INFECTIOUS SALMON ANAEMIA IN ATLANTIC SALMON, *SALMO SALAR* L. IN CHILE

- TRANSMISSION ROUTES AND PREVENTION

Siri Vike

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This has been an interesting period of my life! The study has brought me to the other side of the globe, dealing with a different culture, and fatal earthquakes. I have experienced the brutality among scientists and felt the pettiness of people who tried to make my science invalid. However, also the pleasure of being believed and having my article on the front page of New York Times. Thanks to all of you who cheered for me!

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SUMMARY – in English

Infectious salmon anaemia (ISA) virus causes a severe, commercially significant disease in all countries farming Atlantic salmon (*Salmo salar*). This thesis examines the ISA epizootic in Chile during 2007-09, known as the "ISA crisis". Three important questions were addressed. First, how did the ISA virus arrive in Chile? Second, how was the virus spread within the industry after arrival? And third, what would be the best preventive measures to control the spread of this virus?

Phylogenetic comparison of ISA virus from Chile with all available ISA virus strains showed a close relationship to European ISA virus. The best explanation for this observation is that the ISA virus arrived in Chile with its natural host, Atlantic salmon – through import of embryos. Once in Chile the ISA virus spread rapidly due to the industrial and sanitary practices such as the continuous productions in both freshwater lakes and at sea sites with no generational splits, and movement of fish between sites. At some sites the salmon were weakened due to high stocking densities and the presence of additional pathogens. It was found that the sea louse, *Caligus rogercresseyi* could act as a mechanical vector, and possibly contributed to the spread of the virus. In contrast, the survival time of ISA virions in natural sea water was found to be less than three hours (under experimental conditions), and therefore waterborne transmission over long distances was considered to be unlikely. In addition, ISA mortalities were contagious for up to five days *post mortem,* emphasizing the importance of dead fish removal.

ISA virus is present in the natural wild reservoir of *S. salar* and *S. trutta* in the North Atlantic. However, both in Chile and Norway it is likely that the farming industry accommodate a self-sustaining reservoir of ISA virus. Thus the industry has a possibility to reduce the prevalence by pathogen monitoring and elimination of positive broodfish. Good surveillance programmes can predict ISA outbreaks, and site management can diminish the significance of ISA outbreaks. The key for this is tracing the pathogen throughout the production cycle and implementing appropriate preventative measures, instead of waiting for clinical symptoms to appear and then activate the associated "fire fighting" approach.

SUMMARY – en español

El virus de la anemia infecciosa del salmón (virus ISA, en inglés) causa significantes pérdidas en la acuicultura del salmón del Atlántico (*Salmo salar*). Esta tesis presenta la epizootiología de ISA en Chile durante el 2007 – 2009, período en el que ocurrió la conocida 'crisis del ISA'. Tres importantes materias se incluyen, primero, la forma en que llego llegó el virus ISA a Chile; segundo, cómo se extendió en la acuicultura chilena; y, finalmente, cuáles podrían ser las medidas preventivas para impedir su diseminación.

La comparación filogenética entre las cepas chilenas y las cepas disponibles del virus ISA muestran una relación cercana entre las chilenas y las cepas europeas. La explicación más probable es que el virus arribó a Chile junto con su hospedador natural, el salmón del Atlántico, a través de ovas importadas. Una vez en el país, se propago rápidamente debido a prácticas como la producción continua, en lagos y en el mar, sin realizar separaciones generacionales, y el traslado de peces entre sitios de cultivo. Factores como el debilitamiento sanitario de la biomasa por las altas densidades de cultivo, y la presencia de otros patógenos para peces, posiblemente favorecieron también a la propagación del virus ISA en Chile. La presencia del piojo de mar, *Caligus rogercresseyi,* que podría actuar como vector mecánico, igualmente podría haber contribuido a la dispersión del virus. Por otro lado, se describe que la sobrevivencia del virus de ISA en agua de mar es menor a tres horas, lo que hace improbable la transmisión por esta vía en largas distancias. Además, los peces muertos debido al virus resultan contagiosos por un periodo mayor a cinco días *post mortem* por lo que se deben prontamente removidos.

El virus ISA está presente en sus reservorios naturales, *S. salar* y *S. trutta,* en el Atlántico Norte. Sin embargo, es probable que, tanto en Chile como en Noruega, además los salmónidos de cultivo auto-sustenten reservorios para el virus. Programas de vigilancia y manejo adecuados establecidos por la industria, pueden tanto predecir los brotes de ISA como disminuir los efectos de estos. Así, instaurando programas de monitoreo se pueden excluir los reproductores que resulten positivos. Para controlar el virus ISA es muy importante realizar su trazabilidad a través de todo el ciclo de producción, junto con establecer las medidas apropiadas de prevención, en vez de esperar a que el cuadro clínico este establecido para realizar manejos sanitarios de emergencia en los cultivos.

SUMMARY - på norsk

Infeksiøs lakseanemi (ILA) virus gir en alvorlig sykdom hos Atlantisk laks (*Salmo salar*), med stor økonomisk betydning i alle land med lakseoppdrett. I denne avhandlingen undersøkes ILA-epidemien i Chile mellom 2007-09, også kjent som «ILA-krisen». Tre viktige spørsmål er adressert. Først, hvordan kom ILA viruset til Chile? Dernest, hvordan ble viruset spredd etter ankomst? For det tredje, hva er den beste måten å hindre videre spredning av viruset?

Fylogenetisk sammenligning av alle tilgjengelige ILA virus sekvenser viser at alle virus funnet i Chile er nært beslektet med de europeiske. Den beste forklaringen på denne observasjonen er at ILA viruset har kommet til Chile sammen med sin naturlige vert, Atlantisk laks – gjennom import av embryo. Etter at viruset kom til Chile ble det trolig raskt spredd på grunn av driftsstrukturen og av mangel på forebyggende tiltak. For eksempel var det ved flere anlegg kontinuerlig produksjon av laks både i ferskvann og i sjøen (uten generasjonsskiller) og mye flytting av fisk. Dessuten var laksen ved flere lokaliteter svekket på grunn av høy biomasse og tilstedeværelse av andre patogener. Det ble vist at fiskelusen, *Caligus rogercresseyi*, kan fungere som en mekanisk vektor, og at denne kan ha bidratt til spredningen av ILA virus. Overlevelsen av ILA viruset i naturlig sjøvann, under eksperimentelle forhold, viste seg imidlertid å være begrenset til mindre enn tre timer, noe som gjør det mindre sannsynlig at vannbåren smitte kan skje over lange avstander. I tillegg ble det vist at laks, død av ILA, var smittsom i minst fem dager, noe som viser viktigheten av riktig og rask håndtering av død fisk.

Det naturlige reservoaret til ILA virus er *S. salar* og *Salmon trutta* i Nord-Atlanteren. I chilensk og norsk oppdrett er det imidlertid sannsynlig at det er oppdrettslaksen selv som er reservoaret for ILA viruset. Dette betyr at industrien har en mulighet til å redusere prevalensen ved å overvåke og å eliminere positive stamfisk over flere generasjoner. I tillegg vil gode overvåkningsprogrammer kunne forutse utbrudd av ILA, og basert på disse kan betydningen av utbruddene reduseres. Nøkkelen for dette er å spore ILA viruset gjennom hele produksjonssyklusen og gjøre de riktige forbyggende tiltak, isteden for å vente på at kliniske symptomer skal oppstå med en påfølgende «brannsloknings» strategi.

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ABBREVIATIONS

ISA	= Infectious salmon anaemia
SAV	= Salmonid alphavirus
SRS	= Salmon Rickettsial Syndrome
CPE	= Cytopathogenic effect
HSMI	= Heart- and skeletal muscle inflammation
IPN	= Infectious pancreatic necrosis
IFAT	= Indirect fluorescent antibody test
Ct-values	= Cycle threshold values
F	= Fusion protein
HE	= Hemagglutinin-esterase
HPR	= Highly polymorphic region
IN	= Insert in segment 5
HPR0	= Avirulent ISA virus having a full-length HPR region
HPRΔ	= Virulent ISA virus having deletions in the HPR region
PRV	= Piscine reovirus
RNA	= Ribonucleicacid
Real-time RT-PCR	= Real-time reverse transcriptase polymerase chain reaction
EB	= Emamectin benzoate
UVC	= Ultraviolet radiation (100–280 nm)
Natural seawater	= Seawater sampled direct from sea with no treatments
NA	= North American

LIST OF PAPERS

This thesis is based on the following papers, hereafter referred to in the text by their Roman numerals:

Paper I

Vike, S., Nylund, S., Nylund, A. (2009). ISA virus in Chile: evidence of vertical transmission. Archives of Virology, 154, 1-8.

Paper II

Vike, S., Oelckers, K., Duesund, H., Erga, S. R., Gonzalez, J., Hamre, B., Frette, Ø., Nylund, A. (2013). Salmon Anaemia (ISA) virus - infectivity in seawater under different physical conditions (*in press,* Journal of Aquatic Animal Health).

Paper III

Oelckers, K., Vike, S., Duesund, H., Gonzalez, J., Wadsworth, S., Nylund, A (2014). *Caligus rogercresseyi* as a potential vector for transmission of Infectious Salmon Anaemia (ISA) virus in Chile. Aquaculture, 420-421, 126-132.

Paper IV

Vike, S., Duesund, H., Andersen, L., Nylund, A. (2014). Release and survival of Infectious Salmon Anaemia (ISA) virus during decomposition of Atlantic salmon (*Salmo salar* L.) Aquaculture, 420-421, 119-125.

1. INTRODUCTION

1.1 Salmonid farming in Chile

The three southernmost regions of Chile have good natural conditions for salmonid farming (Figure 1). This is due to stable temperatures (8-16 ºC) optimal for growth in sea locations all year around. Chile has also favourable topography providing protection against rough weather, and excellent water quality. This, and a short distance to suppliers of feed ingredients (fish meal and oil in Chile and Peru), low labour costs, a sizeable value added sector, and favourable trade agreements, has given Chile competitive advantages as a salmonid producer.

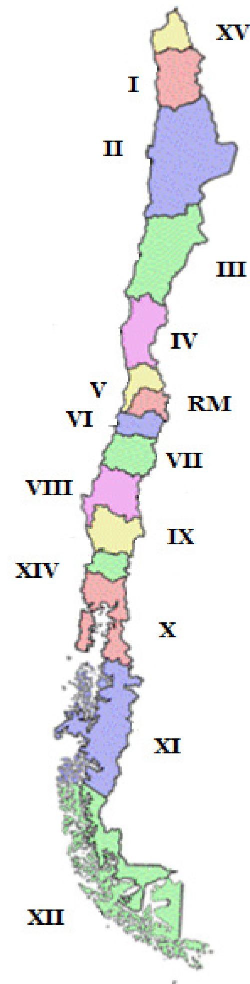


Figure 1. Chile is 4300 km long and divided in 15 regions. Sea sites for production of salmonids are present in Region X (Los Lagos), XI (Aysèn) and XII (Magallanes) (Based on map from Slawojar, public domain, Wikipedia Commons).

Commercial salmonid aquaculture started in Chile in the mid-1980s. The production is mainly based on three species: Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss,* Walbaum) and coho salmon (*Oncorhynchus kisutch,* Walbaum). A few sites also produce Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum).

In 2005, the salmonid production in Chile was the fastest growing worldwide. Chile soon became the world's largest producer of rainbow trout and coho salmon, and the second largest producer of Atlantic salmon. In 2012, 820,735 tonnes were harvested (pers. com. Guerrero, 2013, INTESAL de salmon Chile), making salmonid production the third most important export industry for Chile after copper and cellulose (SalmonChile, 2012). However, as it may be seen in figure 2, the production of Atlantic salmon

suffered more than 60 % decrease from 2008 to 2010. This setback was due to a collapse in the salmon industry caused by a devastating viral epizootic.

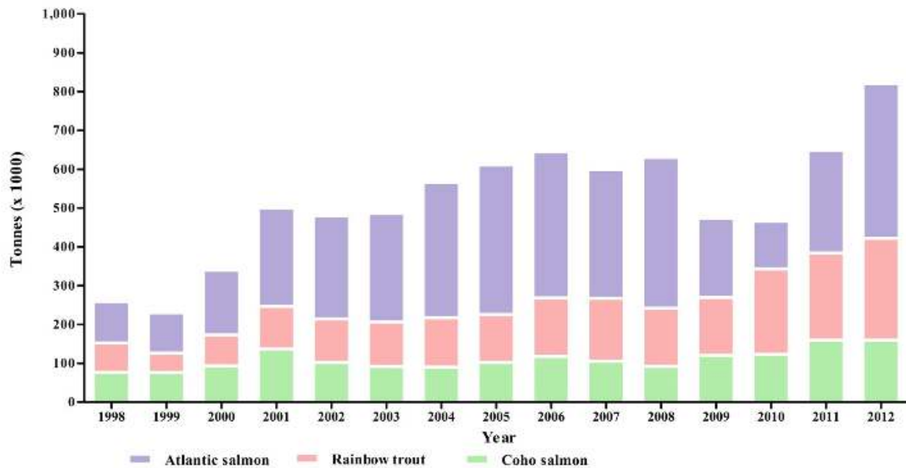


Figure 2. A summary of the yearly production of the three farmed salmonid species in Chile from 1998 – 2012 in tonnes (based on data from: pers. com. Guerrero, 2013, INTESAL de SalmonChile, 2013).

Before farming of salmonids, the southern part of Chile was industrially underdeveloped. In 2007 the same area had 553 marine sites in use, located in regions Los Lagos (72 %) and Aysèn (27 %) (Figure 1). At that time the salmonid industry comprised approximately 30 companies employing close to 25,000 persons directly and more than 20,000 indirectly (Alvial et al., 2012).

Aquaculture all over the world is exposed to infectious pathogens that represent a threat to fish health, fish welfare, the environment and the economies of individual farmers. Viral pathogens are particularly challenging in farming of salmonids, since no effective vaccines (compared to bacterial vaccines) and no medical treatments are available (Sommerset et al., 2005; Crane and Hyatt, 2011; Robertsen, 2011; Kibenge et al., 2012). Due to this it is important to acquire knowledge about reservoirs and

transmission routes, to make it possible to eliminate or avoid the pathogen as early as possible in the production chain. If the pathogen is already present in a fish population, it is important to identify the most important risk factors to avoid triggering disease outbreaks.

This study looks into the ISA epizootic in Chilean salmonid farming during 2007-09, known as the “ISA crisis”. Three important questions were addressed, first how the ISA virus arrived in Chile, second how the virus was spread within the industry after arrival, and third what would be the best preventive measures to control the spread of virus?

1.1.1 Salmon farming and pathogenes

All salmonids are exotic species south of the Equator. Thus, there are no native fish in the genera *Oncorhynchus* or *Salmo* in Chile. The Chilean salmonid farming was developed on genetic material imported from Europe and North America, where these species exist naturally (Gajardo and Laikre, 2003). The first import occurred in the late 19th century, when embryos were brought to Southern Chile and fry released in rivers and lakes for recreational fisheries (Bravo, 2010a). Since the start of the industry more than 1.9 billions of fertilized eggs (embryos) have been introduced into Chile (Ibieta et al., 2011). Several Chilean companies have established their own breeding programmes. However, imports still take place to provide “off season” embryos from the northern hemisphere, as the opposite seasons allow continuous production.

One important risk associated with international trade of biological material is the import/export of exotic pathogens (Johnsen and Jensen, 1991; Bovo et al., 2005; Enzmann et al., 2010). Chile who used to have the competitive advantage of an environment free of salmonid specific pathogens, have lost this advantage due to the import of infected embryos (**Paper I**; Ibieta et al., 2011; Kurath and Winton, 2011; Mutoloki and Evensen, 2011; Kibenge et al., 2013). It is difficult to fully trust preventive bio-security measures, especially when the broodfish have their growth phase in an uncontrolled environment in cages in open sea. Further, it is not possible to

monitor unknown pathogens, and that limitation alone makes transfer of biological material a risk factor.

In Norway most farmed Atlantic salmon are vaccinated with vaccines containing 5-6 components against *Aeromonas salmonicida, Vibrio salmonicida, Listonella anguillarum serotype* O1 and O2a and *Moritella Viscosa,* and infectious pancreatic necrosis (IPN) virus. Vaccination against salmonid alphavirus (SAV) is only performed within a defined zone. A total of 14 vaccines have marketing authorization in 2013 and none of these contain an ISA virus component. Use of ISA virus vaccines in Norway requires special permits (pers. com. Høy, 2013, Norwegian Medicines Agency). In Chile most Atlantic salmon is vaccinated with 4-5 antigens; Atypical *Aeromonas salmonicida, Vibrio salmonicida, Piscericettsia salmonis,* IPN virus and ISA virus. In 2013, fifty vaccines have achieved market authorization for salmonids and sixteen of these include an ISA virus component (SAG, 2011). Thus, the number of available fish vaccines is much higher than in Norway. Moreover, at present nearly all Atlantic salmon in Chile is vaccinated against ISA virus (pers. com. Mendoza, 2013, Mainstream Chile SA).

Traditionally, various strategies for smolt production have been used in Chile, but most often the smolts were produced in open systems in lakes – especially in Lago Llanquihue (860 km^2) in Region Los Lagos. The fry were hatched and grown in closed facilities until reaching about 5 grams, then transported by trucks and placed into cage systems similar to those used in sea. The production period in the lake had several competitive advantages. It was cost efficient due to low investments and low operating cost. Without technological or regulatory limitations, there were nearly unlimited possibilities to produce large numbers of smolts. The smolt was adapted to the “cage-life” (feeding habits, natural light, currents, etc.) before entering the more stressful smoltification period, which is the normal time for transfer to sea cages. Unfortunately, this production style also had some disadvantages. A continuous production with high fish densities and fish numbers, and with three salmonid species in the same area provided excellent conditions for horizontal transmission of

pathogens (Groff and LaPatra, 2000; Murray and Peeler, 2005; Kurath and Winton, 2011). The fry were too small to be vaccinated (Nerland et al., 2011) before entering the uncontrolled environment in the lake and was exposed to a number of different pathogens (pers. obs.). The main challenges were the presence of *Flavobacterium sp.* (Valdebenito and Avendano-Herrera, 2009) and *Saprolegina sp.* (Ibieta et al., 2011). The need for treating the fish with antibiotics and anti-fungal medicaments was relatively high (pers. obs). Other pathogens causing outbreaks in the lakes were *Francisella sp.* (Birkbeck et al., 2007), *Yersinia ruckeri* and IPN virus (Ibieta et al., 2011).

In 2001-2003 ISA virus was detected in healthy Atlantic salmon in Lago Llanquihue (Godoy et al., 2008). A few years later (2007) a virulent strain of ISA virus was found in the same lake in Atlantic salmon smolts (**Paper I**). There are indications that fish infected with virulent ISA virus were later transferred to sea. This was done partly because the fish were not diagnosed to be infected by the ISA virus, and partly since the farmers had experienced, from earlier incidents, that disease problems often decreased after transfer to sea (pers. com.[1]). There are, however, examples of sites that experienced ISA outbreaks only three weeks after sea transfer (Godoy et al., 2008), which suggest that the smolts were infected in the fresh water phase.

Another common strategy used by several companies was to complete the smoltification process in brackish water in the estuaries. This period gave the smolt a milder and smooth adaption to life in seawater. But this production was similar to the production in the lakes, i.e. a continuous production of mixed fish groups, generations and species in the same water body.

In order to have enough freshwater with optimal quality, most of the freshwater sites and hatcheries in Chile are located far away from the sea. Smolt are typically transported over land for 50-300 kilometres followed by wellboat transportation that can last a few hours and up to several days. This is a stressful challenge due to high

[1] From an anonymous source.

fish densities, sub-optimal water quality and variable temperatures that could influence the smolt quality prior to transfer to sea (Iversen et al., 1998; Iversen et al., 2005).

In the marine phase of the production before 2007, there were few regulations and few coordinated activities between companies in the same area. For instance, it was permitted to move fish from site to site, generation shifts were not demanded, and no established standard existed for mortality treatment. A common strategy was to transfer smolt to one starting sea site. When this first site reached its maximum level of density (max 30 kg/m^3 for Atlantic salmon), the fish groups were split and distributed to several sea sites. This production was cost efficient, but also resulted in spreading of pathogens between different locations.

Since the start of the salmonid farming in Chile, piscirickettsiosis / Salmon Rickettsial Syndrome (SRS) has been the main infectious disease in the marine phase of production. SRS is caused by the intracellular bacteria *Piscirikettsia salmonis* (Cvitanich et al., 1991; Fryer et al., 1992), and has been a continuous threat to the sustainability of Chilean aquaculture, because of the relatively high level of antibiotics used to control it. During 2012 mortality from SRS represented 79 % of the mortality in rainbow trout, 60 % in Atlantic salmon and 27 % of coho salmon (pers. com. Godoy, 2012, Etecma).

