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Acknowledgements

This work was carried out during 2006-2014. Financial support was provided by The Research Council of Norway (grants no. 141887/120, 143286/140 and 172542/S40).
My supervisors have been Dr. Ann-Inger Sommer and Professor Jorunn Jørgensen at the Norwegian College of Fisheries Science, UiT The Arctic University of Norway. I would like to thank Ann-Inger Sommer for giving me the opportunity to do a doctoral degree, and for introducing me to the fascinating field of fish health research. I would also like to thank Professor Jorunn Jørgensen, for guiding me through the last part of this long and winding road.

The following people have also given me valuable support in my work:
Audny Johansen has given me invaluable help during our long days in Kårvika and has been a good team worker. She has also been chatting with me so that it seems like the even longer evenings at the lab after sampling in Kårvika just flew away. Saskia Mennen and Lill-Heidi Johansen have given me valuable assistance throughout the different projects and Helene Mikkelsen has taken the time to read the discussion of this thesis.
I would also like to extend my thanks to Professor Michaela Aschan for providing me with a nice office that made it possible for me to finish this thesis.
Marit Seppola my time at Fiskeriforskning/Nofima would not have been the same without you. Thank you for the scientific discussions in the lab, for all the long hours at parties when everyone else had left and for reading this thesis, your help was appreciated more than words can say.
I want to thank my family for all their support. My beautiful girls Margrethe and Maren who is partly the reason why this doctoral degree has taken forever to finish. You fill my life with so much love and fun. To my parents for all the love and support you give me. Last but not least to Jon, for understanding how important it was for me to finish this task and giving me the support to do so.

Kjersti Julin

Sammendrag (summary in Norwegian)

Infeksiøs pankreas nekrose (IPN) er en fiskesykdom som rammer mange fiskeslag over store deler av verden. Det har store økonomiske konsekvenser for norsk lakseoppdrettsnæring. Infeksiøs pankreas nekrose var først kjent som en sykdom i yngel, men i de siste 10 til 20 årene har det vært et like stort problem i post-smolt. Antall utbrudd varierer fra år til år, der 2009 ble registrert som et år med flest utbrudd. Samme år introduserte avlsselskapet AquaGen QTL-rogn. Denne fisken var svært motstandsdyktig mot IPN. Sykdomsproblemene forsvant i de anleggene som benyttet seg av IPN-QTL-rogn, noe som gjenspeiler seg i at det har vært en drastisk nedgang i antall rapporterte sykdomsutbrudd i norsk lakseoppdrettsnæring.

Viruset som forårsaker denne sykdommen, infeksiøs pankreas nekrose virus (IPNV), er et lite dobbeltrådet RNA virus som tilhører familien *Birnaviridae*, genus *Aquabirnavirus*. En infeksjon med IPNV kan gi akutt sykdom med høy dødelighet i atlantisk laks *Salmo salar L.* IPNV-arvematerialet består av to segmenter (A og B) av dobbeltrådet RNA som er pakket i en ikosahedrisk kapsid-struktur, omtrent 60 nm i diameter, og er uten ytre kappe. Segment B består av 2,784 nukleotider (nt) og koder for den RNA-avhengige RNA polymerasen, virus protein 1 (VP1). Segment A består av 3,097 nt og koder for to åpne leserammer. Den lengste åpne leserammen koder for et 107-kDA polyprotein som deles opp av virusets egen protease, VP4, og danner de strukturelle proteinene VP2 og VP3, der VP2 er med å bygge opp den ytre strukturen, IPNV kapsidet. Den korteste koder for VP5, som er et 15-kDa arginin-rikt protein (VP5).

Erfaringer fra naturlige sykdomsutbrudd og fra eksperimentelle smitteforsøk med atlantisk laks viser at dødelighet forårsaket av IPN ofte inntrer 3-8 uker etter at fisk er flyttet til sjøvann. Utbrudd har også vært registrert både før og etter denne perioden. Atlantisk laks som overlever et sykdomsutbrudd kan i ettertid bli bærer av viruset uten symptomer. Reaktivering av IPN har vært demonstrert både under naturlige og eksperimentelle betingelser. Trolig er det reaktivering av IPNV som er ansvarlig for en god del av IPN-utbruddene i oppdrettsnæringen. Fisk som er bærere av IPNV er fri for symptomer og med lavt eller ikke detekterbart nivå av virus. En bærertilstand er ikke en konstant tilstand. Det er antatt at i situasjoner der fisken blir utsatt for stress vil dette kunne påvirke bærertilstanden og føre til økt nivå av virus. Dette kan igjen føre til utbrudd av IPN.

Resultatet av et IPN utbrudd varierer mye, alt fra lav til høy dødelighet. Mye av denne variasjonen er knyttet til de forskjellige virusisolaters sykdomsfremkallende (virulens)

egenskaper. Denne avhandlingen beskriver hvordan molekylære forskjeller i feltisolater er med på å gi virusene forskjellige virulensegenskaper. Ved å samle inn feltisolater, bestemme nukleotidsekvensen til deler av VP2-genet til de forskjellige isolatene og utføre smitteforsøk med de ulike virusisolatene har vi vist at spesifikke aminosyrer er med på å bestemme virusisolatets virulensegenskaper.

Fisk som er bærer av IPNV er symptomfrie med lavt eller ikke detekterbart nivå av virus. Det var derfor nødvendig å utvikle en metode for detektering av virus som var mer sensitiv enn den som tidligere var brukt. En real-time PCR for detektering av transkriptet av VP2-genet ble derfor utviklet. Referansegener ble validert og metoden ble optimalisert for å gi en metode med høy sensitivitet og nøyaktighet.

Når laksen møter IPNV for første gang er resultatet av infeksjonen avhengig både av virulensegenskaper hos viruset og hvor effektivt det medfødte antivirale immunforsvaret til fisken er. Makrofager er viktige celler i det medfødte immunforsvaret, men de representerer også et reservoar for mange persisterende virus, deriblant IPNV. I dette arbeidet har vi smittet smolt i ferskvannsfasen med virus med høy og lav virulens. Vi har sett på forskjeller i utviklingen av sykdom mellom virus isolatene ved å måle mengde virus og se på dødelighet i sjøfasen. Vi har også undersøkt om det antivirale forsvaret til laksen reagerer forskjellig mot de ulike virusisolatene.

List of papers

Paper I. K. Julin, S. Mennen, A.-I. Sommer, (2013). Study of virulence in field isolates of infectious pancreatic necrosis virus obtained from the northern part of Norway. Journal of Fish Diseases 36(2):89-102

Paper II. K. Julin., L.-H. Johansen, A.-I. Sommer, (2009). Reference genes evaluated for use in infectious pancreatic necrosis virus real-time RT-qPCR assay applied during different stages of an infection. Journal of Virological Methods 162: 30–39

Paper III. K. Julin, L-H. Johansen, A.-I. Sommer, J. B. Jørgensen (2014). Persistent infections with infectious pancreatic necrosis virus (IPNV) of different virulence in Atlantic salmon *Salmo salar L*. Journal of Fish Diseases Accepted 03.09.2001

Errata

Paper II. The GenBank accession numbers of the two virus isolates have switched places, the correct is S-IPNV/FS12-01 (GenBank accession no. **DQ536090)** and S-IPNV/SH96 (GenBank accession no. **DQ536091**)

Abbreviations

Ala	Alanine
AP-1	Activator protein 1
Bcl-2	B-cell lymphoma 2
BF-2	Bluegill fry 2
CHSE-214	Chinook salmon embryo
CMS	Cardiomyopathy syndrome
CPE	Cytopathic effects
CpG ODN	CpG oligodeoxynucleotides
DI	Defective interfering
DNA	Deoxyribonucleic acid
dsRNA	double stranded RNA
DXV	Drosophila X virus
eEF1α	Transcription elongation factor 1α
eIF	Elongation factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
He	Hecht
h.p.i.	Hour post infection
HSMI	Heart and skeletal muscle inflammation
HSV-1	Herpes simplex virus type 1
IBDV	Infectious bursal disease virus
IFN	Interferon
IFNAR	IFN- α/β receptor complex
IL	Interleukin-
IPN	Infectious pancreatic necrosis
IPNV	IPN virus
IRF	IFN regulatory factor
ISA	Infectious salmon anemia
ISG	IFN stimulated genes
ISGF3	A heterodimer of STAT1 and STAT2 associates with IRF9
ISRE	IFN stimulated response element
Ja	Jasper
JAK	Janus-activated kinase
kDa	Kilodalton
LG	Linkage group
MABV	Marine birnaviruses
MDA5	Melanoma differentiation associated gene 5
mRNA	messeng RNA
Mx	Myxovirus resistance
NS	Non-structural protein
NF-κB	Nuclear factor-kappa B
NK cells	Natural killer cells
nm	Nanometer
OAS	2',5' oligoadenylated synthase
OIE	OIE-World organisation for animal health
ORF	Open reading frame
PAMPs	pathogen-assosiated molecular patterns
PCR	Polymerase chain reaction
PD	Pancreas disease
pDC	Plasmacytoid dendritic cells
PKR	Protein kinase R
PKZ	Protein kinase Z
Pro	Proline
PRRs	Pattern recognition receptor
pVP2	Precursor VP2
QTL	Quantitative trait loci
RdRp	RNA-dependent RNA- polymerase
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RPS20	Structural ribosomal protein S20
RT	Reverse transcription
RTG-2	Rainbow trout gonad cells
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT-PCR	Reverse transcription PCR
RT-PCR-ELISA	RT-PCR enzyme-linked immunosorbent assay
RT-qPCR	RT-quantitative PCR
STAT	Signal transducer and activator of transcription
Thr	Threonine
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor α
TYK	Tyrosine kinase
VHSV	Viral hemorrhagic septicaemia virus
VPg	Genome-linked protein
VP	Virus protein
VRG	Virus-responsive genes

Introduction

Infectious pancreatic necrosis (IPN)

History

Infectious pancreatic necrosis (IPN) that infects salmonids is an acute and serious disease that is distributed worldwide. IPN is a severe threat, economic and ecologically, to the aquaculture industry. The disease, previously known as acute catarrhal enteritis, was first identified in brook trout *Salvelinus fontinalis* in Canada in the 1940s [1]. In 1955, it was established that the disease was caused by a virus and the disease was named IPN based on lesions observed on the pancreas [2]. IPN virus (IPNV) was the first fish virus to be isolated from cell culture and was initially described in 1958 [3]. This prototype isolate was given the reference number VR299. In 1968, the virus that later on has been referred to as the Sp serotype based on the name of the town Spjarup in Denmark was isolated from rainbow trout *Onchorhynchus mykiss* [4]. IPN was first detected in Norway in 1971, while IPNV was isolated for the first time from healthy rainbow trout in 1975 [5]. The first clinical outbreak of IPN in Norway was diagnosed in 1985 in Atlantic salmon *Salmo salar L.* fingerlings [6] and the disease has been a problem in Norwegian fish farms ever since. The breeding company AquaGen have estimated the economically losses due to IPN to be 4 402 000 000 NOK each year in Norway.

