapid Degradation of Rpb1, a Catalytic Subunit of RNA Polymerase II. *Journal of Virology,* 07, 86(13), pp. 7180-7191.

Atasheva, S., Fish, A., Maarten, F. & Frolova, E. I., 2010. Venezuelan Equine Encephalitis Virus Capsid Protein Forms a Tetrameric Complex with CRM1 and Importin a/b That Obstructs Nuclear Pore Complex Function. *Journal of Virology,* 05, 84(9), pp. 4158-4171.

Bourai, M. et al., 2012. Mapping of Chikungunya Virus Interactions with Host Proteins Identified nsP2 as a Highly Connected Viral Component. *Journal of Virology,* 86(6), pp. 3121-3134.

Chamberlain, R., 1981. Epidemiology of Arthropod-Borne Togaviruses: The Role of Arthropods as Hosts and Vectors and of Vertebrate Hosts in Natural Transmission Cycles. In: *The togaviruses: Biology, Structure, Replication.* New York and London: Academic Press, Inc., pp. 175-228.

Cheng, R. H. et al., 1995. Nucleocapsid and Glycoprotein Organization in an Enveloped Virus. *Cell,* 24 02, Volume 80, pp. 621-630.

Fiskeri og Havbruksnæringens Forskningsfond, 2012. *Transmission Routes and Infection Dynamics of Salmonid Alphavirus (SAV).* [Online] Available at: http://www.fhf.no/prosjektdetaljer/?projectNumber=900258 [Accessed 04 11 2016].

Fringuelli, E. et al., 2008. Phylogenetic analysis and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *Journal of Fish Diseases,* Volume 31, pp. 811-823.

Fros, J. J. et al., 2010. Chikungunya Virus Nonstructural Protein 2 Inhibits Type I/II Interferon-Stimulated JAK-STAT Signalling. *Journal of Virology,* 84(20), pp. 10877-10887.

Fros, J. J. et al., 2015. Chikungunya virus non-structural protein 2-mediated host shut-off disables the unfolded protein response. *Journal of General Virology,* Volume 96, pp. 580-589.

Fros, J. J. & Pijlman, G. P., 2016. Alphavirus Infection: Host Cell Shut-Off and Inhibition of Antiviral Responses. *Viruses,* 11 06, 8(6), p. 166.

Gonzales Vecino, J. L. et al., 2010. *Fôring med spesialtilpasset diett ved utbrudd av PD; pankreas sykdom.* [Online] Available at: http://kyst.no/nyheter/fring-med-spesialtilpasset-diett-ved-utbrudd-av-pd-pankreas-sykdom/ [Accessed 21 09 2016].

Gorchakov, R. et al., 2004. PKR-Dependent and -Independent Mechanisms Are Involved in Translational Shutoff during Sindbis Virus Infection. *Journal of Virology,* 08, 78(16), pp. 8455-8467.

Graham, D. A., Wilson, C., Jewhurst, H. & Rowley, H., 2008. Cultural characteristics of salmonid alphaviruses - influence of cell line and temperature. *Journal of Fish Diseases,* Volume 31, pp. 859-868.

Harding, H. P. et al., 2000. Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Molecular Cell,* 11, Volume 6, pp. 1099-1108.

Hjortaas, M. J. et al., 2012. The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway. *Journal of Fish Diseases,* 36(1), pp. 71-74.

Hodneland, K., 2006. *Salmonid Alphavirus (SAV); Genetic characterization of a new subtype, SAV3, and implementation of a novel diagnostic method.* Bergen: s.n.

Hodneland, K. et al., 2005. New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss in Norway. *Diseases of Aquatic Organisms,* 05 09, Volume 66, pp. 113-120.

Hodneland, K. & Endresen, C., 2006. Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan). *Journal of Virological Methods,* Volume 131, pp. 184-192.

Ingerslev, H.-C.et al., 2006. Expression profilin and validation of reference gene candidates in immune relevant tissues and cells from Atlntic salmon (Salmo salar L.). *Molecular Immunology,* Volume 43, pp. 1194-1201.

Jansen, M. D. et al., 2016. *The epidemiology of pancreas disease in salmonis aquaculture: a summary of the current state of knowledge.* [Online] Available at: http://onlinelibrary.wiley.com/doi/10.1111/jfd.12478/full [Accessed 12 10 2016].

Jansen, M. D. et al., 2015. *Pankreassykdom hos laksefisk - en review med fokus på forebygging, kontroll og bekjempelse,* Oslo: Norwegian Veterinary Institute.

Karlsen, M. et al., 2010. The amino terminus of the salmonid alphavirus capsid protein determines subcellular localization and inhibits cellular proliferation. *Archives of Virology,* Volume 155, pp. 1281-1293.

Kongtorp, R. T. et al., 2010. LAck of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, Salmo salar L., exposed by natural and horizontal routes. *Journal of Fish diseases,* Volume 33, pp. 879-888.

Koonin, E. V., Dolja, V. V. & Morris, J. T., 1993. Evolution and Taxonomy of Positive-Strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences. *Critical Reviews in Biochemistry and Molecular Biology,* 28(5), pp. 375-430.

Kumas Das, P., Merits, A. & Lulla, A., 2014. Functional Cross-talk between Distant Domains of Chikungunya Virus Non-structural Protein 2 is Decisive for its RNA-modulation Activity. *The Journal of Biological Chemistry,* 28 02, 289(9), pp. 5635-5653.