Other pathogens associated with salmonid farming in the sea phase include: *Renibacterium salmoninarum*, *Nucleospora salmonis*, *Kudoa sp*, Atypical *Aeromonas salmonicida*, *Vibrio ordalii*, *Vibrio anguillarum* and IPN virus (Ibieta et al., 2011). In 2007, *Neoparamoeba perurans* causing Amoebic Gill Disease (AGD) was detected in Chile for the first time (Bustos et al., 2011b). Lately piscine reovirus (PRV) has been discovered (Kibenge et al., 2013).

The most challenging parasitic copepode affecting Chilean farming of Atlantic salmon and rainbow trout is the sea louse *Caligus rogercresseyi* (Bravo, 2003). The natural hosts of *C. rogercresseyi* are a wide range of wild marine fish species (Bravo, 2010a). Due to continuous reinfections is it difficult to combat sea lice infestations at the farming sites (Gonzalez and Carvajal, 2003; pers. obs.).

1.1.2 The ISA crisis

The Chilean industry started to experience increased disease problems during 2005-06, after two decades of strong growth and good financial results. The "symptoms" were increasing need for, and use of treatments (antibiotics, anti-fungus and anti-parasitics), and rising mortalities in the freshwater phase as well as the marine production phase. From 2004 to 2007 the average harvest fish weight decreased from 4.5 kg to 2.7 kg, and the average harvest weight per transferred smolt went down from 3.0 kg to 1.8 kg (Alvial et al., 2012).

The most visible fish health challenge at that time was the yearly increase in the number of the sea lice, *Caligus rogercresseyi* (Figure 3).

Figure 3. Atlantic salmon highly infested with *Caligus rogercresseyi*. Photo: Siri Vike

The increase of *C. rogercresseyi* had two main reasons. First, there was only one approved medicament, i.e. oral treatment with emamectin benzoate (EB). The indiscriminate use of this drug over 5-6 years lead to loss in efficacy due to development of resistance in the sea lice population (Bravo et al., 2008). Secondly, a restriction on the use of EB in the last period of the production cycle was implemented due to market demands. This led to an uncontrolled sea lice situation until bath treatment was properly established during 2007-08. The average number of sea lice per Atlantic salmon increased from 5.5 in 2001 to 21 in 2007 (Figure 4). For rainbow trout the average numbers of sea lice were 6.9 (2001) and 21.1 (2007). The high levels

of sea lice infestation also led to increased stress and mucus/skin damage. It is reasonable to assume that this also led to immunosuppression, which consequently made the fish more susceptible to other pathogens (Fast et al., 2004; Johnson et al., 2004; Fast et al., 2007; Tadiso et al., 2011). The first official ISA outbreak came after a period with the highest ever recorded infestations of *C. rogercresseyi* (Figure 4).

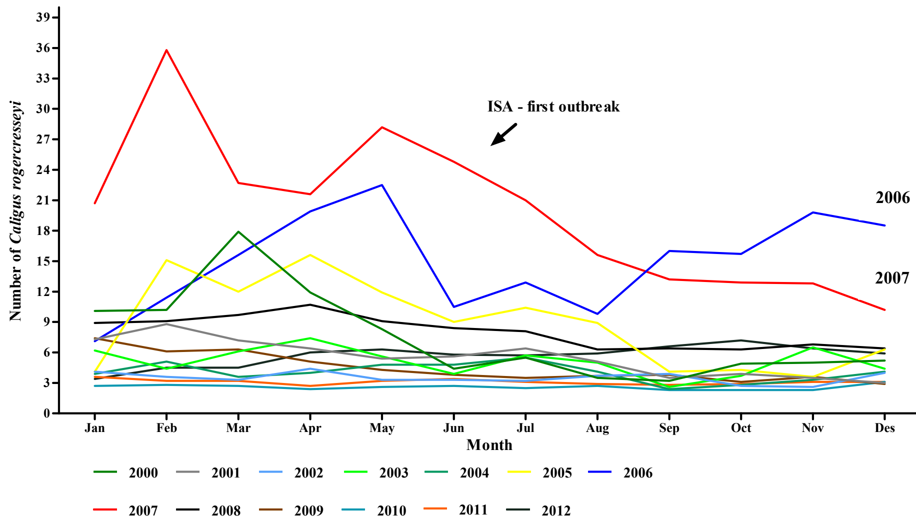


Figure 4. Monthly average number of *C. rogercresseyi* in Chilean Atlantic salmon farming from 2000-2012. The arrow indicates when the first official outbreak of ISA was registered during winter 2007. The top two lines are from 2006 and 2007. The graph represents 75 % of the industry (based on data from: pers. com. Guerrero, 2013, INTESAL de salmon Chile, 2013).

Several farming companies increased their number of smolt transferred to sea, to compensate for the economic losses due to weak biological performance (Alvial et al., 2012; Kibenge et al., 2012). However, it has been shown that intensive production with high fish density may result in higher prevalence as well as higher virulence of pathogens, and consequently a higher risk for disease development (Groff and LaPatra, 2000; Christiansen, 2011; Kurath and Winton, 2011; Nerland et al., 2011; Rimstad, 2011).

In July 2007, the first official outbreak of ISA was reported at a sea site containing Atlantic salmon east of Chiloe. This is regarded as the primary or index case. The site was located in an area with many other fish farms. Prior to the ISA outbreak, the fish population at the site had just recovered from an SRS outbreak (Godoy et al., 2008). In Norway farmers had similar experiences during an ISA epizootic in the 1990-ies, i.e. an ISA outbreak usually occurred just after medication against other diseases or parasites (e.g. Cold Water Vibrosis*, Lepeophtheirus salmonis, Eubothrium sp.*) (Thorud and Håstein, 2003). Also prior to the ISA outbreaks in Scotland in 2008 an increase of *L. salmonis* infestations was observed (Murray et al., 2010).

However, it is likely that this index case in Chile was not the starting point of the epizootic, because:

1) Almost simultaneously ISA was diagnosed in several places;
2) Prior to the index case, ISA was not considered a possible diagnosis, and was likely to be overlooked by fish health personnel. It was believed that ISA virus could only be transmitted horizontally, and therefore could not be present in Chile (see for instance: Rimstad et al., 2007);
3) Lack of sensitive validated diagnostic tools and procedures, combined with several pathogens/diseases present at the same time: *C. rogercresseyi* infestation, SRS outbreaks, co-infection with IPN virus, and amoebic gill disease (*Neoparamoeba perurans*) could mask the ISA symptoms (Bravo, 2010b; Alvial et al., 2012; Cortez-San Martin et al., 2012; pers. obs.);
4) There are some indications about positive ISA virus incidents/findings that were never reported from laboratories nor field, as told by key personnel who suspected that they had observed ISA/ISA virus several years before 2007 (Godoy et al., 2008; Asche et al., 2009; Alvial et al., 2012);
5) Phylogenetic analysis indicates that ISA virus most probably arrived in Chile some years before the index case (**Paper I**; Kibenge et al., 2009; Plarre et al., 2012);
6) Phylogenetic analysis also showed that the ISA virus isolate from the index case could not be the ancestor to subsequent outbreaks, and therefore the following outbreaks could not be a result of horizontal transmission from the index case.

Thus, the time of introductions of the virus remains uncertain (Kibenge et al., 2009; Alvial et al., 2012; Plarre et al., 2012).

As seen from figure 5, the epizootic started in July 2007, but most of the outbreaks appeared one year later. During 2007, diagnostic tools were not properly implemented and experience with ISA was poor; so it is possible that several outbreaks in the first year of the epizootic went unregistered. In addition, the governing authority Sernapesca (the National Fisheries Service) was understaffed and had few personnel with experience in preventive fish health, and thus struggled to handle this huge epizootic. The staff in Sernapesca was increased from 200 in 2007 to 729 during the ISA crisis to implement, control and follow up the new regulations (Alvial et al., 2012).

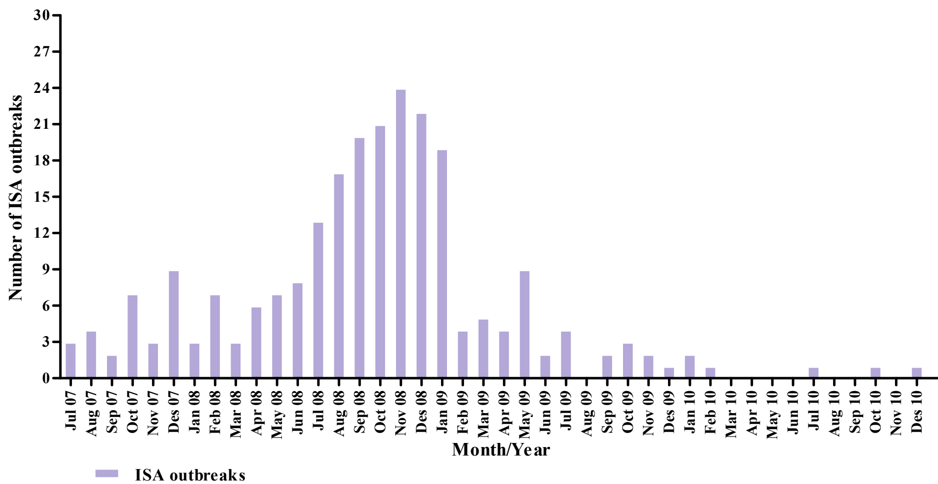


Figure 5. Number of official ISA outbreaks in Chile from July 2007 to October 2010 – total outbreaks of 239 (based on data from: pers. com. Guerrero, 2013, INTESAL de salmon Chile, 2013).

An ISA outbreak varies in clinical manifestations and associated mortality. Some outbreaks have an aggressive development with escalating mortality ending in total "burn-up" of the site (nearly all cages affected at same time). At these sites, fast and proper removal of dead fish is a major challenge to limit further infection. At other sites the mortality can be moderate, and only single cages are affected (Lyngøy, 2002; pers. obs.). Experiences from Norway show that horizontal spread of ISA virus is slow within a farm. It can take a month before neighbouring cages are infected (Thorud and Håstein, 2003; pers. obs.). There are examples of sites where it has taken a year before all cages are affected by clinical ISA with mortality (Poppe et al., 1999). When a single cage is infected, and fish in this cage are eliminated early, the outbreak can be limited to this single cage. In these cases the rest of the site can be harvested at a planned schedule without ISA symptoms. This is common practice in Norway today (pers. com. Ottem, 2013, Mainstream Norway).

The Chilean ISA epizootic had a dramatic impact on the produced volume of Atlantic salmon, which was reduced by 64 % (253.243 tonnes) from 2008 to 2010, as seen in figure 2. Due to economic constrains and uncertainty about the future, the transfer of smolt to sea farms was reduced by more than 80 %, from approximately 250 million in 2007 to less than 50 million in 2009 (Ibieta et al., 2011). This reduction in smolt transferred, meant that the economic crisis lasted longer than just the outbreak period, due to low production volumes in the next generations.

The economic loss has been estimated at USD 2,000 million, representing approximately all the assets that had been developed during the industry's 25 years history (Asche et al., 2009; FIS, 2010). Furthermore, approximately 40 % of workers in the farming companies lost their jobs leading to economic depression and social need in the salmon farming areas (SalmonChile, 2012).

1.2 The ISA virus

The first electron micrograph of the ISA virus, the causative agent of ISA, was taken by Watanabe et al. (1993) (Figure 6). A few years later, several studies had

characterized the virus both morphologically (Hovland et al., 1994; Dannevig et al., 1995; Nylund et al., 1995b; Nylund et al., 1996; Sommer and Mennen, 1996; Falk et al., 1997; Koren and Nylund, 1997) and genetically (Mjaaland et al., 1997; Krossoy et al., 1999; Devold et al., 2001; Krossoy et al., 2001a; Rimstad et al., 2001; Clouthier et al., 2002; Aspehaug et al., 2005). Its biophysical properties, such as sensitivity for changes in temperature, pH and UV has also been investigated (Falk et al., 1997; Torgersen, 1997; MacLeod et al., 2003; Tapia et al., 2013).

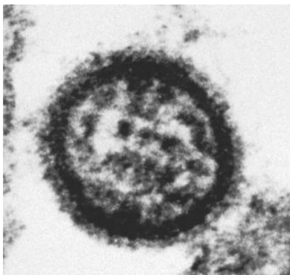


Figure 6. Electron micrograph of an ISA virion. The virus particles are pleiomorphic and enveloped, with a diameter of 100-130 nm. Photo: Koren and Nylund (1997).

ISA virus is an enveloped negative single stranded RNA virus consisting of eight segments (Falk et al., 1997; Mjaaland et al., 1997; Krossoy et al., 1999; Clouthier et al., 2002). It belongs to the aquatic orthomyxovirus assigned to its own genus, *Isavirus* (Sommer and Mennen, 1996; Falk et al., 1997; Koren and Nylund, 1997; Mjaaland et al., 1997; Krossoy et al., 1999; King et al., 2012). Morphologically, the ISA virion has a spherical shape with a diameter of about 100-130 nm (Watanabe et al., 1993; Hovland et al., 1994). ISA virus has been shown to replicate in blood vessel endothelial cells, endocardial cells and leukocytes (Hovland et al., 1994; Dannevig et al., 1995; Nylund et al., 1995b; Nylund et al., 1996).

1.2.1 Biophysical properties of ISA virus

Few studies have examined the biophysical properties of ISA virus, but in general, enveloped virus are usually more sensitive to chemical or physical changes, due to the "fragility" of the lipid bilayer, compared to naked virus. For instance, compared to the naked IPN virus, ISA virus is relatively sensitive to e.g. UVC (Oye and Rimstad, 2001). To inactivate 99.9 % of ISA virus with UVC a dose of 33 ± 3.5 Jm^{-2} is needed, while the dose required for inactivation of IPN virus is substantially higher, 1188 ± 57 Jm^{-2} (Oye and Rimstad, 2001). This means that inactivation of IPN virus demands approximately 36 times the UVC dose than that is needed to inactivate ISA virus. This, together with the findings that ISA virus is inactivated by pH 4 for 30 minutes (Falk et al., 1997), makes the ISA virus possible to handle in practical farming. Normal silage routines at a fish farm uses a pH lower than 4, and a normal UV dose at a site is 45 Jm^{-2} (Lovdata, 2012; pers. obs.). ISA virus has in experiments been shown to have the same optimal temperature for replication as that for growth of Atlantic salmon, 10-15 ^{o}C. At 20 ^{o}C replication is significantly reduced, and at 25 ^{o}C no virus is produced (Falk et al., 1997). Experience from farming sites shows that ISA outbreak mortality decreases when temperatures rise above 15 ^{o}C and increase when the temperature drops under 15 ^{o}C (Poppe et al., 1999).

Limited information is available on how long ISA virions can stay infective outside the host in natural seawater. Tapia et al. (2013) showed that the ISA virions could stay infective longer in freshwater than seawater, but used only sterile water, which makes this study of little relevance for practical farming. MacLeod et al. (2003) indicated that ISA virus *in vitro* could survive in natural seawater for more than one week, while Torgersen (1997) indicated that the survival time was more than 48 hours. However, in both these two studies there are few details about the experiment available and none of them are peer-reviewed. Nylund et al. (1994b) conducted an experiment showing that ISA virus infected blood that was 1:1 diluted with seawater could contain infective ISA virus at least for 20 hours at 6 ^{o}C. However, this study is not relevant for practical fish farming conditions.

1.2.2 ISA pathology

ISA is characterized by severe anaemia (extremely low haematocrit) which is reflected by pale organs, especially the gills. In addition haemorrhages in eyes and skin, exophthalmia, congestion in liver, spleen and intestines and small petechiae in the perivisceral fat may be observed (Figure 7) (Thorud and Djupvik, 1988; pers. obs.).

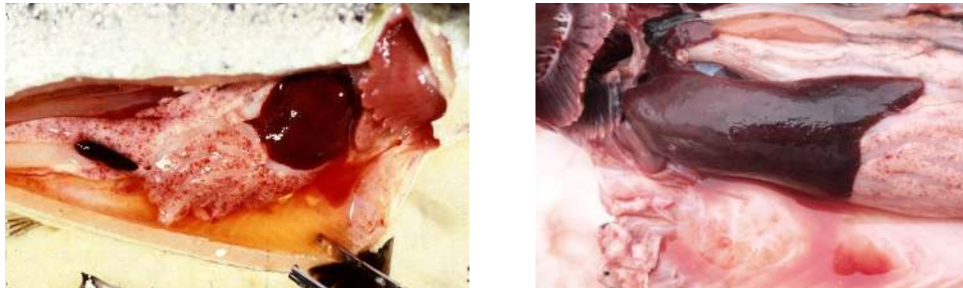


Figure 7. Gross pathology of Atlantic salmon diagnosed with ISA. Note the pale gills and heart, dark liver, numerous of small petechiaes in the perivisceral fat tissue and ascites in the abdominal cavity. Photos: Are Nylund and Siri Vike.

In some cases the liver can be nearly black due to severe haemorrhagic necrosis (Thorud and Djupvik, 1988; Godoy et al., 2008; pers.obs). The pathology and clinical signs of ISA described from outbreaks in Norway (Thorud and Djupvik, 1988) are consistent with the description from the Chilean outbreaks (Godoy et al., 2008; pers. obs). This is not surprising since both the Atlantic salmon and the ISA virus strain originate from the same area (Gajardo and Laikre, 2003; **Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Ibieta et al., 2011; pers. obs).

1.2.3 ISA virus virulence

ISA virus strains vary in virulence (Mjaaland et al., 2005; Kibenge et al., 2007). One definition of virulence is given by Ebert and Herre (1996) described as pathogen induced host mortality. That ISA virus may vary in virulence is observed in field and documented through experiments (Mjaaland et al., 2005; Johnson et al., 2008; Ritchie

et al., 2009; pers.obs). Two potential virulence markers are identified in the ISA virus genome. These two markers are related to the two surface glycoproteins: the hemagglutinin-esterase (HE) protein encoded in segment 6, and the fusion (F) protein encoded in segment 5 (Devold et al., 2006; Markussen et al., 2008; Cottet et al., 2010; Plarre et al., 2012). For other virus in the *Orthomyxoviridae* family (influenza A), it has been shown that the surface proteins are involved in the virulence mechanism (Webster et al., 1992).

The HE protein has a stalk region of variable length, which is called the Highly Polymorphic Region (HPR) (Devold et al., 2001; Krossoy et al., 2001a; Rimstad et al., 2001). It is suggested that all ISA virus strains with a full length HPR (35 amino-acids), named HPR0, are low-virulent (avirulent). This is based on the experience that none of the HPR0 ISA virus identified so far have been associated with disease, while all ISA virus with shorter HPR regions are found to be pathogenic (HPRΔ) (Devold et al., 2001; Krossoy et al., 2001b; Rimstad et al., 2001; Cunningham et al., 2002; Mjaaland et al., 2002; Nylund et al., 2003; Cook-Versloot, 2004; Nylund et al., 2006; Kibenge et al., 2009; Christiansen, 2011; Lyngstad et al., 2011; Lyngstad, 2012; Plarre, 2012; Plarre et al., 2012). In addition, it is not possible to culture HPR0 ISA virus in conventional ISA virus permissive cell lines, thus HPR0 can only be grown in a salmonid host (McBeath et al., 2011). During the ISA epizootic in Chile, 24 different ISA virus HPRs were detected. The ISA virus strains HPR7b were the most dominant, being present in 79.7 % of the outbreaks (Kibenge et al., 2009).

A comparison of the F proteins from HPR0 ISA virus shows that they all share a glutamine amino acid in position 266 (Q^{266}). This is in contrast to virulent ISA virus that have a leucine (L^{266}) in this position, or they have an insert of several amino-acids close to this position (Plarre et al., 2012). There is one exception from this pattern, where a HPR0 ISA virus has a proline in this position (P^{266}) (Kibenge et al., 2007). The fact that HPR0 ISA virus, with the one exception, always have this unique amino acid (Q^{266}) and always have full length HPRs, makes it difficult to know which of these that might be the most important virulence factor, or if it is a combination of the two that determines the virulence (Plarre et al., 2012; pers. obs.).

In farmed Atlantic salmon, HPR0 ISA virus can be found in all life stages (freshwater and seawater), but with large variance in prevalence (Nylund et al., 2006; Lyngstad et al., 2011; Lyngstad et al., 2012; Plarre, 2012; Plarre et al., 2012; pers. obs.). In addition ISA virus HPR0 is found in all major areas with Atlantic salmon farming, except the west coast of Canada (Cunningham et al., 2002; Cook-Versloot, 2004; Plarre et al., 2005; Nylund et al., 2006; Kibenge et al., 2009; McBeath et al., 2009; Christiansen, 2011; Snow, 2011; pers. com. Godoy, 2012, Etecma; Plarre, 2012; Plarre et al., 2012).