Classification

IPNV is the prototype of the virus family *Birnaviridae*, genus *Aquabirnavirus*. The name "birna" highlights the most important features of these viruses. "Bi" signifies the bi-segmented nature of the viral genome (segment A and B) as well as its double-strandedness, while RNA implies the nature of the viral nucleic acid. The *Birnaviridae* contains three families. The other two families are infectious bursal disease virus (IBDV) and drosophila X virus (DXV). IBDV is an *Avibirnavirus* that infects young chicken while DXV is an *Entomobirnavirus* which is infectious to the fruit fly, *Drosophila Melanogaster* [7]. Most knowledge about the birnaviruses is based on studies of IBDV and IPNV [8, 9]. The abbreviation IPNV is used for aquabirnaviruses isolated from salmonids, or viruses that are able to induce IPN in salmonids. Aquabirnaviruses isolated from marine organisms form a

distinct genogroup and are designated marine birnaviruses (MABV) [10]. Aquabirnaviruses are distributed worldwide and can be divided into two distinct serogroups, A and B, based on results from cross-neutralisation assays. There are nine different serotypes (A1-A9) of IPNV within the A serogroup and it contains the isolates associated with disease in salmonids, the serogroup B contain only one antigenically unrelated aquatic birnaviruses [11, 12]. The A1 serotype contains most of the isolates from United States (ref. strain West Buxton (WB)), serotypes A2-A5 are primarly European isolates (ref. strain Sp, Ab, Hecht (He) and Tellina (Te)), serotypes A6-A9 occure in Canada (ref. strain C1, C2, C3, and Jasper (Ja)).

Comparison of the aquabirnaviruses at the genetic level leads to the identification of at least 6 genogroups corresponding to the 9 serotypes in the group A [13]. Thus, genogroup I corresponds to I1 (serotype A9, type strain Ja) and I2 (serotype A1, type strain WB, including most of the isolates from United States); genogroup II corresponds to serotype A3 (Ab); genogroup III corresponds to III1 (serotype A2, type strain Sp) and III2 (serotype B1, type strain TV-1); genogroup IV corresponds to serotypes A5 (type strain Te) and A6 (C1); genogroup V includes serotype A7 (strains C2) and A8 (C3) and genogroup VI corresponds to serotype A4 (He). An additional genogroup VII has also been proposed [14] (Figure 2).

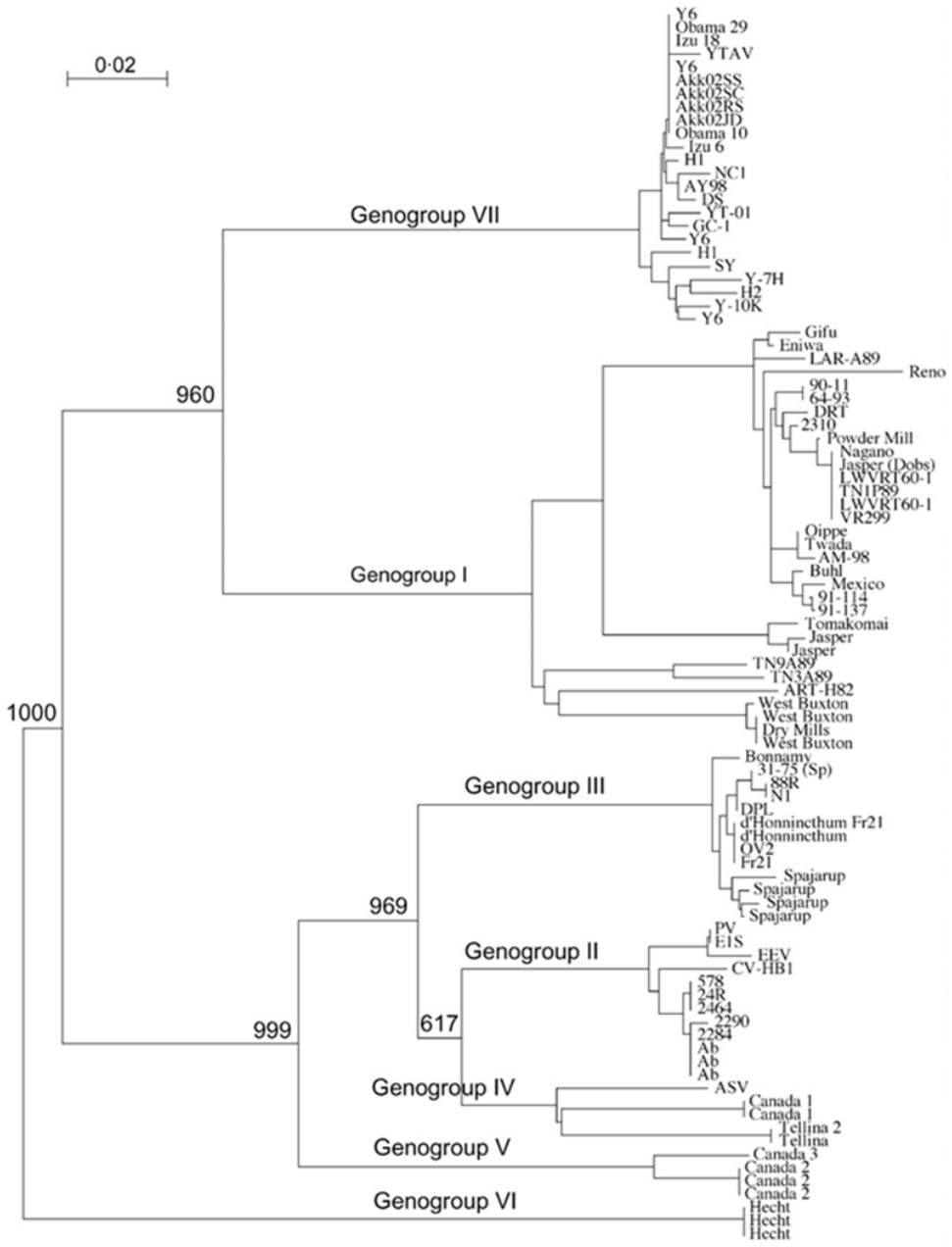

Figure 2.
Molecular phylogenetic tree based on nucleotide sequences of the VP2/VP4 junction region among 93 worldwide isolates of IPNV and other aquabirnaviruses [14].

Infectious pancreatic necrosis virus

The IPNV genome is a bi-segmented (segment A and B) double stranded RNA (dsRNA). It is packed in a medium-sized, non-enveloped, single-shelled T=13, icosahedral capsid 60 nm in diameter composed of 260 trimers of viral protein 2 (VP2) (Figure 1) [15-18].

Segment B encodes a non-structural protein VP1 that has helicase, protease and RNA-dependent RNA-polymerase (RdRp) domains [19]. The VP1 polypeptide is present in low copy numbers in the virion both as free and as a genome-linked protein (VPg). The VPg is attached to the 5' end of both genome segments by a phosphodiester bond [20]. VP1 has been shown to be guanylated *in vitro* and this VP1pGpG complex in turn serves as a primer for RNA synthesis [21].

Segment A encodes a precursor protein. This protein is cotranslationally cleaved by the viral encoded serine-lysine protease (VP4) releasing the proteins precursor of virus protein pVP2 and VP3 [22, 23]. The protease cleavage sites have been localised between amino acids 508 and 509 of the VP2-VP4 junction and between amino acids 734 and 735 of the VP4-VP3 junction [24]. pVP2 is further processed by the host cell proteases to form the mature outer capsid protein VP2 (amino acid 1-442 of the polyprotein) [25].

VP2 is the major outer viral capsid protein and it contains the antigenic regions responsible for induction neutralising antibodies in the host [26]. Residue 221 lies within the central variable domain of VP2 containing the major conformational epitopes recognised by neutralising monoclonal antibodies [27, 28].

VP2 makes up to 62 % of the virion protein by mass, while the VP3 polypeptide is the most abundant one. VP3 is a small protein, only 31-27 kDa compared to VP2 which is 57-54 kDa, VP3 contribute therefore only to 34 % to the mass of the virion protein.

Studies of IBDV have revealed that VP3 is a multifunctional viral component with several activities throughout the viral life cycle [29-32]. VP3 has been suggested to be the key organiser of the birnavirus assembly process, since it maintains critical interactions with other viral components [33]. VP3 is shown to bind VP1 and to self-associate strongly, indicating that it is a matrix protein [34], which implied that VP3 is an internal protein with several roles in organising the IPNV replication cycle.

Further a partly overlapping open reading frame (ORF) encoding an arginin-rich non-structural protein (VP5) has been detected in some viral strains. The VP5 protein has a start codon at nucleotide 112. The first stop codon in this ORF normally appears at nucleotide 511,

resulting in a 15-kDa polypeptide of 133 amino acids. The small ORF is produced in small quantities and is synthesised during the early replication cycles [35-37]. The existence of field strains lacking the VP5 protein initiation codon is also well documented [37, 38]. Strains with premature stop codons at nt 199, 427 and 496 has also been demonstrated [38, 39].

It has been shown that initiation of VP5 translation start at the second in-frame start codon and that the absence of expression does not influence virus growth and it is dispensable for viral replication both *in vitro* and *in vivo*.

VP5 has a Bcl-2 homology domain and Hong *et al.* [40] showed that VP5 is anti-apoptotic, delaying apoptotic cell death in the early replication cycle of IPNV infection. Contrary to this Santi *et al*. [41] failed to show anti-apoptotic activity of VP5. The biological function of IPNV VP5 remains to be determined, but it is still reasonable to believe that the protein has a specific function, particularly since the majority of the IPNV strains encode the protein [37].

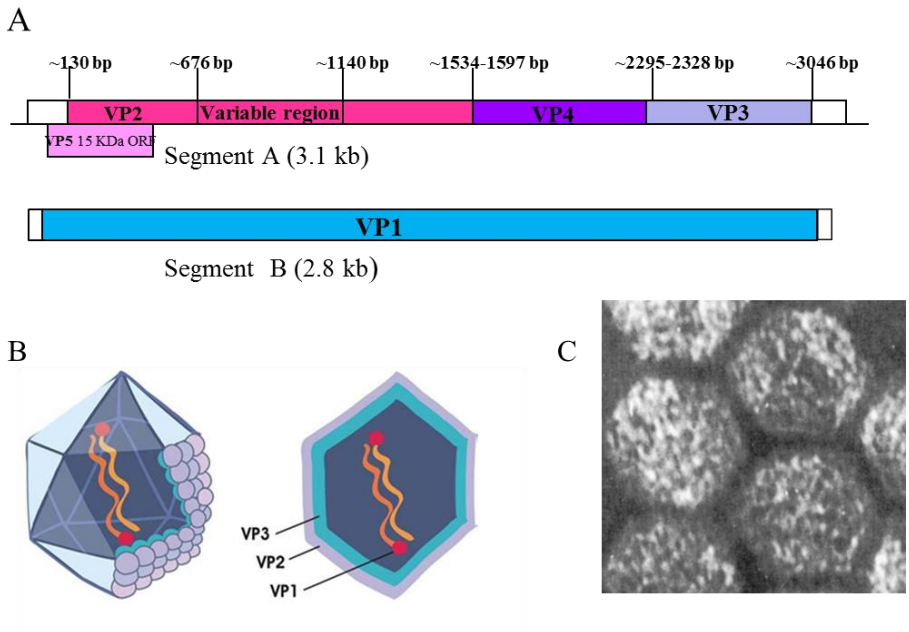

Figure 1.
A. Segment A and B of IPNV encode 5 proteins. Segment A is 3,097 nucleotides (nt) long and encode a 107-kDA precursor protein, which encode VP2, VP3 and VP4. A partly overlapping ORF encode VP5.
Segment B is 2,784 nt long and encode VP1 (94kDa).
B. Schematic diagrams of the IPNV virion.
C. Electronmicrography of IPNV particles [42].