Løvoll, M. et al., 2011. Transcription of reference genes used for quantitative RT-PCR in Atlantic Salmon is affected by viral infection. *Veterinary Research,* 18 01.42(8).

Lester, K., Black, J. & Bruno, D. W., 2011. Prevalence of salmonid alphavirus in Sco ish fish farms from 2006 to 2007. *European Association of Fish Pathologists Bulletin*, 31(5), p. 200.

Liljestrom, P. & Garoff, H., 1991. Internally Located Cleavable Signal Sequences Direct the Formation of Semlike Forest Virus Membrane Proteins from a Polyprotein Precursor. *Journal of Virology,* 65(1), pp. 147-154.

Madhun, A. S. et al., 2016. *Annual report on health monitoring of wild anadromous salmonids in Norway,* s.l.: Norwegian Veterinary Institute, Institute of Marine Research.

McLoughlin, M. F. & Graham, D. A., 2007. Alphavirus infection in salmonids - a review. *Journal of Fish Diseases,* 30(9), pp. 511-531.

McLoughlin, M. F. et al., 2002. Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, Salmo salar L.. *Journal of Fish Diseases,* Volume 25, pp. 33-43.

Merour, E. et al., 2013. A Fully Attenuated Recombinant Salmonid Alphavirus Becomes Pathogenis through a Single Amino Acid Change in the E2 Glycoprotein. *Journal of Virology,* 05, 87(10), pp. 6027-6030.

MicrobeWiki, 2016. *Rubivirus.* [Online] Available at: https://microbewiki.kenyon.edu/index.php/Rubivirus [Accessed 13 10 2016].

Norwegian Veterinary Institute, 2015. *Fiskehelserapporten 2014,* Oslo: Veterinærinstituttet.

Norwegian Veterinary Institute, 2016. *Pancreas Disease - outbreaks and statistics.* [Online] Available at: http://www.vetinst.no/oppdrettsfisk/pankreassykdom-pd-utbrudd-og-statistikk [Accessed 21 09 2016].

OIE, 2016. *Infection with Salmonid Alphavirus.* [Online] Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_salmonid_alphavirus.pdf [Accessed 14 10 2016].

Olsvik, P. A. et al., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology,* 6(21).

Peranen, J., Rikkonen, M., Liljestrom, P. & Kaariainen, L., 1990. Nuclear Localization of Semliki Forest Virus-Specific Nonstructural Protein nsP2. *Journal of Virology,* 05, 64(5), pp. 1888-1896.

Press, M. M., 2013. *Kyststrømmer sprer virus hos oppdrettsfisk.* [Online] Available at: http://forskning.no/fiskesykdommer-oppdrett-virus/2013/10/kyststrommer-sprer-virus-hos-oppdrettsfisk [Accessed 26 10 2016].

Randall, R. E. & Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *Journal of General Virology,* Volume 89, pp. 1-47.

Rathore, A. P. S., Ng, M.-L. & Vasudevan, S. G., 2013. Differential unfolded protein response during Chikungunya and Sindbis virus infection: CHIKV nsP4 supresses eIFa phosphorylation. *Virology Journal,* 10(36).

Schwartz, O. & Albert, M. L., 2010. Biology and pathogenesis of chikungunya virus. *Nature Reviews Microbiology,* 07, Volume 8, pp. 491-500.

Sherman, M. B. & Weaver, S. C., 2010. Structure of the Recombinant Alphavirus Western Equine Encephalitis Virus Revealed by Cryoelectron Microscopy. *Journal of Virology,* 84(19), pp. 9775-9782.

Silver, N., Best, S., Jiang, J. & Thein, S. L., 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BioMed Central Molecular Biology,* 7(33).

Snow, M. et al., 2010. Detection of salmonid alphavirus RNA in wild marine fish: implications for the origins of salmon pancreas disease in aquaculture. *Diseases of Aquatic Organisms,* 17 09, 91(3), pp. 177-188.

Strauss, H. J. & Strauss, G. E., 1994. The Alphaviruses: Gene Expression, Replication, and Evolution. *Microbiological Reviews,* 09, 58(3), pp. 491-562.

Taksdal, T. et al., 2007. Pancreas disease in farmed Atlantic salmon, Salmo salar L., and rainbow trout, Oncorhynchus mykiss (Walbaum), in Norway. *Journal of Fish Diseases,* Volume 30, pp. 545-558.

Ventoso, I. et al., 2006. Translational resistance of late alphavirus mRNA to eIF2a phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes and Development,* 20(1), pp. 1-124.

Villoing, S. et al., 2000. Rainbow Trout Sleeping Disease Virus Is an Atypical Alphavirus. *Journal of Virology,* 01, 74(1), pp. 173-183.

Weston, H. J., Welsh, D. M., McLoughlin, F. M. & Todd, D., 1999. Salmon Pancreas Disease Virus, an Alphavirus Infecting Farmed Atlantic Salmon, Salmo salar L.. *Virology,* 10 04, 256(2), pp. 188-195.

Weston, J. et al., 2002. Comparison of Two Aquatic Alphaviruses, Salmon Pancreas Disease Virus ad Sleeping Sidease Virus, by Using Genome Sequence Analysis, Monoclonal Reactivity, and Cross-Infection. *Journal of Virology,* 06, 76(12), pp. 6155-6163

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