Devold et al. (2001) suggested that all strains of ISA virus HPRΔ could be derived from full length HPR0. It was further hypothesized that HPR0 was the low virulent wild-type that could mutate into HPRΔ causing disease in farmed Atlantic salmon (Cunningham et al., 2002; Nylund et al., 2003). This is based on the fact that only HPR0 ISA virus variants have been found in wild fish (*S. salar* and *S. trutta*) (Cunningham et al., 2002; Plarre et al., 2005). Several authors have confirmed that the most plausible hypothesis for the appearance of virulent ISA virus is that they are derived from low-virulent HPR0 (Nylund et al., 2006; **Paper I**; McBeath et al., 2009; Christiansen, 2011; Snow, 2011; Lyngstad et al., 2012; Plarre, 2012; Plarre et al., 2012). Phylogenetic analysis supports this theory, as low-virulent HPR0 ISA virus is present in the same clades as the virulent strains.

1.3 Transmission and reservoirs of ISA virus

1.3.1 History and distribution of ISA

The official history of ISA starts in 1984 with an outbreak at a freshwater site with Atlantic salmon in Western Norway (Thorud and Djupvik, 1988). Since then, 503 official outbreaks have been reported from Norway. The number of annual outbreaks in Norway has varied between 1-23, except for the peak between 1989-1992, when the farmers experienced an epizootic with 53-80 annual outbreaks (Veterinærinstituttet, 2013). During these four years several conventional hygienic measures were implemented by Norwegian authorities in collaboration with the farmers in order to

reduce the number of ISA virus outbreaks: 1) Separate generations with “all in – all out” production and fallow periods; 2) segregation of production sites (>3 km); 3) obligatory health certificate and health control in hatcheries; 4) ban on moving fish already put in sea; 5) regulations on transport routes; 6) disinfection of wastewater from processing plants and disinfection of the seawater supply in hatcheries; 7) daily removal of dead fish; 8) installation of silage tanks, and; 9) abandonment of unsuitable sites (shallow and/or low-current). At the end of the epizootic in 1992, geographical zones were established as a part of the combating strategy (Lyngøy, 2002; Thorud and Håstein, 2003). Disinfection of eggs to prevent vertical and/or transgenerational transmission became mandatory. All these measures have helped to reduce the number of ISA outbreaks (Lyngstad, 2012).

The disease ISA was present in Norway for a decade before it was detected in other main countries that produce Atlantic salmon; Canada (New Brunswick) (Mullins et al., 1998; Lovely et al., 1999; Ritchie et al., 2001), Scotland (Rodger et al., 1998; Rowley et al., 1999), USA (Maine) (Bouchard et al., 2001), Faroe Islands (Lyngøy, 2002), Ireland (Nylund et al., 2006) and in Chile (Kibenge et al., 2001; Godoy et al., 2008). In addition, avirulent HPR0 strains have been detected from Iceland and from Denmark (pers. com. Godoy, 2012, Etecma).

Figure 8. Geographic distribution of worldwide ISA virus detections. Figure modified from a figure made by Marcos Godoy (Etecma, Chile), used with permission.

The ISA epizootic in the Faroe Islands has many similarities with the Chilean ISA crisis, causing almost a total collapse between 2000 and 2005. Only few of 35 licenses were unaffected (Lyngøy, 2002; Christiansen, 2011). The production volume was reduced by 69 % and the smolt transfer was reduced by 88 % (Patursson, 2010). The new regulations that were implemented were based on the same principles as in Norway (Patursson, 2010), but with more focus on environmental conditions at sea, well boats, mortality treatments and smolt quality.

In Norway there is a tendency that longer production cycles may increase the risk for developing ISA. Broodfish sites represent approximately 1 % of the on-growing sites but had about 5 % of the outbreaks during the last 13 years (pers. com. Binde, 2013, Norwegian Food Safety Authority and own data). Hence, each broodfish facility has approximately 3-4 times higher risk of getting ISA than regular production sites. The

cause of this is not known, but it might be due to the immunosuppression associated with sexual maturation combined with the longer life of the broodfish.

1.3.2 Host range and reservoirs

A total of more than 800 ISA outbreaks have been registered worldwide, and all of them in Atlantic salmon. However, several experimental studies have shown that other salmonids are susceptible to ISA virus, including *Oncorhynchus keta* (Chum salmon), *O. tshawytscha, O. mykiss, O. kisutch, S. trutta* and *Salvelinus alpinus* (Artic charr). The main conclusion from all these studies is that only Atlantic salmon is highly sensitive and can develop disease and mortality. Other salmonid species develop few signs of ISA, but since the virus still seems capable of propagating in these species, they may become asymptomatic carriers (Nylund et al., 1994a; Nylund et al., 1995a; Nylund and Jakobsen, 1995; Nylund et al., 1997; Rolland and Nylund, 1998b; Devold et al., 2000; Snow et al., 2001b; Rolland and Winton, 2003; Kibenge et al., 2006; Biacchesi et al., 2007; MacWilliams et al., 2007). Low mortality has been observed in some studies when *O. mykiss* was challenged with ISA virus (Kibenge et al., 2006; Biacchesi et al., 2007; MacWilliams et al., 2007), but this has not been seen in the field. During the Chilean ISA epizootic, 239 outbreaks occurred at *S. salar* sites, but none at *Oncorhynchus* species sites, even though they were situated at sites close to or between sites with outbreaks (pers. obs). However, five of 128 examined *O. mykiss* farms carried ISA virus during the epizootic (Mardones et al., 2011).

It has been shown that herring, *Clupea harengus*, may support replication of the ISA virus, but without being able to transmit the virus to Atlantic salmon. Herring may clear virus 42 days after challenge (Nylund et al., 2002). In a challenge experiment with Atlantic cod (*Gadus morhua*) no clinical signs of ISA were observed, but ISA virus RNA was identified in the brain 45 days post challenge (Grove et al., 2007). However, cod cohabitating with ISA diseased salmon remained negative (Plarre, 2012). Hence, these two species are not likely asymptomatic carriers of ISA virus, and probably not a threat to salmon farming.

It has also been argued that the saithe (*Pollachius virens*), commonly seen both inside and outside salmon cages, might represent a natural ISA virus reservoir. However, challenge experiments have ruled out this, as ISA virus has not been shown to be able to replicate in saithe (Snow et al., 2002). Saithe sampled inside a cage during an ISA outbreak were, however, ISA virus positive in gills, but not in kidney samples (MacLean et al., 2002; McClure et al., 2004). Blue mussels (*Mytilus edulis*) also co-exist with salmon as they are commonly found growing on the farming cage nets. Skaar and Mortensen (2007) showed that blue mussels rapidly inactivated the ISA virus indicating that they cannot function as a reservoir host or a vector for the virus. This has later been confirmed by Stene et al. (2013).

From farmed Atlantic salmon at the Faroe Island it has been described that HPR0 variants of ISA virus have a rapid increase in prevalence in Atlantic salmon after transfer to sea sites. A possible explanation for this is an unknown marine source of ISA virus, according to Christiansen (2011). However, several surveys of thousands of non-salmonid marine fishes, collected in their natural environment, have so far been unable to identify any marine reservoir for ISA virus (Raynard et al., 2001; MacLean et al., 2002; Snow et al., 2002; McClure et al., 2004; Wallace et al., 2009). A field study done by Stene et al. (2013) investigated the possible presence of ISA virus in sessile organisms and substances, biofilms, sediments and benthic organisms at two Atlantic salmon farming sites during an ISA outbreak. No marine reservoirs were detected. In Chile, Gonzalez et al. (2011) tested 17 different free-living fish species for ISA virus (some commonly found around farming cages), microplankton, fecal material from sea lions (*Otaria flavescens*) (also commonly present around the site), molluscs and crustaceans in an area with salmonid farming. All analysed samples, except one, where negative for ISA virus. The ISA virus positive sample was from a Atlantic salmon (probably escaped) and genotyping of the virus showed 100 % identity with a European genetic variant, based on segment 6.

The fact that ISA virus are only detected in two wild species: *S. salar* and *S. trutta* (Raynard et al., 2001; Cunningham et al., 2002; Plarre et al., 2005) make them the most likely candidates as the natural host (Plarre et al., 2005). However, it looks like

this reservoir of ISA virus in wild *S. salar* and *S. trutta* is of minor importance for the spreading of ISA virus to the farming industry of *S. salar* (Nylund et al., 2006; Johansen et al., 2011; Plarre, 2012; Plarre et al., 2012). Phylogenetic analysis suggests that the farming industry is recirculating the same strains of ISA virus from one generation or fish group to the next, either through vertical or horizontal transmission (Nylund et al., 2006; **Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Lyngstad et al., 2011; Lyngstad et al., 2012; Plarre, 2012; Plarre et al., 2012).

1.3.3 Transmission of ISA virus

The ISA virus from North America are genetically distinct from the ISA virus of European origin, with the exception of one genogroup that occurs on both continents (Devold et al., 2001; Nylund et al., 2003; Devold et al., 2006; Nylund et al., 2006; Plarre et al., 2012). It is not known if this genogroup has been moved between the continents by human activity (movement of embryos) or through the natural migration of wild salmonids in the North Atlantic. The ISA virus isolates among European countries are distinct, but closely related to the first ISA virus detected in Norway (Plarre et al., 2012). In addition, the ISA virus detected during the devastating Chilean 2007-09 epizootic, are all closely related to Norwegian strains (**Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Plarre, 2012). Within the Norwegian Atlantic salmon production there are several distinct clades of ISA virus, but they do not form geographically restricted groups (Nylund et al., 2006).

1.3.4 Horizontal transmission

Horizontal transmission of virus, here defined as transmission from fish to fish, may occur directly, by passive drift in water, or indirectly via vectors or fomites.

In the North Atlantic, the copepod *Lepeophtheirus salmonis* (salmon lice) is the only parasite suggested to act as a mechanical vector for ISA virus (Nylund et al., 1993; Rolland and Nylund, 1998a; Murray et al., 2010). In addition to causing damage to mucus layers and skin, *L. salmonis* may induce immunosuppression (Fast et al., 2007). Anti-lice bath-treatments may also be stressful for the fish. There are few studies from

the field indicating a connection between the parasitic copepod and ISA virus. Hammell and Dohoo (2005) found that strict *L. salmonis* control may reduce probability of infection with ISA at Atlantic salmon farming sites, while Gustafson et al. (2005) found a link between *L. salmonis* infestation and the severity of ISA outbreaks. In Chile the parasitic copepod *Caligus rogercresseyi* is known as a serious problem in salmonid farming (Bravo, 2010a). Epizootic modelling of pathogen data collected by Chilean fish health authorities suggest that there in certain areas of Chile was an increased risk of ISA outbreaks if *C. rogercresseyi* was present. The study did not explain the connection between the pathogens (Valdes-Donoso et al., 2013).

Horizontal transmission of ISA virus between salmonids (*S. salar, O. mykiss* and *S. trutta*) has been demonstrated in experimental tanks and in field cages. Furthermore transmission been suggested to occur between sea sites (Thorud and Djupvik, 1988; Nylund et al., 1993; Dannevig et al., 1994; Nylund et al., 1994a; Nylund et al., 1995a; Nylund and Jakobsen, 1995; Nylund et al., 1995c; Nylund et al., 1997; Rolland and Nylund, 1998b; Devold et al., 2000; Jones and Groman, 2001; Snow et al., 2001a; Snow et al., 2001b; Lyngstad et al., 2008; Mardones et al., 2009; Lyngstad et al., 2011; Mardones et al., 2011). All aspects of the mechanisms for waterborne transmission of ISA virus are not understood. However, ISA virus is present in mucus, feces and urine from Atlantic salmon suffering of ISA, so these secretions most likely play a role (Nylund et al., 1994b; Totland et al., 1996). A recent study from Weli et al. (2013) demonstrates that ISA virus may use the gill epithelial cells in Atlantic salmon as a route of entry. Shedding of virus from ISA diseased salmon may start as early as seven days post challenge and the minimum infective dose of ISA virus is estimated to be $1x10^1$ $TCID_{50}$ mL^{-1} (Gregory et al., 2009). It is possible to detect ISA virus in seawater inside a cage with Atlantic salmon during an ISA outbreak. However, 80-100 meters downstream from the same cage, no ISA virus was detected by using a method with sensitivity of 5.5 ISA virions per mL (Lovdal and Enger, 2002).

Several authors have indicated that waterborne transmission of ISA virus between neighbouring Atlantic salmon sites is the major transmission path together with untreated wastewater from processing plants (Vagsholm et al., 1994; Jarp and Karlsen,

1997; Gustafson et al., 2005; McClure et al., 2005a; Gustafson et al., 2007b; Mardones et al., 2009; Murray et al., 2010). Furthermore, transport of biological material by well-boats (transporting infected fish, discharge from salmon processing plants, or harvested fish) is identified as a possible risk factor (Vagsholm et al., 1994; Murray et al., 2002). It is suggested that there is a risk associated with an increased number of hatcheries delivering smolt to sea sites, due to the possibility of transmission during transport (Vagsholm et al., 1994; Jarp and Karlsen, 1997). Some studies have identified a possible risk related to husbandry practice, e.g. removal of dead fish, rate of sea lice treatment, and divers visiting multiple farms (Jarp and Karlsen, 1997; Gustafson et al., 2005; Hammell and Dohoo, 2005; McClure et al., 2005b). It has also been shown that low fish density at the farming sites may reduce the susceptibility of ISA virus (Hammell and Dohoo, 2005; Scheel et al., 2007).

In order to study transmission patterns of ISA virus a stochastic model was fitted to historical data from ISA outbreaks in Norway between 2002-2005. The authors concluded that waterborne transmission occurs, but a large component of the total risk was related to additional sources, without specifying what these might be (Scheel et al., 2007). A similar model, including three common Atlantic salmon farming diseases, was used for the period 2003-07 with special interest given to seaway distance between sites (Aldrin et al., 2010). The conclusion was that ISA outbreaks occurred more isolated in space and time compared to pancreas disease (PD) and heart and skeletal muscle inflammation (HMSI). The dominant transmission pathway of PD and HSMI seems to be seawater transmission, while only 20-30 % of the ISA outbreaks could be explained by horizontal transmission in seawater (Aldrin et al., 2010). This fits with the finding of Gustafson et al. (2007a) who suggested that ISA manifestations were related to differences in host-susceptibility, because there was a limited connection between cage adjacency and when the fish in the cage developed ISA. A third study extended the model made by Aldrin et al. (2010) to include genetic distance between ISA virus genotypes from different farms in the period 2003-09. Here the conclusion was that 43 % of the ISA-infected farms could have acquired ISA virus from a proximal site. The remaining may have acquired ISA virus from other non-specified sources (Aldrin et al., 2011). None of these studies included information

about freshwater stages or broodfish origins, thus assuming that all transmission occurs in the seawater phase of production.

Lyngstad et al. (2008, 2011) have used genotyping to trace the transmission of ISA in Norway during two periods: 2003-05 and 2007-09. Here the conclusion is that horizontal transmission is the most important infection route, and in the latter study the authors conclude that about half of the outbreaks are a result of ISA virus transmission between proximal sites. Both papers lack data to evaluate the possibility of vertical transmission. However, in the 2011 article low-virulent (HPR0), ISA virus strains have been included, and the authors suggest that these strains could be the source for virulent ISA virus that causes disease outbreaks. Plarre et al. (2012) studied the same outbreaks as Lyngstad et al. (2011), but included data on broodfish origin. All sites with ISA outbreaks received embryos from the same broodfish supplier. Plarre et al. (2012) therefore concluded that the outbreaks might be a result of both horizontal and vertical transmission, for the closely related ISA virus detected in neighbouring farms.

1.3.5 Vertical transmission

In this study vertical or transgenerational transmission of virus has been defined as transmission from either of the parents to the next generation through gonadal products (eggs or milt) or in the reproductive fluid. Such transmission is documented to occur in teleost fish (Mulcahy and Pascho, 1984; Robertsen, 2011). For example it is shown experimentally that betanodavirus can be vertical transmitted from broodfish to egg and larvae of sea bass (*Dicentrarchus labrax*) (Breuil et al., 2002). It has been suggested that IPN virus, IHN virus and PRV virus have been transferred between continents, most likely via salmonid embryos (Mutoloki and Evensen, 2011; Robertsen, 2011; Kibenge et al., 2013). It has also been indicated that vertical transmission of betanodavirus can occur in Atlantic Halibut (*Hippoglossus hippoglossus*) (Grotmol and Totland, 2000).

The first experimental challenge study of vertical transmission of ISA virus was done by Melville and Griffiths (1999), and they concluded that vertical transmission did not occur. However, this result was based on methods that lacked the necessary sensitivity

to identify low levels of virus (carrier fish), and the existence of HPR0 was unknown at this time. Six years later vertical transmission of ISA virus was demonstrated in a field experiment with naturally infected broodfish. In this experiment it was possible to detect ISA virus positive offsprings (egg, embryo and fry), independent of disinfection of eggs with buffodine or not, prior to incubation (Søfteland, 2005). The experiment was ended at fry stage, so it is unknown if the infection could lead to an ISA outbreak. The same year the European Commission sponsored a report on the issue of trading with embryos, where it was concluded that vertical transmission was insignificant for the epidemiology of ISA virus infections, if effective disinfectant was used (Bovo et al., 2005), without documenting it experimentally or in field.

There are also molecular epidemiology studies suggesting that the dominating transmission route for ISA virus in Norway, after implementation of separate generations at sea sites, are vertical transmission and movement of embryos or smolt infected with non-virulent ISA virus (HPR0) (Nylund et al., 2006; Plarre et al., 2012).

Lyngstad et al. (2008) have published a paper, based on genotyping of ISA outbreaks in Norway from 2003 to 2005 where they failed to find evidence for vertical transmission. However, as the authors stated they only had data on broodfish origin for half of the outbreaks included in the study, and therefore it was not feasible to conduct statistical analysis for evaluating vertical transmission of ISAV linked to broodfish origin.

2. AIM OF STUDY

The aim of this study was to generate new knowledge to explain the introduction of ISA virus to Chile and subsequent spread of the virus within the Chilean Atlantic salmon production. This knowledge will be used to recommend changes in the industry practices to prevent future transmission and ISA outbreaks. To address these, four questions were raised:

1. How did ISA virus enter into Atlantic salmon farming in Chile (**Paper I**)?
2. How long can ISA virus stay infective in natural seawater under various physical conditions (**Paper II**)?
3. Is *Caligus rogercresseyi* a potential vector for transmission of ISA virus (**Paper III**)?
4. For how long will dead Atlantic salmon be carrying infective ISA virions and will such virions be released into the environment (**Paper IV**)?

3. OVERVIEW OF PAPERS

Paper I

In 2007 the first outbreaks of ISA in farmed Atlantic salmon was observed in Chile. Several salmon farms in Chile were affected by the disease in the years 2007-2009. In this study, ISA virus has been isolated from salmon in a marine farm suffering an outbreak of the disease in 2008 and from smolts with no signs of ISA in a freshwater lake. Sequencing of the partial genomes of these ISA virus, followed by phylogenetic analysis including genome sequences from members of the North Atlantic and European (EU) genotypes, showed that the Chilean ISA virus belongs to the EU genotype. The Chilean ISA virus groups in a clade with exclusively Norwegian ISA virus. All salmonid species in the southern hemisphere have been introduced from Europe and North America. The absence of indigenous hosts for ISA virus in Chile excludes the possibility of natural reservoirs in this country, and the close relationship between contemporary ISA virus strains from farmed Atlantic salmon in Chile and Norway suggest a recent transmission from Norway to Chile. Norway has exported large amounts of Atlantic salmon embryos to Chile; hence, the best explanation for the presence of a Norwegian ISA virus in Chile is transmission via these embryos, i.e. vertical and/or transgenerational transmission. This supports other studies showing that the ISA virus can be transmitted vertically.

Paper II

This paper investigated the survival time for ISA virions under four different physical conditions: natural seawater and sterilized seawater with and without artificial sunlight (UV radiation). Presence of ISA virus RNA and infectivity of ISA virions were monitored during the 72 hours experiment. The result showed that the infectivity of ISA virions was lost within three hours both in natural seawater and sterile seawater when exposed to UV radiation. The survival time of ISA virions in sterile seawater

was less than 24 hours. However, it was possible to detect ISA virus RNA throughout the experimental period. This indicates that UV radiation and biological activity (in the water) limits the survival time of ISA virions under normal seawater conditions. Based on available literature, results in the present study, the speed and dilution effect in seawater currents, the temperature during the major outbreak periods and the need for an infective dose to reach native salmon, it is unlikely that passive horizontal transmission of ISA virus in seawater over long distances can occur.