Virulence of IPNV

Sequence analysis of different IPNV strains has revealed more genetic variation in the VP2 coding region compared to the VP1, VP3 and the VP4 regions [38, 43] and VP2 region is thus used for phylogenetic analysis. Sano and co-workers [44] were the first to suggest that the virulence of IPNV is associated with Segment A. Specific amino acids at position 217, 286, [43], 199, 217, 221, 247, 500 [45], 217, 221, 247, 500 [38] has been associated with IPNV strains that lead to high mortality in fish.

Recently, it has been shown that threonine a residues at position 217 (Thr217) and alanine at position 221 (Ala221) are the major determinants of virulence in IPNV of the serotype Sp [38, 41]. Highly virulent isolates possess residues Thr217 and Ala221, moderate- to low- virulence strains have proline (Pro) 217 and Ala221. Strains containing Thr221 are almost avirulent, irrespective of the residues at position 217 [46]. The majority of the epitopes recognised by neutralising antibodies are located on VP2 [26-28, 47] and recent findings suggest that there are cross neutralising epitopes also outside those governed by residues 217, 221 and 247 [48]. Both VP1 and VP5 have been indicated to play a role in virulence of IPN. It has been implied that Sp strains of low virulence encode a 15-kDa protein, whereas more virulent strains often contain a truncated VP5 protein gene [38, 45, 49]. Santi et al. found VP5 to be dispensable for virus virulence which is not in accordance with the observations performed by Skjesol et al. [50] showing that VP5 inhibit interferon (IFN) signalling.

Replication

IPNV replicates in cell lines derived from a wide range of freshwater and marine hosts [7] including Chinook salmon embryo (CHSE-214), rainbow trout gonad (RTG-2) and Bluegill fry (BF)-2 cells. It has been shown that IPNV attaches specifically and non-specifically to CHSE cells, while only specific binding leads to a productive infection [51]. Studies have shown that shortly after adsorption, the virus is internalised into vesicular peripheral compartments of cells suggesting that the virus is internalised by receptor-mediated endocytosis [52]. VP2 is suggested to be the cell attachment protein of the virion [7] and a recent study revealed that IPNV binds specifically to membrane proteins of approximately 220 kDa in salmonid cells [53]. There is some controversy whether VP2 is a glycosylated

protein. Previous studies supports that VP2 is indeed a glycosylated protein which is relevant to viral attachment [54].

The virus replicates in cytoplasm and a single cycle of replication takes approximately 24 h in CHSE cells at 15º C [55]. IPNV replicates well in several cell lines derived from fish tissues [56] at temperatures ranging from 15 to 22 °C resulting in characteristic cytopathic effects (CPE) after two to three days [57]. Early after infection (2 and 4 h post infection; h.p.i.), a putative transcription intermediate is initially detected [58], while 4-6 h.p.i. viral mRNA can be identified [58] and viral specific polypeptides. Transcription of the positive strands of IPNV RNA follows a semi-conservative strand-displacement mechanism [59-61]. The reaction is primed by the viral VP1 [7]. The level of virus specific RNA synthesis is maximal at 8-10 h.p.i. and completely diminished after 14 h [7]. Viral proteins are synthesised in the same relative proportions throughout the infective cycle [62]. Immediately after synthesis, non-infectious, immature viral particle appear. These pro-virion particles are detected simultaneously with the viral dsRNA in infected cells suggesting that viral assembly occurs simultaneously as dsRNA replication indicating a temporal and spatial coordination between these events [63]. Subsequently, through proteolytic cleavage of the viral precursors within the capsid the mature infectious virions are made [63]. It has been reported an interaction between the viral RdRp VP1 and VP3 and that the interaction is independent of the presence of dsRNA [34]. VP3 has in addition been reported to interact with itself and with dsRNA, which implies that that VP3 is an internal protein with several roles in organising the IPNV replication cycle. At16 h.p.i, viral progeny are detected implying that complex formation takes place before viral particles appear and thus plays a role in viral assembly [34].

It is uncertain how the viral progeny is released from the cells, since no exocytosis or other virus releasing mechanisms have been observed [64]. Hong et al. [65] found apoptosis to precede necrosis in cultured fish cells infected with IPNV. Apoptosis is a genetically controlled cell suicide process in response to a variety of stimuli [66]. Apoptosis is part of the innate immune response to virus infection, limiting the time and cellular machinery available for viral replication. In asymptomatic infected fish this may be related to the ability of IPNV to hide or escape from the immune system through immune evasion involving inhibition of apoptosis.

Results from *in vitro* and *in vivo* studies of apoptosis give contradictory answers. There is a clear induction of apoptosis in salmon cells *in vitro* following IPNV infection [40, 67, 68], but this effect is unclear in tissue of IPNV-infected fish where usually only a small number of

cells showing apoptosis can be found [69]. Apoptosis is induced in CHSE-214 cells following IPNV infection and the so-called 'McKnight' cells associated with sloughing of mucosal cells in the intestines of juvenile salmonids suffering from IPN resemble apoptotic cells morphologically. Similar apoptotic bodies are in addition found in the liver of Atlantic salmon post-smolts suffering from IPN [49].

Persistent infections

Viral carriers are individuals which harbour a disease agent without manifesting signs or symptoms of disease and may distribute the infectious agents to susceptible species in the population. The carrier state is usually established in a high percentage of Atlantic salmon that survive an IPN outbreak and they are anticipated to become life-long carriers [70]. An IPNV carrier condition at low virus titre does not seem to have a measurable negative impact on the general fish health and it does not influence the mortality rates after secondary infections [71, 72]. Despite that the carrier fish mount a humoral immune response, a carrier condition is not stable and titre levels fluctuate over time from non-detectable to relatively high and are typically increased during periods of stress [73, 74].

In Atlantic salmon and rainbow trout IPNV carriers, the virus have been detected in many visceral organs and in leucocytes of blood and head kidney [75-79]. IPNV can multiply in adherent leucocytes isolated from carriers, despite it does not produce lytic infections [77]. Rønneseth et al. found it unlikely that the main IPNV replication occurred in Atlantic salmon head kidney leucocytes, since the percentage of infected cells was low [80]. In addition Johansen et al. [76] demonstrated that about 1 % of the total amount of head kidney leucocytes are infected with IPNV *in vivo*, while Munro et al. [81] found this number to be much higher.

IPNV is able to induce persistent infection in cell cultures [73], where the persistently infected cells contain and release virus at a low level. The cells are resistant to homologous superinfection and antibodies can cure individual cells [82]. A more recent study has on the other hand shown that vaccine generated antibodies are not sufficient to clear the persistent infections and vaccination do not prevent vaccinated fish from becoming viral carriers [83].

Hedrick [84] proposed that IFN and defective interfering (DI) viruses function either alone or in cooperation to prevent the cell line from cytocidal effects of infectious virus, while others have found that CHSE to a little extent support the replication of DI particles [82].

Santi et al. [41] has implied that strains of IPNV differ in their ability to establish a persistent infection and that the persistent IPN infection is independent of VP5 expression. These authors also suggested that Thr at VP2 residue 221 is a prerequisite for persistent infection, while isolates with Ala at this position do not establish a long-term persistent infection as efficiently as the avirulent virus (Thr221) [41]. Despite all the work done in this field, little is to date known about the molecular and immunological mechanisms involved in establishing the carrier state. Saint-Jean et al. found that the host IFN response may be involved in the maintenance of IPNV persistence [85].

Pathology

The clinical features and pathology of Atlantic salmon fry infected with IPNV is that it is darker in colour, showing in the surface water film or at outflows, making distinctive shimmering movements, whirling or lying on their side and hyperventilating. Whereas infected post smolts may be darker, but generally retain their silver colour. They hang in the water, may spiral with their head up or sink to the bottom where they eventually die. Visible symptoms include abdominal swelling and the presence of faeces trailing from the vent, with internal damage (viral necrosis) to the pancreas and thick mucus in the intestine, pale and friable liver and a pink flush over the pyloric caeca [86, 87]. The histopathological picture in young fry is severe necrosis of the pancreatic acinar cells, while endocrine and most of the limited fatty peripancreatic tissue is normal apart from some lipid necrosis. There is also necrosis of intestinal mucosa with variably intensity and the liver consistently show areas of severe focal or generalised necrosis [88] .

Epidemiology

The mortality of an IPN disease outbreak varies considerably, dependent on the fish species, age and physical condition as well as the virulence of the viral strain [43, 89, 90]. Despite that IPN was originally thought to be solely a disease of salmonid species, the virus have been isolated from a variety of fresh water and marine species, molluscs and crustacean throughout the world [11].

As the name implies, pancreatic tissue in the pylorus is the target for the virus and gets affected during acute IPN [91] and additionally intestine, liver and gastric glands are affected organs for IPNV [38, 92]. It is known that IPNV resides and multiplies within leucocytes isolated from head kidney [76-79]. While IPN formerly was a disease common during the first few weeks after start-feeding of fry and in fingerlings, it is now commonly diagnosed in post-smolts during their first months in seawater [93].

Persistently infected fish represent an major route of horizontal transmission of the virus [91, 94]. However, vertical transmission has not been proven for Atlantic salmon, but it is generally believed that IPNV might transmit through germline from parent to their progeny [95].

Control and vaccination

Virtually all marine farming waters in the North-East Atlantic, from Northern Norway to North-West France appear to be infected with IPNV albeit at different levels [96]. Carrier fish are found in most farms or sea sites and this makes control of IPN a challenging task [70]. Carrier fish commonly release virus in faeces and reproductive products increasing the risk of infecting wild fish stocks and of recurrence of IPN in the carrier population [97]. IPNV is in addition environmentally persistent and can survive for days and months in water and even in air [98].