Paper III

This study showed that *C. rogercresseyi* is capable of being a mechanical vector for ISA virus. Even after 48 hours away from its host, *C. rogercresseyi* can still transmit ISA virus to naïve Atlantic salmon, confirmed by sequencing of the virus. Both the amount and the prevalence of ISA virus RNA decreased the longer the *C. rogercresseyi* was away from its host. This indicates that ISA virus does not replicate in the lice and hence, it is not likely that *C. rogercresseyi* is a biological vector.

ISA virus positive *Caligus sp.* nauplii larva or copepodids were not detected inside or outside the Atlantic salmon farming cage experiencing ISA outbreaks. Nor was it possible to detect ISA virus RNA in nauplii larva or copepodids retained in the lab, even if they were the offspring of ISA virus positive *C. rogercresseyi* population. This indicates that ISA virus is not vertically transmitted from the adult *C. rogercresseyi* to nauplii. Based on these results it is not very likely that *Caligus sp.* nauplii larva or copepodids are vectors for ISA virus.

For the salmonid industry this means that a co-infection with ISA virus and *C. rogercresseyi* increases the risk of transmission of ISA virus within the site and also to other sites in the same hydrographical area.

Paper IV

An ISA outbreak may vary when it comes to clinical manifestations and mortality levels. Some outbreaks have an aggressive development with mortality level up to 90 %. At these sites, efficient removal of dead fish can be a major challenge. Removal of dead fish is an important fish health preventive measure implemented at marine sites. In the present study we examined the infection hazard that dead fish may represent for the remaining fish at the site by examining tissues from fish that died from ISA, and from the surrounding water (0-120 hours *post mortem*). To examine for how long infectious virus particles could be retrieved from dead salmon as this could indicate if such material may pose a risk for spreading of ISA virus. Viral RNA was detectable in water from decomposing salmon by real-time RT-PCR, but no infectious particles could be obtained from water either by inoculating cell cultures or by injecting water samples into fish. Nevertheless, the study showed that infectious virus particles could be retrieved from heart tissue of dead fish for more than 4-5 days *post mortem*.

Thus, daily removal of dead fish is an important preventative measure during ISA outbreaks. However, it is also of importance to remove moribund fish with ISA as these continue to produce and shed virus and may represent a greater risk with respect to the transmission of virus than the fish already dead.

4. GENERAL DISCUSSIONS

4.1 Introduction of ISA virus to Chile

After the first official ISA outbreak in Chile in July 2007, the local farmers had an immediate need for knowledge about ISA virus transmission, and on how to prevent the virus from spreading within the Chilean industry. The first question asked was: Where did the ISA virus come from, a local reservoir or introduced from other areas? This question was addressed in the first studies on the genetics of the emerging ISA virus in the region (Godoy et al., 2008; **Paper I**). It was suggested that the ISA virus had a European origin, but distinct from these, due to one insert in segment five (Godoy et al., 2008). A comparison of ISA virus from two salmon populations in Chile (which originated from Norwegian embryos) with all available previously characterized ISA virus strains, showed a close relationship to Norwegian ISA virus (**Paper I**). Phylogenetic analysis based on three segments, including segment five, showed that the Chilean ISA virus belonged among the Norwegian ISA virus (**Paper I**). The inserts in segment five were not unique for the Chilean isolates, as they already had been described in analyses of Norwegian ISA virus (Devold et al., 2006). The conclusion that the ISA virus isolates detected in Chile had a Norwegian/European origin (**Paper I**) was later confirmed by others Kibenge et al. (2009), Cottet et al. (2011) and Plarre et al. (2012). Later studies have also shown that not all ISA virus from Chile have an insert in segment five and that low-virulent ISA virus (HPR0 variants) are also present in this region (Plarre et al., 2012), i.e. a similar situation to what is seen in Norway (Nylund et al., 2006; Lyngstad et al., 2012; Plarre et al., 2012).

The presence of a Norwegian ISA virus in Chile required an explanation of the virus transfer mechanism from the North Atlantic to the Southern Pacific. A few months before the first ISA outbreak in Chile, the Norwegian Scientific Committee for Food Safety (NSCFS) used the absence of ISA in Chile as one of the arguments against the possibility of vertical transmission of ISA virus, and concluded that screening of broodfish should not be a statutory provision for the industry (Rimstad et al., 2007). A quotation from January 2007: *"(...) lack of ISA disease in some countries that over*

years have imported substantial numbers of eggs from Norway, suggest that the probability of disease emergence following vertical transmission of virus is low". This advice was given despite studies showing the presence of ISA virus in both freshwater and seawater, and in all production stages in Norway (Søfteland, 2005; Nylund et al., 2006). This led to a debate on combating strategies and disease eradication of ISA in Norway - especially whether ISA virus could be transmitted vertically or not. The alternative hypothesis for vertical transmission was the possible existence of an unknown marine reservoir. Such a hypothesis would require introduction of ISA virus into Chile via boat traffic from Norway, i.e. vessels carrying contaminated equipment or ballast water. It is well documented that ballast water may harbour potential pathogens/organisms that could be released into new areas if the necessary environmental conditions during transport are met (Ruiz et al., 2000). However, the sailing time from Norway to Chile is 25-30 days (pers. com. Holm, 2013, EWOS AS), and during this journey the boats will have to cross the Equator with sea temperatures exceeding 28 °C (Lynne et al., 2012). ISA virus are sensitive to temperatures higher than 20 °C (Falk et al., 1997), and appear to have a limited survival time in seawater with biological activity (**Paper II**). Hence, the combined effect of high temperatures, biological activity in the ballast water, and the time of transportation should lead to complete inactivation of any ISA virus present, excluding the possibility of horizontal transmission from an unknown marine reservoir. Moreover, all attempts to identify such a marine reservoir for ISA virus have failed (Raynard et al., 2001; MacLean et al., 2002; Snow et al., 2002; McClure et al., 2004; Wallace et al., 2009; Gonzalez et al., 2011; Stene et al., 2013). Hence, there are no reasons to believe that a salmon specific pathogen should occur in an area with no natural populations of salmonids.

Another hypothesis for the introduction of Norwegian ISA virus into the Chilean salmon industry was presented in **Paper I**. Namely that ISA virus was introduced to Chile from Norway with salmon embryos. This hypothesis was based on the facts that there are no natural populations of salmonids south of the Equator (Gajardo and Laikre, 2003), and that the Chilean industry have imported huge numbers of Atlantic salmon embryos from North America and Europe (Ibieta et al., 2011). Support of the hypothesis was given by a report documenting vertical transmission of ISA virus from

naturally infected broodfish to newly hatched fry, even after disinfection of the ova with buffodine (Søfteland, 2005). This indicates that the ISA virus may be inside or concealed in the surface of the egg shell.

In addition, it had already been shown that ISA virus existed in virulent (HPRΔ) as well as avirulent (HPR0) forms, and that the HPR0 types were quite common in salmon in freshwater (Nylund et al., 2006). The avirulent ISA virus can be transmitted undetected through transport of both salmonids and salmon embryos. Based on these data it was suggested that the ISA virus in Chile had arrived in connection with imported Atlantic salmon embryos from Norway (**Paper I**). Later the same transmission pattern has been suggested for two other common salmon virus, IPN virus and piscine reovirus (PRV) associated with HMSI, which are assumed to have been introduced to Chile by importing embryos from salmonids produced in the North Atlantic (Bustos et al., 2011a; Mutoloki and Evensen, 2011; Kibenge et al., 2013). The best explanation, at present, is that the ISA virus has arrived in Chile together with its natural host (**Paper I**).

Phylogenies (based on segments five and six) including a large number of isolates from Chile also suggest that ISA virus must have been introduced to Chile more than once (Figures 9 and 10). This has already been suggested (**Paper I**; Plarre et al., 2012), and together with the first detection of a North American ISA virus in Chile in 1999 (Kibenge et al., 2001), the existing data support the hypothesis that ISA virus can be transmitted via embryo transport.

The report from the NSCFS was renewed in 2011, but the committee did not change their recommendations regarding the impact of vertical transmission of ISA virus: "*Vertical transmission cannot be ruled out, but has little or no measureable impact for the spread of ISA in Norwegian farming*" (Rimstad et al., 2011). This is an incomprehensible statement. Between 2007 and 2011 it has been well documented that the ISA-crisis in Chile was due to ISA virus originating from Europe, and the only plausible explanation for this is vertical or transgenerational transmission (**Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Plarre et al., 2012). Hence, the fact that such

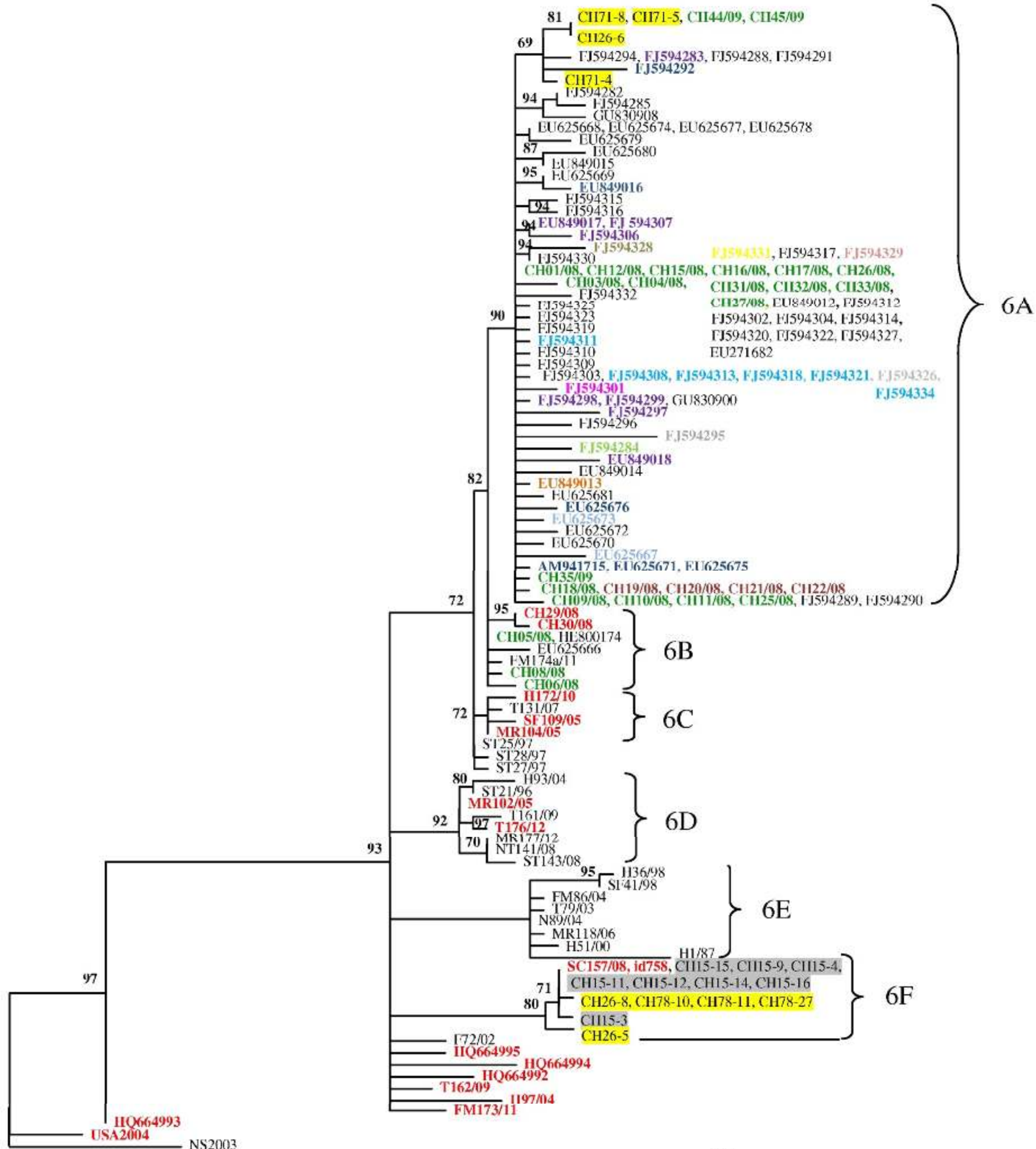
transmission also may occur within the Norwegian salmon industry seems to be ignored.

A successful future strategy for the prevention of spreading of ISA virus should include a strong focus on measures to avoid vertical/transgenerational transmission. To prevent vertical transmission by excluding a small number of broodfish will be far easier and less costly, compared to stamping out salmon with ISA at marine sites and preventing horizontal transmission in local farming areas.

Figure 9. Phylogenetic tree showing the relationship between all available segment six sequences from Chilean ISA virus, combined with a selection based on the isolates presented in Plarre et al. (2012), showing affinity to these. Of the 155 sequenced included are 28 published for the first time in this study. The tree is based on 955 nucleotides excluding three regions: HPR, cytoplasmatic tail and transmembrane. All red virus-ids/accession numbers are sequences from avirulent HPR0 ISA virus. All the other colours illustrate affiliation to a farming company (same colour = same company). The virus-id's highlighted in grey and yellow are sequences from the two Chilean outbreaks in 2013 (kindly provided by Godoy). Overview of the sequences is listed in table A in the Appendix. The phylogeny contains six clades given the names 6A - 6F, and some isolates with unresolved position.

The sequence data were assembled with the help of Vector NTI software (InforMax, Inc.) and the GenBank searches were done with BLAST (2.0). The Vector NTI Suite software package (InforMax, Inc.) was used for the multiple alignments of nucleotide and deduced amino acid sequences. Pairwise comparisons of the different sequences from the two ISA virus segments were performed by the multiple sequence alignment editor GeneDoc (available at: www.psc.edu/biomed/genedoc). Sequences already available on the EMBL nucleotide database were also included in the comparisons.

The tree was constructed using TREE-PUZZLE 5.2 (Available at: http://www.tree-puzzle.de), maximum likelihood (ML). The evolutionary model and substitution rates for the ML analyses were calculated for all datasets using jModelTest (Posada, 2008) with the Akaike Information Criterion option. Both segments were analysed using a GTR nucleotide evolution model with four category gamma rate. The maximum likelihood trees were bootstrapped (50 000 puzzling steps) in TREE_PUZZLE. The ISA virus isolate NS2003 from Nova Scotia, Canada, was used as an out-group in the phylogenetic analysis. Phylogenetic trees were drawn using TreeView (Page, 1996).



81
CH71-8, CH71-5, CH44/09, CH45/09
CH26-6
69
FJ594294, FJ594283, FJ594288, FJ594291
FJ594292
CH71-4
94
FJ594282
FJ594285
GU830908
EU625668, EU625674, EU625677, EU625678
EU625679
87
EU625680
EU849015
95
EU625669
EU849016
FJ594315
94
FJ594316
94
EU849017, FJ 594307
FJ594306
94
FJ594328
FJ594330
FJ594331, FJ594317, FJ594329
CH01/08, CH12/08, CH15/08, CH16/08, CH17/08, CH26/08,
CH03/08, CH04/08,
CH31/08, CH32/08, CH33/08,
CH27/08, EU849012, FJ594312
FJ594302, FJ594304, FJ594314,
FJ594320, FJ594322, FJ594327,
EU271682
FJ594332
FJ594325
90
FJ594323
FJ594319
FJ594311
FJ594310
FJ594309
FJ594303, FJ594308, FJ594313, FJ594318, FJ594321, FJ594326,
FJ594334
FJ594301
FJ594298, FJ594299, GU830900
FJ594297
FJ594296
FJ594295
FJ594284
EU849018
EU849014
82
EU849013
EU625681
EU625676
EU625673
EU625672
EU625670
EU625667
AM941715, EU625671, EU625675
CH35/09
CH18/08, CH19/08, CH20/08, CH21/08, CH22/08
CH09/08, CH10/08, CH11/08, CH25/08, FJ594289, FJ594290
6A
72
95
CH29/08
CH30/08
CH05/08, HE800174
EU625666
FM174a/11
CH08/08
CH06/08
6B
H172/10
T131/07
72
SF109/05
MR104/05
6C
ST25/97
ST28/97
ST27/97
80
H93/04
ST21/96
MR102/05
92
T161/09
97
T176/12
70
MR177/12
NT141/08
ST143/08
6D
93
95
H36/98
SF41/98
FM86/04
T79/03
N89/04
MR118/06
H51/00
H1/87
6E
SC157/08, id758, CH15-15, CH15-9, CH15-4,
CH15-11, CH15-12, CH15-14, CH15-16
71
80
CH26-8, CH78-10, CH78-11, CH78-27
CH15-3
CH26-5
6F
97
F72/02
HQ664995
HQ664994
HQ664992
T162/09
H97/04
FM173/11
HQ664993
USA2004
NS2003
0.01

Figure 10. Phylogenetic tree showing the relationship between all available segment five sequences from Chilean ISA virus, combined with a selection based on the isolates presented in Plarre et al. (2012), showing affinity to these. Of the 129 sequenced included are 28 published for the first time in this study. The tree is based on 844 nucleotides excluding the inserts that occur in some of the sequences. All red virus-id's/accession numbers are sequences from avirulent HPR0 ISA virus. The virus-id's that are highlighted in grey and yellow are sequences from the two Chilean outbreaks in 2013 (kindly provided by Godoy). Overview of the sequences is listed in table A in the Appendix. The phylogeny contains five clades given the names 5A – 5E, and some isolates with unresolved position.

The method for the phylogenetic analysis of segment five are presented in the figure 9 text.

EU130923, EU849005, EU849010
FJ592150
FJ592143
EU849011
EU849006
EU552491
EU449768
EU449767
T10/93
T73/02, T74/03
T167/10
FM168/10
FM174a/11
NT134/08
ST27/97
ST25/97
NT141/08
CH05/08
T90/04
CH25/08
CH06/08, CH08/08, 5CH11/08, FJ592133, GU830907, FJ592141, FJ592139
CH71-4, CH71-6, CH71-8
FJ592135, FJ592138,
CH71-7
H138/08
H97/04
H172-10
USA2004
EU849008
FJ592134
EU849009
MR102/05
MR104/05
CH29/08
CH28/08
CH30/08
ID758
FJ592148
CH01/08, FJ592147, FJ592152, FJ592156, FJ592158, FJ592160,
FJ592140, FJ592165
FJ592163, FJ592168
FJ592142
CH15/08, CH16/08, CH17/08, CH26/08, CH27/08, CH31/08, EU449765, FJ592136,
FJ592157, FJ592161
FJ592137, FJ592145, FJ592149, GU830899,
FJ592154
FJ592151, FJ592153, FJ592155, FJ592159,
FJ592146
FJ592164, FJ592166, FJ592167, FJ592162
FJ592144
EU449766
CH35/09
5A
CH26-7
CH78-8
CH26-6
5B
H02/89
T37/98
H06/91
CH15-12
CH15-16
CH15-14
5C
H93/04
ST44/99
ST26/97
ST21/96, NT81/03
5D
FM114/05, FM116/06, NT115/05
MR139/08
SF57/00, MR61/01
5E
CH26-3
CH87-27
SCOT157/08
SCOT43/98
FM173/11
CH26-8
CH26-5, CH77-16, CH77-18, CH78-16, CH78-9, CH87-25
CH15-9
CH15-4, CH15-5
CH15-15
CH15-11
T162/09
F72/02
NS2003
0.01

4.2 Transmission of ISA virus within Chile

Screening for the presence of fish pathogens using real-time RT-PCR and the identification of signature sequences that may be used in phylogenetic studies, constitutes the basis for molecular epizootiology. This approach has given both scientists and the aquaculture industry a method to identify reservoirs and better understand the distribution and transmission of pathogens. This includes strategies of virus transmission within local populations and between hosts in different areas, i.e. between individual fish, cages within a farm, farming sites, along the coast line, and between continents (**Paper I**). Together, these methods provide a tool, which in some cases can be used to trace a pathogen back to the source of origin and reveal how and when the pathogen entered the farming production chain. This knowledge makes it possible to identify risk factors that can be met by preventive measures reducing the risks of pathogen introduction followed by clinical outbreaks (**Paper I**).