A standardised cell culture method is used to screen for IPN and it is based upon isolation of the virus in tissue culture followed by immunological identification [99]. In addition, more sensitive RT-PCR protocols for IPNV detection have also been developed [100, 101]. Munro et al. [102] found that kidney sampling followed by PCR and cell-based viral titration was the most sensitive method for detection of IPNV carriers. Other methods used for IPNV detection are RT-PCR-ELISA [103], reverse transcription loop mediated isothermal amplification (RT-

LAMP) technology [104], RT-qPCR [105] and flow cytometry assay [106]. However, all these methods involve lethal sampling to obtain the kidney and in some cases, especially with regards to valuable broodstock it is desirable to be able to detect the virus in samples without killing the fish. A highly sensitive, non-lethal and specific RT-qPCR assay has been developed for detection and quantification of IPNV in rainbow trout. IPNV was detected in pectoral fin samples as early as 24 h post-challenge [107], which is desirable in cases where the fish should be kept alive. IPN has huge negative economic impact on the salmonid aquaculture and the development of more effective prohylactics is of high priority.

During the 1980s, the salmon farming industry in Norway experienced huge losses due to bacterial diseases and the use of antibiotics were above acceptable limits. Immersion vaccines based on formalin-inactivated broth cultures were quickly developed against the salmon *vibrio* disease, which gave good efficacy and dramatically reduced the use of antibiotics.

After a few years of further vaccine development, a multivalent vaccine composed of inactivated bacteria and IPNV formulated in an oil-adjuvant was the product of choice and commonly used in aquaculture [108]. The first commercial recombinant IPN subunit vaccine was introduced in Norway in 1995 and has been used throughout the salmon farming industry. The three routes commonly used for administering vaccines in fish include injection, immersion and oral, where injection is the most commonly used administration route [109]. The commercially IPNV vaccines currently available in Norway are multivalent oil-adjuvanted vaccines containing VP2 protein fragments expressed in *E. coli* [110] or formalin inactivated IPNV (PHARMAQ, Novartis). Despite good efficacy in laboratory trials and extensive vaccination, IPN is still a problem.

In 2007 and 2008, it was registered 165 and 158 IPN disease outbreaks in Norway, respectively. In 2009, the number of registered outbreaks peaked with 223, while in the following three years there was a slight decrease with 198, 154 and 119 reported outbreaks. The amount of IPN showed a considerable reduction in 2013, where only 56 outbreaks were registered (Figure 3) [111].

Breeding of Atlantic salmon genetic resistant to IPN is proposed to be the main reason for this positive development. It has been shown that the genetic constitution of the fish has a strong influence on the protective outcome of the vaccine. Both genetic selection and vaccination are powerful prevention strategies to control IPN in Atlantic salmon smolts, as vaccination is capable of providing additional protection even when the fish are partially resistant to IPN [112].

Several studies have showed the existence of genetically determined variations in resistance to infectious diseases [113, 114]. The Norwegian breeding company AquaGen have since 2001 implemented selection for increased genetic resistance of Atlantic salmon to IPN [115]. Salmon full-sibling families have been tested for IPN resistance since 1997, which has demonstrated that mortality range from 10 to 90 % between families [116]. The detection of quantitative trait loci (QTL) is an effective starting point for the application of marker-assisted selection. Identification of the exact region underlying the QTL may lead to fundamental knowledge of genetic regulation of viral disease resistance and of host-virus interactions in fish. The mapping of a major QTL affecting resistance against IPN has been demonstrated and was mapped to linkage group (LG) 21 [117, 118]. The autumn of 2009 AquaGen launched salmon eggs from brood fish, which were chosen based on the genetic marker for IPN resistance. The amount of IPN outbreaks has decreased from 2010 and this decline might be due to the introduction of the QTL salmon.

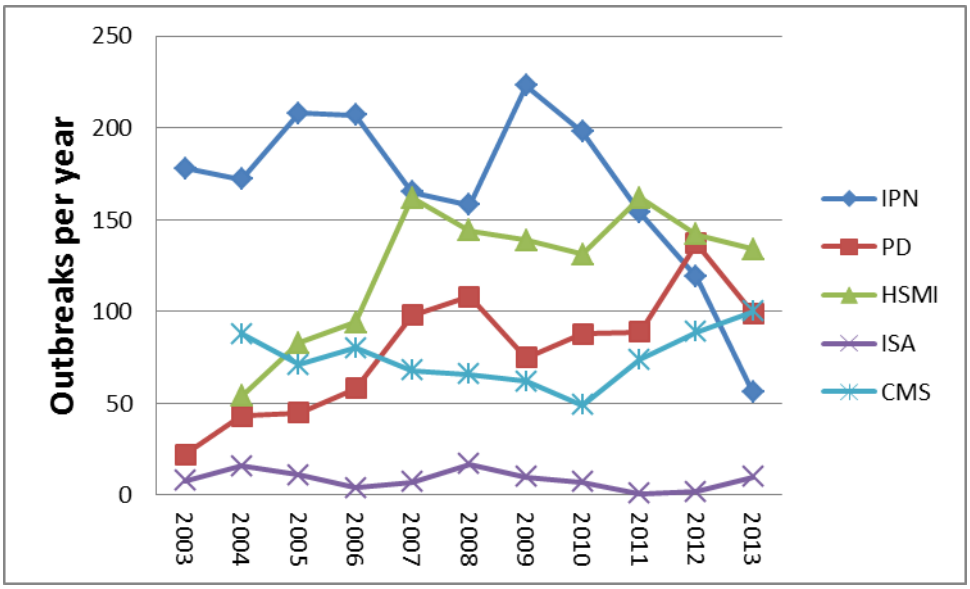

Figure 3.
Disease outbreaks of IPN, PD, HSMI, ISA and CMS reported in Norwegian fish farms in the year 2003-2013 [111].

The innate immune system

The innate immune system is often referred to as the first line of defence against pathogens (virus, bacteria, fungi and protozoa). It comprises the cells and effector proteins that defend the host from infection by other organisms. The innate immunity has limited diversity and depends upon germline-encoded receptors to recognise structures that are shared among many pathogens. The innate immunity specifically targets microbes and is a powerful early defence mechanism capable of limiting and even eradicating infection before adaptive immunity becomes active. In addition to providing early defence against infections, the innate immune response is necessary to activate the adaptive immune response against infectious agents. The first line of defence in innate immunity is provided by epithelial barriers and is the first physical and chemical barrier to pathogens. The epithelial surfaces is covered by mucus containing specialised cells and antibacterial peptides, all of which function to block or kill the entry of microbes [119]. Pathogens that cross these primary barriers are recognised by specialised immune cells with cellular receptors, which trigger the host cell to initiate an innate response. One of the innate immune responses is the IFN system, which provides a powerful and universal intracellular defence mechanism against viruses.

Sensing of viral infection

In order to survive an infection, it is critical for the host cell to detect the invading pathogen. There are several ways in which a cell can recognise a pathogen and multiple signalling pathways that can lead to transcription of antiviral effector molecules. Through cell surface-bound receptors and receptors localised in endosome membranes or in cytosol, the host cell can respond to pathogens at different localisations. Pattern recognition receptors (PRRs) recognise a diverse range of conserved pathogen-associated molecular patterns (PAMPs) shared by infectious disease agents. The main PAMPs of viruses is the viral genomes, mainly dsRNA and ssRNA, but also unmetylated CpG oligodeoxynucleotides present in ds viral genomes.

PRRs can be divided into extracellular/endosomal membrane-bound and intracellular receptors. The most well-known group of PPRs are Toll-like receptors (TLRs) and they are single, membrane-spanning receptors usually expressed in sentinel cells such as macrophages and dendritic cells. The TLRs consists of several members, however, it is mainly TLR3, 7, 8

and 9 that take part in recognition of viruses [120]. Several intracellular receptors for detection of viral nucleic acids have recently been identified. The retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) are thought to be the main PRRs for detection of viral nucleic acids [121, 122].

Recognition of viral PAMP by PRRs-trigger signalling pathways eventually culminates in a strong activation of type I IFN transcription (Figure 4). The main viral PAMP, dsRNA, are recognised by TLR-3, RIG-1 and MDA5 leading to the activation of a signalling cascade involving activation of the transcription factors IFN regulatory factor (IRF)3, IRF7 and nuclear factor-kappa B (NF-κB). These transcription factors then translocate to the nucleus and together IRF3/7 and NF-κB strongly up-regulate the expression of type I IFNs.

Interferons

Interferon's was initially discovered in 1957 and named due to their ability to "interfere" with viral replication and to induce an antiviral state in virally infected cells [123]. Since then, the importance of IFNs has been more elaborated. For example, knockout mice defective in IFN signalling quickly succumb to all kinds of viral infections [124]. Likewise, humans with genetic defects in IFN signalling die of viral disease at an early age [125]. So the type I IFN is crucial for the innate immune systems fight against viral infection. There are three types of IFN described in mammals; IFN-α/β (type I), IFN-γ (type II) and the more recently described IFN-λ 1/2/3 (type III). Mammalian IFN-α/β belongs to a multigene family encompassing several subtypes (IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-κ, IFN-τ and limitin) [126]. Viral infection in animals directly induces IFN type I and III. Type II IFN-γ is mediated by T-cells, neutrophils and natural killer (NK)-cells stimulated by interleukin (IL) 12 and IL-18 [127, 128] and is principally involved in activation of macrophages and T cell differentiation. IFN-λ 1/2/3 are functionally similar to the type I IFNs, but use distinct receptors to mediate their antiviral activity [129]. IFN-λ1/2/3 show little homology to IFN-α, but like IFN-α/β the IFN-λ cytokines activate signal transducer and activator of transcription (STAT) 2 [130].

Binding of dsRNA to RIG-I and MDA5 activates the transcription factors IRF3 and NF-κB which strongly up-regulate IFN-β gene expression, which again triggers the expression of the transcription factor IRF7. IRF7 is responsible for a positive-feedback loop that initiates the synthesis of several IFN-α subtypes (Figure 4).

One of the main differences in mammalian IFN promoters is the presence or absence of a NF-κB-binding element. The NF-κB element in the IFN-β promoter is believed to be essential for an immediate early response to virus infection [131, 132], whereas IFN-α promoters lack the NF-κB element and are usually activated at a later time point.

Most nucleated cells produce IFN-β upon viral infection, while some cell types also produce IFN-λ. In addition, a specific subset of immune cells known as plasmacytoid dendritic cells (pDC) are specialised as high IFN producing cells, which secrete both IFN-α, IFN-β, IFN-ω and IFN-λ upon viral infection [133, 134].

IFN-α/β induces an antiviral state by binding to the IFN-α/β receptor complex 1 and 2 (IFNAR 1/2) that is ubiquitously expressed on the surface of cells [126, 135] (Figure 4). This activation of IFNARs result in tyrosine phosphorylation of STAT1 and 2 by Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) [136, 137]. A heterodimer of STAT1 and STAT2 associates with IRF9, forming the ISGF3 complex. These translocate to the nucleus, bind to promoter motives known as IFN-stimulated response elemants (ISREs) and induce the transcription of effector molecules called IFN stimulated genes (ISGs) [138].

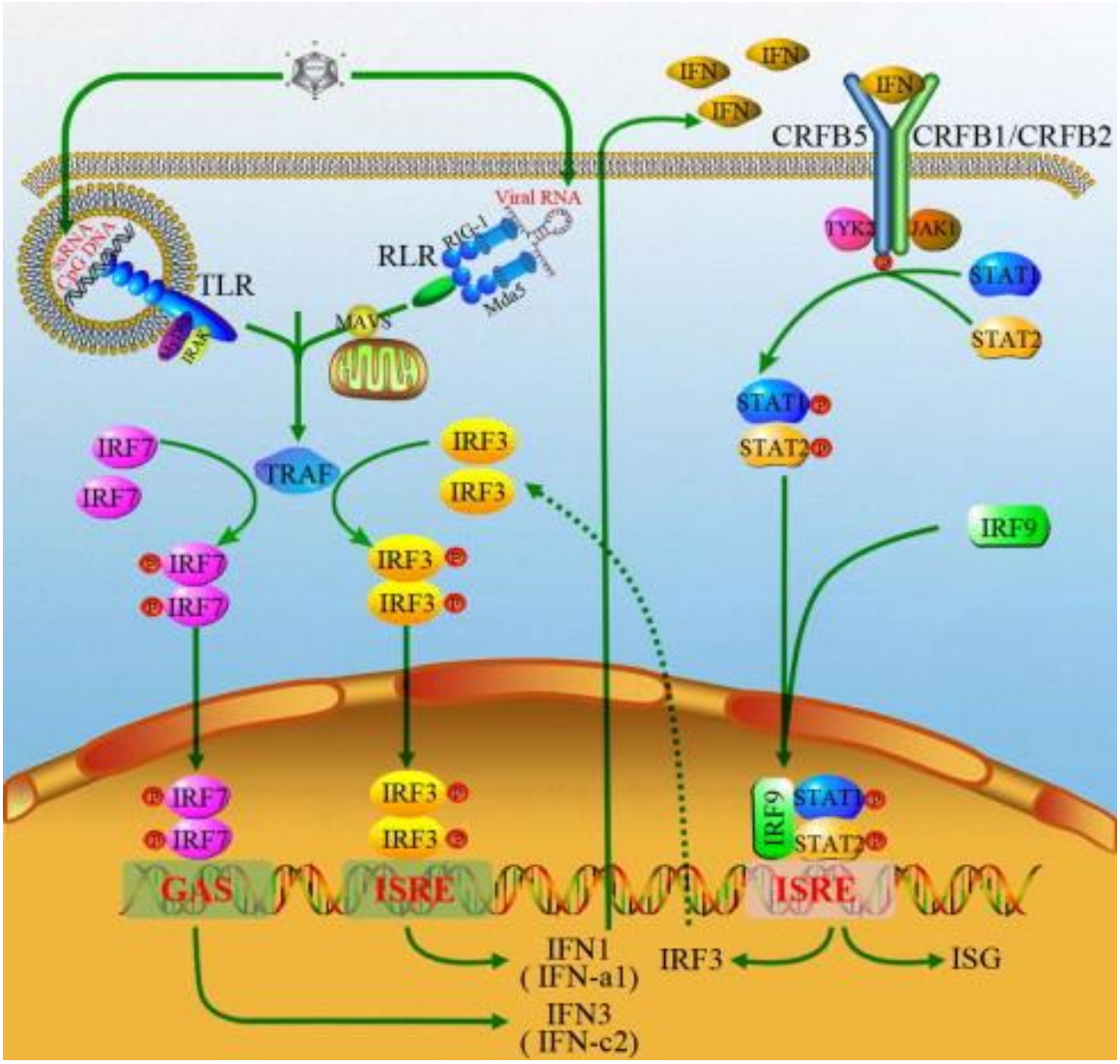

Figure 4. Diagram of the deduced anti-viral signalling of type I IFNs. Virus-infected cells recognise viral PAMP by PRR. Downstream kinases catalyse the phosphorylation of IRF3 or IRF7 respectively. These, in turn, form dimers, translocate into the nucleus, attach to the specific motif in the IFN-a1 or IFN-c2 promoter, and regulate the expression of type I IFNs in collaboration with activator protein (AP)-1 and NF-κB. The newly produced type I IFNs bind to two groups of receptors present on most host cells. After phosphorylation of STAT1 and STAT2 by TYK2 and JAK1 kinases in the cytoplasm, activated transcription factors form a dimer and cross the nuclear membrane associated with IRF9. Following this, the transcription factor complexes bind to ISRE motifs and the ISGs are induced to exert antiviral functions [139].

Interferon stimulated genes

The IFN signalling trough the JAK/STAT pathway and transcription of ISGs leads to an antiviral state in host cells. It is believed that more than 100 ISGs contribute to the antiviral properties of IFNs [140-142]. However, few ISGs have been fully characterised in mammals such as the dsRNA-activated protein kinase R (PKR) [143], the myxovirus resistance gene (Mx) [144], Viperin [145], 2',5' oligoadenylated synthase (OAS) [146] and the ubiquitin-like Interferon stimulated gene 15 kDa protein (ISG15) [147].

PKR

PKR is a serin-threonine kinase with multiple functions in the control of transcription and translation [148] and play a critical role in the antiviral defence mechanism of the host. PKR contain two conserved dsRNA-binding motifs with no sequence specificity required for dsRNA to bind to PKR. PKR is normally inactive, but by binding to dsRNA, it undergoes autophosphorylation and subsequent dsRNA-independent phosphorylation of substrates. The antiviral effect of PKR is due to its phosphorylation of the initiation factor elongation factor (eIF)-2α [149, 150]. This phosphorylation results in the formation of an inactive complex between eIF-2α and eIF-2β, thereby leading to reduced translation initiation and reduced protein synthesis. This inhibits viral replication, but also the cells normal ribosomal function which may lead to apoptosis of the cell [150].

ISG15

The ISG15 protein has high homology to ubiquitin and functions as a protein modifier through covalent conjugation (ISGylation) to more than 200 known proteins, many of these involved in innate antiviral immunity. It was the first ubiquitin-like protein to be discovered [151, 152]. ISG15 contains two ubiquitin-like domains and a C-terminal LRGG motif, where the latter is utilised to conjugate target proteins as a response to viral infections [151, 153]. ISG15 is among the most highly expressed proteins after treatment with IFN-β and expression is increased as early as 2 h following stimulation of mammalian cells [153, 154]. The strong and rapid induction of ISG15 during infections implies an important antiviral role against both RNA and DNA viruses in innate immunity. The importance of ISG15 in antiviral defence is

illustrated by the fact that ISG15−/− mice are deficient in their ability to respond to influenza B virus, herpes simplex virus type 1 (HSV-1) and Sindbis virus infection [155].

Mx

Among the known interferon-induced antiviral mechanisms, the Mx pathway is one of the most powerful. Studies of animal model have established that Mx alone is sufficient to block viral replication in the absence of any other IFN-α/β inducible proteins [156, 157]. Mx proteins belong to the dynamin superfamily of large GTPases. Mx self-assembly and have direct antiviral activity by interfering with viral transcription by inhibiting viral RNA polymerases [149]. In general, Mx proteins were found to bind to essential viral components and to block their function. The Mx protein accumulates in the cytoplasm of the IFN-treated cells and block viral replication soon after cell entry. They inhibit a wide range of viruses and the Mx protein appears to interfere with viral replication at different stages depending on host cell and virus combination. Gene expression is rapidly induced in virus infected cells through the action of virus-induced IFNs. In the absence of IFNs, the Mx gene have a constitutive low expression making Mx transcripts or protein an excellent marker for type I IFN activity [158]. Mx is strictly regulated by type I (α and β) and type III (λ) IFNs [159, 160].

The innate immune defence in Atlantic salmon

In evolutionary terms, teleost's are the first group with the basic aspects of the immune system of higher vertebrates. Despite that the fish immune system are more primitive compared to mammals, there seem to be more similarities than differences. It is important though to keep in mind that fish immunology cannot be considered a homogenous system. The number of fish species is much greater than the number of mammalian species and the evolutionary distance between different teleost families are considered far larger than between mammals.

One of the main differences between the mammals and the teleost's immune system is the difference in hematopoietic organs. The head kidney and spleen constitute as the main hematopoietic organs of teleost's equivalent to the mammalian bone marrow and lymph nodes [161]. The posterior kidney participates in the production of blood cells, while also performing renal functions. Head kidney and spleen are important in the trapping and clearance of pathogens.

Vaccination has successfully brought the main bacterial infections in salmonid farming under control and the use of antibiotics is today almost negligible in this sector. However, vaccination has proven less efficient to limit the amount of viral infections and against some viral diseases there are still no commercial available vaccines.

As a consequence, understanding the fish immune response against viruses is critical to develop more efficient prophylactic and preventive control measures. Numerous reports suggest that the IFN signalling pathway is well conserved among salmonid fish and higher vertebrates. The described IFN signalling elements in mammals are also present and functional in salmonid fish [162-169]. There are many pathways leading to the production of IFN and subsequently many intervening pathways leading to the activation of ISGs. The viruses on the other hand have evolved multiple strategies to inhibit the IFN antiviral response. These mechanisms of action are not well understood, but it is becoming apparent that also fish viruses have developed diverse strategies to counteract the host defence. It is demonstrated that many viruses dedicate parts of their genome to encode gene products able to counteract components of the IFN pathway [137]. They have strategies to suppress IFN synthesis, bind and neutralise secreted IFN molecules, block IFN signalling or inhibit the action of IFN-induced antiviral proteins.

Sensing of viral infection

The generation of antiviral responses during infection requires a rapid viral sensing by PRRs and homologes/orthologs to these receptors are identified in fish. A number of PRRs have been identified in Atlantic salmon including RIG-I [170], TLR3 [171], TRL8 [168] and TRL9 [172] and TRL22. The latter is a dsRNA-specific PRR which has similar functions as TLR3 and found exclusively in lower vertebrates [172, 173]. MDA5 have so far not been described in Atlantic salmon, but have been identified in other species including rainbow trout [167, 174-176] and the gene from rainbow trout has also been used for studies of Atlantic salmon . PKR has been cloned in Atlantic salmon, but has not yet been functionally characterised [177].

Expression profiling of Atlantic salmon challenged with IPNV showed that TLR8, 9, RIG-I and MDA5 were up-regulated and that for most of the fish the immune gene expression mirrored the virus levels in pancreas and head kidney [39]. PKR on the other hand was unexpectedly down-regulated at the studied time points in the challenge experiment.

Interferons

Teleost fish are armed with a well-functioning innate antiviral defence mechanism based on IFN production [178, 179]. The first fish IFN sequences (from zebrafish, pufferfish and Atlantic salmon) were published in 2003 [180-182]. The type I IFNs from fish and mammals are very different. Despite that fish type I IFNs have amino acid sequences more similar to mammalian IFN-α/β, type I IFN genes of teleost fish possess a gene structures similar to IFN-λ [179-181]. These findings have inspired the hypothesis that both fish IFN and IFN-λ genes possess the ancestral IFN gene structure, whereas IFN-α/β is thought to have arisen by retrotransposition insertion of an ancestral IFN cDNA into the genome during the evolution of tetrapods [182].