By comparing homologous sequences in the genomes of virus phylogenetic trees can be constructed, providing valuable information on the diversification of a particular virus and its evolutionary history (Snow, 2011). To estimate relationships among ISA virus strains, the main targets in most studies have been segments five and six, the two surface protein coding genes (F and HE). These sequences exhibit the largest variations and hence they give the best resolution and most relevant phylogenetic information (Devold et al., 2001; Krossoy et al., 2001b; Nylund et al., 2003; Plarre et al., 2005; **Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Christiansen, 2011; Lyngstad et al., 2011; Plarre et al., 2012). The estimated time of separation between Chilean and Norwegian ISA virus suggests that the introduction to Chile must have occurred in the period from 1995 until as late as 2007 (Kibenge et al., 2009; **Paper I**). It has been suggested that segment five was introduced between 1996 and 2002 and segment 6 between 1995 – 1999 (Plarre et al., 2012). Hence, based on these two segments, the virus could have been present in the Chilean industry some years before the first official outbreak of ISA.

During the ISA outbreaks in Chile in 2007 it soon became evident that the ISA virus was widely spread throughout the salmon industry. When the epizootic peaked in 2009 up to 60 % of all marine sites were affected together with an unregistered number of freshwater sites (Mardones et al., 2011; Alvial et al., 2012; Valdes-Donoso et al., 2013; pers. obs.). After the arrival of ISA virus in Chile, the virus met an optimal environment for replication and spreading due to the structure of the Chilean salmon industry. For instance, many smolts were continuously produced in lakes and at sites in brackish water before transportation to sea sites. In some areas fish were kept at high densities without separating generations or subsequent fallow periods, providing excellent conditions for transmission. In addition, at marine sites many fish were already compromised mainly due to the presence of *Caligus rogercresseyi* and *Piscirickettsia salmonis*, and in freshwater the fry and smolt were frequently infected with *Flavobacterium psychrophilum* and *Saprolegina sp.* (Valdebenito and Avendano-Herrera, 2009; Bravo, 2010a; Ibieta et al., 2011; pers. obs.).

Based on a phylogenetic analysis of segment six the majority of the ISA virus (N = 82) detected in the Chilean production of Atlantic salmon in the period from 2007 – 2009, group closely together (clade 6A), with a sister-group consisting of a collection of Norwegian and Chilean isolates, group 6B (Figure 9). Clade 6A consists of virulent ISA virus only, both from fresh- and seawater, collected from salmon belonging to at least 13 different companies in region X and XI (Figure 1) (Kibenge et al., 2009; present study). The fact that different companies share the same ISA virus could suggest a common origin of broodfish, but the structure of the industry suggests that horizontal transmission must have played a major role. Due to limited epizootic data available, it is not possible to map the transmission routes in detail. At this time several companies could operate in lakes, in brackish water and also at marine sites with short geographical distance between them. It is very likely that the close proximity played an important role for the horizontal transmission of the virus. However, the survival time of the ISA virions in sea water is limited (less than three hours) due to sunlight (UV) and biological activity in the water (**Paper II**). It is theoretically possible that transmission of ISA virus has occurred through escaped or introduced salmonids, or by using *C. rogercresseyi* as a vector (**Paper III**). In addition

can the sub-optimal treatment of dead fish, at some sites, have contributed to transmission of ISA virus (**Paper IV**). New outbreaks of ISA in Chile in 2013 with virus that grouped in clade 6A show that this group of virus is still maintained in the salmon population (Figure 9).

A possible example of vertical transmission during the epizootic in Chile is provided by sequence CH25/08 (Figure 9), which came from an ISA outbreak in a closed hatchery with no connection to other fish or sea sites. The ISA virus sequences CH09/08, CH10/08 and CH11/08 from salmon in seawater sites are identical to CH25/08 at segment 6. This might be a result of horizontal transmission of ISA virus through the movement of smolt.

Group 6B consists of the ISA virus sequences that are the closest relatives to the members of clade 6A, and includes both virulent and avirulent ISA virus from fresh- and seawater in Chile and two virulent ISA virus from seawater in Norway. The close affinity with the Norwegian virus could suggest a more recent introduction to Chile than the isolates in clade 6A. It seems even more likely that the Chilean isolates in clade 6F, isolated from ISA outbreaks in 2013, must represent a different introduction compared to members of clade 6A. The isolates in this clade (6F) group closely together with an avirulent HPR0 ISA virus collected from broodfish site in Scotland and a HPR0 sequence (id758) that has been found in Chile.

Most of the groups/clades in the phylogeny based on segment five (Figure 10) are poorly supported and provide limited additional information compared to the analysis of segment six (Figure 9). The most interesting observation is that the majority of the ISA virus from the 2013 outbreaks of ISA in Chile are different from the dominating group of virus from the 2007 – 2009 outbreaks. However, due to poor resolution it is not possible to detect any clear affinity to ISA virus from a specific European country. Some of the differences observed between the phylogenies based on segments six and five could be due to re-assortment (Devold et al., 2006; Markussen et al., 2008; Plarre et al., 2012).

The conclusion from the phylogenetic analysis of ISA virus from Chile is that there must have been more than one introduction from Europe to Chile (Plarre et al., 2012; present study).

The only natural hosts for the ISA virus, detected so far, are Atlantic salmon (*S. salar*) and trout (*S. trutta*) in the North Atlantic (Raynard et al., 2001; Plarre et al., 2005). Challenge experiments have confirmed that these species are susceptible to ISA virus and may act as hosts (Thorud and Djupvik, 1988; Nylund et al., 1994a; Nylund et al., 1994b; Nylund et al., 1995a; Nylund and Jakobsen, 1995; Devold et al., 2000; Gregory et al., 2009). It has been suggested that the life cycle of the ISA virus must reflect the biology of these two hosts (Nylund et al., 2003; Nylund et al., 2006; Plarre et al., 2012). No marine fish species in the North Atlantic or on the coast of Chile have been shown to be reservoir for ISA virus (Raynard et al., 2001; MacLean et al., 2002; Snow et al., 2002; McClure et al., 2004; Wallace et al., 2009; Gonzalez et al., 2011). This means that on the Chilean coast there are no known natural hosts, and the only reservoir for the virus should be farmed salmonids and salmonids introduced or escaped into the wild. This artificial (man-made) reservoir follows the structure of the industry and movement of embryos, fry and smolt, and possibly the number of escaped salmonids in the region. Hence, except for possible new introductions of ISA virus from the Northern hemisphere, the reservoirs and transmission of the virus must be solely related to how the industry is operating.

It is known that low virulent virus in nature can become more virulent when they are exposed to artificially high densities of the host (Kurath and Winton, 2011; Nerland et al., 2011). This is also suggested for the ISA virus, as there is strong support for the hypothesis that HPR0 ISA virus can mutate into virulent variants (Nylund et al., 2006; McBeath et al., 2009; Christiansen, 2011; Snow, 2011; EFSA, 2012; Lyngstad et al., 2012; Plarre, 2012; Plarre et al., 2012). The trigger or triggers for such mutations might be a high number of hosts which make it more likely that the mutation occur or different types of stress. The stress link is seen in trout (*S. trutta*), where the production of ISA virus increased during stress and sexual maturation (Nylund et al., 1994a). In nature this stress stimulus can be maturation or escaping from predators,

while in farming stress is induced through for instance: high rearing densities, exposure to bath treatments, periods with low oxygen, change in feeding regimes, transportation, handling, vaccination, forced hierarchy, infections with parasites and other pathogens etc. Taken together these may lead to chronic stress, which may make the fish more susceptible to pathogens (Kurath and Winton, 2011; Nerland et al., 2011; Kibenge et al., 2012) and may also promote replication and mutations of HPR0 ISA virus resulting in virulent strains. Many production related stressors were present in Chile before the ISA crisis, probably giving the ISA virus an optimal environment and might explain the rapid development of the epizootic.

Many epizootic studies use the ISA diagnosis as the starting point for tracing transmission routes, and estimate where the fish were infected by studying outbreaks in neighbouring sites in a defined area (Gustafson et al., 2007b; Scheel et al., 2007; Lyngstad et al., 2008; Mardones et al., 2009; Aldrin et al., 2010; Aldrin et al., 2011; Lyngstad et al., 2011; Mardones et al., 2011). However, when considering the presence of HPR0 this might be misleading, since the fish could have been infected months or years before the outbreak. This means that these studies, designed to study virus transmission patterns, might in fact have been studying ISA disease triggers instead of transmission patterns. Almost simultaneous outbreaks in a certain area or a "hotspot" might then be the result of similar environmental stressors such as; rapid rise in temperature, algae blooms, bad weather causing change in feeding habits or high prevalence of parasites as *C. rogercresseyi* – as seen during the ISA crisis (**Paper III**). This means that it is very important to know the ISA virus status/prevalence in a fish group/site before the outbreak occurs if any conclusions about the transmission pattern should be drawn.

4.2.1 *Caligus rogercresseyi* a vector for ISA virus

The possible mechanisms for horizontal transmission of ISA virus between marine production sites are not well documented, but it has been suggested that passive transmission through water or by vectors/fomites is important (Nylund et al., 1993; Lyngstad et al., 2008; Mardones et al., 2009). There is no definitive proof of fish virus

being obligate users of arthropod vector transmission (Nerland et al., 2011), but knowledge from the North Atlantic shows that the copepod *Lepeophtheirus salmonis* can be a mechanical vector for ISA virus (Nylund et al., 1993). Could the large populations of *C. rogercresseyi* at the marine production sites for Atlantic salmon in Chile have a similar vector role? Was it a coincidence that the ISA epizootic happened during a period with the highest ever recorded infestation of *C. rogercresseyi* on farmed Atlantic salmon? These were questions raised by the industry during the ISA crisis (**Paper III**).

It is well documented that *Caligus* spp. parasitize a wide variety of hosts (Kabata, 2003). Moreover, it is well documented that *C. rogercresseyi* uses a wide range of marine fish in Chilean waters as hosts (Bravo, 2010a). *C. rogercresseyi* has also shown a high preference for the introduced Atlantic salmon as host, and it can be found in high abundance on this farmed species (Pino-Marambio et al., 2007; Mordue and Birkett, 2009). **Paper III** showed that *Caligus* spp. larvae, nauplii and copepodids (collected in the field during ISA outbreak and hatched in the laboratory from ISA virus positive lice) were all negative for ISA virus, suggesting that the spreading stage of the lice do not transmit ISA virus. There is no detailed knowledge about the factors that trigger adult lice to change hosts, but farmed salmon are almost certainly an attractive host (high numbers and rearing densities). There seem to be few reasons for leaving the host, except when the host is dying (pers. com. Bravo, 2013, Universidad Austral de Chile), which is the case during an ISA outbreak. This means that *C. rogercresseyi* infected with ISA virus could be moving between hosts within a farm, but may also end up on wild marine species on the outside of the cages. These wild fish species may contribute to the movement of lice between neighbouring farming sites. Since the lice feed on mucus and blood from the salmon host, they could be carrying ISA virus. In **Paper III** it was shown that *C. rogercresseyi* was positive for ISA virus 48 hours after they were removed from the Atlantic salmon host and they were able to infect Atlantic salmon with the virus. This shows that a co-infection with *C. rogercresseyi* and ISA virus may increase the risk of transmission of the virus both within and between production sites for Atlantic salmon. It has also been shown that lice may have an immunosuppressive effect on its host (Fast et al., 2004; Fast et al.,

2007; Tadiso et al., 2011), thus increasing the chances for establishing infections with ISA virus.

It is interesting to note that the fish groups that were diagnosed with ISA in Chile in April 2013, had two additional diagnoses; Caligidosis and SRS (Godoy et al. *submitted*), i.e. the same situation as during the ISA epizootic in 2007 - 2009 (Godoy et al., 2008; Alvial et al., 2012; Valdes-Donoso et al., 2013).

This means that a successful preventive strategy to reduce spreading of ISA virus in Chile should also include control with *C. rogercresseyi* (**Paper III**).

4.2.2 Passive waterborne transmission

Hydrodynamic transport is regarded as a key element for transmission of ISA virus (Gustafson et al., 2007b), and nearly all advisory reports and epizootic studies of ISA transmission assume that the ISA virus retains its infectivity for a long period in natural water. Hence, knowledge about the potential of the ISA virus to maintain its infectivity in natural seawater is an important factor for combating ISA. However, when studying the literature most often referred to, the documentation of survival time in natural water is lacking (Nylund et al., 1994b; Torgersen, 1997; Rimstad and Mjaaland, 2002; MacLeod et al., 2003; Tapia et al., 2013). The studies presented by Tapia et al. (2013) and Nylund et al. (1994b) do not use natural seawater and are therefore of limited relevance for the farming industry. In a review it is claimed that the ISA virus had a 3-$\log_{10}$ reduction titre after four months, but there is no data included to support this statement (Rimstad and Mjaaland, 2002). A none peer-reviewed study of MacLeod et al. (2003) concludes that ISA virions remain infective for one week in both seawater and freshwater at 15°C and 4°C. Torgersen (1997) concluded that the ISA virus survival time in sea water was more than 48 hours, but also this study is not peer-reviewed. Moreover, none of these studies have published reproducible results. Fish farmers in Chile during the ISA crisis pointed to this weakness in the documentation of ISA virus survival, and an experiment was therefore initiated (**Paper II**).

The results presented in **Paper II** suggested that the ability of the ISA virus to maintain is infectivity in natural seawater was limited to less than three hours. While the survival time of ISA virions in sterile seawater was less than 24 hours. UV radiation simulating natural sunlight had approximately the same inactivating effect as natural seawater. There is a great variability spatially and temporally in surface current speed (5 m deep) in salmon farming areas. The distance between farming sites also varies – but today is seldom less than 2-3 km. Some areas have typical current speeds from 1-10 cm s^{-1}, while in more exposed sites the average current speed may exceed >40 cm s^{-1} (pers. com. Ellingsen, 2013, SINTEF). If the current is stable in one direction, between two farming sites, then it will take approximately eight hours at a speed of 10 cm s^{-1} and approximately two hours if the speed is 40 cm s^{-1}, to cover a distance of 3.0 km. When considering the high dilution effect (Oye and Rimstad, 2001) in such strong current and the need for an infective dose (Gregory et al., 2009), is it not likely that a infective dose of ISA virus can be transmitted from one site to the other through passive waterborne transport (**Paper II**).

Removal of dead fish is looked upon as an important fish health preventive husbandry practice implemented at marine sites (Vagsholm et al., 1994; Lovdata, 2012). There are several articles on how to inactivate ISA virus (Falk et al., 1997; Oye and Rimstad, 2001), but no studies have focused on how long salmon that have died of ISA may release virus into the environment. Before the ISA crisis in Chile there was no standardized system for mortality management, and some sites did not have closed mortality containers or proper inactivation routines. Thus, it was discussed how quickly dead fish should be removed to avoid the spread of the virus. This was based on the fact that viral replication will not occur in a dead fish. Could it be likely that the ISA virions, covered with a sensitive lipid membrane, would stay infectious inside a dead fish during the decomposing process? The experiment conducted to answer this question showed that ISA virus could remain infectious within a decomposed salmon for at least five days at 7 °C (**Paper IV**). During practical farming conditions there is no doubt that fish dead of ISA are contagious, and need to be removed promptly for ISA virus inactivation. However, moribund fish continue to produce and shed virions (Thorud and Djupvik, 1988; Nylund et al., 1994b; Jones and Groman, 2001; Thorud

and Håstein, 2003; Gregory et al., 2009), and fast removal of these is even more important than removal of dead fish. So when prioritizing management of a marine site experiencing an ISA outbreak, the infected and moribund fish should be removed first.

4.3 Chile after the ISA crisis

The ISA crisis irrevocably changed the Chilean salmon industry's and authorities attitude to preventive fish health in a fast and brutal way in all steps of production. The most important changes were (**Paper I**; Alvial et al., 2012; **Paper II**; **Paper III**; **Paper IV**):

a. Secure the production of ISA virus free eggs/smolt by better disinfection procedures and implementation of broodfish screening as criteria for choice of embryos – both locally produced and imported (**Paper I**);
b. Establish on-shore facilities for broodfish and smolt production to improve and control pathogen status and quality of the smolt (**Paper I**);
c. Introduce generation split and fallow period – no mixing of fish groups or species sharing the same body of water, together with restrictions on moving fish (**Paper I**);
d. Reduce biomass and stocking densities at Atlantic salmon farming sites (max 17 kg/m^2 in sea);
e. Execute proper mortality treatment for silage and transport (**Paper IV**);
f. Improve bio-security procedures especially between farming sites, well boats and fish processing plants;
g. Implement micro and macro zones with coordinated activity;
h. Improve surveillance/monitoring of several pathogens and expand availability of better diagnostic tools and preventive approach (**Paper III**);
i. Use of ISA vaccines;
j. Increase awareness of preventive fish health by the management of the farming companies.

The cost of all these changes, together with higher debts, resulted in an increase of the production cost by nearly 50 %, from 1.83 USD per kilo in 2006 to 3.63 USD per kilo

in 2011 (Hersoug, 2012). As a consequence, the Chilean farmers lost much of their competitive advantage as salmonid producers. However, the period after the ISA crisis showed improved biological performance with low mortality, especially for *S. salar* (Figure 11). It is reasonable to believe that all the preventive measures introduced as a result of the ISA crisis have had a positive impact on other fish health challenges as well. In addition, after the ISA crisis there was a low demand for smolt and only healthy, high quality fish were transferred to sea. This gave a low biomass at salmon farming sites with relatively robust fish, which most likely has contributed to the improved biological performance since the ISA crisis.

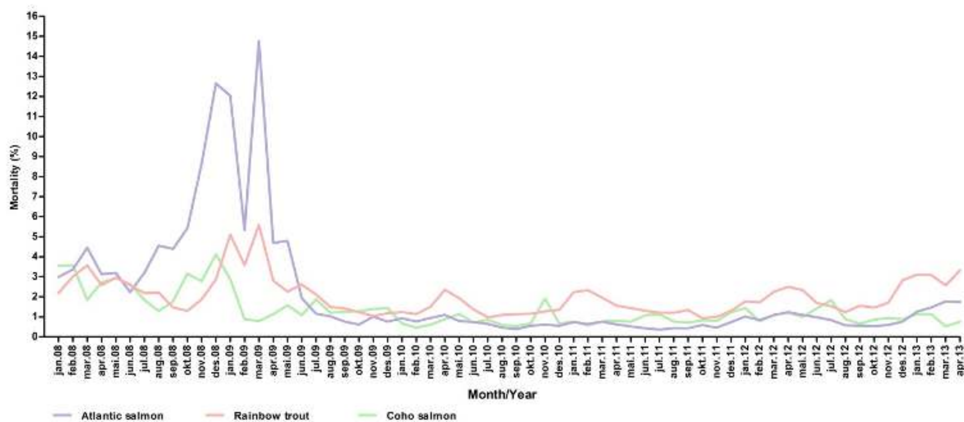


Figure 11. Percentage mortality per month for each farmed species. The ISA epizootic lasted from June 2007 to June 2009 (based on data from: pers. com. Esquivel, 2013, AquaBench, representing 85.6 % of the Chilean industry).

4.4 Controlling ISA virus – a personal view and advice

From today's research-based knowledge, it is likely that the salmon farming industry is circulating the ISA virus in the production cycle (Nylund et al., 2006; **Paper I**; Kibenge et al., 2009; Cottet et al., 2010; Lyngstad et al., 2011; Lyngstad et al., 2012; Plarre, 2012; Plarre et al., 2012; Stene et al., 2013), and that transmission from wild fish or other reservoir is limited (Johansen et al., 2011; Plarre et al., 2012). The best argument for this is that there are no new main ISA virus strains detected in the last 20 years in farmed salmonids (Plarre et al., 2012; present study). Assuming this to be correct, it gives the farmers a possibility to predict and control ISA outbreaks in salmon farming by using modern molecular epizootiology tools, such as real-time RT-PCR and sequencing. By systematic use of these tools through monitoring programs, the farmers will have information about the presence, strains, virulence and prevalence of ISA virus and can apply a preventive approach from these particulars (**Paper I**), instead of waiting for an outbreak and the associated "fire fighting" approach. To some extent this approach has been implemented in Chilean salmon farming, but only to a limited degree in the Norwegian industry.

Preventive fish health starts with broodfish, and at this stage the logic is easy, since an ISA virus negative broodfish will not give ISA virus positive offspring. This is the only stage in the production cycle where individual screening can be done, since all fish are killed during stripping. This is also assumed to be a good time point for screening, since viral load is expected to increase the closer to maturation the fish comes (Nylund et al., 1994a). The cost of screening a broodfish population producing embryos for half a million smolts will be approximately 40-50,000 NOK in Norway, while an ISA outbreak may incur costs exceeding millions of NOK. The average loss during an ISA outbreak site during the ISA crisis in Chile was 15-25 million NOK per site (pers. obs.).