Atlantic salmon possesses four different subtypes of type I-like IFN and these are encoded by several genes: IFNa (two genes), IFNb (four genes), IFNc (five genes) and IFNd (one gene) [171, 183], where IFN a, b and c according to their signalling pathway would be ortholougs to the human IFNα/β [171]. IFNa1 and IFNa2 are believed to be the most important in the early phase of infection, similar to human IFN-β [171]. However, Chang et al. [184] recently demonstrated that IFNd and IFNc plasmids induces systemic up-regulation of antiviral genes

in live Atlantic salmon indicating that the distinct roles of the different salmon IFNs are not yet revealed. Salmon IRF1, IRF2, IRF3 and IRF7 are important regulators of the Atlantic salmon IFNa1 promoter and they may have similar roles as their mammalian counterparts, which are key regulators of virus induced IFN-α/β transcription [185]. The salmon IRFs have expression properties similar to their mammalian homologs, except for IRF3, which is up-regulated by stimuli known to induce type I IFN or IFN inducible genes, while mammalian IRF3 is ubiquitously expressed [185]. IRF1 have a early and high expression upon virus associated stimuli which indicate that it is of significant importance in innate virus signalling and defence [185].

All the different IFNs are constitutively expressed in head kidney and spleen and reveal a distinct inducible expression pattern depending on which RNA-ligand that is used [183].

Numerous studies have demonstrated that the level of IFNa transcripts are induced in spleen and head kidney of salmon infected with virus [69, 186, 187] and this elevated expression is also found in head kidney of carrier fish [188]. However, the antiviral response is not sufficient to eliminate the virus.

The IFN are expressed at very low levels in unstimulated cells and organs of untreated fish, however both IFNa, IFNb and IFNc are strongly induced upon stimulation with double-stranded RNA poly I:C in salmon head kidney leucocytes [171].

IFNa1 and IFNc have been shown to possess strong and similar antiviral activity against IPNV, while inducing similar transcription levels of antiviral genes in cell lines. The IFNb also possess antiviral activity however at a lower level and delayed compared to IFNa1 and IFNc whereas IFNd show no antiviral activity against IPNV in salmon cells line [183].

Slightly different results were obtained *in vivo*, where IFNa1 was induced by two isolates (high and low virulent), while IFNb and c was not up-regulated and IFNc was even slightly down-regulated by the virulent isolate [39]. For both viral isolates a major up-regulation of the IFN-induced gene Mx was found.

IFNa1 and IFNa2 have been shown to provide protection against IPNV in salmonid cells [180, 189], whereas other studies of IPNV infected cell lines have shown inhibition of IFN signalling [50, 85, 190, 191].

It has been suggested that type I IFN is able to protect cells from IPNV infection either directly or indirectly via the production of type II IFN [192].

During persistent infections the damage of the infected cells or their functions must be limited, so the virus must be able to balance the host immune system. It has been suggested

that viral evasion of the IFN system could contribute to *in vitro* viral persistence and development of healthy carrier fish [85].

Pre-treatment with IFN-a1 delays the maturation of pVP2 into VP2 and thereby the synthesis of viral protein is inhibited by IFN [50, 193]. While IPNV is able to inhibit IFN signalling in host cells when treated with IFN after infection, which then again lower the Mx induction in these cells [50, 190, 191]. This suggests that IPNV encode proteins that interfere with the establishment of an antiviral state normally induced by IFN, or counteract the antiviral activity of ISGs. Skjesol et al. [50] also found VP4 and VP5 to be the most probable candidates responsible of interfering with the IFN-signalling pathway in salmon.

Interferon stimulated genes

Among the ISGs that exist in mammals, the best described in salmonids are Mx, ISG15, viperin and PKR. IFNa1, IFNb and IFNc induce several ISGs including Mx, ISG15, ISG58, viperin and PKR as the antiviral response against IPNV, whereas IFNd neither display antiviral activity against IPNV nor induce Mx protein [183]. And there are found high correlation in expression of virus-responsive genes (VRG; genes with specialised antiviral response and other genes that are activated in response to viruses) and IFNa which is in line with the important role of IFNs in orchestration of antiviral responses [194]. It is thus an interesting observation that VRGs were up-regulated only in fish infected with a high virulent IPNV and not in fish infected with a low virulent IPNV [39].

Production of ISGs can be induced independent of IFN signalling [195, 196]. However, IPNV is unable to initiate expression of the antiviral Mx protein by itself in CHSE cells and is therefore considered a poor inducer of the Mx promoter [50, 190, 193].

Mx play a major role in protection against viruses and the wide spread use of this gene as a marker for IFN activity both *in vitro* and *in vivo* experiments has resulted in a considerable amount of data on its induction [69, 197-201]. The Atlantic salmon Mx gene are expressed as three isoforms [202], where Mx1 and Mx2 have similar expression pattern and are localised in cytosol, while Mx3 have a sub-cellular localisation. Mx gene induction can be found in all fish tissues following viral infection, revealing a wide spread and systemic release of IFN.

IPNV is strongly inhibited in salmonid cells expressing high levels of Mx proteins after treatment with IFN [180, 193, 201]. Furthermore, the Atlantic salmon Mx protein has been

shown to directly inhibit IPNV protein synthesis [203], which confirms that Mx1 protein possess antiviral activity against IPNV [180].

IPNV induce Mx both *in vitro* and *in vivo*. IPNV was found to suppress activation of the Mx promotor in RTG-2-P1 and TO cells [190, 193] whereas IPNV induced IFN-transcripts in RTG-2 cells and IFN-like activity in both the fibroblast-like RTG-2 cells and Atlantic salmon macrophages [190, 204]. Earlier a link between Mx protein expression and inhibition of IPNV has been shown *in vitro* [193, 201, 203, 205], while *in vivo* there is no clear correlation between the expression of antiviral genes and protection [39].

It is known that head kidney leucocytes express the antiviral Mx gene following infection with IPNV *in vitro* [206]. Importantly, the IPNV used in these experiments where of different origin. These data suggest that IPNV's affects the IFN system in a cell type-dependent manner, which is dependent of the virus serotype and/or level of virulence.

IPNV induces both IFN and Mx in Atlantic salmon *in vivo* [186]. Mx is highly induced after challenge with IPNV and the level of induction of Mx differ between the IPNV isolates [39]. The Mx induction was both higher and earlier in fish challenged with a high virulent IPNV isolate compared with fish infected with the low virulent isolate.

The expression of the Mx gene is characterised by extremely high individual variation among fish challenged at the same time point and in contrast to other immune genes the expression of Mx seem to stay up-regulated compared to the control group with uninfected fish [187].

A gene coding for both ISG15 [207] and PKR [177] has been cloned in Atlantic salmon, but the detailed function of them are scarce. Also antiviral proteins specific to fish have been revealed, one of which is the PKR-like eIF2α-kinase protein kinase Z (PKZ) [208]. PKZ encodes proteins with two Zα binding motifs instead of dsRNA-binding motifs found in PKR. In Atlantic salmon cells PKZ show up regulation by IFN, and PKZ phosphorylate eIF2α *in vitro* [208].

Cytokines in Atlantic salmon

An inflammatory reaction is critical to the efficiency of the innate responses to any type of infection including viral infections [209]. It is characterised by the systemic release of specific cytokines such as IL-1β, tumor necrosis factor (TNF)α, and chemokine's such as IL-8 to the site of inflammation. Many of the inflammatory cytokines have been identified in salmonid fish.

IL-1β and IL-8 transcripts are constitutively expressed in spleen, head kidney and gills of all fish [188, 210]. Some have demonstrated that IL-1β is produced in head kidney in response to the IPNV infection [188], while others [186, 187] found IL-1β not to be or only weakly induced by IPNV infection.

The expression of IFNα1 and the anti-inflammatory cytokine IL-10 increase in head kidney and spleen of IPNV infected salmon in response to the infection [187].

Also persistently infected salmon showed an up-regulation of IL-10 expression, whereas the lack of induction of IL-1β and IL-8 seem to be a common feature of acute and chronic IPNV infection in salmon. The increased expression of IL-10 in IPNV-infected salmon, accompanied by the absence of IL-1β and IL-8 indicates that the virus triggers a clear anti-inflammatory response that may be part of the viral mechanisms to establish persistence [188, 211].

TNFα is a cytokine involved in apoptotic processes as well as in the activation of numbers of other cytokines. In particular, the capacity of IPNV to activate TNFα in connection with induction of apoptosis in some cell systems has been reported [212]. TNFα is often associated with type I IFN production following viral infection. McBeath et al. demonstrated that Atlantic salmon induce type I IFN response, however IPNV infected salmon did not induced expression of TNFα [186].

Objective of the study

The objective of this work was to study genetic differences among IPNV strains isolated from farmed salmon and how these differences influence the host viral carrier condition and the antiviral immune response.
The sub goals were:

- Genetic characterisation of virulence in field isolates of IPNV.

- Develop, optimise and validate a RT-qPCR for IPNV quantification and to use this method for viral detection during different stages of IPNV infection.

- Use a head kidney leukocyte *ex vivo* model to study how difference in IPNV virulence influences the ability to establish a persistent infection, and whether immune stimulation of IPNV carrier cells may limit the infection.

- Identify if there exists genotypic pathogenic properties of selected IPNV strains that may activate or evade antiviral responses**.**

Discussion

Several IPNV field isolates was gathered from disease outbreaks at different locations in the Northern part of Norway. The IPNV were isolated and genetically characterised (Paper I). In an IPNV carrier condition the virus level is commonly below the detection limit of the traditional micro-titration assay and necessitates the development of a more sensitive assay. RT-qPCR is a powerful and reproducible method for the detection and quantification of RNA with a broad detection range and a high throughput capacity. However, to obtain reliable results optimisation of the RT-qPCR assay and validation of the reference genes are essential (Paper II).

In paper III, Atlantic salmon fry were challenged with two different IPNV isolates with either a high or a low virulence motif in the gene coding VP2. To follow virus replication and subsequent immune response an IPNV challenge was performed. The challenge was done in the freshwater phase to establish a persistent IPNV infection, while viral amounts were measured during both the fresh and the seawater phase. Simultaneously, measurements of Mx gene expression, which is used as an indirect indicator of the type IFN response, were used to study the effect of the different IPNV strains on the antiviral response. Further *ex vivo* head kidney leukocytes derived from salmon infected with the two viral isolates were treated with a known immune stimulant (CpG DNA) to study the effect on Mx expression.