If all the broodfish are screened and the ISA virus positive fish are eliminated, the next check point should be at pre-transfer smolt stage, where each genetically distinct fish group has to be tested. If every farmer present in a hydrographical sea zone only

transfers ISA virus negative smolts, and the assumption that farmed salmon have a self-sustaining cycle of the virus is correct, then where can the ISA virus come from? One source could be escaped farmed salmon or possibly wild fish. However, when a salmon has developed ISA it has problems controlling swimming behaviour and has poor appetite (Thorud, 1991; Poppe et al., 1999; pers. obs.). Thus the likelihood that ISA diseased fish could infect a cage of farmed salmon is small. It may be supposed that wild or escaped salmonids that are asymptomatic carriers of ISA virus can have a transmission role between sites, however no reports or studies support this hypothesis (Johansen et al., 2011).

Based on today's knowledge it is conceivable that the ISA virus can be eliminated from populations of farmed Atlantic salmon if a strict monitoring strategy is implemented in production and if all ISA virus positive broodfish are eliminated (**Paper I**). There will be a transitional period with some outbreaks, but if the assumption (that elimination is possible) is correct, the frequency of outbreaks will gradually be reduced.

Preventative strategies to combat ISA need to be accommodated to local needs and conditions, but in general a hazard plan (based on today's status) can be divided into three, depending on the ISA virus status in a fish group;

> Hazard plan A) to be executed if the *fish group is negative for ISA virus*. This fish group should be treated as normal and preferably never mixed with ISA virus positive fish groups.
>
> Hazard plan B) to be executed if the *fish group is infected with ISA virus HPR0 strain (low-virulent)*. Positive broodfish should always be eliminated. However, having an HPR0 infected fish group later in production leaves two choices: eliminate the fish, or avoid ISA triggers (stress) leading to both increased replication and risk of mutation to virulent strains. Since the chances of mutation are believed to be low (Christiansen, 2011; Lyngstad et al., 2012; Plarre et al., 2012), it might be justifiable to keep these fish for normal production. However, this fish group needs to be monitored more frequently

than an ISA virus negative group, and moribund fish should be included in the screening procedure.

Hazard plan C) to be executed if the *fish group is infected with ISA virus HPRΔ strain (virulent strains)*. If this occurs during the freshwater stage, the entire fish group must be eliminated. If this occurs in sea phase, elimination should start if increased mortality is seen. Before elimination, of fish in the cage, high emphasis must be given to proper removal of moribund and dead fish (**Paper IV**). Only the positive cage with increased mortality needs to be eliminated, while all other cages at the site must be monitored carefully.

To succeed with an ISA virus elimination strategy, a reliable screening method is a prerequisite. To achieve the best sensitivity and specificity of the real-time RT-PCR analysis, attention to detail in every step of the way is important. The least desirable error in practical farming is a false negative result where infected fish continue into production. In contrast, a false positive result can be rechecked to avoid unnecessary measures to be implemented if the recheck is negative. Today there are some weaknesses in the screening practice, e.g. there is no common consensus of what is the best tissue to screen when stripping broodfish. At present heart, gills, kidney, and milt as well as ovarian fluid are used by the farmers. In addition there are variations in platforms and assays used which might influence the results. False negative results might also be due to the inappropriate use of a cut-off value in these assays (pers. obs.) and mixing of tissues lowering the sensitivity (Lyngstad et al., 2011; pers. obs.). Continuing with today's slightly different use of the same method and practice can undermine the method, and it can be concluded that the method is not reliable enough (detecting ISA virus) and not giving enough cost/benefit in practical farming.

Another factor affecting success of such screening is the attitude and support provided by the regulating and controlling authorities. They need to encourage farmers to be at the front in the search for new knowledge and having approved screening programs for all relevant pathogens.

4.5 End notes

Farming of salmonids will continue to grow and movement of fish and embryos in different life stages will continue, hence facilitating the spread of pathogens. ISA virus is present in the natural wild reservoir of *S. salar* and *S. trutta* in the North Atlantic. However, both in Chile (as in Norway) it is likely that the farming industry has a self-sustaining reservoir of ISA virus. By proper monitoring to reduce horizontal spread and by eliminating positive broodfish to reduce vertical spread **(Paper I)**, the farmers can reduce the prevalence to a minimum. With a good surveillance program it should be possible to predict ISA outbreaks, and site management can diminish the significance of ISA through good husbandry and fish health practices. Important husbandry practices are for example to control the number of sea lice (**Paper III**), appropriate removal of dead fish (**Paper IV**) and a sufficient distance between sites (**Paper II**). The key for this is proactively tracing the pathogen throughout the production cycle and making decisions based on this, instead of waiting for clinical symptoms to appear and the associated "fire fighting" approach.

5. FUTURE R&D WORK

A future aim is to develop better and standardized tools and protocols for the farming industry to screen and monitor ISA virus in production of Atlantic salmon. This is needed to reduce further the number of false negatives and to avoid undermining of the monitoring method. Molecular biology is a rapidly progressing field and it is likely that the present real-time RT-PCR method will be replaced in some years. It is important for fish farmers to maintain good contact with researchers that follow technology development and are able to test out and implement improved and new methods.

The most needed work to be done is tissue tropism studies for all sizes of salmon, but especially for HPR0 ISA virus and broodfish, to secure the correct tissue to be sampled. Improved diagnostic tools to distinguish between HPR0 and HPRΔ would also be valuable. Developing cell lines to grow HPR0 variants of ISA virus would improve knowledge about persistent infections and make diagnostic work easier.

More knowledge about ISA virus biophysical qualities and especially survival time in natural water is needed. **Paper II** suggested that maximum survival time for ISA virus in natural seawater was less than three hours at 10 °C, but the exact survival potential was not determined. This is important basic knowledge that should be available for researchers and authorities, as well as farmers.

6. SOURCE OF DATA

Aldrin, M., Lyngstad, T.M., Kristoffersen, A.B., Storvik, B., Borgan, O., Jansen, P.A., 2011. Modelling the spread of infectious salmon anaemia among salmon farms based on seaway distances between farms and genetic relationships between infectious salmon anaemia virus isolates. Journal of the Royal Society Interface 8, 1346-1356.

Aldrin, M., Storvik, B., Frigessi, A., Viljugrein, H., Jansen, P.A., 2010. A stochastic model for the assessment of the transmission pathways of heart and skeleton muscle inflammation, pancreas disease and infectious salmon anaemia in marine fish farms in Norway. Preventive Veterinary Medicine 93, 51-61.

Alvial, A., Kibenge, F., Forster, J., Burgos, J., Ibarra, R., St-Hilaire, S., 2012. The Recovery of the Chilean Salmon Industry - the ISA crisis and its consequences and lessons. Global Aquaculture Alliance. http://www.gaalliance.org/cmsAdmin/uploads/GAA_ISA-Report.pdf.

Asche, F., Hansen, H., Tveteras, R., Tveteras, S., 2009. The Salmon Disease Crisis in Chile. Marine Resource Economics 24, 405-411.

Aspehaug, V., Mikalsen, A.B., Snow, M., Biering, E., Villoing, S., 2005. Characterization of the infectious salmon anemia virus fusion protein. Journal of Virology 79, 12544-12553.

Biacchesi, S., Le Berre, M., Le Guillou, S., Benmansour, A., Bremont, M., Quillet, E., Boudinot, P., 2007. Fish genotype significantly influences susceptibility of juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), to waterborne infection with infectious salmon anaemia virus. Journal of Fish Diseases 30, 631-636.

Binde, M., 2013. Norwegian Food Safety Authority, *pers. com.*

Birkbeck, T.H., Bordevik, M., Froystad, M.K., Baklien, A., 2007. Identification of Francisella sp from Atlantic salmon, *Salmo salar* L., in Chile. Journal of Fish Diseases 30, 505-507.

Bouchard, D.A., Brockway, K., Giray, C., Keleher, W., Merrill, P.L., 2001. First report of Infectious Salmon Anemia (ISA) in the United States. Bulletin of the European Association of Fish Pathologists 21, 86-88.

Bovo, G., Hill, B., Husby Håstein, T., Michel, C., Olesen, N.J., Storset, A., Midtlyng, P.J., 2005. Orthomyxovirus infections: Infectious salmon anaemia (ISA). In: Work Package 3 report: Pathogen Survival Outside the Host, and Susceptibility to Disinfection. VESO, Oslo, Norway, 13–15. http://www.eurl-fish.eu/~/media/Sites/EURL-FISH/english/activities/scientific%20reports/fisheggtrade%20wp_3.ashx.

Bravo, S., 2003. Sea lice in Chilean salmon farms. Bulletin of the European Association of Fish Pathologists 23, 197-200.

Bravo, S., 2010a. Caligidae ectoparasites on farmed salmonids in Chile: Classification, distribution, biology, control and resistance development. Universidad Austral de Chile and Norwegian School of Veterinary Science, *Dr. philos thesis*.

Bravo, S., 2010b. The reproductive output of sea lice *Caligus rogercresseyi* under controlled conditions. Experimental Parasitology 125, 51-54.

Bravo, S., 2013. Universidad Austral de Chile, *pers. com.*

Bravo, S., Sevatdal, S., Horsberg, T., 2008. Sensitivity assessment of *Caligus rogercresseyi* to emamectin benzoate in Chile. Aquaculture 282, 7-12.

Breuil, G., Pepin, J.F.P., Boscher, S., Thiery, R., 2002. Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, Dicentrarchus labrax (L.). Journal of Fish Diseases 25, 697-702.

Bustos, P., Rozas, M., Bohle, H., Ildefonso, R., Sandoval, A., Gaete, A., Araya, C., Grothusen, H., Tapia, E., Gallardo, A., Rojas, A., Lara, M., Labra, A., Galvez, C., 2011a. Primer reporte de piscine reovirus en salmon del Atlantico, *Salmo salar*, cultivado en Chile. *VD Julio Magazine.*

Bustos, P.A., Young, N.D., Rozas, M.A., Bohle, H.M., Ildefonso, R.S., Morrison, R.N., Nowak, B.F., 2011b. Amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) farmed in Chile. Aquaculture 310, 281-288.

Christiansen, D.H., Ostergaard, P. S., Snow, M., Dale, O. B., Falk, K., 2011. A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (*Salmo salar* L.) in the Faroe Islands. Journal of General Virology 92, 909-918.

Clouthier, S.C., Rector, T., Brown, N.E.C., Anderson, E.D., 2002. Genomic organization of infectious salmon anaemia virus. Journal of General Virology 83, 421-428.

Cook-Versloot, M., Griffiths, S., Cusack, R., McGeachy, S., Ritchie, R., 2004. Identification and characterisation of infectious salmon anaemia virus (ISAV) haemagglutinin gene highly polymorphic region (HPR) type 0 in North America. Bulletin of the European Association of Fish Pathologists 24, 203-208.

Cortez-San Martin, M., Rivas-Aravena, A., Guajardo, S., Castillo, M.T., Jashes, M., Sandino, A.M., Spencer, E., 2012. Simultaneous detection of the IPN and ISA viruses in outbreaks of clinical disease and mortality in farmed Atlantic salmon, *Salmo salar* L., in Chile. Journal of Fish Diseases 35, 461-465.

Cottet, L., Martin, M.C.-S., Tello, M., Olivares, E., Rivas-Aravena, A., Vallejos, E., Sandino, A.M., Spencer, E., 2010. Bioinformatic Analysis of the Genome of Infectious Salmon Anemia Virus Associated with Outbreaks with High Mortality in Chile. Journal of Virology 84, 11916-11928.

Cottet, L., Rivas-Aravena, A., Cortez-San Martin, M., Maria Sandino, A., Spencer, E., 2011. Infectious salmon anemia virus-Genetics and pathogenesis. Virus Research 155, 10-19.

Crane, M., Hyatt, A., 2011. Viruses of Fish: An Overview of Significant Pathogens. Viruses-Basel 3, 2025-2046.

Cunningham, C.O., Gregory, A., Black, J., Simpson, I., Raynard, R.S., 2002. A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bulletin of the European Association of Fish Pathologists 22, 366-374.

Cvitanich, J.D., Garate, O.N., Smith, C.E., 1991. The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch Postulate. Journal of Fish Diseases 14, 121-145.

Dannevig, B.H., Falk, K., Namork, E., 1995. Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cellline from Atlantic salmon head kidney. Journal of General Virology 76, 1353-1359.

Dannevig, B.H., Falk, K., Skjerve, E., 1994. Infectivity of internal tissues of atlantic salmon, *Salmo salar* L, experimentally infected with the etiologic agent of infectious salmon anemia (ISA). Journal of Fish Diseases 17, 613-622.

Devold, M., Falk, K., Dale, O.B., Krossoy, B., Biering, E., Aspehaug, V., Nilsen, F., Nylund, A., 2001. Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. Diseases of Aquatic Organisms 47, 119-128.

Devold, M., Karlsen, M., Nylund, A., 2006. Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. Journal of General Virology 87, 2031-2040.

Devold, M., Krossoy, B., Aspehaug, V., Nylund, A., 2000. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Diseases of Aquatic Organisms 40, 9-18.

Ebert, D., Herre, E.A., 1996. The evolution of parasitic diseases. Parasitology Today 12, 96-101.

EFSA, 2012. Panel on Animal Health and Welfare (AHAW); Scientific Opinion on infectious salmon anaemia. EFSA Journal 2012;10(11):2971. http://www.efsa.europa.eu/en/efsajournal/doc/2971.pdf.

Ellingsen, I., 2013. SINTEF, *pers. com.*

Enzmann, P.J., Castric, J., Bovo, G., Thiery, R., Fichtner, D., Schutze, H., Wahli, T., 2010. Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. Diseases of Aquatic Organisms 89, 9-15.

Esquivel, R., 2013. AquaBench, *pers.com.*

Falk, K., Namork, E., Rimstad, E., Mjaaland, S., Dannevig, B.H., 1997. Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). Journal of Virology 71, 9016-9023.

Fast, M.D., Johnson, S.C., Eddy, T.D., Pinto, D., Ross, N.W., 2007. *Lepeophtheirus salmonis* secretory/excretory products and their effects on Atlantic salmon immune gene regulation. Parasite Immunology 29, 179-189.

Fast, M.D., Ross, N.W., Craft, C.A., Locke, S.J., MacKinnon, S.L., Johnson, S.C., 2004. *Lepeophtheirus salmonis*: characterization of prostaglandin E-2 in secretory products of the salmon louse by RP-HPLC and mass spectrometry. Experimental Parasitology 107, 5-13.

FIS, 2010. Salmon Industry Lost USD 2,000 Million Due to ISA Virus. Fish Information & Services, Tokyo, Japan. http://fis.com/fis/worldnews/worldnews.asp?monthyear=11-2010&day=11&id=39160&l=e&country=0&special=&ndb=1&df=0.

Fryer, J.L., Lannan, C.N., Giovannoni, S.J., Wood, N.D., 1992. *Piscirickettsia Salmonis* GEN-NOV, SP-NOV, the causative agent of an epizootic disease in salmonid fishes. International Journal of Systematic Bacteriology 42, 120-126.

Gajardo, G., Laikre, L., 2003. Chilean aquaculture boom is based on exotic salmon resources: a conservation paradox. Conservation Biology 17, 1173-1174.

Godoy, M., 2012. Etecma, *pers. com.*

Godoy, M.G., Aedo, A., Kibenge, M.J.T., Groman, D.B., Yason, C.V., Grothusen, H., Lisperguer, A., Calbucura, M., Avendano, F., Imilan, M., Jarpa, M., Kibenge, F.S.B., 2008. First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (*Salmo salar*) in Chile. Bmc Veterinary Research 4.

Gonzalez, L., Carvajal, J., 2003. Life cycle of *Caligus rogercresseyi,* (Copepoda : *Caligidae*) parasite of Chilean reared salmonids. Aquaculture 220, 101-117.

Gonzalez, R.R., Ruiz, P., Llanos-Rivera, A., Cruzat, F., Silva, J., Astuya, A., Grandon, M., Jara, D., Aburto, C., 2011. ISA virus outside the cage: Ichthyofauna and other possible reservoirs to be considered for marine biosafety management in the far-southern ecosystems of Chile. Aquaculture 318, 37-42.

Gregory, A., Munro, L.A., Snow, M., Urquhart, K.L., Murray, A.G., Raynard, R.S., 2009. An experimental investigation on aspects of infectious salmon anaemia virus (ISAV)

infection dynamics in seawater Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 32, 481-489.

Groff, J.M., LaPatra, S.E., 2000. Infectious diseases impacting the commercial culture of salmonids. Journal of Applied Aquaculture 10, 17-90.

Grotmol, S., Totland, G.K., 2000. Surface disinfection of Atlantic halibut Hippoglossus hippoglossus eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. Diseases of Aquatic Organisms 39, 89-96.

Grove, S., Hjortaas, M.J., Reitan, L.J., Dannevig, B.H., 2007. Infectious salmon anaemia virus (ISAV) in experimentally challenged Atlantic cod (*Gadus morhua*). Archives of Virology 152, 1829-1837.

Guerrero, X., 2013. INTESAL de salmon Chile, *pers. com.*

Gustafson, L., Ellis, S., Robinson, T., Marenghi, F., Merrill, P., Hawkins, L., Giray, C., Wagner, B., 2007a. Spatial and non-spatial risk factors associated with cage-level distribution of infectious salmon anaemia at three Atlantic salmon, *Salmo salar* L., farms in Maine, USA. Journal of Fish Diseases 30, 101-109.

Gustafson, L.L., Ellis, S.K., Bartlett, C.A., 2005. Using expert opinion to identify risk factors important to infectious salmon-anemia (ISA) outbreaks on salmon farms in Maine, USA and New Brunswick, Canada. Preventive Veterinary Medicine 70, 17-28.

Gustafson, L.L., Ellis, S.K., Beattie, M.J., Chang, B.D., Dickey, D.A., Robinson, T.L., Marenghi, F.P., Moffett, P.J., Page, F.H., 2007b. Hydrographics and the timing of infectious salmon anemia outbreaks among Atlantic salmon (*Salmo salar* L.) farms in the Quoddy region of Maine, USA and New Brunswick, Canada. Preventive Veterinary Medicine 78, 35-56.

Hammell, K.L., Dohoo, I.R., 2005. Risk factors associated with mortalities attributed to infectious salmon anaemia virus in New Brunswick, Canada. Journal of Fish Diseases 28, 651-661.

Hersoug, B., 2012. Lakseoppdrett i Chile - et større comeback enn Lazarus? . In, Sjømatsrådets Lakseseminar, Oslo.

Holm, Y., 2013. EWOS AS, *pers. com.*

Hovland, T., Nylund, A., Watanabe, K., Endresen, C., 1994. Observation of infectious salmon anenia virus in Atalantic salmon, *Salmo Salar* L. Journal of Fish Diseases 17, 291-296.

Høy, T., 2013. Scientific Director in the Norwegian Medicines Agency, *pers. com*.

Ibieta, P., Tapia, V., Venegas, C., Hausdorf, M., Takle, H., 2011. Chilean Salmon Farming on the Horizon of Sustainability: Review of the Development of a Highly Intensive Production, the ISA Crisis and Implemented Actions to Reconstruct a More Sustainable Aquaculture Industry, Aquaculture and Enviroment - A Shared Destiny, DR Barbara Sladonja (Ed.) ISBN: 978-953-307-749-9. http://cdn.intechweb.org/pdfs/25465.pdf.

Iversen, M., Finstad, B., McKinley, R.S., Eliassen, R.A., Carlsen, K.T., Evjen, T., 2005. Stress responses in Atlantic salmon (*Salmo salar* L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. Aquaculture 243, 373-382.

Iversen, M., Finstad, B., Nilssen, K.J., 1998. Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture 168, 387-394.

Jarp, J., Karlsen, E., 1997. Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 28, 79-86.

Johansen, L.H., Jensen, I., Mikkelsen, H., Bjorn, P.A., Jansen, P.A., Bergh, O., 2011. Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. Aquaculture 315, 167-186.

Johnsen, B.O., Jensen, A.J., 1991. The Gyrodactylus story in Norway. Aquaculture 98, 289-302.

Johnson, A., Binette, S.L., Cook-Versloot, M., Beattie, M., McGeachy, S., Gagné, N., T., M.J., R.J., R., 2008. Association between ISAV mortalities and ISAV molecular type in the Bay of Fundy, Canada. Canadian Technical Report of Fisheries and Aquatic Sciences 2782.

Johnson, S.C., Treasurer, J.W., Bravo, S., Nagasawa, K., Kabata, Z., 2004. A review of the impact of parasitic copepods on marine aquaculture. Zoological Studies 43, 229-243.

Jones, S.R.M., Groman, D.B., 2001. Cohabitation transmission of infectious salmon anemia virus among freshwater-reared Atlantic salmon. Journal of Aquatic Animal Health 13, 340-346.