Genetic characterisation of IPNV virulence

Before this project was initiated (2001) little was known of what determined that some IPNV isolates resulted in disease outbreaks with high mortality, while others gave low mortality. In paper I, the main goal was to determine by sequence analyses of the viral strains followed by challenge experiment with different IPNV Sp isolates the mortality rates and which virulence traits could be detected among the isolates. In an earlier study, Sano et al. [44] had generated a reassortant virus between virulent and avirulent strains of two different serotypes and demonstrated that the virulence of IPNV is associated with segment A. Variation in virulence described by use of challenge experiment and serological classification has not only been found between serotypes, but also within the same serotype [90, 213]. Nucleotide sequence analyses have revealed that VP2 might be the major determinant of virulence [43]. With this in mind, collaboration with the Fish Health Services was initiated and field samples from

disease outbreaks of IPN were collected from different locations in the Northern region of Norway. Pyloric tissue was collected from fish where IPNV had been identified either by IPNV co-agglutination test by veterinary services, or by immunohistochemistry.

The IPNV strains (n=18) were isolated and a partial sequence of VP2 was sequenced (578 bp). The isolates showed very little genetic variation, although all sampled fish came from different smolt producers, farms and locations. However, it was detected variations in amino acids 217, 221, 247 and 252 in VP2. A challenge experiment was performed and the results were compared with mortality from the field outbreak. Fourteen of the isolates had residues Thr-Ala at position 217 and 221 which correlated with high virulence, while three had the Pro-Ala which correlated moderate to low mortality and one isolate had a mix between the two types. Field isolates of IPNV has been collected from different parts of the country and when results were compared there were few differences in amino acid sequence [38, 45].

Aquaculture of farmed salmon is important in many countries worldwide and comparable studies has also been performed in Scotland, Ireland, Turkey, Chile, Iran, Australia, Mexico, USA, Korea and Spain [12, 214-224]. The isolates from Scotland, Ireland, Turkey, Chile, Iran and Australia were all classified as belonging to the Sp serotype showing that this serotype has diverged broadly geographically.

It is now a common understanding that viruses of the Sp strain encoding Thr217-Ala221 are highly virulent, while isolates encoding Pro217-Ala221 are moderately virulent. The Thr217-Thr221 isolates results in low virulence and Pro217-Thr221 isolates can be classified as avirulent. However, conflicting data have recently been reported which described strains with Pro217-Ala221 to be highly virulent both in experimental conditions and in field [214-216]. This support the notion that viral, host and environmental factors as well as amino acid residues influence pathogenicity [89, 90, 225, 226].

For the time being it is not known why these molecular differences have such a huge impact on the virulence of IPNV. However, there have been some speculations of which features the amino acids 217 and 221 possess that influence such differences between various virus isolates. Structural analysis of the VP2 subviral particle (SVP) shows that residues 217 and 221 are located in close proximity on the P-domain of the VP2 capsid [227]. It is thus suggested that the Thr217-Thr221 residues give an outwardly projecting hydrogen bond, while Pro217-Ala221 give an inwardly projecting hydrogen bond. As a consequence, the Thr217-Thr221 may have a higher binding capacity to host cell receptors than Pro217-Ala221, which result in higher host cell invasion and intracellular replication of high virulent

IPNV strains. This theory is supported by structural studies performed on other viruses, which found that substitution of amino acids reduced the viral binding avidity [228, 229]. However, to fully understand the mechanism behind these molecular differences and how this influence virulence there is an indispensable need for detailed molecular studies of the virus-host interactions.

Viruses tend to adapt their virulence to changes in the host and this is especially common for RNA viruses. This can be explained by the absent proofreading activity in RNA virus polymerases resulting in a constant generation of new genetic viral variants. It is an unquestionable fact that RNA virus populations exist as swarms of mutant genotypes and that these viruses readily adapt to changing environmental conditions. Therefore, the high mutation rate of RNA viruses is the major factor responsible for their enormous adaptive capacity in contrast to viruses that are composed of genomic DNA with an intact proofreading activity. Since 2001, the Norwegian breeding company AquaGen has implemented selection of Atlantic salmon with increased genetic resistance to IPN [115]. An interesting question is whether this selection has contributed to adaption of the genotypic pathogenic properties of the IPNV over the years?

The IPNV N1 strain was isolated from Atlantic salmon during an IPN outbreak in the 80s in Norway [42]. The N1 isolate encode the motif Pro217-Thr221 (classified as avirulent) and in earlier bath challenge experiments with this isolate mortality has been obtained (A.-I. Sommer, personal communication). In contrast, when the N1 isolate later on was used in bath challenges, it gave no mortality (own data, results not published). These results could indicate that breeder's attempt to select for salmon that are resistant to IPNV has been successful. However, outbreaks of IPN are still a problem in the salmon farming industry even though the salmon are more resistant against IPNV. In the 18 field isolates that we collected all the isolates had amino acid sequence differing from the N1 isolate (Paper I). This indicates that when salmon with increased resistance to IPN was implemented, the virus also adapted to these changes. However, it also has to be mentioned that other not yet identified viral motives throughout the IPNV genome could be responsible for the conflicting mortality results obtained with virulent and avirulent strains.

IPN has for decades been recognised as the most common disease and received much focus due to its huge negative economic impact on Atlantic salmon production in Norway and Scotland [96]. In the latest fish health report from the Norwegian Veterinary Institute [111] it is shown that the number of outbreaks has declined the last couple of years. The development

of the QTL salmon has been given much of the honour for this promising development together with the fight against IPNV in the hatcheries.

Detection methods for IPNV in head kidney

The use of large challenge experiments with many samples necessitates the development of assays with high throughput capacity. The RT-qPCR is a sensitive and reproducible method for detection and quantification of mRNA transcripts and was therefore choosen to analyse the samples from the challenge experiment. The main goal in Paper II was therefore to compare the stability of RT-qPCR reference genes in salmon and to optimise IPNV detection to identify viral carriers.

Titration vs. RT-qPCR

In paper II when IPNV titres were compared with RT-qPCR for the same head kidney samples there was a clear correlation of the measured viral amounts. However, viruses could not be detected by the micro-titre assay in many of the samples, while the RT-qPCR detected low virus RNA levels.

In paper III we show that neither the micro-titre assay nor the RT-qPCR was sensitive enough to detect viral RNA (VP2) in head kidney from salmon persistently infected with the high virulent virus. The head kidney leucocytes, which is one of the cell types where IPNV are reported to persist [76], had to be isolated and cultivated before it was possible to detect VP2 RNA. Munro et al. [75] have previously shown that IPNV could be detected in head kidney macrophages that were negative by the standard carrier tests by using lysates from the cells.

In paper II it is emphasised that IPNV titres determined in the samples by micro-titration assay give an estimate of the number of infectious virus particles, whilst RT-qPCR detects VP2 RNA transcript level, which not necessarily reflects the quantity of infectious virus particles. However, our results from the *ex vivo* leucocyte study indicate that the high virulent virus replicate in the adherent leukocytes. Furthermore, the challenge experiment demonstrated that IPNV infection was activated after transfer to seawater. Together, these findings demonstrate that infectious viruses are produced in the leucocytes.

One of the conclusions in paper II is that relative quantitation by RT-qPCR might not be sufficiently accurate to determine low IPNV amounts in carrier fish. The fluorescent signals

in RT-qPCR commonly show high individual variations, especially high variations are detected when the viral amounts is low which makes the obtained results less reliable. Thus, at the carrier stage of an infection there appear to be a question of whether virus can be detected or not. However, in most of the experiments it is not the relative IPNV amount that is of interest, but rather whether the fish is infected or healthy. In order to answer this question, the RT-qPCR assay seems to be the best alternative, since the detection limit of the micro-titration assay is too high. Similar results are later reported by Ørpetveit et al. [230] which showed that RT-qPCR is equally or slightly more sensitive than the OIE-recommended cell culture method for detection of IPNV in carriers.

The same conclusion was drawn concerning accurate detection and quantification of the fish viral hemorrhagic septicemia virus (VHSV) [231]. In this paper they found that cell culture cultivation of the virus (the approved diagnostic method) was unreliable, while two-color fluorometric RT-qPCR was highly sensitive, accurate and free of false negatives.

Evaluation of reference genes

The achievement of reliable RT-qPCR results is only possible after application of an appropriate normalisation method to correlate for the different amount of input RNA among samples. It is an absolute necessity because the technique poses problems at various stages of sample preparation and processing. The most commonly mentioned problems are: RNA extraction procedure along with sample storage and its quality, the process of reverse transcription (cDNA synthesis) including poorly selected target primers/absent validation of reference genes and inappropriate statistical analysis [232]. The selection and validation of reference genes is essential for RT-qPCR data normalisation and the selection of suitable reference genes therefore remains critical [233]. Reference genes are an internal reaction control that encompasses sequences different from the target. For a gene to be regarded as a reliable reference it must meet several important criteria [234], where the most important is that the expression level is unaffected by experimental factors.

In paper II we studied the effect of IPN infection on the transcriptional level of the reference genes such as 18S rRNA, β-actin, structural ribosomal protein S20 (RPS20), transcription elongation factor 1α (eEF1α), glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in different organs and cells. The most stably expressed reference genes in the different organs and cells were β-actin. eEF1 and

RPS20 was ranked the most stable transcribed genes among the head kidney groups, while GAPDH was the least stably expressed gene. In many of the groups, the reference gene G6PHD was among the most stable expressed. However the results showed that G6PHD were highly affected by the IPNV infection and therefore not an appropriate candidate for accurate quantification by RT-qPCR in infected salmon.

Others have reported similar results describing significant changes in transcription of typical reference genes (eEF1αB, 18S rRNA, β-actin and RPS20) during salmon pancreas disease (SPD) [235]. Comparable experiments have been performed in humans, which gave similar results during viral infection [236]. This shows that change in transcription of typical reference genes due to virus infection not only apply for virus infections in fish, but is also independent of the host.

There are reported several studies of reference genes evaluation in salmon [235, 237-240] describing different experimental conditions that affect gene expression such as immune stimulation, infection with different viruses and smoltification. All these studies conclude that the different conditions seem to have little influence on the gene expression of EF1α and it is therefore regarded as an appropriate candidate for data normalisation.

Innate immune response in Atlantic salmon persistently infected with IPNV

In paper III, the virulence factor study (Paper I) and the optimised and validated RT-qPCR assay (Paper II) was used to investigate how persistent IPNV infections impact the innate immune response and *vice versa*. The occurrence of IPNV with different virulence as well as the innate immune marker Mx was studied throughout an experimental challenge covering both a fresh and a seawater phase. This was performed to identify whether there are differences in the innate immune response in salmon that was persistently infected with IPNV of high and low virulence.

An interesting outcome of the study was that both the high virulent strain (Thr217-Ala221) and the low virulent strain (Thr217-Thr221) established persistent infections at the same efficiency. The results from the isolated leucocytes revealed that both isolates still persisted in the fish after 10 and 16 weeks post challenge. However, at these time points the high virulent virus persisted at a slightly lower level than the low virulent virus. Others have in previously

studies indicated that virulent strains (Thr217-Ala221) do not establish a persistent infection as effectively as the low virulent (Thr217-Thr221) [41, 46] and they suggested that a Thr at 221 is linked to IPNV persistent infection [227]. Our challenge experiment is conducted in a similar way as the experiment reported by Santi et al. [41]. However, there are some differences such as that Santi et al. used fry while we used smolts for the challenge experiment. Furthermore, they used the whole fish for virus isolation while we used a specific organ (head kidney). The high and low virulent virus used in our experiment differed in the length of the VP5. The high virulent field isolate had a truncated VP5 of 12 kDa while the attenuated low virulent isolate do not encode a VP5. However it has earlier been demonstrated that VP5 has no function as a virulence factor and that the establishment of persistent IPNV infection is independent of VP5 expression [41] implying that these differences should not affect the outcome of the infection. The main difference between these two studies is the virus isolates used. The high virulent isolate with the Thr217-Ala221 motif was used in both experiments. However, there might be other unknown differences that affect the ability of IPNV to persist in the Atlantic salmon.

To promote their survival, viruses have evolved multiple strategies for evasion of the host immune response. Avoidance of host immune responses is essential for viruses to persist in their hosts for long periods. Even for viruses that cause acute infections, immune evasion strategies are important to prolong infection and increase the opportunities for transmission to new hosts. Impairing of the host response, avoiding recognition by the host immune defences and resisting control by immune effector mechanisms are some of the strategies the viruses use to evade the immune responses. One objective of Paper III was to identify if there exists genotypic pathogenic properties of selected IPNV strains that may activate or evade antiviral responses. The Mx-promoter assay was used to indirectly measure IFN activity in immune stimulated head kidney derived leukocytes persistently infected with IPNV of high and low virulence. Immune stimulation with the TLR9-agonist CpG ODN class C showed that supernatants derived from leucocytes infected with the high virulent isolate induced significant lower Mx reporter activity than the leucocytes infected with the low virulent IPNV. These results suggest that the high virulent IPNV isolate may possess properties that inhibit the IFN antiviral response resulting in evasion of host immune responses. This evasion might offer one explanation why this viral strain is classified as high virulent.

Another objective was to use *ex vivo* studies of leucocytes from challenged fish to investigate whether immune stimulation with CpG motives of the IPNV carrying cells may "cure" the infection. These TLR/PRR ligands have previously been used as immune stimulants to activate the immune defence against IPNV [193, 241]. In paper III we show that after CpG stimulation of head kidney leucocytes infected with the low virulent isolate the mean level of detected VP2 RNA transcripts was higher in the CpG stimulated leucocytes compared to the unstimulated cells. The low virulent virus apparently do not replicate in leucocytes at any of the studied time points. In contrast, in the head kidney leucocytes infected with the high virulent isolate the situation was opposite. The data indicate that the virus replicates both in the unstimulated and in the CpG stimulated leucocytes within the experimental period. However, it seems as though the virus replication is slower in the CpG-stimulated leucocytes when compared to untreated cells. Although the CpG treatment does not "cure" the infection in the leucocytes infected with high virulent virus, this treatment may induce IFN-induced genes or other players in the immune system that activate inhibition of some of the "events" necessary for viral replication. Attempts to cure persistently infected Atlantic salmon using poly I:C has been reported previously [242]. In this study they obtained comparable results in respect to increased IFN response, however, IPNV still persisted in the fish sampled at later time points. Altogether, these and our results strongly indicate that immune stimulation neither by poly I:C nor CpG can efficiently cure an IPNV carrier state.

When these results are summarised we find, in agreement with several other studies, that changes in amino acid residues 217 and 221 of VP2 have profound impact on the outcome of the infection. The high virulent virus replicate in the leucocytes even though the replication is modestly inhibited by CpG treatment. On the other hand these replicating viruses inhibit some of the effect of the CpG stimulation, which result in less Mx promoter activation and thus is an indication of reduced IFN activity. These results are in accordance with previously reported results, which demonstrated that IFNa1 do not completely inhibit IPNV growth, but causes a delay in viral protein synthesis and suggest that IPNV-encoded proteins may be involved in weakening of IFN signalling [50]. There are no indications that the low virulent virus in the isolated leucocytes is replicating and also there are no signs that the virus inhibit activation of the Mx promoter. These data are in accordance with results published by Skjesol et al. which found that VRG were differently expressed in head kidney infected with high virulent IPNV compared to low virulent IPNV [39].

Concluding remarks

1. A correlation between the different amino acid patterns and virulence of the IPNV was revealed, where specific amino acid residues at position 217 and 221 were manifested as being important for IPNV virulence

2. The evaluation of reference genes and optimisation of a RT-qPCR assay has developed a more sensitive and accurate method for quantification of IPNV in persistently infected salmon.

3. Studies of IPNV with high and low virulence have revealed that

- Both low and high virulent IPNV can infect Atlantic salmon persistently
- The high virulent IPNV in a persistent infection seem to evade the host's innate immune responses
- The high virulent IPNV persists at a lower amount in the freshwater phase than the low virulent virus and as a consequence the immune response is either not triggered or it is inhibited by the HV virus
- Results from both challenge experiments and RT-qPCR show that the HV isolate replicate faster than the LV isolate
- There are modest, but significant differences in the cells ability to produce IFNs between the HV and LV infected groups.

Future perspectives

Viral persistence has been characterised in mammals were it has been observed that susceptible hosts may have an infection associated with inhibition of cytokine activity [243]. For example modulation of the host's expression of anti-inflammatory cytokines can help the establishment of chronic infections [244, 245]. IL-10 is a potent anti-inflammatory cytokine that during an infection are involved in regulation and inhibition of pro-inflammatory cytokine expression. Reyes-Cerpa et al. [188] found that salmon persistently infected with IPNV presented a high IL-10 level, which distinguished this group from the uninfected group. This indicates that the virus triggers an anti-inflammatory response that may be part of the mechanisms to establish persistence. The same authors later demonstrated that IPNV infection in rainbow trout occurs with a distinct cytokine expression profile depending on the type of infection, i.e. acute or persistent infection [246]. Both IPNV itself and the immune responses the virus elicit have been investigated in numerous reports. A few studies have been conducted with IPNV isolates of differing virulence, however for most of the experiments the infection has been carried out with only one isolate of IPNV, either a high or a low virulent isolate or with unknown virulence [39, 50, 69, 85, 186-188, 194, 197, 203, 207, 246-249].

The challenge experiment described in paper III was completed in 2006/2007. The knowledge about the immune system of bony fish have expanded since then and in the subsequent years more Atlantic salmon immune genes have been identified and further characterised, including type I IFNs, PKR, ISG15, IL-10, IL1β and IL8 and many more [177, 179, 180, 202, 207, 250, 251]. If this experiment had been performed today more genes which are known to be important in the defence against viral infection, like those mentioned above, would have been added to the RT-qPCR measurements.

The data in paper III lay a ground for further studies aimed at elucidating difference in the transcription of these immune genes and in addition the data presented may be fundament for further functional studies of selected immune genes, to find their impact related to IPNV infections, aimed at widening the understanding about their roles in antiviral defence.
This and work performed by others on molecular characterisation of IPNV into high and low virulent strains have laid an important foundation for further work. To bring this further reverse genetic must be implemented so construction of different recombinant virus can be

performed. Conducting experiments with these recombinant viruses might uncover some differences in the underlying mechanisms (IFN pathway) and reveal some of the molecular and immunological mechanisms involved in establishing the carrier state.

Summary of papers

Paper I. Study of virulence in field isolates of infectious pancreatic necrosis virus obtained from the northern part of Norway.

Kjersti Julin, Saskia Mennen, Ann-Inger Sommer.

In paper 1 we focused on the virulence of different isolates of infectious pancreatic necrosis virus (IPNV). In order to study the variety of IPNV strains involved in outbreaks of infectious pancreatic necrosis (IPN) in Atlantic salmon fish farms, samples were collected from 19 different outbreaks of IPN in the northern part of Norway. The main objective of this study was to examine whether IPNV isolates of different virulence were involved in the outbreaks and could explain the variable IPN protection observed in vaccinated post-smolts in the field. Both the molecular basis of virulence of all field isolates and virulence expressed by mortality after bath challenge of unvaccinated post-smolts with eight of the isolates were studied. Very little variation among the field isolates was detected when the 578-bp variable region encoding the VP2 protein known to be involved in virulence was sequenced. The cumulative mortality after experimental challenge with field isolates genetically characterised as highly virulent was always high (40–56%), while the cumulative mortality of the same strains in vaccinated post-smolts during the field outbreaks varied from 1 to 50%. Although the tested samples came from fish vaccinated with the same vaccine product, the protection against IPN varied. These results demonstrate that differences in virulence of the isolates were not the main reason for the variation in mortality in the field outbreaks. Most of the field isolates were of high virulence, which is shown in experimental challenges to be important for mortality, but clearly other factors that might affect the susceptibility of IPN also play an important role in the outcome of an IPNV infection.

Paper II. Reference genes evaluated for use in infectious pancreatic necrosis virus retime RT-qPCR assay applied during different stages of an infection.

Kjersti Julin, Lill-Heidi Johansen and Ann-Inger Sommer.

When performing a RT-qPCR it is important to determine the most suitable reference genes to use for normalisation of the data to get reliable results. In paper 2 the stability of six reference genes, 18S, β-actin, RPS20, eEF1α, G6PDH and GAPDH, was examined in tissues from Atlantic salmon (Salmo salar) and Chinook salmon embryo cells (CHSE-214). The tissue samples selected for analysis were taken from head kidney and pylorus and collected at different time points during a challenge experiment with IPNV. The stability of some of the reference genes was also studied in infected CHSE-214 cells. The ranking of the genes examined was carried out using the geNorm program. This program determines the most stable genes from a set of genes tested in a given cDNA sample. The stability of the reference genes varied in different tissues and in the cell line at different stages of infection with IPNV. This study demonstrated that tissue-specific combinations of reference genes must be used to normalise real time data for use for quantitation of IPNV.

Paper III. Persistent infections with infectious pancreatic necrosis virus (IPNV) of different virulence in Atlantic salmon *Salmo salar L*.

Kjersti Julin, Lill-Heidi Johansen, Ann-Inger Sommer and Jorunn B. Jørgensen.

In paper 3 we investigated the occurrence of the virus as well as the innate immune marker Mx in the HK throughout an experimental challenge covering both a fresh and a seawater phase. The fish were challenged with two IPNV isolates, possessing high and low virulence motifs in VP2. While no sign of disease and mortality were observed in the freshwater phase, both isolates caused mortality due to reactivation of the virus after transfer to seawater. In accordance with earlier reports the high virulent (HV) strain exhibited higher mortality than the low virulent (LV) strain. Interestingly, in the freshwater phase higher levels of virus transcripts were detected in the HK of fish infected with LV IPNV compared to HV, suggesting that the HV isolates is able to limit its own replication to a level where the innate immune system is not alerted. To test this hypothesis further ex vivo HK leukocytes derived from fish infected with the two isolates and uninfected controls were stimulated with CpG DNA, a TLR9 ligand, known to induce the IFN pathway in salmon. Upon stimulation, significant higher IFN-levels were found in the LV compared to the HV group in the freshwater phase. This suggests that the viruses attenuate the antiviral host immune response in different manners which may contribute to the observed differences in disease outcome.

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