Kabata, Z., 2003. Copepods parasitic on fishes. Keys and notes for the identification of British species. Synopses of the British Fauna New Series 47, i-viii, 1-274.

Kibenge, F., Kibenge, M., Groman, D., McGeachy, S., 2006. In vivo correlates of infectious salmon anemia virus pathogenesis in fish. Journal of General Virology 87, 2645-2652.

Kibenge, F.S.B., Garate, O.N., Johnson, G., Arriagada, R., Kibenge, M.J.T., Wadowska, D., 2001. Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Diseases of Aquatic Organisms 45, 9-18.

Kibenge, F.S.B., Godoy, M.G., Fast, M., Workenhe, S., Kibenge, M.J.T., 2012. Countermeasures against viral diseases of farmed fish. Antiviral Research 95, 257-281.

Kibenge, F.S.B., Godoy, M.G., Wang, Y., Kibenge, M.J.T., Gherardelli, V., Mansilla, S., Lisperger, A., Jarpa, M., Larroquete, G., Avendano, F., Lara, M., Gallardo, A., 2009. Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virology Journal 6.

Kibenge, F.S.B., Kibenge, M.J.T., Wang, Y., Qian, B., Hariharan, S., McGeachy, S., 2007. Mapping of putative virulence motifs on infectious salmon anemia virus surface glycoprotein genes. Journal of General Virology 88, 3100-3111.

Kibenge, M.J.T., Iwamoto, T., Wang, Y., Morton, A., Godoy, M.G., Kibenge, F.S.B., 2013. Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family Reoviridae and its genome segment S1 sequences group it into two separate sub-genotypes. Virology Journal 10.

King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2012. Virus Taxonomy. Classification and Nomenclature of Virus. Ninth Report of the International Committee on Taxonomy of Virus. ELSEVIER ACADEMIC PRESS Inc. USA. 749 - 761.

Koren, C.W.R., Nylund, A., 1997. Morphology and morphogenesis of infectious salmon anaemia virus replicating in the endothelium of Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 29, 99-109.

Krossoy, B., Devold, M., Sanders, L., Knappskog, P.M., Aspehaug, V., Falk, K., Nylund, A., Koumans, S., Endresen, C., Biering, E., 2001a. Cloning and identification of the infectious salmon anaemia virus haemagglutinin. Journal of General Virology 82, 1757-1765.

Krossoy, B., Hordvik, I., Nilsen, F., Nylund, A., Endresen, C., 1999. The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. Journal of Virology 73, 2136-2142.

Krossoy, B., Nilsen, F., Falk, K., Endresen, C., Nylund, A., 2001b. Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. Diseases of Aquatic Organisms 44, 1-6.

Kurath, G., Winton, J., 2011. Complex dynamics at the interface between wild and domestic viruses of finfish. Current Opinion in Virology 1, 73-80.

Lovdal, T., Enger, O., 2002. Detection of infectious salmon anemia virus in sea water by nested RT-PCR. Diseases of Aquatic Organisms 49, 123-128.

Lovdata, 2012. Forskrift om drift av akvakulturanlegg (akvakulturdriftsforskriften) § 16, http://www.lovdata.no/for/sf/fi/ti-20080617-0822-002.html.

Lovely, J.E., Dannevig, B.H., Falk, K., Hutchin, L., MacKinnon, A.M., Melville, K.J., Rimstad, E., Griffiths, S.G., 1999. First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. Diseases of Aquatic Organisms 35, 145-148.

Lyngstad, T., 2012. Tracing transmission pathways for infectious salmon anaemia (ISA) virus. *Dr. scient theis,* Norwegian School of Veterinary Science.

Lyngstad, T.M., Hjortaas, M.J., Kristoffersen, A.B., Markussen, T., Karlsen, E.T., Jonassen, C.M., Jansen, P.A., 2011. Use of Molecular Epidemiology to Trace Transmission Pathways for Infectious Salmon Anaemia Virus (ISAV) in Norwegian Salmon Farming. Epidemics 3, 1-11.

Lyngstad, T.M., Jansen, P.A., Sindre, H., Jonassen, C.M., Hjortaas, M.J., Johnsen, S., Brun, E., 2008. Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003-2005. Preventive Veterinary Medicine 84, 213-227.

Lyngstad, T.M., Kristoffersen, A.B., Hjortaas, M.J., Devold, M., Aspehaug, V., Larssen, R.B., Jansen, P.A., 2012. Low virulent infectious salmon anaemia virus (ISAV-HPR0) is prevalent and geographically structured in Norwegian salmon farming. Diseases of Aquatic Organisms 101, 197-206.

Lyngøy, C., 2002. Infectious Salmon Anemia in Norway and the Faroe Islands: An Industrial Approach. In, Miller O, Cipriano RC (Eds.), International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech.Bull.No.1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior; U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, 97-109.

Lynne, D.T., Gerard, F., Rick, L., 2012. Physical Oceanography of the tropical Pacific - chapter 2: Geography of the Pacific Islands. M. Rapaport, editor. Bess Press, Honolulu, HI. Pp: 19-32. http://sam.ucsd.edu/papers/talley_tropical_pacific.html.

MacLean, S.A., Bouchard, D.A., Ellis, S.K., 2002. Survey of Nonsalmonid Marine Fishes for Detection of Infectious Salmon Anemia Virus and Other Salmonid Pathogens. Miller O, Cipriano RC (Eds.), International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech.Bull.No.1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior; U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, 135-153.

MacLeod, L.A., Raynard, R.s., Murray, A.G., Gregory, A., 2003. Survival of infectious salmon anaemia virus (ISAV) in aquatic environments. Poster from 11th EAFP-meeting (non-peer reviewed), Malta.

MacWilliams, C., Johnson, G., Groman, D., Kibenge, F.S.B., 2007. Morphologic description of infectious salmon anaemia virus (ISAV)-induced lesions in rainbow trout *Oncorhynchus mykiss* compared to Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 78, 1-12.

Mardones, F.O., Perez, A.M., Carpenter, T.E., 2009. Epidemiologic investigation of the re-emergence of infectious salmon anemia virus in Chile. Diseases of Aquatic Organisms 84, 105-114.

Mardones, F.O., Perez, A.M., Valdes-Donoso, P., Carpenter, T.E., 2011. Farm-level reproduction number during an epidemic of infectious salmon anemia virus in southern Chile in 2007-2009. Preventive Veterinary Medicine 102, 175-184.

Markussen, T., Jonassen, C.M., Numanovic, S., Braaen, S., Hjortaas, M., Nilsen, H., Mjaaland, S., 2008. Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. Virology 374, 515-527.

McBeath, A., Fourrier, M., Munro, E., Falk, K., Snow, M., 2011. Presence of a full-length highly polymorphic region (HPR) in the ISAV haemagglutinin-esterase does not affect the primary functions of receptor binding and esterase activity. Archives of Virology 156, 2285-2289.

McBeath, A.J.A., Bain, N., Snow, M., 2009. Surveillance for infectious salmon anaemia virus HPR0 in marine Atlantic salmon farms across Scotland. Diseases of Aquatic Organisms 87, 161-169.

McClure, C., Hammell, K., Dohoo, I., 2005a. Risk factors for outbreaks of infectious salmon anemia in farmed Atlantic salmon, *Salmo salar*. Preventive Veterinary Medicine 72, 263-280.

McClure, C.A., Hammell, K.L., Dohoo, I.R., Gagne, N., 2004. Lack of evidence of infectious salmon anemia virus in pollock Pollachius virens cohabitating with infected farmed Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 61, 149-152.

McClure, C.A., Hammell, K.L., Stryhn, H., Dohoo, I.R., Hawkins, L.J., 2005b. Application of surveillance data in evaluation of diagnostic tests for infectious salmon anemia. Diseases of Aquatic Organisms 63, 119-127.

Melville, K.J., Griffiths, S.G., 1999. Absence of vertical transmission of infectious salmon anemia virus (ISAV) from individually infected Atlantic salmon Salmo salar. Diseases of Aquatic Organisms 38, 231-234.

Mendoza, J., 2013. Mainstream Chile, *pers. com.*

Mjaaland, S., Hungnes, O., Teig, A., Dannevig, B.H., Thorud, K., Rimstad, E., 2002. Polymorphism in the infectious salmon anemia virus hemagglutinin gene: Importance and possible implications for evolution and ecology of infectious salmon anemia disease. Virology 304, 379-391.

Mjaaland, S., Markussen, T., Sindre, H., Kjoglum, S., Dannevig, B.H., Larsen, S., Grimholt, U., 2005. Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates. Archives of Virology 150, 2195-2216.

Mjaaland, S., Rimstad, E., Falk, K., Dannevig, B.H., 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): An orthomyxo-like virus in a teleost. Journal of Virology 71, 7681-7686.

Mordue, A.J., Birkett, M.A., 2009. A review of host finding behaviour in the parasitic sea louse, Lepeophtheirus salmonis (Caligidae: Copepoda). Journal of Fish Diseases 32, 3-13.

Mulcahy, D., Pascho, R.J., 1984. Adsorption to fish sperm of vertically transmitted fish viruses. Science 225, 333-335.

Mullins, J.E., Groman, D., Wadowska, D., 1998. Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. Bulletin of the European Association of Fish Pathologists 18, 110-114.

Murray, A.G., Munro, L.A., Wallace, I.S., Berx, B., Pendrey, D., Fraser, D., Raynard, R.S., 2010. Epidemiological investigation into the re-emergence and control of an outbreak

of infectious salmon anaemia in the Shetland Islands, Scotland. Diseases of Aquatic Organisms 91, 189-200.

Murray, A.G., Peeler, E.J., 2005. A framework for understanding the potential for emerging diseases in aquaculture. Preventive Veterinary Medicine 67, 223-235.

Murray, A.G., Smith, R.J., Stagg, R.M., 2002. Shipping and the spread of infectious salmon anemia in Scottish aquaculture. Emerging Infectious Diseases 8, 1-5.

Mutoloki, S., Evensen, O., 2011. Sequence similarities of the capsid gene of Chilean and European isolates of infectious pancreatic necrosis virus point towards a common origin. Journal of General Virology 92, 1721-1726.

Nerland, A.H., Øvergård, A.C., Patel, S., 2011. Viruses of fish. In: Studies in Viral Ecology. ed Christon J. Hurst. Wiley-Blackwell, ISBN: 978-1-1180-2458-4.

Nylund, A., Alexandersen, S., Lovik, P., Jakobsen, P., 1994a. The response of brown trout (Salmo trutta L.) to repeated challenge with infectious salmon anaemia (ISA). Bulletin of the European Association of Fish Pathologists 14, 167.

Nylund, A., Alexandersen, S., Rolland, J.B., Jakobsen, P., 1995a. Infectious salmon anemia virus (ISAV) in brown trout. Journal of Aquatic Animal Health 7, 236-240.

Nylund, A., Devold, M., Mullins, J., Plarre, H., 2002. Herring (*Clupea harengus*): A host for infectious salmon anemia virus (ISAV). Bulletin of the European Association of Fish Pathologists 22, 311-318.

Nylund, A., Devold, M., Plarre, H., Isdal, E., Aarseth, M., 2003. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. Diseases of Aquatic Organisms 56, 11-24.

Nylund, A., Hovland, T., Hodneland, K., Nilsen, F., Lovik, P., 1994b. Mechanisms for transmission of infectitous salmon anemia (ISA). Diseases of Aquatic Organisms 19, 95-100.

Nylund, A., Hovland, T., Watanabe, K., Endresen, C., 1995b. Presence of infectious salmon anemia virus (ISAV) in tissueof Atlantic salmon , *Salmo salar*, collected during 3 separate outbreaks of the disease. Journal of Fish Diseases 18, 135-145.

Nylund, A., Jakobsen, P., 1995. Sea-trout as a carrier of infectious salmon anemia virus. Journal of Fish Biology 47, 174-176.

Nylund, A., Krossøy, B., Watanabe, K., Holm, J.A., 1996. Target cells for the ISA virus in Atlantic salmon (*Salmo salar* L.). Bulletin of the European Association of Fish Pathologists 1996 Vol. 16 No. 2 pp. 68-72 16, 68-72.

Nylund, A., Kvenseth, A.M., Krossoy, B., 1995c. Susceptibility of wild salmon (*Salmo salar* L.) to infectious salmon anaemia (ISA). Bulletin of the European Association of Fish Pathologists 15, 152-156.

Nylund, A., Kvenseth, A.M., Krossoy, B., Hodneland, K., 1997. Replication of the infectious salmon anaemia virus (ISAV) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 20, 275-279.

Nylund, A., Plarre, H., Karlsen, M., Fridell, F., Ottem, K., Bratland, A., Saether, P., 2006. Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Archives of Virology 152, 151-179.

Nylund, A., Wallace, C., Hovland, T., 1993. The possible role of *Lepeophtheirus salmonis* (Kroyer) in the transmission of infectious salmon anaemia. In: *Pathogens of wild and farmed fish: Sea Lice* (ed. by G. A. Boxshall & D. Defaye). Ellis Horwood Limited UK.

Ottem, K.F., 2013. Mainstream Norway, *pers. com.*

Oye, A.K., Rimstad, E., 2001. Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. Diseases of Aquatic Organisms 48, 1-5.

Page, R.D.M., 1996. TreeView: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12, 357-358.

Patursson, Ø., 2010. Sustainable aquaculture on Islands - the example from the Faroe Islands. Aquaculture Research Station of the Faroes. http://fiskaaling.fo/upload/patursson_ae2010.pdf.

Pino-Marambio, J., Mordue, A.J., Birkett, M., Carvajal, J., Asencio, G., Mellado, A., Quiroz, A., 2007. Behavioural studies of host, non-host and mate location by the Sea Louse, Caligus rogercresseyi Boxshall & Bravo, 2000 (Copepoda : Caligidae). Aquaculture 271, 70-76.

Plarre, H., 2012. Infectious salmon anaemia virus (ISAV): Evolution, genotyping, reservoirs and transmission. University of Bergen, PhD thesis.

Plarre, H., Devold, M., Snow, M., Nylund, A., 2005. Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Diseases of Aquatic Organisms 66, 71-79.

Plarre, H., Nylund, A., Karlsen, M., Brevik, O., Saether, P.A., Vike, S., 2012. Evolution of infectious salmon anaemia virus (ISA virus). Archives of Virology 157, 2309-2326.

Poppe, T., Bergh, Ø., Espelid, S., Nygaard, S., 1999. Fiskehelse og fiskesykdommer (*in Norwegian*). Universitetsforlaget AS, 128-134 Norway.

Posada, D., 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25, 1253-1256.

Raynard, R.S., Murray, A.G., Gregory, A., 2001. Infectious salmon anaemia virus in wild fish from Scotland. Diseases of Aquatic Organisms 46, 93-100.

Rimstad, E., 2011. Examples of emerging virus diseases in salmonid aquaculture. Aquaculture Research 42, 86-89.

Rimstad, E., Biering, E., Brun, E., Falk, K., Kibenge, F., Mjaaland, S., Snow, M., Winton, J.R., 2007. Norwegian Scientific Committee for Food Safety: Opinion of the Panel on Animal Health and Welfare. Which risk factors relating to spread of Infectious Salmon Anaemia (ISA) require development of management strategies? http://www.vkm.no/dav/edccd8ceaa.pdf.

Rimstad, E., Dalsgaard, I., Hjeltnes, B., Håstein, T., 2011. Risikovurdering - stamfiskovervåkning og vertikal smitteoverføring (*in Norwegian*). Norwegian Sceintific Committee for Food Safety. 11.01.11.

Rimstad, E., Mjaaland, S., 2002. Infectious salmon anaemia virus - An orthomyxovirus causing an emerging infection in Atlantic salmon. Apmis 110, 273-282.

Rimstad, E., Mjaaland, S., Snow, M., Mikalsen, A.B., Cunningham, C.O., 2001. Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. Journal of Virology 75, 5352-5356.

Ritchie, R.J., Cook, M., Melville, K., Simard, N., Cusack, R., Griffiths, S., 2001. Identification of infectious salmon anaemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain differences. Diseases of Aquatic Organisms 44, 171-178.

Ritchie, R.J., McDonald, J.T., Glebe, B., Young-Lai, W., Johnsen, E., Gagne, N., 2009. Comparative virulence of Infectious salmon anaemia virus isolates in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 32, 157-171.

Robertsen, B., 2011. Can we get the upper hand on viral diseases in aquaculture of Atlantic salmon? Aquaculture Research 42, 125-131.

Rodger, H.D., Turnbull, T., Muir, F., Millar, S., Richards, R.H., 1998. Infectious salmon anaemia (ISA) in the United Kingdom. Bulletin of the European Association of Fish Pathologists 18, 115-116.

Rolland, J.B., Nylund, A., 1998a. Infectiousness on organic materials originating in ISA-infected fish and transmission of the disease via salmon lice (*Lepeophtheirus salmonis*). Bulletin of the European Association of Fish Pathologists 18, 173-180.

Rolland, J.B., Nylund, A., 1998b. Sea running brown trout: carrier and transmitter of the infectious salmon anemia virus (ISAV). Bulletin of the European Association of Fish Pathologists 18, 50-55.

Rolland, J.B., Winton, J.R., 2003. Relative resistance of Pacific salmon to infectious salmon anaemia virus. Journal of Fish Diseases 26, 511-520.

Rowley, H.M., Campbell, S.J., Curran, W.L., Turnbull, T., Bryson, D.G., 1999. Isolation of infectious salmon anaemia virus (ISAV) from Scottish farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 22, 483-487.

Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A., Colwell, R.R., 2000. Global spread of microorganisms by ships - Ballast water discharged from vessels harbours a cocktail of potential pathogens. Nature 408, 49-50.

SAG, 2011. Productos biológicos inmunológicos con registro provisional uso en salmónidos, Servicio Agrícola y Ganadero, 17.01.13. Servicio Agrícola y Ganadero http://www.sag.gob.cl.

SalmonChile, 2012. Chilean Salmon Industry Brief. http://www.salmonchile.cl/frontend/seccion.asp?contid=397&secid=33&secoldid=33&subsecid=117&pag=1.

Scheel, I., Aldrin, M., Frigessi, A., Jansen, P.A., 2007. A stochastic model for infectious salmon anemia (ISA) in Atlantic salmon farming. Journal of the Royal Society Interface 4, 699-706.

Skaar, C.K., Mortensen, S., 2007. Fate of infectious salmon anaemia virus (ISAV) in experimentally challenged blue mussels *Mytilus edulis*. Diseases of Aquatic Organisms 74, 1-6.

Snow, M., 2011. The contribution of molecular epidemiology to the understanding and control of viral diseases of salmonid aquaculture. Veterinary Research 42.

Snow, M., Raynard, R., Bruno, D.W., van Nieuwstadt, A.P., Olesen, N.J., Lovold, T., Wallace, C., 2002. Investigation into the susceptibility of saithe Pollachius virens to infectious salmon anaemia virus (ISAV) and their potential role as a vector for viral transmission. Diseases of Aquatic Organisms 50, 13-18.

Snow, M., Raynard, R.S., Bruno, D.W., 2001a. Comparative susceptibility of Arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. Aquaculture 196, 47-54.

Snow, M., Raynard, R.S., Inglis, J., Bruno, D.W., 2001b. Investigation into the potential for seawater rainbow trout (*Oncorhynchus mykiss*) to act as vectors of infectious salmon anaemia virus (ISAV). Bulletin of the European Association of Fish Pathologists 21, 252-262.

Sommer, A.I., Mennen, S., 1996. Propagation of infectious salmon anaemia virus in Atlantic saimon, *Salmo salar* L., head kidney macrophages. Journal of Fish Diseases 19, 179-183.

Sommerset, I., Krossoy, B., Biering, E., Frost, P., 2005. Vaccines for fish in aquaculture. Expert Review of Vaccines 4, 89-101.

Stene, A., Hellebø, A., Aspehaug, V., Devold, M., 2013. Virusreservoarer på norske oppdrettslokaliteter - med fokus på PD og ILA. NF Xpert (Norsk Fiskeoppdrett) Juli 2013 40-41.

Søfteland, E., 2005. Experiments with ISAV infected broodfish (Atlantic salmon). Can the ISAV be transmitted via gonadal products? Mattilsynet, Universitetet i Bergen,

Akvaforsk, Fomas, Veterinærinstituttet, Bolaks, Salmobreed. (in Norwegian), http://www.salmobreed.no, 1–31.

Tadiso, T.M., Krasnov, A., Skugor, S., Afanasyev, S., Hordvik, I., Nilsen, F., 2011. Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. Bmc Genomics 12.

Tapia, E., Monti, G., Rozas, M., Sandoval, A., Gaete, A., Bustos, P., 2013. Assessment of the *in vitro* of survival of the Infectious Salmon Anaemia (ISAV) under difterent water types and temperature. Bulletin of the European Association of Fish Pathologists 33, 3-12.

Thorud, K., Djupvik, H.O., 1988. Infectious anaemia in Atlantic salmon (*Salmo Salar* L.). Bull Eur Assoc Fish Pathol 8:109–111.

Thorud, K.E., 1991. Infectious salmon anemia. Norwegian College of Veterinary Medicine. Dr. Scientiarum thesis, Oslo, Norway.

Thorud, K.E., Håstein, T., 2003. Experiences With Regulatory Responses to Infectious Salmon Anemia in Norway. In: Miller O, Cipriano RC (Eds.), International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech.Bull.No.1902. Washington, DC. U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior; U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service, pp. 155-159.

Torgersen, Y., 1997. Physican and chemical inactivation of the Infectious salmon anaemia (ISA) virus. Workshop on Infectious salmon anaemia. St. Andrews, New Brunswick, 26. November 1997, 44-53.

Totland, G.K., Hjeltnes, B.K., Flood, P.R., 1996. Transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smelts of Atlantic salmon *Salmo salar* during their presymptomatic phase. Diseases of Aquatic Organisms 26, 25-31.

Vagsholm, I., Djupvik, H.O., Willumsen, F.V., Tveit, A.M., Tangen, K., 1994. Infectious salmon anemia (ISA) epidemiology in Norway. Preventive Veterinary Medicine 19, 277-290.

Valdebenito, S., Avendano-Herrera, R., 2009. Phenotypic, serological and genetic characterization of Flavobacterium psychrophilum strains isolated from salmonids in Chile. Journal of Fish Diseases 32, 321-333.

Valdes-Donoso, P., Mardones, F.O., Jarpa, M., Ulloa, M., Carpenter, T.E., Perez, A.M., 2013. Co-infection patterns of infectious salmon anaemia and sea lice in farmed Atlantic salmon, *Salmo salar* L., in southern Chile (2007-2009). Journal of Fish Diseases 36, 353-360.

Veterinærinstituttet, 2013. Infeksiøs lakseanemi (ILA) (in Norwegian). http://www.vetinst.no/Temasider/Fiskesykdommer/Infeksioes-lakseanemi-ILA.

Wallace, I.S., Munro, L.A., Pert, C.C., Macintoch, A.m., Kilburn, R., 2009. Shetland wild fish ISAV investigation. Marine Scotland Internal Report 18/09.

Watanabe, K., Hovalnd, T., Nylund, A., Endresen, C., 1993. Påvisning og lokalisering av ILA viruset hos laks. Norsk Fiskeoppdrett 8, 64-65.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza-A viruses. Microbiological Reviews 56, 152-179.

Weli, S.C., Aamelfot, M., Dale, O.B., Koppang, E.O., Falk, K., 2013. Infectious salmon anaemia virus infection of Atlantic salmon gill epithelial cells. Virology Journal 10, 5.

7. SUPPLEMENTARY MATERIAL

Table A. An overview of ISA virus sequences included and their GenBank accession numbers in the phylogenies in figure 9 and 10. The name of origin country and virus-id is given in the first column. The second and third column shows year of collection, HPR in segment 6 and presence of insert in segment 5 (IN), respectively, where this info was available. Column six gives a code for the company where the virus strain was sampled. The last two columns present the source of the ISA virus sequences.

Country/virus-id	Year	HPR-IN	Acc.# Seg. 5	Acc.# Seg. 6	Company	Seg. 5 source	Seg. 6 source
Chile							
CH71-8	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH71-5	2013	3-?					Godoy et al. Submitted
CH71-4	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH71-6	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH71-7	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH77-16	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH77-18	2013	3-?				Godoy et al. Submitted	
CH78-27	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH78-8	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH78-9	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH78-10	2013	3-?					Godoy et al. Submitted
CH78-11	2013	3-?					Godoy et al. Submitted
CH78-16	2013	3-?				Godoy et al. Submitted	
CH87-25	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH26-8	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH26-7	2013	3-?				Godoy et al. Submitted	
CH26-3	2013	3-?				Godoy et al. Submitted	
CH26-5	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH26-8	2013	3-?				Godoy et al. Submitted	
CH26-6	2013	3-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-15	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-4	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-9	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-11	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-3	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-12	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-14	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
CH15-16	2013	14-?				Godoy et al. Submitted	Godoy et al. Submitted
ID758	2012	0					Godoy et al. Submitted
CH44/09	2009	7b-IN			3	Present study	Present study
CH45/09	2009	7b-IN			3	Present study	Present study
VT04222008-106	2008		EU849005	EU849012		Kibenge et al. 2009	Kibenge et al. 2009
VT05202008-111	2008		EU849010			Kibenge et al. 2009	
Biovac 307418	2008		FJ592150			Kibenge et al. 2009	
Biovac31606-H	2008			FJ594282			Kibenge et al. 2009
Biovac 307403	2008		FJ592143			Kibenge et al. 2009	

Country/virus-id	Year	HPR-IN	Acc.# Seg. 5	Acc.# Seg. 6	Company	Seg. 5 source	Seg. 6 source
VT11282007-37	2007		EU849006			Kibenge et al. 2009	
Biovac 26415-3	2008		EU449768			Kibenge et al. 2009	
Biovac 26830	2008		EU449767			Kibenge et al. 2009	
Biovac32980-5	2008			FJ594294			Kibenge et al. 2009
31682-10	2008	1c		FJ594283	6		Godoy et al. 2008
U24636	2007		EU130923			Godoy et al. 2008	
Biovac32980-5	2008			FJ594288			Kibenge et al. 2009
Biovac31606-L	2008			FJ594291			Kibenge et al. 2009
Biovac30942/943	2008	7b-IN		FJ594292	2		Kibenge et al. 2009
Biovac31606-H	2008	1c		FJ594282	11		Kibenge et al. 2009
Biovac33004-2	2008			FJ594285			Kibenge et al. 2009
isolate 901				GU830908			Cottet et al. 2010
Biovac32916-1	2008			FJ594315			Kibenge et al. 2009
Biovac 31606H	2008		FJ592135			Kibenge et al. 2009	
Biovac 315924	2008		FJ592138			Kibenge et al. 2009	
Biovac 315922	2008		FJ592139			Kibenge et al. 2009	
Biovac316825	2008		FJ592141			Kibenge et al. 2009	
Biovac 2006B13364	2008		FJ592134			Kibenge et al. 2009	
Biovac 317903GH	2008		FJ592148			Kibenge et al. 2009	
Biovac 317909GH	2008		FJ592147			Kibenge et al. 2009	
Biovac 316478GH	2008		FJ592152			Kibenge et al. 2009	
Biovac 319059Cz	2008		FJ592156			Kibenge et al. 2009	
Biovac 316485GH	2008		FJ592158			Kibenge et al. 2009	
Biovac 30942/30943	2008		FJ592140			Kibenge et al. 2009	
Biovac 315878	2008		FJ592165			Kibenge et al. 2009	
PM4165 #11	2008		FJ592142			Kibenge et al. 2009	
Biovac 319057Cz	2008		FJ592160			Kibenge et al. 2009	
PM4165 #8	2008		FJ592163			Kibenge et al. 2009	
Biovac 316483GH	2008		FJ592168			Kibenge et al. 2009	
Biovac 322322044K	2008		FJ592157			Kibenge et al. 2009	
Biovac 322322032LK	2008		FJ592161			Kibenge et al. 2009	
Biovac 26572	2007		EU449765			Kibenge et al. 2009	
Biovac 316873	2008		FJ592136			Kibenge et al. 2009	
Biovac 316875	2008		FJ592137			Kibenge et al. 2009	
Biovac 316894	2008		FJ592145			Kibenge et al. 2009	
Biovac 316673GH	2008		FJ592149			Kibenge et al. 2009	
Biovac 316675GH	2008		FJ592151			Kibenge et al. 2009	
Biovac 316853	2008		FJ592153			Kibenge et al. 2009	
Biovac 3159020	2008		FJ592155			Kibenge et al. 2009	
Biovac 315917	2008		FJ592159			Kibenge et al. 2009	
Biovac 3291366	2008		FJ592162			Kibenge et al. 2009	
Biovac 316851	2008		FJ592164			Kibenge et al. 2009	
Biovac 3159018	2008		FJ592166			Kibenge et al. 2009	
Biovac 315916	2008		FJ592167			Kibenge et al. 2009	
isolate 752			GU830899			Cottet et al. 2010	
Biovac 316473GH	2008		FJ592154			Kibenge et al. 2009	
Biovac 32089P1	2008		FJ592146			Kibenge et al. 2009	
Biovac 316891	2008		FJ592144			Kibenge et al. 2009	
Biovac 26829-2	2008		EU449766			Kibenge et al. 2009	
Biovac32916-1	2008			FJ594316			Kibenge et al. 2009
VT05202008-111	2008	7b-IN		EU849017	6		Kibenge et al. 2009
Biovac31667-5GH	2008	7f-IN		FJ594307	6		Kibenge et al. 2009
Biovac30741-8	2008	7b-IN		FJ594306	6		Kibenge et al. 2009
Biovac31587-8	2008	7b-IN		FJ594328	8		Kibenge et al. 2009

Country/virus-id	Year	HPR-IN	Acc.# Seg. 5	Acc.# Seg. 6	Company	Seg. 5 source	Seg. 6 source
Biovac31587-9	2008			FJ594330			Kibenge et al. 2009
CH03/08	2008	7c			3	Present study	Present study
CH04/08	2008	7c			3	Present study	Present study
Biovac33004-21	2008			FJ594332			Kibenge et al. 2009
Biovac32719-108	2008			FJ594325			Kibenge et al. 2009
Biovac32913-66	2008			FJ594323			Kibenge et al. 2009
Biovac33064-107	2008			FJ594319			Kibenge et al. 2009
Biovac31647-3	2008	7b-IN		FJ594311	10		Kibenge et al. 2009
Biovac31685-3	2008	7b-IN	FJ592133	FJ594310		Kibenge et al. 2009	Kibenge et al. 2009
Biovac33059-2	2008			FJ594309			Kibenge et al. 2009
Biovac29560-2H	2008			FJ594303			Kibenge et al. 2009
Biovac31647-8GH	2008	7b-IN		FJ594308	10		Kibenge et al. 2009
Biovac31905-9Cz	2008	7b-IN		FJ594313	10		Kibenge et al. 2009
Biovac31648-5GH	2008	7b-IN		FJ594318	10		Kibenge et al. 2009
Biovac31905-7Cz	2008	7b-IN		FJ594321	10		Kibenge et al. 2009
PM-4165 #8	2008	7b-IN		FJ594326	1		Kibenge et al. 2009
Biovac31648-3GH	2008	7b-IN		FJ594334	10		Kibenge et al. 2009
Biovac31591-6	2008	7b-IN		FJ594331	13		Kibenge et al. 2009
Biovac31589-17	2008			FJ594317			Kibenge et al. 2009
Biovac31590-18	2008	7b-IN		FJ594329	4		Kibenge et al. 2009
CH01/08	2008	7b-IN	EU851042	EU851043	3		Vike et al. 2009
CH12/08	2008	7b			3	Present study	Present study
CH15/08	2008	7b-IN			3	Present study	Present study
CH16/08	2008	7b-IN			3	Present study	Present study
CH17/08	2008	7b-IN			3	Present study	Present study
CH26/08	2008	7b-IN			3	Present study	Present study
CH27/08	2008	7b-IN			3	Present study	Present study
CH31/08	2008	7b-IN			3	Present study	Present study
CH32/08	2008	7b			3	Present study	Present study
CH33/08	2008				3	Present study	Present study
Biovac31590-20	2008			FJ594312			Kibenge et al. 2009
Biovac31590-20	2008			FJ594302			Kibenge et al. 2009
Biovac31790-3GH	2008			FJ594304			Kibenge et al. 2009
Biovac32232-2044K	2008			FJ594314			Kibenge et al. 2009
Biovac31591-7	2008			FJ594320			Kibenge et al. 2009
Biovac32232-2032LK	2008			FJ594322			Kibenge et al. 2009
Biovac32232-2032LK	2008			FJ594327			Kibenge et al. 2009
Biovac31685-1	2008			EU271682			Kibenge et al. 2009
Biovac32089-P1	2008	7b-IN		FJ594301	12		Kibenge et al. 2009
Biovac31689-4	2008	7b-IN		FJ594298	6		Kibenge et al. 2009
Biovac31689-1	2008	7b-IN		FJ594299	6		Kibenge et al. 2009
isolate 752			GU830907	GU830900		Cottet et al. 2010	Cottet et al. 2010
Biovac30740-3	2008	7b-IN		FJ594297	6		Kibenge et al. 2009
Biovac32325-4	2008			FJ594296			Kibenge et al. 2009
PM-4165 #11	2008	7b-IN		FJ594295	1		Kibenge et al. 2009
13364-2006B	2008	9		FJ594284	8		Kibenge et al. 2009
VT05202008-114	2008	7b-IN	EU849011	EU849018	6	Kibenge et al. 2009	Kibenge et al. 2009
CH35/09	2008	7b-IN			3	Present study	Present study
CH18/08	2008	7b			7	Present study	Present study
CH20/08	2008	7b			7	Present study	Present study
CH28/08	2008	0			3	Present study	Present study
CH21/08	2008	7b			7	Present study	Present study
CH22/08	2008	7b			7	Present study	Present study
CH09/08	2008	36			3	Present study	Present study

Country/virus-id	Year	HPR-IN	Acc.# Seg. 5	Acc.# Seg. 6	Company	Seg. 5 source	Seg. 6 source
CH10/08	2008	36			3	Present study	Present study
CH11/08	2008	36			3	Present study	Present study
CH25/08	2008	36			3	Present study	Present study
Biovac31592-4	2008			FJ594289			Kibenge et al. 2009
Biovac31592-2	2008			FJ594290			Kibenge et al. 2009
CH29/08	2008	0			3	Present study	Present study
CH30/08	2008	0			3	Present study	Present study
CH05/08	2008	35			3	Present study	Present study
CH08/08	2008	35			3	Present study	Present study
CH06/08	2008	35			3	Present study	Present study
VT11282007-35	2007			EU625668			Kibenge et al. 2009
VT11282007-38	2007		EU849008	EU625677		Kibenge et al. 2009	Kibenge et al. 2009
VT11282007-040	2007			EU625678			Kibenge et al. 2009
VT11282007-033	2007			EU625674			Kibenge et al. 2009
VT11282007-042	2007			EU625679			Kibenge et al. 2009
VT11282007-043	2007			EU625680			Kibenge et al. 2009
VT11282007-034	2007			EU849015			Kibenge et al. 2009
VT11282007-36	2007	7b-IN	EU552491	EU849016	2	Kibenge et al. 2009	Kibenge et al. 2009
VT11282007-032	2007			EU849014			Kibenge et al. 2009
VT04222008-107	2008	7f-IN	EU849007	EU849013	9	Kibenge et al. 2009	Kibenge et al. 2009
VT11282007-044	2007			EU625681			Kibenge et al. 2009
VT11282007-037	2007	2/7b-IN		EU625676	2		Kibenge et al. 2009
VT11282007-39	2007	5	EU849009	EU625673	5	Kibenge et al. 2009	Kibenge et al. 2009
VT01302008-068	2007			EU625672			Kibenge et al. 2009
VT11152007-30	2007			EU625670			Kibenge et al. 2009
VT11282007-38	2007	5		EU625667	5		Kibenge et al. 2009
NO/1720/07	2007	7b-IN		AM941715	2		Orpetveit, not publisched
VT11152007-031	2007	7b-IN		EU625671	2		Kibenge et al. 2009
VT11052007-27	2007			EU625675			Kibenge et al. 2009
VT11282007-35	2007			EU625666			Kibenge et al. 2009
Norway							
FM173/11	2011	0	JN711019	JN711060	3	Plarre et al. 2012	Plarre et al. 2012
FM174a/11	2011	14b	JN711020	JN711061	3	Plarre et al. 2013	Plarre et al. 2013
FM168/10	2010	14c	JN711018			Plarre et al. 2012	
T161/09	2009	5		JN711090		Plarre et al. 2012	Plarre et al. 2012
FM116/06	2006	9	JN711058			Plarre et al. 2012	
FM114/05	2005	3	JN711016			Plarre et al. 2012	
FM86/04	2004	1		AY971659			Nylund et al. 2006
T167/10	2010	5	JN711054			Plarre et al. 2012	
ISA9 09A	2009			HE800174			Lyngstad et al. 2012
T162/09	2009	0	JN711053				Plarre et al. 2012
T131/07	2007	2		JN711078		Plarre et al. 2012	Plarre et al. 2012
T10/93	1993	4a	EU449767			Krossøy et al. 2001	Krossøy et al. 2001
T73/02	2002	14	AY853923			Nylund et al 2006	
T90/04	2004	16	JN711055			Plarre et al. 2012	
T74/03	2003	14	AY853924	AY971664		Devold et al. 2006	Devold et al. 2006
T79/03	2003	14		AY971665			Nylund et al. 2006
T176/12	2012	0				Present study	
T37/98	1998	2	AY853934			Devold et al. 2006	
N89/04	2004	14		AY971662			Devold et al. 2006
NT141/08	2008	2	JN711031	JN711070		Plarre et al. 2012	Plarre et al. 2012
NT134/08	2008	34	JN711030			Plarre et al. 2012	
NT115/05	2005	3	JN711029			Plarre et al. 2012	
NT81/03	2003	7b	AY853955			Devold et al. 2006	

Country/virus-id	Year	HPR-IN	Acc.# Seg. 5	Acc.# Seg. 6	Company	Seg. 5 source	Seg. 6 source
5ST44/99	1999	14	AY853954			Devold et al. 2006	
ST143/08	2008	35		JN711073		Plarre et al. 2012	Plarre et al. 2012
ST25/97	1997	6	AY853926	AF364885		Devold et al. 2006	Devold et al. 2001
ST26/97	1997	14	AY853951			Devold et al. 2006	
ST28/97	1997	6		AY853929			Devold et al. 2006
ST27/97	1997	6	AY853929	AF364897		Devold et al. 2006	Devold et al. 2001
ST21/96	1996	14	AY853952	AF364886		Devold et al. 2006	Devold et al. 2001
MR177/12	2012	8				Present study	
MR139/08	2008	35	JN711027			Plarre et al. 2012	
MR118/06	2006	30				Present study	
MR104/05	2005	0	JN711026	DQ108607		Plarre et al. 2012	Nylund et al. 2006
MR102/05	2005	0	EU851044	DQ108605		Nylund et al. 2006	Nylund et al. 2006
5MR61/01	2001	11a-IN	AY853935			Devold et al. 2006	
SF109/05	2005	0		DQ108610			Nylund et al. 2006
5SF57/00	2000	11b-IN	AY853939			Devold et al. 2006	
SF41/98	1998	15		AF364871		Devold et al 2001	
H172/10	2010	0	JN711023	JN711064		Plarre et al. 2012	Plarre et al. 2012
H138/08	2008	0	JN711022			Plarre et al. 2012	
5H93/04	2004	10	AY853948	AY973179		Devold et al. 2006	Nylund et al. 2006
H51/00	2000	7b		AF364882			Devold et al 2001
H06/91	1991	2	AY853934			Devold et al. 2006	
H02/89	1989	2	AY853930			Devold et al. 2006	
H36/98	1998	15	AY853958	AF302799		Devold et al. 2006	Krossøy et al, 2001
H97/04	1997	0	JN711024			Plarre et al. 2012	
H1/87	1987	1		AF364893			Devold et al. 2001
Scotland							
SCOT157/08	2008	0	JN711010	JN711096		Plarre et al. 2012	Plarre et al. 2012
SCOT43/98	1998	7b	AF302802				Krossøy et al 2001
Faeroe Islands							
F72/02	2002	3	AY853917	AF526263		Devold et al. 2006	Nylund et al 2003
FO/01b/07	2007	0		HQ664995			Christiansen et al. 2011
FO/01a/07	2007	0		HQ664994			Christiansen et al. 2011
FO/01/06	2006	0		HQ664992			Christiansen et al. 2011
T162/09	2009	0		JN711091		Plarre et al. 2012	Plarre et al. 2012
H97/04	2004	0		DQ108604		Plarre et al. 2012	Nylund et al. 2006
FO/03/06	2006	0		HQ664993			Christiansen et al. 2011
North America							
USA2004	2004	0	JN711056	AY973194		Plarre et al. 2012	Nylund et al. 2006
NS2003	2003	0	JN711056	AY973182		Plarre et al. 2012	Nylund et al. 2006